

Interferon Regulatory Factor 5 (IRF5):

An important player in

macrophage polarization

and

TNF regulation

Thomas Krausgruber

A thesis submitted for the degree of Doctor of Philosophy at the
Kennedy Institute of Rheumatology
Faculty of Medicine
Imperial College London

Supervisors:

Dr Irina A Udalova

Dr Jonathan Dean

London, May 2011

Abstract

Macrophages are dynamic and heterogeneous cells that can be divided into specific, phenotypic subsets. Based on Th1/Th2 polarization concept they are referred to as pro-inflammatory classical M1 (IL-12^{high}, IL-23^{high}, IL-10^{low}) macrophages and anti-inflammatory M2 (IL-12^{low}, IL-23^{low}, IL-10^{high}) macrophages. In contrast to T lymphocyte subsets, the transcription factor(s) underlying macrophage polarization remain largely unknown.

My research has highlighted the importance of Interferon regulatory factor 5 (IRF5) for establishing the pro-inflammatory M1 macrophage phenotype. I was able to show that high expression of IRF5 is characteristic of M1 macrophages, in which it transcriptionally regulates M1-specific cytokines, chemokines and co-stimulatory molecules. Consequently, the depletion of IRF5 in human M1 macrophages results in down-regulation of M1-specific cytokines and further evidence for a role of IRF5 in effective immunity stems from my work using an *in vivo* model of polarizing inflammation. IRF5 deficient mice showed a significant reduction in serum levels of M1-specific cytokines compared to wild-type littermate controls. Therefore, the suppression of macrophage function via inhibition of IRF5 provides a new approach to attenuate the inflammatory response.

Tumor necrosis factor (TNF) plays an essential role in the host defence against infections but is a major factor in the pathogenesis of chronic inflammatory diseases. The expression of TNF is therefore tightly regulated. I was able to demonstrate that IRF5 is not only involved in the induction of human TNF gene expression but also crucial for the late phase secretion of TNF by human myeloid cells. IRF5 is using a complex molecular mechanism to control the TNF gene with two spatially separated regulatory regions (5' upstream and 3' downstream of the gene) and two independent modes of action (direct DNA binding and formation of IRF5/RelA complex) being involved. The manipulation of the IRF5/RelA interaction could be a putative target for cell-specific modulation of TNF gene expression.

Declaration

The experimental data described in this thesis are original and have been performed by myself, with exceptions indicated. All collaborations have been acknowledged in the appropriate places, either in the materials and methods section or in the result chapters.

This work has been carried out at the Kennedy Institute of Rheumatology Division and was funded by the Arthritis Research UK, Medical Research Council, the European Community Seventh Framework Programme FP7/2007-2013 and the Kennedy Institute trustees.

Thomas Krausgruber

May, 2011

Acknowledgements

First and foremost, I would like to thank Dr Irina Udalova for her outstanding supervision during the course of my PhD and for providing the concept of this study. I would like to thank you for your teaching, enthusiasm, inspiration throughout, motivation when needed and guidance, but also for giving me the freedom to explore and pursue my own ideas. Your overall influence helped me to develop into a mindful and independent scientist.

I thank Dr Jonathan Dean who has always taken an interest in my work, providing critical and constructive suggestions. I also thank the late Prof Brian Foxwell for guidance in the first year of my PhD.

The technical advice and expertise, that made the generation of the data in this thesis possible, were kindly provided by the following people: Miss Katrina Blazkova (so helpful throughout and in particular with murine work), Dr David Saliba (investigated the IRF5/RelA interaction), Mrs Alessandra Lanfrancotti (virus purification and EMSA), Mrs Helen Lockstone and Miss Natasha Sahgal (Micro array analysis), Dr Dilair Baban (RNA hybridisation), Dr Daniel Wong (purification of recombinant IRF5 DBD), Dr Irina Udalova (EMSA), Dr Tim Smallie (provided luciferase constructs and performed luciferase experiments), Dr Saba Alzabin (proliferation assays and help with FCM analysis), Dr Lynn Williams (scientific advice), Prof Tracy Hussell (scientific advice) and last but not least, Prof Sir Marc Feldmann (scientific as well as personal advice and help).

I would like to thank everyone in my group, both current (Katrina, Scott, David, Hayley, Grisha) and past (Fergie, Lionel, Helen) for their help, support and for making those long hours in the lab much more enjoyable. Thanks to Tim Smallie, Matt Pierce and Andy Kinloch for offering scientific advice and for encouragement when needed.

The compilation of this thesis was only possible because of the proof-reading and constructive comments kindly provided by Irina, Jon, Katrina, Hayley, Scott and David.

Most of all, I would like to extend my thanks and love to my now fiancée Julia, for her seemingly endless patience and constant support. It was your love and optimism that helped me through all this.

Nothing is impossible with you at my side :-)

Table of contents

ABSTRACT	2
DECLARATION	3
ACKNOWLEDGEMENTS	4
TABLE OF CONTENTS	5
LIST OF FIGURES	10
LIST OF TABLES	12
1. INTRODUCTION	13
1.1 The immune system	13
1.1.1 Granulocytes.....	16
1.1.2 Monocytes	17
1.1.3 Dendritic cells.....	18
1.1.4 Macrophages	19
1.1.5 Natural killer cells	26
1.1.6 T Lymphocytes.....	27
1.1.7 B-cells.....	30
1.2 The Interferon regulatory factor family of transcription factors	31
1.3 Regulation of the innate immune response by IRF and NF-κB family members	32
1.3.1 Activation of NF- κ B proteins by TLR signalling.....	33
1.3.2 IRF3/IRF7: master regulators of type I IFN induction.....	35
1.3.3 IRF4/IRF8: essential factors for DC development and function	36
1.4 Interferon regulatory factor 5	37
1.4.1 IRF5 gene structure	37
1.4.2 IRF5 protein structure	39
1.4.3 Expression of IRF5	40
1.4.4 Activation of IRF5.....	40
1.4.5 Role of IRF5 in antiviral immunity and cytokine induction.....	41
1.5 Dysfunction of immunity – Role of IRF5 in autoimmune diseases	43
1.5.1 Systemic lupus erythematosus.....	43
1.5.2 Inflammatory bowel disease.....	45

1.5.3 Rheumatoid arthritis	47
1.6 Hypothesis of investigation	52
1.6.1 Specific aims for TNF study (Chapter 3):.....	52
1.6.2 Specific aims for macrophage polarization study (Chapter 4 and 5)	53
2. METHODS	54
2.1 Cell culture	54
2.1.1 Maintenance of cell lines	54
2.1.2 Cryopreservation of cells.....	55
2.2 Work with primary human myeloid cells	55
2.2.1 Isolation of human peripheral blood mononuclear cells	55
2.2.2 Isolation of human monocytes.....	55
2.2.3 <i>In vitro</i> differentiation of human monocytes	56
2.3 Work with mice and murine cells	56
2.3.1 Isolation of bone marrow cells	56
2.3.2 <i>In vitro</i> differentiation of bone marrow cells	57
2.3.3 Isolation and culture of spleen cells.....	57
2.3.4 <i>In vivo</i> experiment with IRF5 knock-out mice	58
2.4 MTT cell viability assay	58
2.5 Quantitative real-time PCR	59
2.5.1 RNA extraction and quantification	59
2.5.2 cDNA synthesis.....	59
2.5.3 Quantitative real-time PCR 2 standard curve method.....	59
2.6 Micro array analysis	62
2.7 Enzyme linked immunosorbent assay (ELISA)	62
2.7.1 BD™ Cytrometic bead assay (CBA).....	65
2.8 Western blotting	66
2.8.1 Preparation of whole cell protein extracts.....	67
2.8.2 Protein quantification: BCA assay	67
2.8.3 SDS-PAGE	67
2.8.4 Protein transfer	67
2.8.5 Immunoblotting and protein detection	68
2.9 Electrophoretic mobility shift assay (EMSA)	69
2.9.1 DNA probe labelling	69

2.9.2 Binding reaction and gel run.....	69
2.10 Cloning and genetic constructs used in this study	70
2.10.1 Gene reporter and expression constructs used in this study	70
2.10.2 PCR.....	72
2.10.3 Restriction digests.....	72
2.10.4 DNA purification	72
2.10.5 DNA ligation	72
2.10.6 Transformation of chemically competent cells	73
2.10.7 Amplification and isolation of plasmids	73
2.10.8 Identification of positive clones.....	73
2.11 Transfection and adenoviral infection of cell cultures	73
2.11.1 Transfection of siRNA oligonucleotides into cell lines.....	74
2.11.2 Transfection of siRNA oligonucleotides into primary human cells.....	74
2.11.3 Transfection of plasmids into cell lines	74
2.11.4 Adenoviral infection of primary human myeloid cells	75
2.12 Luciferase gene reporter assays	76
2.13 Immuno-precipitation	76
2.14 Chromatin immuno-precipitation	77
2.14.1 Fixation and preparation of nuclear extracts.....	77
2.14.2 Sonication	78
2.14.3 Immuno-precipitation.....	78
2.14.4 DNA purification	79
2.14.5 ChIP primer design	79
2.14.6 ChIP primer testing and optimisations	80
2.14.7 Real-time PCR analysis	82
2.15 Flow cytometry (FCM) analysis	83
2.16 Mixed lymphocyte reactions (MLR)	84
2.16.1 MLR with MDDCs as APCs.....	84
2.16.2 MLR with macrophages as APCs.....	85
2.17 Bioinformatic analysis	85
2.18 Statistical analyses	85
2.19 Buffers and Solutions	86
2.19.1 General buffers	86
2.19.2 SDS-PAGE and Western blotting	86
2.19.3 ELISA buffers.....	88

2.19.4 EMSA.....	88
2.19.5 Chromatin immuno-precipitation.....	88
2.19.6 Molecular biology solutions	89
3. IRF5-MEDIATED HUMAN TNF GENE EXPRESSION	91
3.1 Introduction	91
3.2 Results	92
3.2.1 IRF5 expression is high in MDDCs and controls late phase TNF secretion	92
3.2.2 si-RNA mediated depletion of IRF5 reduces late-phase TNF secretion	98
3.2.3 IRF5 is involved in transcriptional regulation of TNF	100
3.2.4 Computational analysis of the human TNF locus	103
3.2.5 Design of ChIP primers	105
3.2.6 IRF5 is recruited to a 5' upstream and 3' downstream region of TNF	105
3.2.7 IRF5 forms specific physical interactions with RelA.....	109
3.2.8 RelA is required for IRF5-dependent transactivation of the TNF gene.....	112
3.3 Conclusion	115
4. IRF5 PROMOTES INFLAMMATORY MACROPHAGE POLARIZATION	119
4.1 Introduction	119
4.2 Results	120
4.2.1 IRF5 expression is high in human M1 macrophages	120
4.2.2 IRF5 expression is plastic in human macrophages	123
4.2.3 IRF5 influences the polarization of human macrophages	126
4.2.4 IRF5 induces the mRNA expression of macrophage lineage-specific cytokines.....	129
4.2.5 IRF5 directly regulates mRNA expression of lineage-specific cytokines	135
4.2.6 IRF5 inhibits the transcription of the human IL-10 gene	141
4.3 Conclusion	143
5. IRF5 PROMOTES TH1/TH17 RESPONSES AND ITS IMPORTANCE FOR <i>IN VIVO</i> INFLAMMATION	147
5.1 Introduction	147
5.2 Results	148

5.2.1 IRF5 promotes human T lymphocyte proliferation and Th1 responses	148
5.2.2 IRF5 promotes human Th17 responses	151
5.2.3 IRF5 regulates the phenotype of mouse macrophages	155
5.2.4 Importance of IRF5 in a mouse model of M1 inflammation.....	157
5.3 Conclusion	161
6. DISCUSSION	165
7. ABBREVIATIONS	176
8. REFERENCES	180
9. SUPPLEMENTARY MATERIAL - PUBLICATIONS	206

List of Figures

Figure 1.1 Recognition of pathogens by innate cells	14
Figure 1.2 Simplified overview of the interplay between innate and adaptive immunity	16
Figure 1.3 Simplified schematic of myeloid differentiation	17
Figure 1.4 Inducers and selected properties of polarized macrophages.....	22
Figure 1.5 <i>In vitro</i> polarization of macrophages using growth factors	24
Figure 1.6 Domain model of IRF family members	32
Figure 1.7 Overview of transcription factor activation by TLR signalling	33
Figure 1.8 Human IRF5 gene structure	38
Figure 1.9 Schematic representation of healthy versus RA joint	50
Figure 2.1 Melt curve analysis of ChIP primer pairs	81
Figure 2.2 Improvement of C _t values with Takara master mix	82
Figure 3.1 IRF5 is highly expressed in MDDCs.....	92
Figure 3.2 Prolonged TNF secretion in MDDCs	93
Figure 3.3 Late phase TNF secretion is required for potent T lymphocyte response	95
Figure 3.4 Ectopic expression of IRF5 in MDMs results in prolonged TNF secretion and increased Th1 responses.....	97
Figure 3.5 siRNA-mediated depletion of IRF5 is not possible in HEK-293-TLR4 cells.....	98
Figure 3.6 siRNA-mediated depletion of IRF5 affects late phase secretion of TNF	100
Figure 3.7 LPS-induced expression of TNF mRNA is IRF5 dependent	101
Figure 3.8 IRF5 is involved in transcriptional regulation of TNF	103
Figure 3.9 Schematic of the human TNF locus	104
Figure 3.10 Recruitment of IRF5 to 5' upstream and 3' downstream region of TNF	106
Figure 3.11 <i>In vitro</i> binding of IRF5 to 5' upstream and 3' downstream regions of TNF....	108
Figure 3.12 IRF5 and RelA are recruited to 5' upstream and 3' downstream regions of the TNF gene in MDDCs	108
Figure 3.13 Co-recruitment of RelA and IRF5 to region H.....	109
Figure 3.14 Ectopic IRF5 specifically interacts with RelA in HEK-293 cells	110
Figure 3.15 Endogenous IRF5 interacts with RelA in MDDCs.....	111
Figure 3.16 RelA is required for IRF5-binding to region H of the TNF gene	113
Figure 3.17 RelA is required for IRF5-mediated activation of TNF	114
Figure 3.18 Modes of IRF5 recruitment to regions of the human TNF locus.....	116
Figure 4.1 The cytokine environment determines macrophage phenotype.....	121
Figure 4.2 High expression of IRF5 in M1 macrophages and upregulation by GM-CSF ...	122

Figure 4.3 IRF5 expression is induced by M1 macrophage maturation protocols	123
Figure 4.4 Plasticity of macrophages in respond to environmental changes	124
Figure 4.5 Plastic expression of IRF5 in macrophages	125
Figure 4.6 Ectopic IRF5 influences the production of IL-12p70 and IL-23	126
Figure 4.7 Ectopic IRF5 induces IL-1 β and TNF but inhibits IL-10 secretion	127
Figure 4.8 siRNA-mediated knock-down of IRF5 in M1 macrophages	128
Figure 4.9 Depletion of IRF5 influences the production of lineage-specific cytokines	129
Figure 4.10 IRF5 induces the mRNA expression of lineage-specific cytokines	130
Figure 4.11 IRF5 depletion influences mRNA expression of lineage-specific cytokines ...	130
Figure 4.12 IRF5 influences the global expression profile of macrophage subsets	132
Figure 4.13 IRF5 drives the production of selected lineage specific cytokines	134
Figure 4.14 LPS-induced recruitment of IRF5 to promoter regions of lineage-specific cytokines	141
Figure 4.15 IRF5 inhibits the transcriptional activation of the human IL-10 gene	142
Figure 4.16 Transcription factors underlying macrophage polarization	146
Figure 5.1 IRF5 induced T lymphocyte proliferation	149
Figure 5.2 IRF5-expressing macrophages induce Th1 activation	150
Figure 5.3 IRF5-expressing macrophages induce Th1 development	151
Figure 5.4 IRF5-expressing macrophages induce Th17 activation	152
Figure 5.5 IRF5-expressing macrophages induce Th17 development	153
Figure 5.6 IRF5-expressing macrophages induce Th17 cytokine profile	154
Figure 5.7 IRF5-expressing macrophages do not induce the Treg or Th2 lineage	155
Figure 5.8 IRF5 expression is high in GM-CSF differentiated BMDMs	156
Figure 5.9 Impaired induction of M1 cytokines in BMDMs of IRF5 ^{-/-} mice	156
Figure 5.10 Impaired induction of M1 cytokines in LPS challenged <i>Irf5</i> ^{-/-} mice	158
Figure 5.11 Impaired mRNA induction of M1-specific cytokines in peritoneal cells from <i>Irf5</i> ^{-/-} mice	159
Figure 5.12 Reduction in IFN- γ and IL-17 production in <i>ex vivo</i> splenocyte cultures	160
Figure 5.13 Potential feedback loop involving IRF5, IL-23 and GM-CSF	163
Figure 6.1 Interplay between M1 macrophages, T lymphocytes and infiltrating cells	172

List of Tables

Table 2.1: Cell lines used in this study	54
Table 2.2: TaqMan primers used in this study.....	60
Table 2.3: Antibodies used for ELISA	63
Table 2.4: Recombinant proteins used as standards for ELISA	65
Table 2.5: Antibodies and standards used for CBA.....	66
Table 2.6: Antibodies used for western blotting.....	68
Table 2.7: Oligonucleotide sequences used for generating EMSA probes	70
Table 2.8: Expression constructs used in this study.....	71
Table 2.9: Luciferase gene reporters	71
Table 2.10: Antibodies used for ChIP.....	79
Table 2.11: ChIP primers used for analysing human TNF locus.....	82
Table 2.12: Primers used for ChIP analysis	83
Table 2.13: Antibodies used for FCM analysis	84
Table 4.1 Newly identified IRF5 target genes.....	133
Table 4.2 Putative IRF5 binding sites in -2000/+2000 bp relative to the TSSs of selected genes	135

1. Introduction

1.1 The immune system

The immune system has been shaped by evolution to allow multicellular organisms to live together with microorganisms, e.g. in the microbiota of the gut. However, many microorganisms, such as viruses, bacteria, fungi and parasites, have the potential to cause disease and threaten host viability. Accordingly, the immune system has evolved to protect the body from invasion and infection by pathogenic organisms and employs various mechanisms to distinguish particular features of the pathogens from healthy host tissue. In vertebrates, the immune system is highly dynamic, using several cell types, tissues and organs to provide multiple levels of defence against varying types of pathogens. However, the immune system not only provides protection against pathogens but also from “self”. It puts the host under permanent surveillance in order to recognise and eliminate unhealthy or damaged tissues therefore protecting the body from itself. The immune system has to evolve constantly as pathogens develop new ways to avoid detection by the immune system and adapt new mechanisms to infiltrate and infect their host.

There are two main arms of the immune system which respond in coordination to challenge or invasion - the innate and the adaptive response. All multicellular organisms possess innate immunity whereas adaptive immunity appeared during the evolution of vertebrates. The innate immune system is composed of a humoral arm, which consists of antimicrobial peptides and opsonins, and a cellular arm, which involves specialized cells. These cells are able to internalise and digest bacteria and other cells, to scavenge toxic compounds produced during metabolism and to produce inflammatory mediators that can kill invading pathogens.

The innate arm of the immune response provides the initial line of defence against invading microorganisms and is also responsible for initiating and ‘instructing’ the adaptive system through antigen presentation. It is triggered by germline encoded pattern recognition receptors (PRRs) which detect relatively invariant molecular patterns (pathogen-associated molecular patterns, PAMPs) found in most microorganisms [1, 2]. This makes the innate response largely non-specific as it will react to a wide range of infectious pathogens as well as to host tissue damage [3]. The activation of the innate immune system is not only based on the recognition of PAMPs but also occurs in the presence of danger signals or danger-

associated molecular patterns (DAMPs) released by injured cells. The release of DAMPs is a common event as tissue damage and cell lysis are often associated with infections that lead to the release of host molecules. Recognition of these DAMPs by the immune system not only allows the sensing of an ongoing infection and subsequent recruitment of more immune cells but can also initiate the repair of the damaged tissue [4, 5]. Therefore, the innate arm of the immune system not only scans the cellular environment for signs of invading pathogens but also recognises the damage caused by them.

Binding of PAMPs to innate PRRs will initiate a rapid and powerful response, associated with the initiation of gene expression and the release of inflammatory mediators (Figure 1.1). Inflammation is a key innate defence mechanism that is characterised by heat, redness, swelling, pain, increased blood flow and permeability of local blood vessels as well as recruitment of immune cells.

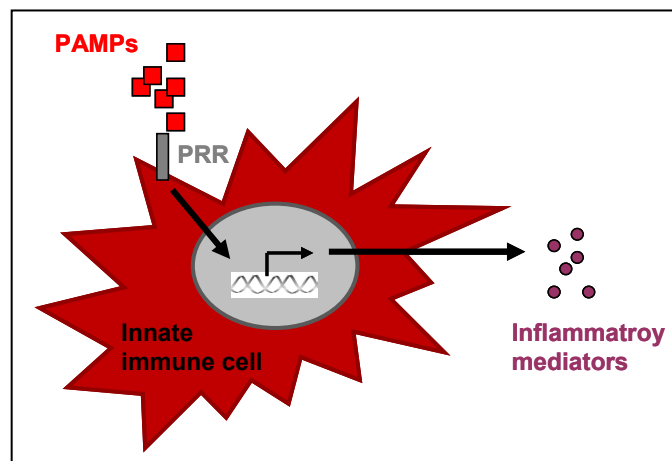


Figure 1.1 Recognition of pathogens by innate cells

Pathogens are recognised via PRRs expressed on innate immune cells. PRR signalling leads to the initiation of gene expression and subsequent secretion of inflammatory mediators.

PRRs can be broadly divided into 3 classes, transmembrane, cytosolic and secreted receptors [6]. Transmembrane PRRs include the Toll-like receptor (TLR) family and the C-type lectins. TLRs are either expressed on the plasma membrane or in endosomal/lysosomal organelles [7]. Membrane-bound TLRs recognise PAMPs that are accessible on the cell surface whereas endosomal TLRs mainly detect microbial nucleic

acids such as double-stranded RNA. The expression of TLRs is cell-type specific, which allows the distribution of recognition functions to various cell types [8]. The C-type lectin family consists of Dectin-1 and Dectin-2 that are able to detect compounds of fungal cell walls [9].

Cytosolic PRRs include the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NOD-like receptors or NLRs). Unlike TLRs, most cell types express RLRs which detect viral pathogens [10]. NLR family members are intracellular sensors that can detect degradation products of pathogens, stress signals as well as microbial products. NLR signalling results in the activation of a multiprotein complex called the inflammasome [11]. Lastly, secreted PRRs bind to microbial cell surfaces and can activate the classical as well as lectin pathways of the complement system and opsonise pathogens for phagocytosis by macrophages and neutrophils [6].

Unlike innate immunity, adaptive immunity is highly specific for a particular pathogen but may take extended periods of time to fully initiate an effective response. It requires specific “instructions” from innate cells in order to be activated. The activation occurs through highly variable, non-germline encoded receptors that bind a specific antigen. The antigens are presented as peptide fragments in the context of major histocompatibility complex molecules (MHC) by specialised antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs). Thus, the inflammatory environment provided by innate immune cells influences the activation, expansion and selection of pathogen-specific adaptive immune cells. The adaptive system is largely mediated by the actions of B and T lymphocytes and is characterised by immune specific memory. Memory is generated towards a specific pathogen and a repeated encounter usually evokes a more rapid immune response.

Taken together, protective immunity is divided into two systems that are in constant interplay with each other: the non-specific innate and the pathogen-specific adaptive immunity (Figure 1.2). The cellular repertoire of the immune system and their functions are described below.

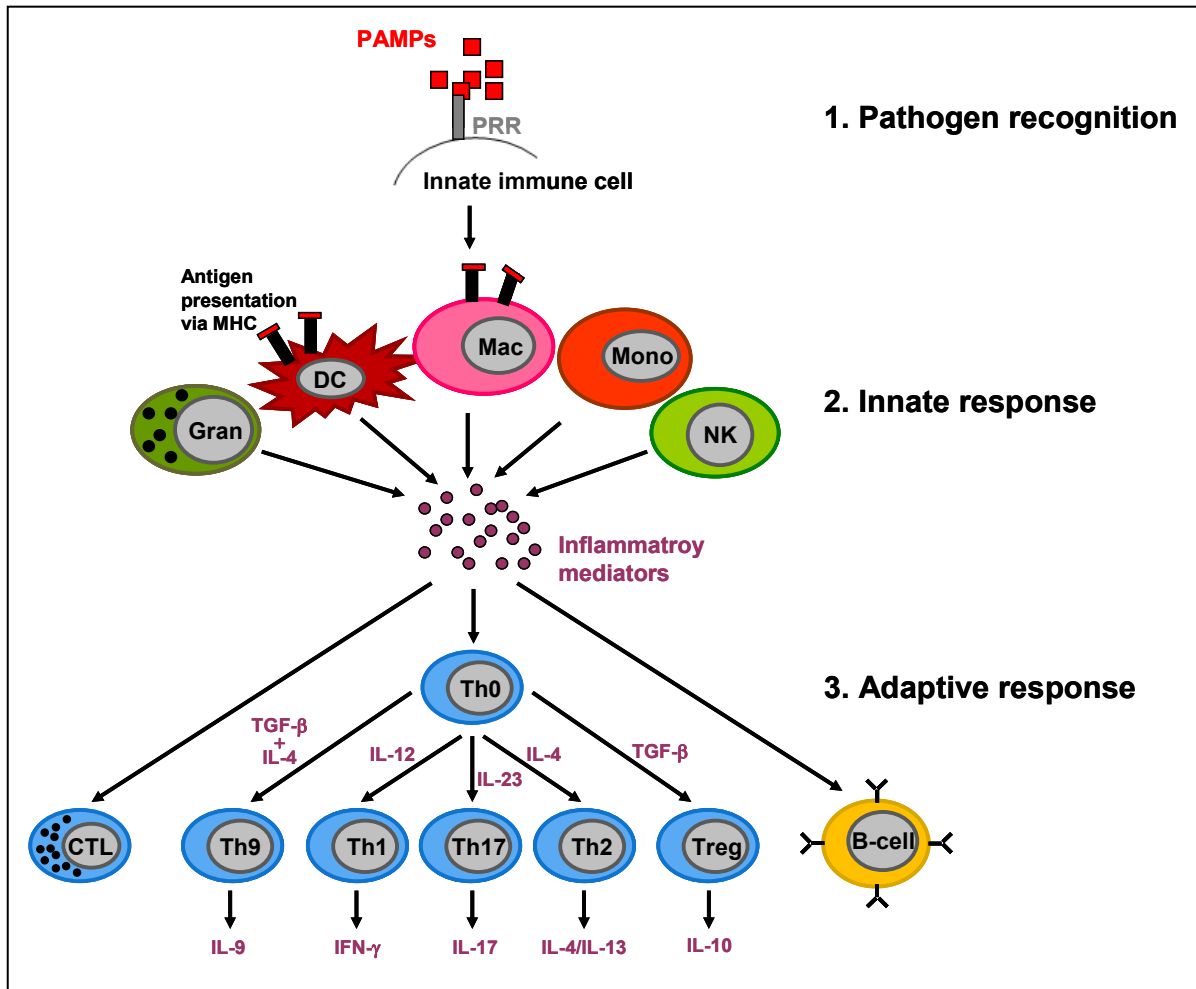


Figure 1.2 Simplified overview of the interplay between innate and adaptive immunity

Invading pathogens are recognised by innate immune cells leading to the release of inflammatory mediators and antigen presentation via MHC. The inflammatory environment provided by the innate cells determines the adaptive response, e.g. high levels of IL-12 will provoke a Th1 response whereas high levels of IL-4 will lead to a Th2 response. DC - dendritic cell; Mac - macrophage; Gran - Granulocyte; Mono - monocyte, NK - natural killer cell; Th0 - naïve T-helper lymphocyte; Th1 - T-helper 1 lymphocyte; Th17 - T-helper 17 lymphocyte; Th2 - T-helper 2 lymphocyte, CTL - cytotoxic T- lymphocyte

1.1.1 Granulocytes

Granulocytes are a category of white blood cells characterised by the presence of granules in their cytoplasm which contain a cocktail of toxic compounds that are harmful to bacteria and fungi. Granulocytes are involved in the clearance of pathogens by phagocytosis and

recruitment of additional immune cells to the site of infection through the release of chemo-attractants. They are among the first circulating cell types recruited to sites of infection or tissue damage. There are three types of granulocytes: neutrophil granulocytes, eosinophil granulocytes and basophil granulocytes. Neutrophils are the most abundant type of circulating white blood cells, constituting 50% to 60% of the total cell count, and are professional phagocytes. Eosinophils play a crucial part in the killing of parasites because of their unique granule composition. Basophils are one of the least abundant cells in the blood and contribute to an increased blood flow to the site of infection [12].

1.1.2 Monocytes

Monocytes are derived from a common myeloid progenitor (CMP) and comprise approximately 5-10% of circulating leukocytes. They show some typical morphological features such as irregular cell shape, kidney-shaped nucleus, cytoplasmic vesicles and a high cytoplasm-to-nucleus ratio. Both human and mouse monocytes can be defined by the expression of macrophage-colony stimulating factor (M-CSF) receptor 1 (M-CSFR, CD115) and the absence of T and B lymphocyte markers such as cluster of differentiation 4 (CD4) and CD19. Monocytes are the progenitors of tissue resident macrophages as well as DCs and differentiate into macrophages or DCs *in situ* during an inflammatory response (Figure 1.3) [13].

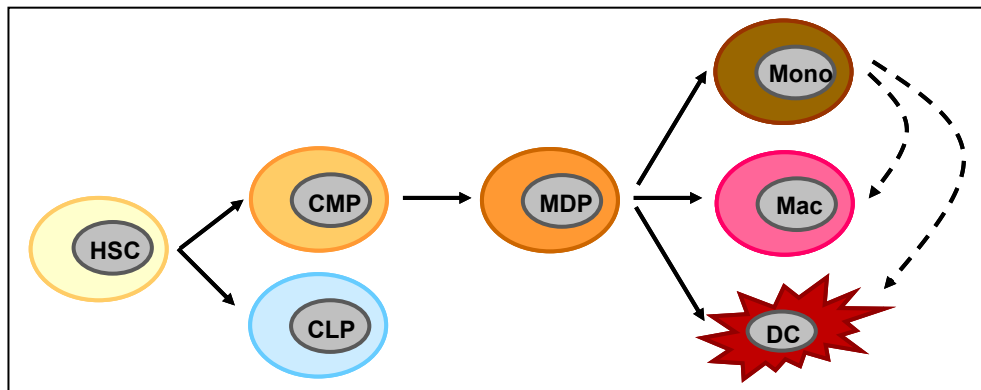


Figure 1.3 Simplified schematic of myeloid differentiation

Hematopoietic stem cells (HSC) produce common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). CMPs give rise to the monocyte/macrophage and DC progenitor called MDP. During inflammation, monocytes (Mono) can differentiate into monocyte-derived DCs (DC) and macrophages (Mac) indicated by dashed lines.

The development of blood monocytes is dependent on the growth factor M-CSF as mice deficient in either M-CSF or M-CSFR show a reduced number of monocytes [14, 15]. M-CSFR is a hematopoietic growth factor receptor expressed not only on monocytes but also macrophages, DCs and their progenitors [16], a population of cells often referred to as the mononuclear phagocyte system (MPS). The two known ligands for M-CSFR include M-CSF [17] and the more recently described IL-34 [18] which are both important for the development of the MPS lineage as M-CSF-deficient mice have a milder phenotype than M-CSFR-deficient mice [14].

Monocytes can be divided into three distinct subpopulations which differ in size, cell surface marker expression and chemokine receptors expression [13]. Human CD14⁺CD16⁻ monocytes represent 80% to 90% of blood monocytes. They express high levels of the chemokines receptor 2 (CCR2) but low levels of chemokine (C-X3-C motif) receptor 1 (CX3CR1), produce Interleukin 10 (IL-10) upon stimulation rather than proinflammatory cytokines and are excellent phagocytes [19-21]. In contrast to this major subset, CD14⁺CD16⁺ monocytes express high levels of CX3CR1 and low levels of CCR2 [19]. It has been shown that it is this population that is responsible for the production of TNF and IL-1 β upon bacterial lipopolysaccharides (LPS) stimulation [22]. Furthermore, these cells express the Fc-gamma receptors CD32 and CD64 which enable the CD14⁺CD16⁺ monocytes to phagocytose [23]. Interestingly, the proportion of CD14⁺CD16⁺ relative to the rest of the monocyte population is increased at sites of acute inflammation [24]. The last subset of monocytes expresses CD16 but low levels of CD14 (CD14^{dim}CD16⁺) and lack the expression of Fc-gamma receptors. These cells are poor phagocytes and weak responders to cell surface TLR stimulation. Instead, they produce TNF and IL-1 β in response to viruses and immune complexes containing nucleic acids [25].

1.1.3 Dendritic cells

Dendritic cells are specialised antigen-processing and -presenting cells that are equipped with high phagocytotic activity as immature cells and high cytokine-producing capacity as mature cells. They serve as major APCs and are therefore crucial for an effective and pathogen-specific activation of adaptive immunity. DCs are highly migratory cells that can move from tissues to lymphoid organs both in steady state (negative selection of T lymphocytes, see section 1.1.6) and during infections. DCs mature in the presence of infectious agents and release a huge arsenal of inflammatory mediators and upregulate

CCR7 which enables rapid migration into lymphoid organs. It is this migratory capacity that separates DCs from other APCs in their ability to stimulate naïve T lymphocytes. DC maturation is accompanied by up-regulation of MHC class II and co-stimulatory molecules as well as increased antigen presentation to naïve T lymphocytes [26, 27]. These differentiate into the appropriate effector cell type, dependent on the origin of the antigen presented and cytokine environment provided by DCs, followed by multiple rounds of proliferation before migrating to the site of infection. Both the DC-dependent activation and the requirement for proliferation are the reasons for the delayed response of adaptive immunity compared to innate immunity.

Like macrophages, DCs are resident within most tissues and are a heterogeneous population. The pathways leading to DC differentiation are not entirely understood but the current model suggests that monocytes, macrophages and most DCs originate *in vivo* from a hematopoietic stem cell-derived progenitor with myeloid-restricted differentiation potential called the common myeloid progenitor (CMP). However, there is ongoing debate whether DCs can differentiate from lymphoid progenitors that usually give rise to T and B lymphocytes. Steady state DCs can broadly be divided into plasmacytoid DCs (pDCs) and conventional DCs (cDCs) [28, 29]. pDCs differ from cDCs in that they are long-lived and specialised to respond to viral infection with a massive production of type I interferons (see section 1.3.2). cDCs can be further divided into distinct subtypes depending on the tissue location (e.g. Langerhans cells in the epidermis of the skin and lamina propria DCs in the gut) or surface marker expression (see section 1.3.3).

1.1.4 Macrophages

The zoologist Elie Metchnikoff was the first one to recognise macrophages as phagocytic cells responsible for pathogen elimination and formed the basis of macrophage biology and innate immunity over 100 years ago. The definition of a macrophage was originally based on phagocytosis which led to the term macrophages (from the Greek for large eaters) to distinguish these cells from the polymorphonuclear microphages (from the Greek for small eaters; later neutrophils). Macrophages represent a heterogeneous population of immune cells that evolve within a specific microenvironment out of the MDP progenitors or monocytes [30]. Transcription factors that control the development of macrophages from progenitors include the Ets family member PU.1 and the Interferon Regulatory Factor (IRF) family member IRF8.

PU.1 can induce myeloid commitment in immature multipotent progenitor cells [31] and is required for the generation of CMPs. In addition, PU.1 controls several cell fate decisions along the lineage pathway, particularly during the macrophage vs. DC choice of monocytes [13, 28]. The function of PU.1 at a certain progenitor stage depends on its balance with antagonistic factors that drive alternative fates. For instance, PU.1 antagonizes C/EBP α thus driving monocytic differentiation at the expense of granulocyte differentiation [32]. Furthermore, high expression of PU.1 is required to induce DC fate in monocytes by antagonizing macrophage-inducing transcription factors such as MafB and c-Maf [33]. Therefore, PU.1 induces cell development by overruling key regulatory factors of other pathways. In addition to these antagonizing functions, PU.1 also controls cell development via direct transcriptional activity. As indicated above, the growth factor M-CSF is critical for MPS differentiation. Reddy *et al* showed that PU.1 can transactivate the M-CSF promoter [34] and a subsequent study showed that PU.1-deficient myeloid progenitors do not express M-CSF and show absence of the monocyte/macrophage lineage [35]. However, ectopic expression of M-CSF in PU.1-deficient cells can not rescue macrophage differentiation [35], indicating that M-CSF signalling is not sufficient to drive macrophage commitment in the absence of PU.1.

A role of IRF8 in myeloid cell development was first suggested by the fact that *Irf8*^{-/-} mice exhibit a systemic expansion of neutrophils [36]. Subsequent studies showed a cell-intrinsic function of IRF8 in the differentiation of myeloid cells. Progenitor cells of *Irf8*^{-/-} mice are hyperresponsive to granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) but their response to M-CSF is markedly reduced [37]. Therefore, *Irf8*^{-/-} progenitor cells differentiate into granulocytes even in the presence of M-CSF indicating that IRF8 is important for the differentiation of macrophages by inhibiting that of granulocytes. Consistent with that, IRF8 expression is detected in myeloid progenitor populations and macrophages but declines in granulocytes [38, 39]. The precise molecular function of IRF8 in cell development is not entirely clear but it has been shown that IRF8 protects the activated M-CSFR from degradation and thus prolongs M-CSF signalling [40]. In addition, IRF8 can interact with PU.1 which greatly increases the efficiency of target DNA-binding of IRF8 [41].

Among the cells classified as macrophages are many different subtypes with distinct immune functions. Many of these functions appear to be opposing in nature: proinflammatory vs. anti-inflammatory, immunogenic vs. tolerogenic and tissue-destructive

vs. wound healing [42]. Furthermore, macrophages demonstrate a remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype and physiology in response to cytokines and microbial stimuli [43]. Based on the Th1/Th2 polarization concept [44] these cells can roughly be classified as M1 (classically activated) macrophages, which produce proinflammatory cytokines ($IL-12^{high}$, $IL-23^{high}$, $IL-10^{low}$) and mediate resistance to pathogens and tissue destruction; and M2 (alternative activated) macrophages, which produce anti-inflammatory cytokines ($IL-12^{low}$, $IL-23^{low}$, $IL-10^{high}$) and promote tissue repair and remodelling as well as tumour progression [45, 46].

Historically, the first cytokine shown to convert resting macrophages into “active cells” was the Th1-derived cytokine IFN- γ . IFN- γ increased the antigen presenting capacity as well as proinflammatory cytokine production of macrophages and enhanced their ability to kill intracellular pathogens [47]. Thus, the activation of macrophages with IFN- γ became known as “classical activation” of macrophages also referred to as the M1 phenotype. The immunological counterpart of IFN- γ was identified as IL-4 which is a Th2-derived cytokine. Stein *et al* demonstrated that the expression of mannose receptor (MRC1 or CD206) was characteristic of IL-4 activated macrophages [48] and subsequent studies established that IL-4 could upregulate a distinctive set of genes, establishing a functional pattern quite different from that induced by IFN- γ [49]. The distinctive effects of IL-4 on macrophages lead to the term “alternative activation” or M2 macrophage phenotype, emphasising the contrast with “classical activation” by IFN- γ . Th2 lymphocytes also secrete IL-13, a cytokine that shares a receptor complex with IL-4, both signalling through the common IL-4 receptor α chain. Not surprising, it was found that IL-13 has a redundant as well as a nonredundant role in shaping the macrophage phenotype compared to IL-4 [50].

Other groups have extended the definition of alternative macrophage activation very loosely to additional mediators of macrophage function, such as IL-10, glucocorticosteroids and TGF- β [51]. There was a need for a more refined nomenclature of M2 macrophages as each stimulus induces both unique and overlapping gene expression repertoires. Mantovani and colleagues suggested a further subdivision of M2 macrophages into M2a (IL-4/IL-13 stimulated, alternatively activated macrophages), M2b (immune complexes, TLR ligand or IL-1 β stimulated, type II activated macrophages) and M2c (IL-10 stimulated, deactivated macrophages) [52] (Figure 1.4). In addition to the M2 macrophage subtypes discussed above, tumor-associated macrophages (TAMs) are considered to have an M2-like

phenotype. TAMs are recruited into the tumor environment as monocytes from the bloodstream by chemoattractants by both malignant and stromal tumors. Several studies have indicated that tumors provide a cytokine environment which favours an alternative activation of macrophages. For example, TAMs upregulate the expression of IL-10, TGF- β and other M2-associated genes while downregulating IL-12 expression, antigen presentation, antimicrobial and antitumor properties [53-56].

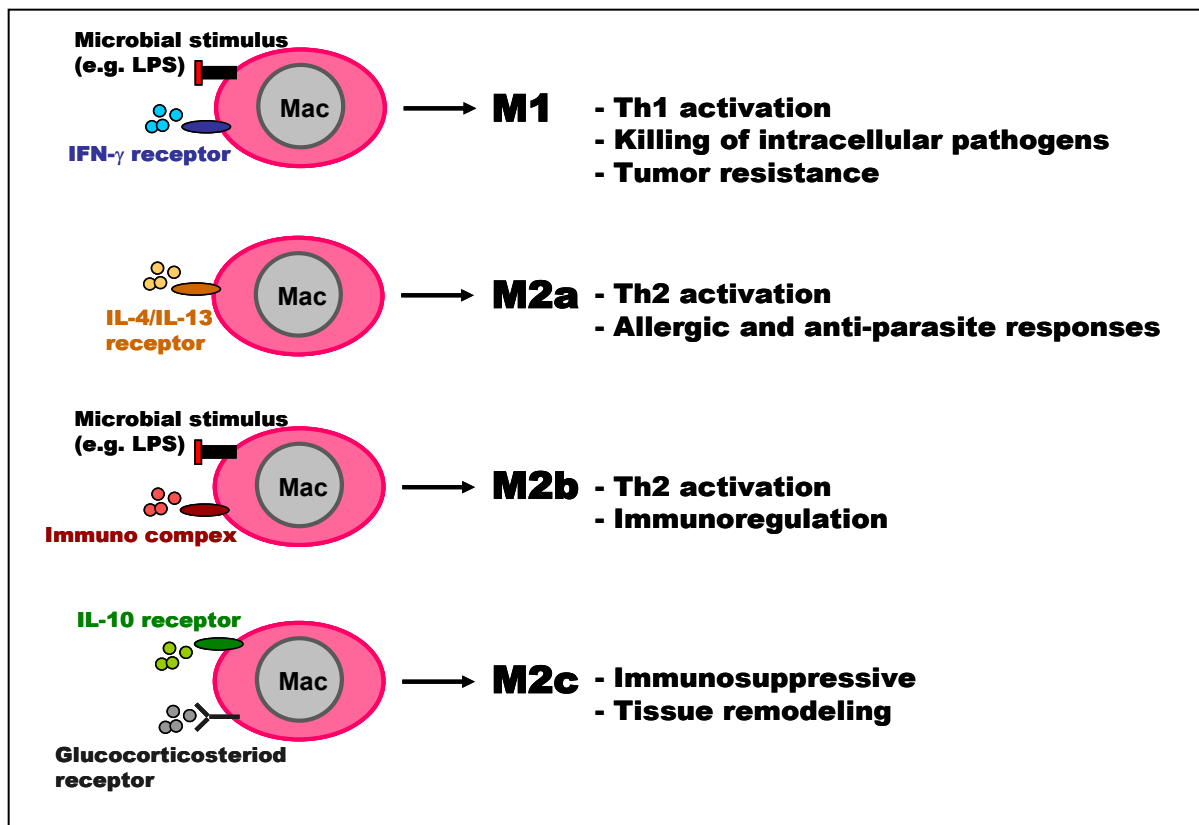


Figure 1.4 Inducers and selected properties of polarized macrophages

Macrophage exposure to IFN- γ and LPS drives M1 polarization equipping them with cytotoxic and antitumoral properties. In contrast, M2 macrophages are in general more prone to immunoregulatory and immunosuppressive functions. In particular, M2a (exposure to IL-4/IL-13) and M2b (exposure to immune complexes and TLR ligands) exert immunoregulatory functions and drive Th2 responses, whereas M2c (exposure to IL-10 or Glucocorticosteroids) are more related to suppression of immune responses and tissue remodelling.

It should be realised though, that any form of classification that results from *in vitro* experiments underscores the complexity of the *in vivo* situation, where macrophages are exposed to a mixture of stimuli and will therefore adopt mixed functional profiles accordingly. Furthermore, despite obvious phenotypic differences in M1 vs. M2 macrophages, the term “alternatively activated” to describe IL-4/IL-13 treated macrophages is somewhat misleading since IL-4/IL-13, like IFN- γ , should probably be considered a priming rather than an activation stimulus for macrophages. Therefore, a direct comparison of IFN- γ primed and LPS stimulated macrophages to IL-4/IL-13 primed only macrophages might not be correct. The observed differential production of proinflammatory cytokines of M1 and M2 macrophages may actually solely depend on the presence or absence of a microbial stimulus. In fact, there is substantial evidence that IL-4 and IL-13 do not necessarily inhibit but may enhance proinflammatory cytokine production, such as IL-12 and TNF, when used as a priming agent followed by microbial stimulation [57-60]. Furthermore, some investigators use macrophage-like cell lines, such as THP-1, to characterise macrophage polarization and formulate one to one correlation to the human/mouse system, which is very debatable. Thus, a unified polarization protocol indicating appropriate cell types and clarifying priming time as well as the microbial stimulus used might be useful.

One way to overcome the discrepancies in having priming and activation, or priming alone, is to use a different polarization protocol. The development of macrophages from monocytes depends on lineage-determining growth factors, with M-CSF and GM-CSF among the most important ones [61]. As discussed above, disruption of M-CSF signalling affects the MPS lineage with a widespread effect on macrophage-lineage development. By contrast, GM-CSF-deficient mice show no defects in myeloid development with the exception of alveolar macrophage maturation, which is compromised [62]. This points towards a significant contribution of M-CSF to general lineage commitment whereas GM-CSF might be more important for defining macrophage subsets. *In vitro* culture systems, for both human and mouse monocytes, were developed to obtain different types of macrophages resulting in the M-CSF and GM-CSF method [63, 64]. By using these culture methods monocyte-derived macrophage (MDM) subsets were defined by comparing their cytokine profiles following exposure to TLR ligands, such as LPS [63-67]. Similar to IFN- γ /LPS, treatment of monocytes with GM-CSF leads to the differentiation of M1 macrophages with an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype upon LPS stimulation whereas the employment of M-CSF, followed by LPS stimulation, results in an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype (M2 macrophages) (Figure 1.5). Using this protocol, any differences in cytokine

production should reflect the treatment or “priming” with different growth factors (GM-CSF for M1 and M-CSF for M2 respectively) as both types receive a microbial stimulation.

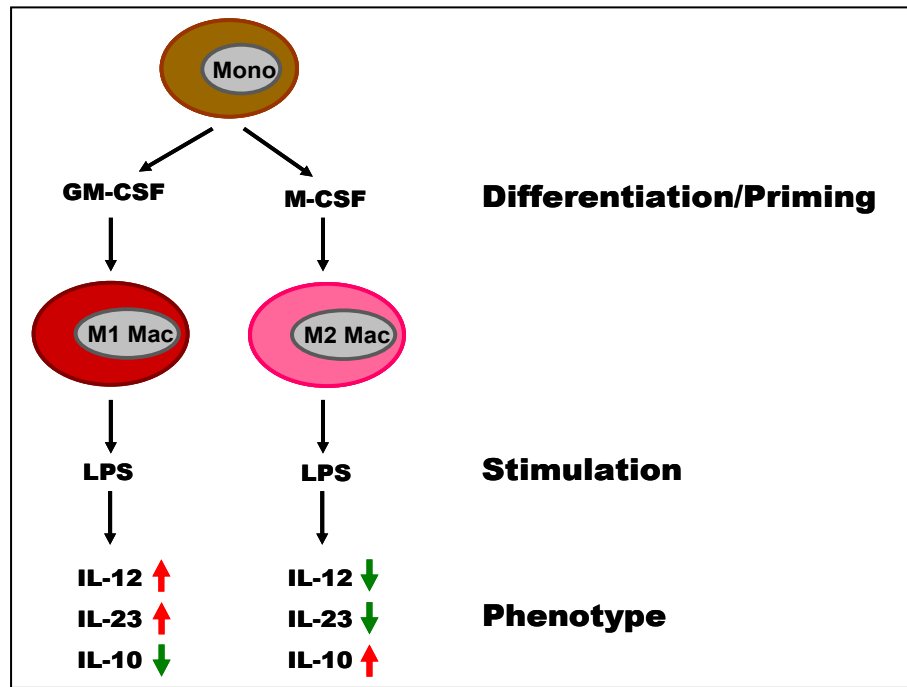


Figure 1.5 *In vitro* polarization of macrophages using growth factors

Human monocytes (Mono) are cultured either in the presence of GM-CSF or M-CSF for 4-6 days to obtain M1 (M1 Mac) and M2 macrophages (M2 Mac) respectively. After differentiation/priming both subsets are exposed to TLR ligands such as LPS resulting in an M1 cytokine profile (IL-12^{high}, IL-23^{high}, IL-10^{low}, GM-CSF treatment) or M2 cytokine profile (IL-12^{low}, IL-23^{low}, IL-10^{high}, M-CSF treatment). The same protocol is applicable to the murine system where bone-marrow progenitors can be used to generate macrophage subsets.

In addition to the differences in the cytokine profile of macrophage subsets mentioned above, polarized macrophages also differ in the expression of their cell surface receptors. M1 macrophages are characteristic of high expression of MHC II, IL-1R1, IL-18R, co-stimulatory molecules, such as CD80 and CD86, and the chemokine receptor CCR7. M2 macrophages on the other hand are associated with high levels of scavenger receptors (MSR1 and CD36), CD14, CD163, CD209 (DC-SIGN), MRC1, CCR2 as well as the decoy IL-1R2 [52, 67-69].

The M1 program of macrophages is usually associated with protection during acute infectious diseases by inducing a strong Th1/Th17 response. Consequently, the protective role of M1 macrophages has been exemplified in mice deficient for components of the IL-12 pathway [70]. An example of a pathogen-induced M1 macrophage polarization and thus stimulation of intracellular killing of bacteria includes *Listeria monocytogenes* which causes disease in immunocompromised patients and pregnant women [71]. Similarly, *Salmonella typhi* (typhoid fever) and *Salmonella typhimurium* (gastroenteritis) induce the M1 polarization of human and murine macrophages and this induction is associated with the control of the infection [72]. In addition, *Mycobacterium tuberculosis* induces a gene signature that corresponds to that of IFN- γ and therefore polarizes toward the M1 profile [73]. Other mycobacterial diseases such as Buruli disease (*Mycobacterium ulcerans*) and opportunistic infections (*Mycobacterium avium*) are also characterised and controlled by M1 polarization of macrophages [72].

As mentioned above, M1 polarization supports resistance to intracellular bacteria and controls the acute phase of infection. However, an excessive or prolonged M1 program is potentially harmful for the host, as demonstrated in acute infections with *Escherichia coli* or *Streptococcus* species. *E. coli* infection can cause many diseases, including sepsis, which induces a systemic inflammatory response and immune dysregulation that lead to tissue damage and potentially multiple organ failure [74]. *In vitro*, *E. coli* induces a typical M1 profile through the recognition of LPS by TLR4 and it has been demonstrated that M1 macrophage polarization and sepsis severity are related. For instance, patients with severe sepsis show high concentrations of M1-specific cytokines that correlates with mortality [75, 76]. *Streptococcus* species can cause amongst other things meningitis as well as pneumonia in humans and other animals. Host responses are generally characterised by an intense inflammatory reaction and an M1 polarization of macrophages. In a murine model of pneumonia, mortality correlates with lung inflammation and the presence of M1-specific cytokines, like TNF, IL-1 β and IL-6 [77].

As opposed to M1, M2 macrophages promote Th2 responses, resulting in effector functions such as parasite encapsulation and killing, but also exacerbation of Th2 cytokine-associated pathologies, such as allergies. Nematode and trematode worms like *Nippostrongylus*, *Toxocara*, *Schistosoma*, and *Taenia* induce a strong M2 response and require an IL-4/IL-13-driven Th2 immune response to clear the infection [68, 78]. Several murine models have been used to study the immune response and the contribution of polarized macrophages to

control these parasites. Administration of *N. brasiliensis* induces a strong Th2 response and lung pathology is subsequently associated with M2 macrophage polarization [79, 80]. Other models include the infection of mice with the parasite *S. mansoni* which also results in a Th2 cytokine response, generally important for the clearance of parasitic infections [81]. Not surprisingly, IL-4R α -deficient animals, which lack M2 macrophages, do not survive *S. mansoni* infection as a consequence of a shift from Th2 to Th1 response [82]. In humans, *S. mansoni* infection results in the production of IL-4 and the IL-4 protein levels correlate with the severity of the disease [83].

It is well established that pathogens have evolved different strategies to interfere with macrophage polarization. For instance, the dengue viruses benefit from a Th2 response and subsequent M2 environment. There is a shift in cytokine expression from type I and II IFN response to enhanced expression of Th2 cytokines upon virus transmission [84]. These Th2 cytokines not only dampen the Th1 response required for viral elimination but also induce the M2-specific MRC1, which binds all four serotypes of dengue virus and can be exploited by the virus to infect M2 macrophages [85]. Chronic inflammation is also associated with M2 macrophage polarization and asthma is the most prominent example. Asthma is characterized by enhanced expression of Th2 cytokines in the airway tissues of asthmatic patients and murine models of asthma demonstrated the presence of M2 polarized macrophages [68, 86, 87]. As mentioned above, TAMs have also been described as M2 polarized although there are no consistent correlations with IL-4/IL-13 expression and tumor progression. This suggests that the tumor environment includes other cytokines and mechanisms to influence the phenotype of recruited macrophages.

1.1.5 Natural killer cells

Natural killer (NK) cells are a type of cytotoxic lymphocytes that function in the innate immune system. They play a major role in the rejection of tumors and the targeted destruction of virus-infected cells through the release of granules. NK cells are activated in response to interferons and macrophage-derived cytokines and provide an early source of inflammatory mediators such as IFN- γ , TNF, GM-CSF and chemokines which all support a Th1 response [88]. Human NK cells comprise approximately 15% of all lymphocytes and are defined phenotypically by their expression of cluster of differentiation 56 (CD56) and lack of expression of CD3. Furthermore, NK cells are defined by the expression of the Fc-gamma receptor III (CD16) which enables them to attack an antibody-coated cell [89].

Furthermore, NK cells detect the lack of MHC class I (usually highly expressed on cells) which can occur when cells are perturbed by viral infection or cellular transformation. Thus, NK cells can distinguish between healthy cells that express MHC class I molecules but selectively kill target cells “in distress” that down-regulate MHC class I molecules [90].

1.1.6 T Lymphocytes

T lymphocytes (or T cells) are defined by the expression of the T-cell receptor (TCR), a transmembrane immunoglobulin-like protein capable of recognising pathogen-derived antigens [91]. The ability of the TCR to recognise an almost infinite array of antigens is due a unique system of random mutation and recombination events, commonly referred to as V(D)J recombination, of the genes coding for the TCR [92]. T lymphocytes originate from a common lymphoid progenitor in the bone marrow and migrate to the thymus where they undergo the final stages of their differentiation, the negative selection. The random generation of the TCR can lead to recognition of “self” antigens from the host which would induce an immune response against the body's own tissue. APCs therefore present “self” antigens to the lymphocytes in the thymus. The recognition of “self” antigens by T lymphocytes results in the initiation of apoptosis of the reacting cell and therefore only T lymphocytes that don't recognise self antigens complete their maturation in the thymus [93]. The remaining T lymphocytes migrate to the peripheral lymphoid organs on the patrol for foreign antigens as either cytotoxic CD8⁺ T-lymphocytes (CTLs) or CD4⁺ T-helper (Th) lymphocytes [94].

T helper lymphocytes are naïve cells that can only be activated if they receive two distinct signals. The first is the recognition of foreign antigens presented by APCs on MHC II by the TCR. In addition, a signal from co-stimulatory molecules, such as CD80 and CD86 which are expressed by activated APCs, is required. This system is important for the maintenance of peripheral tolerance and T lymphocytes will undergo apoptosis if both signals are not received [95]. T helper cells expand and differentiate into various effector T lymphocyte subsets and depending on the cytokines they produce, these T lymphocyte subsets have very different properties and functions. T helper lymphocytes include the well-defined effector subsets Th1 lymphocytes and Th2 lymphocytes, as well as the more recently described Th17 and Th9 lymphocytes, but also regulatory subsets like regulatory T lymphocytes (Tregs) [96, 97].

Naïve T helper lymphocytes differentiate into Th1 lymphocytes in the presence of IL-12 [98]. Th1 lymphocytes contribute to cell-mediated immunity and are characterised by the secretion of IFN- γ which in turn acts on DCs, macrophages, CTL and natural killer cells to increase their respective effector functions [97]. Th1 lymphocytes are important for host defence against intracellular pathogens and induction of delayed type hypersensitivity responses. However, uncontrolled Th1 responses against self-antigens can lead to the development of autoimmunity. Studies showing that mice deficient in the Th1 transcription factor T-bet [99] are resistant to the development of experimental autoimmune encephalomyelitis (EAE) suggesting that Th1 lymphocytes are the major effector T lymphocytes responsible for inducing MS [100]. Because Th1 effector mechanisms seemed to explain many histopathological and clinical features of EAE and other autoimmune diseases, including type I diabetes and rheumatoid arthritis, Th1 lymphocytes became the archetypical inducer of organ-specific autoimmunity [96].

The differentiation of Th2 lymphocytes is dependent on the transcription factor GATA3 [101] and occurs in the presence of IL-4. They are involved in humoral-mediated immunity and are essential for clearing extracellular organisms like parasites as well as helminthes. In addition, Th2 lymphocytes play an important role in eosinophilic inflammation and IgE production in allergic reactions and asthma. Th2 lymphocytes are characterised by the production of IL-4, IL-5 as well as IL-13 and regulate macrophage and B cell behaviour [102].

More recently, 3 additional helper T lymphocyte subsets have been described: Th17 lymphocytes, Th9 lymphocytes and regulatory T lymphocytes (Tregs). The hypothesis of Th1 lymphocytes being solely responsible for induction of organ-specific autoimmunity was challenged when it was shown that animals lacking the Th1 signature cytokine IFN- γ are not resistant but in fact are more susceptible to multiple autoimmune diseases including EAE [103] and collagen-induced arthritis [104]. This raised the question whether another subset of T lymphocytes might be required for the induction of EAE and other organ-specific autoimmune diseases.

The Th1 inducing cytokine IL-12 is composed of the subunits p35 and p40. While IL-12p35 deficient mice were more susceptible to EAE, surprisingly loss of the IL-12p40 chain made mice highly resistant to EAE [105]. In 2000 a novel cytokine chain p19 was discovered and Oppmann *et al* showed that IL-12p40 is not only essential for forming IL-12 but can also pair

up with p19 to form a novel cytokine called IL-23 [106]. Thus all previous approaches that targeted the p40 chain of IL-12 would also affect IL-23 production. Subsequent experiments with p19, p35 and p40 deficient mice showed that *p19*^{-/-} and *p40*^{-/-} animals were resistant to EAE, whereas *p35*^{-/-} animals, which lacked IL-12 and Th1 responses but were able to form IL-23, remained susceptible to EAE [107]. A follow-up study showed that IL-23 is important for the expansion of IL-17-producing T lymphocytes, later called Th17 lymphocytes, which are capable of inducing EAE in an adoptive transfer model [108]. Expression of the transcription factor ROR γ T is characteristic of Th17 lymphocytes [109]. The cytokine environment required for Th17 differentiation is under debate as there are differences between human and murine development [110]. One cytokine that is equally important for both species is IL-23 [111-114]. Th17 lymphocytes produce IL-17A and IL-17F, which belong to the same family and are partly redundant in their effector functions. Both cytokines induce pro-inflammatory cytokines like IL-6, IL-1 and TNF as well as pro-inflammatory chemokines like CXCL1 and IL-8 and thus promote tissue inflammation and recruitment of neutrophils to the site of inflammation [115].

Th17 lymphocytes play an important role in mediating host defences and have a specialized role in clearing pathogens that are not adequately handled by Th1 or Th2 lymphocytes including bacteria like *Citrobacter*, *Klebsiella pneumoniae* and *Borrelia burgdorferi*, but also fungi such as *Candida albicans*. The receptors for IL-17 are expressed on both hematopoietic and non-hematopoietic cells [96, 115]. Consequently, IL-17 cytokines promote inflammation on several levels making Th17 lymphocytes particularly suited for the promotion of autoimmunity. Elevated levels of IL-17 were detected in several autoimmune diseases including MS [116] and rheumatoid arthritis [117, 118] and IL-17 deficiency leads to suppression of both CIA [119] and EAE [120]. These studies demonstrated the importance of Th17 lymphocytes in autoimmune diseases and led to a reevaluation of the role of other effector T lymphocytes in the induction of autoimmune tissue inflammation.

The most recent addition to effector T lymphocytes is the Th9 subset which is induced in the presence of TGF- β plus IL-4. Th9 lymphocytes are characterized by the secret IL-9 and thought to have effector rather than regulatory properties [121, 122]. A bona fide subset-defining transcription factor for the Th9 lineage is yet to be determined although it has been shown that IRF4 is essential for IL-9 production by Th9 lymphocytes [123].

While effector T lymphocytes promote inflammation, Tregs serve to control it. Tregs play a very important role in autoimmune pathogenesis by maintaining self-tolerance and by controlling expansion and activation of autoreactive CD4⁺ T effector lymphocytes. They are characterised by the expression of CD4, CD25 as well as the expression of the transcription factor FOXP3 and are thought to suppress autoimmunity by the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)- β [124].

CD8⁺ T lymphocytes emerging from the thymus are predestined to become cytotoxic T lymphocytes (CTLs). Like T-helper lymphocytes, CTLs require TCR and co-stimulatory receptor stimulation to become activated. However, the TCR on CTLs is activated by antigens presented in the context of MHC class I explaining the relevance of CTLs for the control and clearance of viral infection. They mediate their antiviral effects either by direct lysis of infected target cells or by the secretion of potentially toxic cytokines such as IFN- γ and TNF. Because of their memory cell progeny, CTLs also contribute to protection against subsequent encounters with the same foreign agent(s) [125, 126].

1.1.7 B-cells

B lymphocytes, or B cells, are derived from common lymphoid progenitor cells in the bone marrow and undergo maturation in the spleen. The hallmark of B cells is their ability to produce antibodies which are soluble immunoglobulin proteins that are composed of 2 light and 2 heavy chains. The light chain is hyper-variable which allows the recognition of a huge number of antigens therefore conveying unique specificity. The light chain is epitope specific and binds to pathogens which neutralises their effects and marks them for phagocytosis by other cells of the immune system. In contrast, the number of heavy chains, determining the isotype of the antibody which is important for different effector functions, is limited [127]. B cells can switch the heavy chain of an antibody (class switch) while the light chain remains unchanged resulting in an antibody that still recognises the same antigen but will initiate a different effector function due to the new isotype. B cells retain an immunological memory after antibody production by forming memory B cells which enable a quicker response when an antigen is encountered again [128].

1.2 The Interferon regulatory factor family of transcription factors

Host defence serves two main functions: the generation of an immune response to invading pathogens and the suppression of tumor development. These are achieved by the efficient coordination of genetic networks in which transcription factors control the expression of a distinct set of target genes, depending on the cell type and/or the nature of the stimuli.

Interferon regulatory factors (IRFs) were originally thought to regulate the transcription of type I Interferons (IFN) and IFN-inducible genes but members of this family are now recognised as major regulators of many aspects of innate and adaptive immune responses. The IRF family consists of nine members in human and mice: IRF1, IRF2, IRF3, IRF4 (also known as LSIRF, PIP or ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP) and IRF9 (also known as ISGF3 γ) [129, 130]. The first member, IRF1, was discovered in 1988 and IRF10 was identified in chickens recently, although it is absent in humans and mice [131].

Each IRF protein contains a well-conserved DNA binding domain (DBD) of about 120 amino acids at its N-terminus (Figure 1.6). The DBD forms a helix-turn-helix motif and mediates specific binding to NGAAANNGAAACT, termed the IFN-stimulated regulatory element (ISRE) [129] because it was first identified in the promoters of genes induced by type I IFN, namely IFN- α s and IFN- β [131]. An analysis of the crystal structure of the DBD revealed that GAAA is the core DNA recognition sequence of the helix-turn-helix motif [132]. ISREs can also be found in the promoters of the genes that encode type I IFNs themselves, as well as in the promoters of many other genes that are involved in immunity and oncogenesis. The sequence domain in the 5' region of the IFN genes, termed the virus responsive elements (VRE), contains multiple ISREs which are highly conserved both in IFN- α and IFN- β gene promoters [133-135]. The C-terminal regions of IRFs, except for IRF1 and IRF2, contain an IRF association domain (IAD) that is responsible for homo- and heteromeric interactions with other family members as well as other transcription factors such as PU.1 and signal transducer and activator of transcription (STAT) [129, 130]. These interactions can further define the nucleotide sequences adjacent to the core IRF-binding motif to which the protein complex binds.

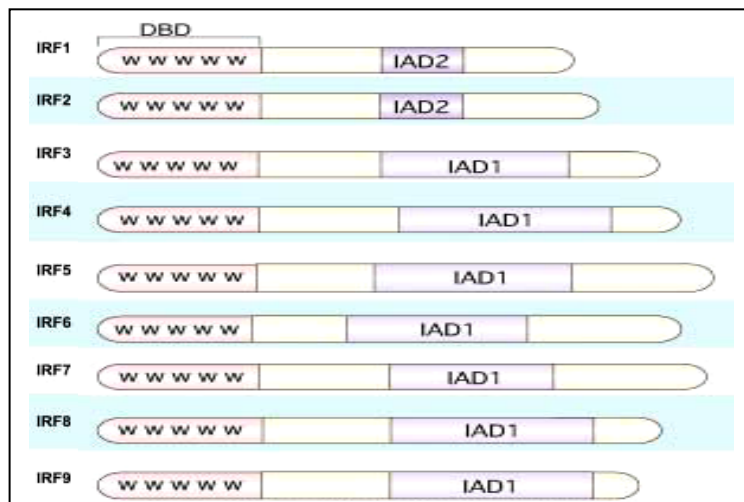


Figure 1.6 Domain model of IRF family members

As shown in this schematic domain model, all IRF members carry a N-terminal DBD which contains repeated tryptophan residues (represented by W). All IRFs, except IRF1 and IRF2, have an IRF association domain (IAD1) that is needed for interactions with other family members or other transcription factors. Another association domain (IAD2) that is just present in IRF1 and IRF2 is important for forming homodimers. Adopted and modified from [136].

1.3 Regulation of the innate immune response by IRF and NF- κ B family members

Recognition of invading pathogens is central to the host immune system. The innate immune system depends on a limited number of germline-encoded PRRs. These recognise PAMPs such as bacterial LPS and viral nucleic acids [137]. Three classes of PRRs, cytosolic PRRs, membrane-bound TLRs and secreted receptors have been identified. Depending on the nature of the pathogen and the cell type involved, signal transduction through PRRs leads to the transcriptional induction of various target genes including type I IFNs, pro-inflammatory cytokines and chemokines. One of the major, best characterised transcription factors that is activated by virtually all PRRs, and which orchestrates the expression of these genes, is nuclear factor κ B (NF- κ B). In the past few years extensive studies have revealed that IRFs are also widely involved in most PRR signalling events leading to gene activation and thus, important in linking innate and adaptive immune responses.

1.3.1 Activation of NF- κ B proteins by TLR signalling

Signalling through TLRs can be broadly categorised into two pathways, namely the myeloid differentiation primary-response protein 88 (MyD88) -dependent and the TIR domain containing adapter-inducing IFN β (TRIF) -dependent pathways (Figure 1.7). All TLRs, except TLR3, activate the MyD88 pathway; whereas TLR3 and TLR4 can activate the TRIF pathway. Both pathways commonly activate NF- κ B and IRF family members [137, 138].

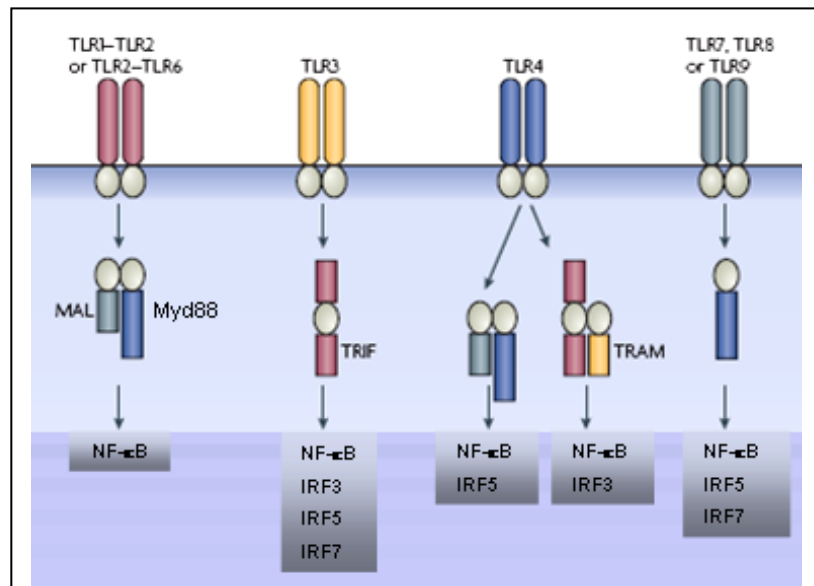


Figure 1.7 Overview of transcription factor activation by TLR signalling

TLR1, TLR2, TLR4 and TLR6 activation leads to the recruitment of MyD88-adaptor-like (Mal) and MyD88 whereas TLR7, TLR8 and TLR9 recruit MyD88 alone. All TLRs, except TLR3, utilise the MyD88-dependent pathway which activates NF- κ B, IRF5 and IRF7. TLR3 recruits TRIF while TLR4 can recruit the TRIF-related adaptor molecule (TRAM) which in turn recruits TRIF. Signalling via the TRIF-dependent pathway leads to the activation of NF- κ B as well as IRF3, IRF5 and IRF7. Adopted and modified from [139].

The NF- κ B family of transcription factors consists of 5 members: RelA (p65), RelB, c-Rel, NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100). They are characterised by the presence of a Rel homology domain (RHD) at the N-terminus which is responsible for DNA binding, dimerisation and interaction with the inhibitor of NF- κ B (I κ B) proteins [140]. NF- κ B proteins have to form homo- or hetero-dimers in order to bind

DNA and activate transcription. RelA:p50 is the most abundant and therefore most widely studied NF- κ B complex. It is expressed in most cell types and is essential for the induction of innate immunity and pro-inflammatory cytokine production. NF- κ B complexes containing RelA, RelB or c-Rel are able to directly activate transcription, since these proteins contain a C-terminal trans-activation domain (TAD) [141]. In contrast, p50 and p52 lack the TAD and their respective homodimers can compete with TAD-containing complexes and function as transcriptional repressors [142].

The I κ B proteins interact with NF- κ B complexes and retain them in the cytoplasm by interfering with the nuclear localization sequence of NF- κ B proteins. The “classical” I κ B proteins include I κ B α , I κ B β , I κ B ϵ , I κ B ζ and BCL-3 which are characterised by the presence of an ankyrin repeat domain (ARD) within their C-terminus [143]. The C-terminal halves of p105 and p100 also harbor multiple ankyrin repeats that allow them to serve an I κ B-like function [144].

Activation of NF- κ B is regulated through the inhibitor of κ B kinase (IKK) complex, which consists of 2 kinases, IKK α and IKK β , and a regulatory protein IKK γ (or NF- κ B essential mediator, NEMO) [145]. Two major signalling pathways (the canonical and the non-canonical) which result in the activation of NF- κ B complexes have been characterized [146]. The canonical NF- κ B signalling pathway is the most well studied and results mainly in RelA:p50 and c-Rel:p50 heterodimer activation. During canonical NF- κ B signalling, upstream signals result in phosphorylation of IKK β , which in turn phosphorylates the I κ B, resulting in its proteosomal degradation. This releases the NF- κ B heterodimers, allowing its nuclear translocation and promoter binding for inflammatory gene transcription [143, 145, 146].

The non-canonical NF- κ B pathway is activated in response to a small subset of TNF family members, including CD40L, Lymphotoxin α and β as well as B cell-activating factor (BAFF) [143, 145], and is based on inducible processing of p100 and activation of RelB:p52 heterodimers. It is strictly dependent on IKK α and its phosphorylation by NF- κ B-inducing kinase (NIK) [147]. NIK-activated IKK α dimers phosphorylate p100 at specific serine residues within its C-terminal ARD resulting in the release of the N-terminal p52 fragment bound to RelB and consequently NF- κ B activation [148].

1.3.2 IRF3/IRF7: master regulators of type I IFN induction

The initial hypothesis for the model whereby IRF3/IRF7 induces type I IFN genes was the positive-feedback model, in which IRF3 is primarily responsible for the induction of IFN- β in the early phase of a response, whereas IRF7, whose expression is induced by IFN- β , functions in the later phase of a response to induce IFN- α expression [149]. This early view was supported by several lines of evidence. IFN- α gene induction is reduced in *Ifn β ^{-/-}* mouse embryonic fibroblasts (MEFs) [150], and IFN α/β expression is impaired in *Irf3^{-/-}* MEFs upon Newcastle disease virus (NDV) infection [149]. However, the generation of *Irf7^{-/-}* mice allowed the rigorous assessment of the above model of positive-feedback regulation. In MEFs from these knock-out mice, type I IFN gene induction by viruses such as vesicular stomatitis virus (VSV), herpes simplex virus-1 (HSV-1) and encephalomyocarditis virus (EMCV) is more severely impaired than in *Irf3^{-/-}* MEFs. Consistently, *Irf7^{-/-}* mice are more vulnerable than *Irf3^{-/-}* mice to viral infections, which correlate with a marked decrease in serum IFN levels [151]. Thus, although IRF7 is initially expressed at a low level, the formation of a heterodimer between IRF7 and IRF3, rather than an IRF3 homodimer, is presumed to be more important for the production of IFN- α and IFN- β . The positive-feedback regulation of IRF7 then comes into effect to achieve the full induction of type I IFN genes during the later phase of the response.

Recently, pDCs which are capable of high-level induction of type I IFN genes have gained a lot of attention [152-154]. In contrast to cDCs and MEFs, this small DC subset expresses high levels of TLR7 and TLR9 in endosomes and produces large amounts of type I IFNs in response to corresponding TLR ligands. TLR7 recognises single-stranded RNA (ssRNA) derived from genomic RNA of viruses such as influenza virus and VSV, whereas TLR9 recognises hypomethylated CpG oligodeoxynucleotides in DNA from bacteria or DNA viruses such as HSV [155-157]. The induction of type I IFNs is crucially dependent on the activation of IRFs and this raises the question of how these TLRs, which exclusively utilise MyD88, can activate IRFs. Investigations have shown that IRF7, but not IRF3, directly interacts with the death domain of MyD88 in pDCs [158]. Furthermore, IRF7 interacts with tumor necrosis factor receptor-associated factor 6 (TRAF6) and another study has revealed that Inhibitor of NF- κ B kinase- α (IKK α) phosphorylates IRF7 [158, 159].

It is unclear why pDCs but not other cell types such as cDCs and macrophages produce large amounts of type I IFNs in response to TLR7 and TLR9 ligands. The finding that pDCs have a higher constitutive expression level of IRF7 compared with other cell types does not fully account for the robust IFN production [160]. Instead, the localisation of TLR ligands appears to be critical feature. The TLR9 ligand CpG preferentially co-localises to MyD88-IRF7-containing endosomal compartments whereas the same ligand mainly localises to lysosomes in cDCs and Mphs. This was confirmed by targeting CpG to endosomes which resulted in a robust production of type I IFNs in cDCs and Mphs [161, 162]. pDCs have therefore a unique mechanism for retaining TLR ligands in the endosomes which accounts for the high levels of type I IFN gene induction in these cells.

1.3.3 IRF4/IRF8: essential factors for DC development and function

DCs are professional antigen-presenting cells (APCs) that are crucial for the innate and adaptive response to infection. They can sense invading pathogens through PRRs and responds by secreting inflammatory mediators as well as by upregulating the surface expression of MHC II and costimulatory molecules. DCs also capture, process antigens and present antigenic peptides on MHC molecules to T lymphocytes, thereby triggering Th1/Th17 and Th2 responses or inducing tolerance. Among the cells classified as DCs are many subtypes with distinct immune functions [26]. For Instance, mouse splenic DCs can be classified into four subsets: CD4⁺ DCs, CD8 α ⁺ DCs (cDCs), CD4⁻ CD8 α ⁻ (double negative, DN) DCs and pDCs [29].

These subsets express different sets of genes and the expression of two structurally related IRFs, IRF4 and IRF8, is subset-selective [163, 164]. IRF4 is expressed at high levels in DN DCs and CD4⁺ DCs but low in pDCs. As a consequence, the CD4⁺ DC population is absent in *Irf4*^{-/-} [163]. Conversely, IRF8 is expressed at high levels in pDCs and CD8 α ⁺ DCs, thus *Irf8*^{-/-} mice are largely devoid of these DC subsets leading to an impaired production of IFN- α [165, 166]. In support of the complementary control of DC subsets by IRF4 and IRF8, *Irf4/Irf8*^{-/-} double KO mice have only a few DN DCs and are missing all other DC subtypes in the spleen [167]. Furthermore, the full differentiation and function of epidermal Langerhans cells and dermal DCs require IRF8 [168].

Thus, IRF4 and IRF8 play a critical role in the regulation of DC development and function. Through their specific activities, IRFs appear to equip DCs with the diversity required for directing optimal immune responses. However, the involvement of IRFs in human DC subtypes is less clear than it is in mouse DCs and further studies are required.

1.4 Interferon regulatory factor 5

IRF5 is a particularly interesting IRF family member with various activities, including activation of type I IFN genes, inflammatory cytokines and tumor suppressors. Although type I IFN responses are beneficial to the host, inflammatory cytokines that are stimulated by IRF5 can be a major contributor to the morbidity and mortality associated with autoimmune diseases. For instance, mutations in IRF5 are associated with systemic lupus erythematosus [169-171], rheumatoid arthritis [172-175] and inflammatory bowel disease [176-178] in several case-control studies.

1.4.1 IRF5 gene structure

The human IRF5 gene was mapped to the chromosome 7q32 and consists of nine exons with the first acting as an alternative non-coding exon (Figure 1.8). Four alternative exons for the 5' UTR (named exon1A, 1B, 1C and 1D) have been identified with the majority of the transcripts containing exon 1A [179, 180]. Interestingly, the alternative first exons give rise to different 5'UTRs that could have effects on mRNA stability, mRNA localisation or translation. Analysis of the translational efficiency of the alternative 5'UTRs revealed that exon 1A is the most efficient for protein synthesis while exon 1C has an inhibitory effect [179].

Exon 6 also participates in the formation of IRF5 isoforms. Two constitutively active alternative in-frame splice sites have been identified in this exon leading to the formation of two families of isoforms. The splicing affects a repetitive sequence and determines the number of sequence repeats in each IRF5 isoform family. The isoforms 5 and 6, with four repeats, are expressed in the absence of splicing whereas splicing results in the expression of isoform 1 and 4 with two repeats respectively. Both acceptor sites have equal strength and therefore equal amounts of alternative transcripts are produced [181]. The repeat motif

encodes for a proline-rich region located in a putative PEST domain and could thereby participate in protein-protein interactions [182].

In addition, it has been shown recently that IRF5 has two alternative polyA sites generating transcripts with either short or long 3'UTRs [183, 184]. As a consequence, isoforms with a long 3'UTR have two AU-rich elements (ARE) which are known to be responsible for rapid mRNA turnover and therefore affect mRNA degradation. On the other hand, isoforms with a short 3'UTR have a longer half-life and the expression levels of these transcripts are upregulated in IFN- α stimulated cells [179].

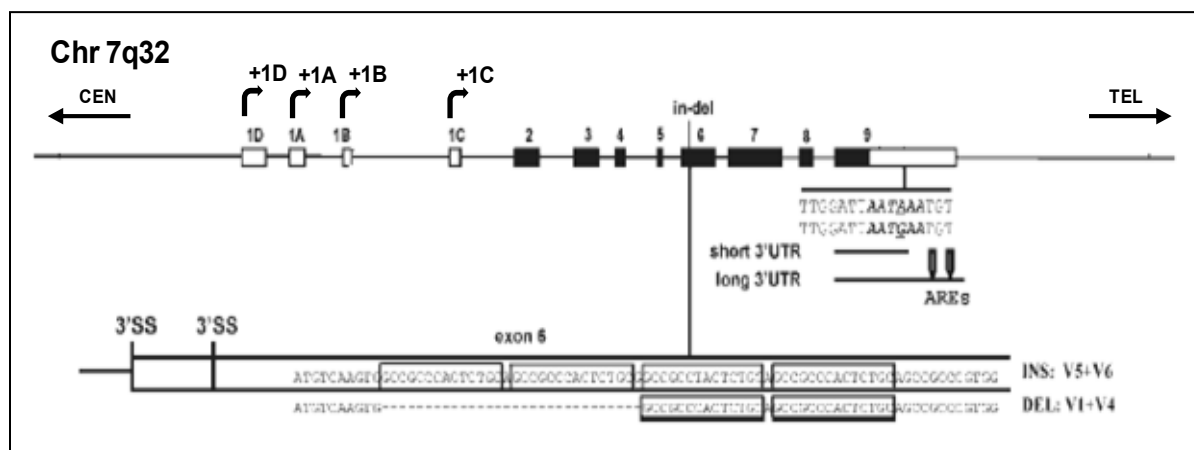


Figure 1.8 Human IRF5 gene structure

Protein coding exons are allocated as black and non-coding exons as white squares. The four non-coding alternative first exons 1A, 1B, 1C and 1D and the associated transcription start sites (TSSs, indicated as +1) are shown. Exon 6 has two constitutively active alternative acceptor splice sites and the details are shown below the gene structure. Splicing results in the expression of isoforms 1 and 4 whereas the absence of splicing leads to the expression of isoforms 5 and 6. The alternative polyA sites are also shown. The preservation of this site results in a stop codon and transcripts with short 3'UTR resulting in mRNA that is more stable compared to transcripts with long 3'UTR. Adopted and modified from [181].

In summary, human IRF5 exists as multiple isoforms generated by the usage of different 5' and 3'UTRs as well as alternative splicing of exon 6. The resulting isoforms may have distinct functions as it has been shown for IRF3 isoforms [185]. It should be mentioned here that no alternative splicing of IRF5 occurs in mice and therefore there is only one isoform present.

1.4.2 IRF5 protein structure

The most obvious difference between IRF5 and other family members is highlighted by two nuclear localisation signals (NLS), one in the N- and the other in the C-terminus of the protein. It has been shown that the 3'NLS located in the DNA binding domain (DBD) is responsible for low levels of nuclear translocation and therefore weak transactivation activity of IRF5 in unstimulated cells, while the 5'NLS is masked and becomes available only after phosphorylation which drives its nuclear translocation and strong transactivation function [186]. In addition, the IRF5 protein contains a constitutively active nuclear export signal (NES) which controls trafficking between nucleus and cytoplasm. The NES is dominant over the NLS resulting in cytoplasmic retention of most of the IRF5 protein in unstimulated cells. Mutation of the four leucine residues within NES resulted in the nuclear accumulation of IRF5 but not stimulation of type I IFN genes, suggesting that nuclear translocation alone is not sufficient for IRF5 function [187].

Although a putative autoinhibitory domain close to the C-terminus was discovered some time ago [186], its function was revealed only recently. Unphosphorylated IRF5 remains in the cytoplasm in an autoinhibited state where binding sites for other proteins, such as CREB binding protein (CBP), at the C-terminus are masked. Phosphorylation induces an unfolding of the C-terminal region that triggers the formation of homo- or heterodimers with other IRFs and this unmasking the CBP binding site. IRF dimers then translocate to the nucleus where they interact with CBP and other transcription factors to form complexes that bind to promoters and enhancers which contain ISREs. Further experiments showed phosphorylation increases the affinity of IRFs for CBP but the response of IRF5 and IRF3 to phosphorylation is very different. The increase in affinity of IRF3 for CBP is much greater than that for IRF5 [188]. The differences are consistent with the fact that an unphosphorylated autoinhibitory domain cannot completely inhibit activation of IRF5 [186], and therefore the phosphorylation-dependent switch between autoinhibition and activation is more finely tuned in IRF5 than in IRF3. These differences in sensitivity to activation between IRF5 and IRF3 could represent distinct functional requirements and are manifested in the different physiological roles of IRF family members. For instance, IRF3 is constitutively expressed in all cells and acts upon viral infections. It must be strongly autoinhibited and activated only in response to a clear signal. Therefore, the sensitivity of a given IRF member is likely to be controlled through intermolecular auto-inhibition as phosphorylation-induced activation of IRF5 and other IRF family members leads to a structural change of the C-terminal autoinhibitory region.

1.4.3 Expression of IRF5

So far little is known about the expression of IRF5 protein in humans. It has been shown that IRF5 transcripts can be detected in unstimulated peripheral blood mononuclear cells (PBMCs), pDCs, monocytes, monocyte-derived DCs (moDCs), natural killer cells (NK) and B lymphocytes but not in T lymphocytes. Furthermore, treatment of these cells with either IFN- α or HSV-1 cell type specifically enhanced the levels of distinct IRF5 isoform transcripts [160, 180]. Although the relative levels of IRF5 mRNA were similar in pDCs, MDDCs and monocytes the levels of IRF5 protein were not examined previously.

1.4.4 Activation of IRF5

IRF family members are targets of both Myd88 and TRIF signalling pathways in the TLR-dependent gene induction programme. It was shown that IRF5 directly interacts with the central region of MyD88 as well as with TNF receptor-associated factor 6 (TRAF6) and interleukin 1 receptor-associated kinase 1 (IRAK1) [189, 190]. Downstream targets of TRAF6 and IRAK1 are the IKK-related kinases IKK ϵ (also called IKKi) and TANK binding kinase 1 (TBK1) which are known to phosphorylate IRF3 and IRF7 in response to certain viruses, dsRNA or LPS signalling. It has been shown that IRF5 is also a substrate for both kinases after NDV, VSV and HSV-1 (but not SeV) infection and in *in vitro* experiments using kinase overexpression [187, 191, 192]. Nuclear localisation of IRF5 can be observed after phosphorylation by IKK ϵ /TBK1 at 4 distinct leucine residues within the NES (at position 150, 154, 157 and 159) and substitutions of these residues to phosphomimetic aspartic acid leads to nuclear retention in unstimulated cells [187, 193]. However, nuclear translocation did not result in gene activation suggesting that other posttranslational modifications or other co-factors are needed to fully activate IRF5. Additional experiments revealed putative phosphorylation sites in the transactivation domain which contains serine-rich domains. Phosphorylation of Ser425, Ser427 and Ser430 is likely to contribute to activation by destabilising the autoinhibitory conformation of the C-terminus [188] and substitution of Ser451, Ser453, Ser456 and Ser462 to aspartic acid produced a protein that functioned as a constitutively active transcription factor [193].

1.4.5 Role of IRF5 in antiviral immunity and cytokine induction

IRF5 was first linked to innate immunity in 2001 when it was shown that NDV but not SeV could activate IRF5, leading to the induction of distinct IFN- α genes [192]. Follow up studies revealed that also VSV and HSV-1 can both activate IRF5 resulting in synthesis of active IFN- α proteins [186]. The experiments described above were conducted with the 2fTGH cell line, which expresses IRF3 but not IRF5 or IRF7 and is therefore unable to induce IFN- α gene transcription upon viral infection. Overexpression of IRF5 was sufficient for reconstitution of the anti-viral response and the induction of functional IFN- α proteins. The authors also examined possible redundancies in the function of IRF5 and IRF7 and found that IFN- α gene subtypes induced by IRF5 in NDV-infected cells are distinct from the ones induced by NDV-infected IRF7-expressing cells. This suggests that the expression profile of IFN- α genes is dependent on different IRFs expressed in infected cells. A more recent study tried to reveal the target genes of IRF5 and IRF7 in BJAB cells after NDV infection [194]. It was shown that the genes are only partially overlapping and therefore, IRF5-induced genes are not a subgroup of genes induced by IRF7. Strong expression of antiviral early inflammatory genes was observed in infected IRF5-expressing cells whereas the transcripts induced specifically by IRF7 were mitochondrial genes and genes affecting the DNA structure.

A number of papers have linked TLR signalling to activation of IRF5 protein. Ligation of TLR3, TLR4, TLR5, TLR7/8 and TLR9 with their ligands resulted in the formation of a complex composed of MyD88, TRAF6 and IRF5 [189, 190]. Signalling via TLR7/TLR8 activated IRF5 and IRF7 but not IRF3 in HEK-293-TLR4-CD14/Md2 and 2fTGH cell lines and activated IRF5 is needed for production of type I IFNs in these cells. Silencing of IRF5 with siRNA disrupted signalling from TLR7/TLR8 and significantly reduced the IFN response. However, IFN expression was not completely abolished, suggesting that IRF7 also plays a role in induction of type I IFN in these cells [189].

In 2005 Takaoka *et al.* generated *Irf5*^{-/-} mice which developed normally, had no sign of abnormal haematopoiesis and almost normal activation of NF- κ B, p38 or JNK signalling pathways. However, myeloid cells (spleen-derived cDCs, pDCs and macrophages) from IRF5-deficient mice displayed a significant decrease in expression and secretion of pro-inflammatory cytokines, namely TNF, IL-6 and IL-12p40, in response to TLR3, TLR4, TLR5 and TLR9 signalling. Consequently, *Irf5*^{-/-} mice survived lethal shock induced by CpG and

were resistant to LPS-induced endotoxic shock due to marked decrease of serum cytokine concentrations of TNF, IL-6 and IL-12p40. Using ChIP, the authors showed CpG-induced recruitment of IRF5 to the promoter of IL-12p40 and suggested IRF5-mediated transcriptional activation of IL-12p40. Bioinformatic analysis of the TNF and IL-6 promoters identified several putative ISREs prompting Takaoka *et al* to propose that these genes are activated by direct binding of IRF5 similarly to IL-12p40. However, the authors provided no clear evidence for a direct role of IRF5 in the transcriptional regulation of IL-12p40 as IRF5 ChIPs were not performed in combination with RNA polymerase II (Pol II) ChIPs (as a measurement of active transcription upon cell activation) and the involvement of IRF5 in regulating TNF as well as IL-6 expression remains purely speculative.

Surprisingly, TLR9-mediated induction of type I IFNs in pDCs was not impaired [190], suggesting that IRF5 may not participate in the induction of type I IFNs in IRF5-deficient mice contradicting the studies of others performed in human cells [195]. The authors mentioned differences between mice and human (IRF5 isoforms) or the possible role of other unknown factors. However, recent experiments with *Irf5*^{-/-} mice confirmed the dependence of IFN- α 4 and IFN- β promoters on IRF5 after NDV infection. As a consequence, NDV-induced type I IFN serum levels were decreased in *Irf5*^{-/-} mice compared to wild type mice [196]. A different study performed simultaneously by another group revealed that *Irf5*^{-/-} mice also have lower type I IFNs serum levels after VSV and HSV-1 infection [195].

To conclude, it has been shown that IRF5 is important for the host response to viral infections and it has been suggested that IRF5 is involved in the induction of pro-inflammatory cytokines. Yet, to date there are no studies linking IRF5 with direct transcriptional regulation of cytokines like TNF or IL-6. Furthermore, IRF5 functions seem to be cell type-specific in mice but data obtained to date are not always consistent between humans and mice. In general, most studies investigating IRF5 function were performed using IRF5 overexpression in various cell lines but its role in primary human cells remains elusive. Clearly, there are still open questions and further investigation is needed to clarify the role of IRF5 in modulation of the human innate immune response and its contribution to autoimmunity. The regulation or manipulation of IRF5 protein levels and its function may provide a potential therapeutic target for inflammatory disease as well as viral infections.

1.5 Dysfunction of immunity – Role of IRF5 in autoimmune diseases

The immune system is the defence mechanism of the body. It senses the invasion of “foreign” pathogens (non-self recognition) and tissue damage (altered-self recognition) and protects the host by initiation of an inflammatory, anti-microbial and anti-stress response. Dysfunction of the immune system is at the centre of a wide variety of diseases including autoimmunity, allergy, infections and cancer.

Like most diseases of the immune system, autoimmune diseases are complex and depend on the interplay between environmental, epigenetic and genetic factors all of which result in dysregulation of downstream biological networks. Invading pathogens or tissue damage generally results in a state of inflammation which involves the production of proinflammatory cytokines as well as chemokines and the subsequent recruitment of immune cells to the site of inflammation. This inflammatory process is then resolved by the production of negative regulators of immunity which limit the inflammatory response in order to avoid excessive tissue and host damage. Both phases must be precisely coordinated and are therefore tightly controlled. This control is disrupted in environmentally and/or genetically pre-disposed individuals leading to a state of chronic inflammation. This results in severe and often irreversible damage to the tissue causing the development of autoimmune diseases. An integrative evaluation of the complex network alterations underlying the pathogenesis of autoimmune diseases was until recently difficult. However, technical advances now enable the analysis of DNA, RNA or proteins in patient samples on a genome wide level (genome-wide association studies, GWAS). These techniques, combined with bioinformatics, can assess the activity of the entire transcriptome in a single sample, enabling the identification of genes and single-nucleotide polymorphisms (SNPs) associated with disease pathology.

1.5.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease with heterogeneous clinical manifestations. The spectrum of human SLE ranges from solely skin involvement to systemic disease which is characterised by relapsing and remitting course with high morbidity. Therefore, SLE patients can have surprisingly different phenotypes, with one patient suffering from malar rash whereas another may experience life-threatening manifestations such as nephritis [197]. SLE patients are predominantly women and

confirming the diagnosis of SLE requires the fulfilment of 4 out of 11 criteria with one being the presence of anti-nuclear antibodies (ANA). These ANA are detected in 95% of patients but in only less than 0.5% of patients with other autoimmune diseases like rheumatoid arthritis (RA) [198]. ANAs are directed against the host's chromatin components such as double-stranded DNA (released from apoptotic cells), histones and nucleosomes and cause the activation of the innate immune system. In addition to these direct effects of ANAs on immunity, is thought that many SLE symptoms result from the deposition of antigen-antibody complexes on tissues [198, 199]. For example, immune complexes can end up in the renal glomerular basement membrane resulting in the infiltration of monocytes and macrophages and subsequent production of proinflammatory cytokines that ultimately induce glomerulonephritis [200].

Several genetic associations with SLE susceptibility have been identified using a candidate gene approach where a gene is either hypothesized to play an etiological role based on its function or due to its location within a chromosomal region identified by linkage studies. The first genes shown to be risk factors for SLE were those encoding human leukocyte antigen (HLA) genes [201] followed by the identification of IRF5 [169], protein tyrosine phosphatase nonreceptor type 22 (PTPN22) [202] and signal and transducer and activator of transcription 4 (STAT4) [203] as risk genes. Four SLE GWAS were published in 2008 [204-207] providing not only confirmation of previously identified loci (HLA, IRF5 and STAT4) but also led to the identification of many novel loci including ITGAM, TNFAIP3 and BANK1. Although these GWAS resulted in identification of new risk genes more work is needed to elucidate the functional mechanisms of the causal variants.

An example where the causal variant has likely been identified includes IRF5. IRF5 is one of the most strongly and consistently SLE-associated loci outside the HLA region and was detected using both candidate gene and GWAS approach. Association studies derived from multiple ethnic backgrounds ranging from Sweden, Finland, Spain, Argentina and USA to Mexico have identified four functional IRF5 variants:

- a 5 bp insertion/deletion (in/del) near the 5' untranslated region (UTR)
- rs2004640 in the first intron
- a 40 bp in/del in the sixth exon
- rs10954214 in the 3' UTR

The strongest association in all cohorts was with SNP rs2004640, with the highest risk haplotype in Mexico and consistent association in Europeans [169-171, 184]. However, there are population differences as analysis of cases and controls from China revealed only a very weak correlation [208]. The haplotypes defined by different combinations of the above SNPs are associated with increased, decreased or neutral levels of risk for SLE. Important for the work presented in this thesis, increased risk haplotypes are associated with an increased expression of IRF5 mRNA and subsequent increase in IRF5 protein levels resulting in functional changes in IRF5-mediated signalling [209-212].

The use of IRF5 deficient mice in murine lupus models further established a role for IRF5 in disease pathology. Phenotypes of *Irf5*^{-/-} mice in the lupus models *FcγRIIB*^{-/-} *Yaa* and *FcγRIIB*^{-/-} have been described. These mice showed diminished autoantibody production, attenuated glomerulonephritis and reduced serum IgG autoantibody levels [213]. The decrease in autoantibody production found by Richez *et al* was confirmed by another group. Savitsky *et al* injected *Irf5*^{-/-} and wild-type mice with pristane oil and found reduced IgG glomerular deposits and antinuclear autoantibodies as well as a lack of IgG2a autoantibody secretion in the *Irf5*^{-/-} mice and suggested a role for IRF5 in B cell maturation and differentiation [214]. Furthermore, a recent study analysed the phenotype of IRF5-deficient MRL/lpr animals which showed again reduced autoantibody production, milder glomerulonephritis and consequently improved mouse survival [215].

1.5.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises of Crohn's Disease and ulcerative colitis, which are severe inflammatory disorders of the gastrointestinal tract. They affect as many as 1 in 250 people in both Europe and the US and patients often suffer from debilitating symptoms including abdominal cramping and bloody diarrhoea [216]. IBD is associated with an overactive immune response to the intestinal environment and a consequence of chronic inflammation in the gut is the promotion of tumorigenesis. In fact, patients with IBD have a higher risk of developing colitis-associated colorectal cancer [217, 218]. There is no current cure for the disease, and treatment consists of long-term immunosuppressive therapy with a potential requirement for surgical intervention.

Commensal microorganisms contribute to host defence by limiting the growth of potentially harmful pathogens via crosstalk with the innate immune system. Despite this mutualism between the microbiota and the host, changes in the composition of the intestinal environment or colonisation with specific pathogens may alter intestinal homeostasis and the nature of the immune response resulting in spontaneous colitis and/or tumorigenesis [217, 218]. Therefore, the unique microbial environment of the intestine places the innate immune system at the heart of intestinal homeostasis. Several host innate immune mechanisms have evolved to deal with the challenge posed by the microbiota. Anatomically, a physical barrier formed by a single layer of intestinal epithelial cells (IECs) shields the rest of the body from commensal microorganisms in the intestinal lumen. A balance between cell death via apoptosis and survival is important for the maintenance of intestinal homeostasis as well as barrier function and innate immune cell-derived cytokines are regulators of apoptosis. It has been shown that the proinflammatory mediator TNF induces the apoptosis of IECs leading to barrier disruption and subsequently sustained inflammation in the intestine [219, 220]. Consequently, TNF-targeting biologics confer protection in IBD by modulating apoptosis in the gut and restoring barrier function [221-224]. Other innate immune cell-derived cytokines associated with IBD pathology include IL-1, IL-6, IL-10, IL-18 and IL-23 [217] all of which are associated with the M1 macrophage phenotype. In summary, environmental triggers as well as genetic predispositions affect intestinal maintenance and cells of the innate immune system are central in orchestrating intestinal homeostasis.

GWAS have identified several susceptibility loci for IBD including innate receptors (NOD2 and TLRs) [225-227], innate-derived cytokines (IL-12p40 and IL-10) and transcriptional regulators of the immune response (IRF5) [176-178]. Of those, IL-12p40 is of particular interest due to its involvement in the formation of IL-23. IL-23 is a heterodimeric cytokine comprising of IL-12p40 and IL-23p19 [106] and is critical in the pathogenesis of a number of murine models of autoimmune disease and inflammatory conditions such as EAE [107], collagen induced arthritis (CIA) [112] and intestinal inflammation [113]. The functional activity of IL-23 has been linked to the maintenance and population expansion of Th17 lymphocytes, which have an increased expression of the IL-23 receptor (IL-23R) [110]. In addition, it has been shown recently that IL-23 can antagonise the development of FoxP3⁺ Treg lymphocytes therefore facilitating intestinal inflammation [228]. Human IBD is associated with increased expression of IL-23 and Th17 signature cytokines such as IL-17A and IL-17F [229]. GWAS have identified SNPs conferring risk to both Crohn's disease and

ulcerative colitis in the gene coding for the human IL-23R which further highlights the role of IL-23-mediated signalling in IBD [230, 231]. Consequently, neutralisation of IL-23 has been shown to be beneficial in numerous murine models of IBD [232, 233]. Although it is generally accepted that IL-23 and its effect on Th17 lymphocytes is crucial in mouse models of IBD [228, 234], the innate cells that produce IL-23, primarily M1 macrophages, are less well studied in this context.

The association of IRF5 with IBD is also unclear although it has recently been described that the 5 bp in/del within the 5'UTR creates an additional binding site for the transcription factor SP1 resulting in increased expression of IRF5 mRNA [178]. However the data presented here could provide first insights into the role of IRF5 in the pathogenesis of IBD and explain its association with the disease.

1.5.3 Rheumatoid arthritis

Rheumatoid arthritis was first described more than 200 years ago in the doctoral dissertation of Augustin Jacob Landré-Beauvais [235]. Where SLE is inflammation of the skin and kidneys, and IBD of the gut, RA is a chronic inflammatory disease of the synovial tissue. It affects approximately 1% of the population worldwide [236] and is associated with inflammation of the synovial joints as well as progressive destruction of surrounding cartilage and bone [237].

Although the etiology of RA remains undefined, the contribution of genetic risk factors has been established as the prevalence of the disease is higher in monozygotic twins than in the general population [238-240]. In particular, genes of the major histocompatibility complex (MHC) on chromosome 6 are tightly linked to RA. This data led to the shared epitope hypothesis whereby the DR4 and DR1 subtypes of the DRB1 chain, which is part of the MHC class II complex, shared amino acids 70-74 and were most commonly associated with RA [241]. GWAS and GWAS meta-analysis identified additional genetic risk factors although the association with RA was found to be weaker for all of those risk genes [242-244].

The effects of IRF5 polymorphisms on susceptibility to RA have also been investigated and the results were inconsistent. The first two studies conducted could find no significant correlation between SNP rs2004640 and RA patients from France, Spain, Sweden and Argentina [245, 246]. Nevertheless, recent analysis of four different case-controls cohorts, including Korea, Tunisia and Japan, found a very strong association between the IRF5 SNP rs2004640 and RA [172-175, 247, 248]. The authors also suggested that this SNP contributes more to RA susceptibility in patients that have the shared epitope and/or are positive for anti-citrullinated protein antibodies. Moreover, a recent GWAS meta-analysis identified IRF5 as a risk gene for RA further supporting its role in this disease [244].

Considering that women are 3 times more susceptible to RA than men, the possible role of hormonal factors is at the centre of ongoing discussion and investigation [249, 250]. In addition to genetic risk factors, environmental risk factors, most prominently infections and smoking [251, 252], have been identified. Infections clearly linked to RA include mycobacteria, *Escherichia coli* and Epstein-Barr virus [253]. Infections may be of particular interest as infectious agents can trigger the activation of the innate immune system via TLR signalling highlighting the role of pattern recognition. There is a growing body of evidence that endogenous ligands of TLRs may contribute to inflammatory cytokine production and therefore prolonging the inflammatory responses in RA. For example, murine models of RA displayed reduced symptoms when lacking TLR4 [254], or when treated with TLR4 antagonists [255]. Furthermore, depletion of TLR8 was shown to significantly reduce inflammatory cytokine production in *ex vivo* cultures of synovial membranes derived from RA patients [256].

Tissue biopsies of RA patients undergoing joint replacement therapy have constituted a major tool for studying characteristics of the disease *ex vivo* as well as mechanisms of dysregulation of inflammation. A hallmark of RA is the increase in size and thickening of the synovial membrane due to the recruitment of various cell types, mainly macrophages, fibroblasts and T lymphocytes (Figure 1.9) [257, 258]. These cells spontaneously release proinflammatory cytokines such as, TNF, IL-1, IL-6 and GM-CSF even after removal from the joint [259-262]. Anti-inflammatory molecules such as IL-10, which are expressed as part of normal acute inflammatory responses, can also be detected in RA synovial joints and the *ex vivo* cultures [263].

The infiltrating cells were characterised based on surface marker expression and macrophages were of particular interest. Synovial macrophages are a major source of proinflammatory cytokines in the joint and their cell number in the synovium correlates well with the degree of inflammatory disease activity in RA [264]. Recent data suggest that synovial macrophage numbers can be used to evaluate disease severity and as predictors of responsiveness to therapy. Gerlag and colleagues were the first to identify CD68⁺ macrophages as an RA biomarker associated with clinical responsiveness to corticosteroids [265]. Their studies were extended over a wide range of interventions and a strong link between the mean change in the Disease Activity Score and the mean change in the number of macrophages was noted [264]. In addition to the high sensitivity of the CD68⁺ macrophage population, their numbers showed a distinction between effective and ineffective treatment [264].

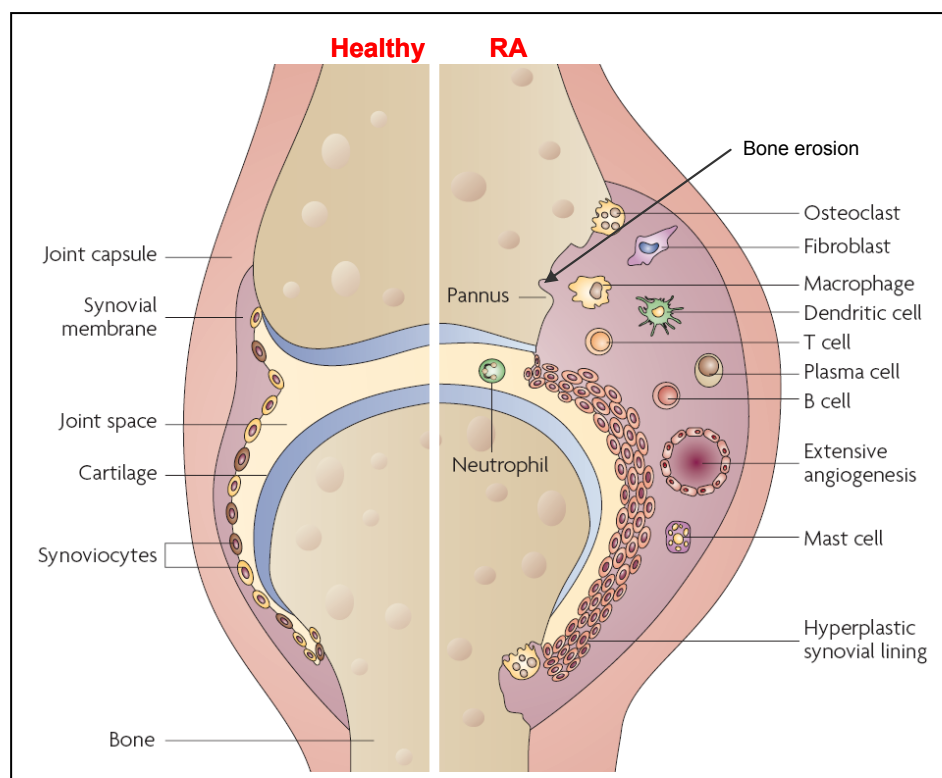


Figure 1.9 Schematic representation of healthy versus RA joint

The RA joint is characterised by the infiltration of immune cells into the synovial membrane resulting in hyperplasticity of the synovial lining and ultimately bone erosion. Adopted and modified from [266].

Comparison of macrophage numbers in the joint of RA and spondylarthritis patients showed that the total number of macrophages is similar in both forms of arthritis, but the subset expressing the M2 specific surface marker, CD163, is clearly increased in spondylarthritis [267, 268]. Moreover, the presence of distinct macrophage subsets in both diseases seems to be associated with different local inflammatory milieus as the M1 cytokine signature can be found in the inflamed joints of RA patients but is absent in spondylarthritis [269]. It is desirable to further evaluate the contribution of macrophage subsets to disease severity and potentially use them as even better predictors of therapeutic success compared to the general macrophage staining with CD68 described above. However this is not possible yet due to the lack of a clear distinguishing marker for human macrophage subsets.

The cause behind the spontaneous release of cytokines in the joint is currently not known, however the production of TNF has been placed at the centre of disease pathology. First support for a dominant role of TNF was provided by both *in vitro* and *in vivo* studies. Production of a range of proinflammatory cytokines by cultured cells from the joints of RA patients can be decreased by a neutralizing antibody to TNF [270, 271]. Murine models of arthritis such as the collagen-induced arthritis (CIA) model were used to show that treatment with anti-TNF antibodies or soluble TNF receptor abrogated inflammation in the synovium [272]. An open Phase I/II clinical trial involving 20 patients with active RA showed that the treatment was safe, tolerated and, more importantly, resulted in significant clinical as well as laboratory improvements [273]. The efficacy of anti-TNF therapy in RA was subsequently confirmed in randomised controlled trials which further established the central role of TNF in the disease [274-276]. There are currently three drugs (Humira [adalimumab], Remicade [infliximab] and Enbrel [etanercept]) licensed as TNF-blocking agents and used to treat RA and other inflammatory diseases. However, one limitation of anti-TNF treatment of RA is that approximately only 60% of patients respond. Furthermore, the high cost, limited convenience of injections and risk of systemic side-effects are the reason for further research into the molecular mechanisms of the inflammatory response in RA leading to identification and validation of new therapeutic targets.

Overall, SNPs in the gene coding for the transcription factor IRF5 are clearly associated with a number of autoimmune diseases in several case-control studies. The SNPs seem to predominantly affect either the transcription of the IRF5 gene itself or the stability of the resulting mRNA and it has been suggested that these changes lead to an increase in the amount of IRF5 protein. However, the effect of increased IRF5 protein on downstream

signalling is mostly unknown due to an absence of defined IRF5-specific target genes. Furthermore, as shown in murine cells, the expression of IRF5 protein is cell-type specific but we currently lack knowledge of IRF5 protein expression in human immune cells. It is therefore important to identify IRF5 target genes and determine the IRF5 protein expression profile in human cells in order to get a better understanding of its association with as well as contribution to autoimmune diseases.

1.6 Hypothesis of investigation

TNF is the major cytokine associated with chronic inflammatory diseases such as inflammatory bowel diseases and rheumatoid arthritis and tight regulatory control must be maintained over its production. Many investigators have focused on NF- κ B proteins, a dominant transcription factor family in the production of TNF and other proinflammatory cytokines, but recent studies suggest a role for IRF5 in regulating inflammatory processes. As mentioned previously, the genes that are regulated in an IRF5-dependent manner are ill-defined but studies in IRF5-deficient mice suggest that the TNF gene is a possible target of IRF5. However, the molecular mechanisms of IRF5-mediated TNF gene expression are not known.

The initial focus of this study was to dissect the role of IRF5 in regulating human TNF gene expression in human myeloid cells with the working hypothesis that **“IRF5 is required for TNF transcription in human MDDCs in response to LPS”**.

1.6.1 Specific aims for TNF study (Chapter 3):

1. Characterise the expression of IRF5 protein in primary human myeloid cells during cell differentiation and upon activation. The identification of a particular level of IRF5 expression amongst the myeloid lineage will determine the cell type(s) for future experiments.
2. Investigate the requirement of IRF5 for activation of human TNF gene expression. In particular, determine if IRF5 is directly regulating TNF transcription or perhaps is involved in post-transcriptional/translational processes.
3. If aim 2 indicates a direct transcriptional regulation of TNF gene expression by IRF5, I intend to identify regulatory elements in the human TNF locus to which IRF5 binds *in vivo*.
4. Search for protein-protein interactions between IRF5 and other transcription factors with a focus on NF- κ B family members.

During this part of the project I observed a low expression of IRF5 in monocyte-derived macrophages (differentiated in M2 polarizing conditions) which prompted me to investigate IRF5 levels under M1 conditions. M1 macrophages are in many ways similar to monocyte-derived DCs, thus I expected a high expression of IRF5 in these cells and hypothesised that **“IRF5 is a determining factor of human macrophage polarization”**.

1.6.2 Specific aims for macrophage polarization study (Chapter 4 and 5)

1. Investigate the expression of IRF5 during macrophage polarization using different protocols. There is limited knowledge about environmental signals that induce IRF5 expression in human macrophages, thus every trigger of IRF5 expression would be novel.
2. Determine if IRF5 expression is static during macrophage polarization. Macrophages can adopt their phenotype in response to environmental changes and a subset-defining transcription factor should also change accordingly.
3. Investigate whether any subset-defining cytokines are under the transcriptional control of IRF5. Determine if IRF5 is directly regulating these cytokines, demonstrated for human TNF, and if yes, identify regulatory elements to which IRF5 binds *in vivo*.
4. Characterise if ectopic expression of IRF5 or siRNA-mediated inhibition has adverse effects on macrophage phenotype and cytokine profile.
5. Results from Chapter 3 indicated that ectopic expression of IRF5 promotes a potent Th1 response. However, it is important to further characterise the effect of ectopic IRF5 on T lymphocyte fate and expand to other known T lymphocyte subsets with a focus on the Th1/Th17 lineage.
6. Determine the phenotype of macrophages obtained from wild-type and IRF5-deficient mice. If IRF5 functions as a subset-defining transcription factor in both man and mouse, I intend to investigate the *in vivo* role of IRF5 in macrophage polarization using wild-type and IRF5-deficient mice.

2. Methods

2.1 Cell culture

All cell cultures were maintained at 37°C in 5% CO₂ and 95% humidity.

2.1.1 Maintenance of cell lines

Cell lines used in this study were purchased from Invitrogen, USA, and are listed in Table 2.1. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; PAA, USA) supplemented with 10% foetal calf serum (FCS; Gibco, USA) and 1% penicillin/streptomycin (PAA, USA). In addition, HEK293-TLR3 cells were supplemented with 10 mg/ml of blasticidin (Invitrogen, USA) and HEK-293-TLR4-CD14/Md2 cells were supplemented with 10 mg/ml and 50 mg/ml of blasticidin and HygroGold™ (Invivogen, USA) respectively. Cells were passaged upon reaching a confluency of approximately 70-80%. The culture media was aspirated and cells were washed once with Dulbecco's phosphate buffered saline (PBS; PAA, USA) before addition of trypsin/EDTA (PAA, USA) for 5-10 minutes at 37°C. Afterwards cells were resuspended in a suitable volume of media to obtain an approximate dilution of 1 in 6.

All cell lines were regularly screened for mycoplasma infection while they remained in long term culture. This procedure was kindly performed by Mrs P Amjadi.

Table 2.1: Cell lines used in this study

Cell line	Description
HEK293-TLR3	Human embryonic kidney (HEK)-293 cells that stably express toll-like receptor 3
HEK293-TLR4	Human embryonic kidney (HEK)-293 cells that stably express toll-like receptor 4 as well as the co-receptors CD14 and Md2
HT1080	Human fibrosarcoma cell line

Cells were stimulated where indicated with either 100 ng/ml LPS (Alexis Biochemicals, USA) or 25 µg/ml Polyinosinic Polycytidylic Acid (pl:C; Invitrogen, USA).

2.1.2 Cryopreservation of cells

Cell lines subjected to cryopreservation were first collected and then resuspended in a mixture of 90% FCS and 10% dimethylsulfoxide (DMSO; Invitrogen, USA) at a concentration of 10^7 cells/ml. They were then transferred into cryovials in 1ml aliquots and placed at -70°C in an insulated container to ensure slow freezing. The cryovials were subsequently transferred into a liquid nitrogen tank for long term storage. Upon re-culture, cryovials were placed in a waterbath at 37°C until the contents were thawed after which cells were quickly washed to remove the DMSO and resuspended in the appropriate culture media.

2.2 Work with primary human myeloid cells

2.2.1 Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation of platelet-pheresis residues from healthy donors that were purchased from North London Blood Transfusion Service (Colindale, UK). Heparinised residues were diluted 1:1 with Hank's Balanced Salt Solution (HBSS; PAA, USA) and layered over an equal volume of Ficoll-Hypaque lymphoprepTM (Axis-Shield, UK) in sterile 50 ml tubes. Centrifugation was performed at 2,200 revolutions per minute (rpm) for 20 min at room temperature in a Sorvall RT7 (Kendro Laboratory products, UK) with a minimum deceleration setting on the brake. The interface layer was collected and washed twice with HBSS followed by centrifugation for 10 min at 1,800 rpm. PBMCs were then resuspended in 45 ml of HBSS before being passed through a cell strainer (BD Bioscience, UK) to remove any clumps.

2.2.2 Isolation of human monocytes

Enriched populations of monocytes were obtained by centrifugation at 2,500 rpm in a Beckman JE6 elutriator (Beckman Coulter, UK). Elutriation is a process that separates a heterogeneous cell population based on size. Cells are loaded into a specialised chamber and subjected to a centrifugal field, which is counter-balanced by pumping media through the chamber at a constant rate. As the flow rate gradually increases cells of increasing size are ejected from the chamber and collected. Monocytes were ejected when the flow rate

was increase to 18-22 ml/minute and their purity was determined by analysis of cell size and granularity using flowcytometry. Only fractions containing more than 80% monocytes and less than 5% granulocytes were used in experiments.

2.2.3 *In vitro* differentiation of human monocytes

Upon collection of monocyte-enriched fractions cells were counted and resuspended in Roswell Park Memorial Institute media 1640 (RPMI; PAA, USA) supplemented with 10% FCS, 1% penicillin/streptomycin to a concentration of 10^6 cells/ml. 10 ml of this cell suspension was seeded per 10 cm tissue culture dish (Falcon, UK).

For differentiation of monocytes into DCs, GM-CSF and IL-4 (Peprotech, UK) were added at a final concentration of 50 ng/ml and 10 ng/ml respectively. 5-7 days later non-adherent cells were harvested, resuspended in fresh culture media to a concentration of 10^6 cells/ml and seeded appropriately. Macrophages were generated from monocytes either by addition of 100 ng/ml M-CSF (for M2 differentiation) or 50 ng/ml GM-CSF (for M1 differentiation) (Peprotech, UK). 4-6 days later adherent cells were harvested by scraping, resuspended in fresh culture media to a density of 10^6 cell/ml and seeded appropriately.

2.3 Work with mice and murine cells

I would like to thank Miss Katrina Blazkova (Kennedy Institute of Rheumatology, London, United Kingdom) who routinely performed isolation of bone marrow cells as well as organ extraction. Katrina was also instrumental in establishing and optimising *in vivo* experiments.

2.3.1 Isolation of bone marrow cells

C57BL/6 wild type or *Ir55*^{-/-} (on a C57BL/6 background) were sacrificed by cervical dislocation and the abdomen as well as the hind legs were sterilised with 70% ethanol. Skin was removed from hind limbs and bones (femur and tibia) were removed by cutting through the bones at the ankle and as near the pelvis as possible. Scissors were used to trim as much muscle and fat from the bones as possible. Next, bones were separated by cutting through the knee joint resulting in an opening into the bone marrow cavity at both ends of

each bone. Bone marrow progenitors were flushed out of the bones into 50 ml tubes with RPMI media using a 5 ml syringe and a 25 gauge needle. Bone marrow progenitors were pipetted up and down to bring the cells into a single cell suspension. Cells were then passed through a cell strainer and the strainer washed with 5 ml RPMI. Cells were pelleted by centrifugation at 1,800 rpm for 5 min and resuspended in 1 ml of RPMI supplemented with 10% FCS, 1% penicillin/streptomycin and 0.01% 2-mercaptoethanol (bone marrow media, BM media). The bone marrow progenitors were counted and adjusted to a concentration of 10^6 cells/mL in BM media and subsequently used for *in vitro* differentiation.

2.3.2 *In vitro* differentiation of bone marrow cells

Bone marrow cells were isolated as described in section 2.3.1, counted and 5×10^6 cells in 10 ml BM media were seeded in 10 cm bacterial dishes (Falcon, UK). Macrophages were generated either by addition of 50 ng/ml murine M-CSF (for M2 differentiation) or 20 ng/ml murine GM-CSF (for M1 differentiation) (Peprotech, UK). On day 3, 10 ml of BM media supplemented with either M-CSF (50 ng/ml) or GM-CSF (20 ng/ml) was added to the cultures. On day 6, 10 ml of media was collected and cells were pelleted by centrifugation at 1,800 rpm for 5 min. Pellets were resuspended in 10 ml of BM media supplemented with either M-CSF (50 ng/ml) or GM-CSF (20 ng/ml) and transferred back to the original culture dishes. Cells were harvested between days 8-9, resuspended in fresh BM media (without cytokines) to a density of 10^6 cell/ml and seeded appropriately.

2.3.3 Isolation and culture of spleen cells

Mice were sacrificed by cervical dislocation and the abdomen sterilised with 70% ethanol. The spleen was subsequently removed and put in RPMI at 4°C. Spleens were then homogenised and passed through a cell strainer and the strainer washed with 5 ml BM media. Cells were pelleted by centrifugation at 1,500 rpm for 5 min, resuspended in 2 ml of Red Blood Lysis Buffer (Sigma, UK) and left for 15 min at RT. The buffer was diluted with 20 ml of BM media followed by centrifugation at 1,500 rpm for 5 min. Cells were washed twice with 20 ml of BM media, counted and adjusted to a density of 6×10^6 cells/ml. Of this suspension, 0.5 ml was plated per well in 24 well plates and the media supplemented with antibody to CD3 (anti-CD3; 10 ng/ml; 145-2c11; BD Bioscience) where indicated. In other applications, 0.5 ml of suspensions were pelleted and used directly for RNA extraction.

2.3.4 *In vivo* experiment with IRF5 knock-out mice

For *in vivo* experiments, *Irf5*^{-/-} mice and their wild-type littermates were injected intraperitoneally with 20 µg LPS in 200 µl sterile PBS. Mice were sacrificed after 3 h, blood collected by cardiac puncture and peritoneal cells collected by performing peritoneal lavage using sterile PBS. The blood was collected in 1.5 ml tubes, stored at 4°C overnight and then centrifuged next day at 14,000 rpm for 15 min. The serum (top layer) was carefully transferred to a new tube and stored at -20°C until further use. The peritoneal cells were counted and equal amounts (usually 5 x 10⁵ cells) used for RNA extraction. Spleens and mesenteric lymph nodes were removed and either snap frozen and stored at -80°C until further use or cultured for 48 h in BM media supplemented with antibody to CD3 (anti-CD3; 10 ng/ml; 145-2c11; BD Bioscience). Following culture, supernatants were collected and stored at -80°C and cells used for RNA extraction. The experimental protocol was approved by the UK Home Office.

2.4 MTT cell viability assay

The 3-(4,5,-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide cell viability assay is based on the principle that tetrazolium salts are reduced to water-insoluble formazan crystals in metabolically active cells as a function of redox potential of mitochondria. The overall activity of mitochondrial dehydrogenases is directly correlated with the number of viable cells. Thus, this assay can be used to measure factor-induced cytotoxicity or non-specific cell death in a population of cells, since treatment with a cytotoxic factor will affect the rate of formazan crystal formation.

Following the end of the experimental conditions, supernatants were removed and replaced with fresh culture medium containing 5 µg/ml MTT and cultured at 37°C for 4 h. Cells were then lysed by addition of one volume of 10M hydrochloric acid containing 10% SDS and placed at 37°C overnight. Absorbance was read at 620nm on a Multiskan Biochromatic™ plate reader (Labsystems, UK) using Ascent 2.4.2 software (Labsystems, UK) to measure the levels of viable cells. All samples were analysed in triplicate.

2.5 Quantitative real-time PCR

Real-time PCR allows the quantification of mRNA levels within distinct populations of cells. All gene expression analyses carried out in this study used TaqMan primer probes to amplify mRNA which had previously been reverse-transcribed to generate cDNA.

2.5.1 RNA extraction and quantification

Total RNA was extracted from cells using either a QiaAmp RNA Blood mini kit or RNeasy mini Kit (both from Qiagen, Germany) as per manufacturer's instructions. Contaminating genomic DNA was removed from RNA samples using RNase-Free DNase Set (Qiagen, Germany) which can be used in combination with the RNA extraction kits mentioned above. The procedure routinely removed DNA contamination to levels below the detection limits of a standard real-time PCR. RNA concentration was quantified using a nanodrop 1000 (Thermo Scientific, USA).

2.5.2 cDNA synthesis

cDNA synthesis reactions contained 0.1-1 µg of total RNA and cDNA was synthesised using SuperScript® III Reverse Transcriptase (Invitrogen, USA) and 18-mer oligo dT primers (Eurofins MWG, UK) as per manufacturer's instructions. Following synthesis samples were diluted 1 in 4 in nuclease free water and stored at -20°C until required.

2.5.3 Quantitative real-time PCR 2 standard curve method

Real-time PCR 2-standard curve method requires cDNAs of both the gene of interest and a housekeeping gene to generate a standard curve. A human TNF cDNA clone was kindly provided by Mrs N. Ito (Kennedy Institute of Rheumatology, London, United Kingdom). A human ribosomal protein (PO) cDNA clone was created as follows. Total RNA was extracted from human M2 macrophages and 1 µg of total RNA was used to synthesise cDNA. PO cDNA was amplified by PCR using the following primers: F: 5'TGCCCAGGGAAGACAGGGCG and R: 5'- ATATGGGATTTGGTCTCTTTGACTAA; then cloned into TOPO TA vector (Invitrogen, USA).

The cDNA clones were serially diluted 1 in 25 to generate standard curves that covered a range of cycle thresholds (Cts) that reflected the heterogeneity of gene expression within the samples. The top value for each standard curve was arbitrarily chosen but the values of the each consecutive point on the standard curve reflected the dilution factor 1 in 25. The Ct values of both the gene of interest and the house-keeping gene were then converted into concentrations according to their respective standard curve. Finally, mRNA levels were normalised by dividing the calculated value of the gene of interest by the calculated value of the house-keeping gene within each sample.

Real-time PCR reactions were performed in a Corbett Rotor-gene 6000 machine (Corbett Research Ltd, Cambridgeshire, UK) and analysed using Corbett Rotogene 6000 software or in a ABI 7900HT machine (Applied Biosystems, USA) and analysed manually. TaqMan PreAmp Master Mix (Applied Biosystems, USA) and TaqMan gene expression assays (Applied Biosystems, USA) were used as per the manufacturer's instructions. Table 2.2 contains a detailed list of TaqMan probes used in this study. Reactions were either carried out in 10 µl volumes (Corbett Rotor-gene 6000) or 6 µl volumes (ABI 7900HT machine) in duplicate. Thermocycling conditions were as follows:

10 min at 95°C

45 cycles of 10s at 95°C + 35s at 60°C

Table 2.2: TaqMan primers used in this study

Gene	Species	Catalogue no.
Arg1	Mouse	Mm00475988_m1
Chi3l3	Mouse	Mm00657889_mH
FOXP3	Human	Hs01085834_m1
GATA3	Human	Hs00231122_m1
Hprt1	Mouse	Mm00446968_m1
IFNG	Human	Hs00174143_m1
Il1b	Mouse	Mm99999061_mH
IL6	Human	Hs00174131_m1
Il6	Mouse	Mm00446190_m1
IL10	Human	Hs00174086_m1

Il10	Mouse	Mm00439616_m1
IL12A	Human	Hs00168405_m1
Il12a	Mouse	Mm99999066_m1
IL12B	Human	Hs00233688_m1
Il12b	Mouse	Mm01288990_m1
IL17A	Human	Hs00174383_m1
IL17F	Human	Hs00369400_m1
IL21	Human	Hs00222327_m1
IL22	Human	Hs01574154_m1
IL23A	Human	Hs00372324_m1
Il23a	Mouse	Mm00518984_m1
IL23R	Human	Hs00332759_m1
IL26	Human	Hs00218189_m1
IRF5	Human	Hs00158114_m1
Irf5	Mouse	Mm00496477_m1
Mrc1	Mouse	Mm00485148_m1
RelA	Human	Hs00153294_m1
Retnla	Mouse	Mm00445109_m1
RPLPO	Human	4326314E
TBX21	Human	Hs00203436_m1
TNF	Human	Hs00174128_m1
Tnf	Mouse	Mm00443258_m1

ROR γ t gene expression was detected by SybrGreen with a primer set specific for human RORC2 (RORC2_F1, TGAGAAGGACAGGGAGCCAA; and RORC2_R1: CCACAGATTTTGCAAGGGATCA).

2.6 Micro array analysis

The quality control of the RNA samples and the hybridisation was carried out by Dr Dilair Baban (Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom). Micro array analysis was kindly performed by Mrs Helen Lockstone and Miss Natasha Sahgal (both Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom).

Gene expression data were obtained by hybridising RNA from a total of 24 samples from 6 experimental groups (n=4 per group) to Illumina HumanHT-12 Expression BeadChips. Raw data were exported from the Illumina GenomeStudio software (v1.0.6) for further processing and analysis using R statistical software [277] (v2.10) and BioConductor packages [278]. Raw signal intensities were background corrected using array-specific measures of background intensity based on negative control probes, prior to being transformed and normalised using the 'vsr' package [279]. Quality control analyses did not reveal any outlier samples. The dataset was then filtered to remove probes not detected (detection score <0.95) in any of the samples, resulting in a final dataset of 25,620 probes.

Statistical analysis was performed using the Linear Models for Microarray Analysis (limma) package [280]. Differential expression between the experimental groups was assessed by generating relevant contrasts corresponding to the relevant comparisons. Raw p-values were corrected for multiple testing using the false discovery rate controlling procedure of Benjamini and Hochberg [281], adjusted p-values below 0.01 were considered significant. Significant probe lists were then annotated using the relevant annotation file (HumanHT-12_V3_0_R2_11283641_A) that was downloaded from the Illumina website (<http://www.illumina.com>) for further biological investigation.

2.7 Enzyme linked immunosorbent assay (ELISA)

ELISA allows the quantification of proteins that have been secreted into the supernatant by cultured cells. Antibodies were used to specifically capture the protein of interest from culture supernatants. Biotin-antibody conjugates were subsequently incubated with the immuno-complexes before the addition of streptavidin-HRP. Finally HRP substrate was added to the samples which induced a colour change that can be quantified. Running a standard curve of known protein concentrations in parallel to the samples enabled absolute

concentrations of secreted proteins to be inferred. All ELISA antibodies used in this thesis are listed in Table 2.3 and recombinant proteins are listed in Table 2.4.

Capture antibodies were diluted in PBS and applied to 96-well plates overnight at 4°C to adhere. The PBS was then removed and plates were blocked with PBS containing 2% Bovine Serum Albumin (BSA) for 1 h at room temperature. In between all subsequent steps, plates were washed 3 times with PBS containing 0.05% tween. A 7-point standard curve was generated by 1 in 3 serial dilutions of the protein of interest in PBS containing 0.5% BSA, with the top concentration ranging from 4-10 ng/ml. After removing the blocking solution from the plates, standards and samples of interest were placed on top of the capture antibody and incubated for 2 h at room temperature or at 4°C over night. The biotinylated detection antibody was diluted in PBS containing 0.05% BSA and then applied to the plates for 1 h at room temperature after washing. This procedure was repeated except streptavidin-HRP was incubated with the immuno-complexes instead of the biotinylated detection antibody. Finally, the plates were washed, TMB Microwell Peroxidase Substrate (KP Inc, USA) was added to the appropriate wells and the enzymatic reaction was terminated by addition of 1M H₂SO₄. Absorbance was read at 450nm by a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic) and analysed using Ascent Labsystems software. All samples were analysed in triplicate in a volume of 50 µl. A list of all buffers and their composition used in ELISA experiments can be found in section 2.19.3.

Table 2.3: Antibodies used for ELISA

Antigen type	Species	Clone/Lot. number	Catalogue no.	Supplier
CCL2 capture	Human	5D3-F7	14-7099	eBioscience
CCL2 detect	Human	2H5	13-7096	eBioscience
CCL5 capture	Human	21418	MAB678	R&D Systems
CCL5 detect	Human	VC08	BAF278	R&D Systems
CCL13 capture	Human	73506	MAB327	R&D Systems
CCL13 detect	Human	Lot. No. BJ103	BAF327	R&D Systems
CCL22 capture	Human	57226	MAB336	R&D Systems
CCL22 detect	Human	Lot. No. C1G02	BAF336	R&D Systems
IFN- γ capture	Human	NIB42	551221	BD Bioscience

IFN- γ detect	Human	4S.B3	554550	BD Bioscience
IFN- γ capture	Mouse	AN-18	551309	BD Bioscience
IFN- γ detect	Mouse	R4-6A2	551506	BD Bioscience
IL-1 β capture	Human	2805	MAB601	R&D Systems
IL-1 β detect	Human	802700	BAF201	R&D Systems
IL-4 capture	Human	8D4-8	500701	BioLegend
IL-4 detect	Human	MP4-25D2	500803	BioLegend
IL-10 capture	Human	JES3-19F1	554705	BD Bioscience
IL-10 detect	Human	JES3-12G8	554499	BD Bioscience
IL-10 capture	Mouse	JES5-16E3	14-7101	eBioscience
IL-10 detect	Mouse	JES5-2A5	33-7102	eBioscience
IL-12/IL-23 p40 capture	Human	C8.3	551227	BD Bioscience
IL-12p70 capture	Human	20C2	555065	BD Bioscience
IL-12/IL-23 p40 detect	Human	C8.6	554660	BD Bioscience
IL-12/IL-23 p40 capture	Mouse	C15.6	14-7125	eBioscience
IL-12p70 capture	Mouse	C18.2	14-7122	eBioscience
IL-12/IL-23 p40 detect	Mouse	C17.8	13-7123	eBioscience
IL-17A capture	Human	BL127	512602	BioLegend
IL-17A detect	Human	BL23	512703	BioLegend
IL17a capture	Mouse	TC11-18H10	555068	BD Bioscience
IL17a detect	Mouse	TC11-8h4.1	555067	BD Bioscience
IL-23p19 capture	Human	eBio473P19	14-7238	eBioscience
IL-23p19 capture	Mouse	G23-8	14-7232	eBioscience
IP-10 capture	Human	4D5	555046	BD Bioscience
IP-10 detect	Human	6D4/D6/G2	555048	BD Bioscience
TNF capture	Human	MAB1	551220	BD Bioscience
TNF detection	Human	MAB11	554511	BD Bioscience

Table 2.4: Recombinant proteins used as standards for ELISA

Protein	Species	Catalogue no.	Supplier
CCL2	Human	14-8398	eBioscience
CCL5	Human	278-RN-010	R&D Systems
CCL13	Human	327-P4	R&D Systems
CCL22	Human	336-MD	R&D Systems
IFN- γ	Human	554617	BD Bioscience
IFN- γ	Mouse	554587	BD Bioscience
IL1 β	Human	201-LB-005	R&D Systems
IL-4	Human	560602	BioLegend
IL-10	Human	554611	BD Bioscience
IL-10	Mouse	39-8181	eBioscience
IL-12p40	Human	554633	BD Bioscience
IL-12p70	Human	554613	BD Bioscience
IL-12p40	Mouse	398122	eBioscience
IL-12p70	Mouse	14-8121	eBioscience
IL-17a	Human	570509	BioLegend
IL-17a	Mouse	421-ML-025	R&D Systems
IL-23	Human	14-8239	eBioscience
IL-23	Mouse	14-8231	eBioscience
IP-10	Human	551130	BD Bioscience
TNF	Human	554618	BD Bioscience

2.7.1 BD™ Cytometric bead assay (CBA)

The CBA system (BD Bioscience, UK) uses the sensitivity of amplified fluorescence detection by flow cytometry to measure a soluble protein. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The CBA capture bead is a single bead population with distinct fluorescence intensity that allows the detection of a specific protein in a small sample volume. Each bead population is given an alpha-numeric position indicating its position relative to other beads in the CBA system. Beads with different positions can be combined in assays to create a multiplex assay. In a CBA assay the capture bead, PE-conjugated detection reagent, and

standard or test samples are incubated together to form sandwich complexes. The sample results obtained from flow cytometry are analysed using the FCAP Array™ software (BD Bioscience, UK). Assays were performed as per manufacturer's instructions and details of antibodies as well as standards used are shown in Table 2.5.

Table 2.5: Antibodies and standards used for CBA

Antigen type	Species	Bead position	Catalogue no.	Supplier
IL-1 β capture	Mouse	E5	51-9005790	BD Bioscience
IL-1 β detect	Mouse		51-9005788	BD Bioscience
IL-1 β standard	Mouse		51-9005500	BD Bioscience
IL-6 capture	Mouse	B4	51-9005236	BD Bioscience
IL-6 detect	Mouse		51-9004153	BD Bioscience
IL-6 standard	Mouse		51-9003526	BD Bioscience
IL-10 capture	Mouse	C4	51-9005234	BD Bioscience
IL-10 detect	Mouse		51-9004152	BD Bioscience
IL-10 standard	Mouse		51-9003528	BD Bioscience
TNF capture	Mouse	C8	51-9005324	BD Bioscience
TNF detect	Mouse		51-9004161	BD Bioscience
TNF standard	Mouse		51-9003536	BD Bioscience

2.8 Western blotting

Western blotting allowed the identification and semi-quantitation of specific proteins and specific post-translational modifications of proteins e.g. phosphorylation. Following lysis of cellular membranes, total protein concentration was determined and equal amounts were resolved by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). Resolved proteins were then transferred onto a membrane prior to immunoblotting with antibodies specific for the protein of interest. All antibodies used for western blotting in the study are listed in Table 2.6. A list of all the buffers and their composition used in western blotting experiments can be found in section 2.19.2.

2.8.1 Preparation of whole cell protein extracts

Cells were harvested, transferred to 1.5 ml tubes and washed once with ice cold PBS. Cells were then resuspended in 50-100 µl of the appropriate lysis buffer containing protease and phosphatase inhibitors and the tubes were placed on ice for approximately 20 min. Cellular debris was then pelleted by centrifugation and the lysate was transferred into new tubes and stored at -80°C until required.

2.8.2 Protein quantification: BCA assay

Protein concentrations were determined using BCA Protein Assay Reagent (Thermo Scientific, USA) as per the manufacturer's instructions. Briefly, a 7-point standard curve was generated by 1 in 2 serial dilutions of BSA in water, with the top standard at a concentration of 4 µg/ml. BCA reagent A and B were mixed at a ratio of 50:1 and 200 µl was dispensed into an appropriate number of wells in a 96-well plate. 2 µl of sample and 10 µl of standard was added per well. Additionally, 2 µl of lysis buffer only served as a blank. The plate was incubated for 1 h at 37°C, after which the absorbance was read at 540nm on a Multiskan Biochromatic™ plate reader (Labsystems, UK). Protein concentrations were calculated according to the standard curve using Ascent 2.4.2 Labsystems software (Labsystems, UK). All samples were analysed in duplicate.

2.8.3 SDS-PAGE

10-15 µg of total protein and an appropriate volume of 4X loading buffer were boiled at 95°C for 10 min. The denatured proteins were then separated at 150-200 volts on a pre-cast NuPAGE® Novex 4-12% Bis-Tris gel (Invitrogen, USA) that was immersed in NuPAGE® MOPS SDS Running Buffer (Invitrogen, USA). A Full-Range Rainbow Molecular Weight Marker (RPN800; GE Healthcare, UK) was run in parallel so that the molecular weight of visualised proteins could be estimated.

2.8.4 Protein transfer

Following SDS-PAGE, resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, UK). PVDF membranes were cut to a suitable size, dehydrated in methanol and then equilibrated in transfer buffer. The PVDF membrane was

placed on top of the gel containing resolved proteins, then sandwiched between 4 pieces of transfer-buffer soaked filter paper. The assembly was then placed in a transfer cassette in between two transfer soaked sponges and inserted into a transfer tank filled with pre-cooled transfer buffer. A constant voltage of 30V was applied for 2 h at room temperature.

2.8.5 Immunoblotting and protein detection

After protein transfer membranes were incubated in blocking buffer and placed on a shaker at RT for at least 1 h. Primary antibodies were diluted in blocking solution and usually incubated with the membranes at 4°C over night with gentle agitation. In between all subsequent steps membranes were subjected to 4x 15 min washes with washing buffer. HRP-conjugated secondary antibodies were diluted in blocking buffer and sequentially incubated with the membranes for 1 h at room temperature with gentle agitation. Immuno-complexes were detected using the chemiluminescent substrate solution ECL (GE Healthcare, UK), visualised using Hyperfilm MP (Amersham Pharmacia Biotech, UK) and developed using an AGFA Cruis-60 automatic film processor (AGFA-Gaevert, UK). When multiple protein detections were required on the same membranes, antibodies were stripped from the membrane using ReBlot Plus Strong Antibody Stripping Solution (Chemicon, UK) as per the manufacturer's instructions.

Table 2.6: Antibodies used for western blotting

Antibody	Source	Dilution	Catalogue no.	Supplier
anti-HA HRP	Rat	1:1.000	12013819001	Roche
anti-FLAG HRP	mouse	1:1.000	A8592	Sigma
β-Actin	Mouse	1:10.000	65541	Sigma
IRF1	Rabbit	1:5000	sc-479	Santa Cruz
IRF3	Rabbit	1:4.000	sc-9082	Santa Cruz
IRF4	Rabbit	1:5000	sc-28696	Santa Cruz
IRF5	Goat	1:1.000	ab2932	Abcam
IRF5	Rabbit	1:5.000	ab21689	Abcam
IRF5	mouse	1:5000	sc-56714	Santa Cruz
IRF7	Rabbit	1:5.000	sc-9083	Abcam
IRF8	Rabbit	1:5000	sc-13043	Santa Cruz
IRF9	Rabbit	1:5.000	sc-10793	Santa Cruz
p50/p105	Rabbit	1:5.000	sc-114	Santa Cruz

p52/p100	Rabbit	1:10.000	sc-298	Santa Cruz
p65/RelA	Rabbit	1:500	sc-372	Santa Cruz
RelB	Rabbit	1:5.000	sc-226	Santa Cruz
c-Rel	Rabbit	1:5.000	sc-17	Santa Cruz

2.9 Electrophoretic mobility shift assay (EMSA)

EMSAs were kindly performed by Dr Irina Udalova and Mrs Alessandra Lanfrancotti (both Kennedy Institute of Rheumatology, London, United Kingdom). A list of all the buffers and their composition used in western blotting experiments can be found in section 2.19.4.

2.9.1 DNA probe labelling

The two complementary oligonucleotides (Table 2.7) were dissolved in TE to a final concentration of 15 pmole/ μ l (15 μ M). 10 μ l of each oligonucleotide is mixed with 10 μ l of 10x buffer H (Boehringer, UK) and 70 μ l of water in a 1.5 ml tube. The mixture was then layered with 50 μ l of mineral oil. Primers were annealed by placing tubes in a beaker with approximately 1 L of boiling water for 5 min and then allowed to cool down to room temperature overnight. 2 μ l of the annealed duplex (6 pmoles of ends) were mixed with 2 μ l (6 pmoles) of [α - 32 P] dCTP (Perkin Elmer, USA), 1 μ l of a dNTP mixture (containing 3 mM of each dATP, dGTP, dTTP), 2 μ l of 10x Klenow enzyme buffer, 12 μ l of water and 1 μ l of Klenow enzyme. The mixture was incubated at room temperature for 30-45 min and the radiolabelled oligoduplexes were purified through Chroma spin TE-10 spin columns (Takara Bio, USA). The level of incorporation was estimated by monitoring the radioactivity left in the column and the radioactivity recovered in the flowthrough. The probes were diluted down to approximately 20000 CPM/ μ l.

2.9.2 Binding reaction and gel run

The binding reaction contained 50 ng of bacterially expressed and purified IRF5 DBD, 2 μ l of radiolabelled probe and 4 μ l of 2x EMSA binding buffer and was incubated at room temperature for 10-15 min. The reaction was analyzed by electrophoresis in a

nondenaturing 5% polyacrylamide gel (0.5 x TBE) at 200 V for 2 hours at 4°C. The gels were then dried and quantified using the PhosphorImager (FujiFilm, Japan).

Table 2.7: Oligonucleotide sequences used for generating EMSA probes:

Name	Sequence (5' – 3')
κB4_for	agctGGGCATGGGAATTTCCAACCTCT
κB4_rev	agctGAGTTGGAAATTCCTATGCCC
κB4a_for	agctAACTCTGGGAATTCCAATCCTT
κB4a_rev	agctAAGGATTGGAATTCCTCAGAGTT
κB4b_for	agctCTTGCTGGGAAAATCCTGCAG
κB4b_rev	agctGCTGCAGGATTTTCCCAGCAAG
ISRE1_for	agctGAAGCCAAGACTGAAACCAGCATT
ISRE1_rev	agctTAATGCTGGTTTCAGTCTTGGCTTC
ISRE2_for	agctCCGGGTCAGAATGAAAGAAGAAGG
ISRE2_rev	agctCCTTCTTCTTTTATTCTGACCCGGT
ISRE5_for	agctGGAGAAGAAACCGAGACAGAAGGTG
ISRE5_rev	agctCACCTTCTGTCTCGGTTTCTTCTCC
ISRE16_for	agctTTTGCTTAGAAAAGAAACATGGTCTC
ISRE16_rev	agctGAGACCATGTTTCTTTTCTAAGCAAA
ISRE17_for	agctACATAAACAAAGCCCAACAGAATATTCC
ISRE17_rev	agctGGAATATTCTGTTGGGCTTTGTTTATGT
PRDI-III (IFNβ)_for	agctGGGAAACTGAAAGGGAAAGTGAAGTGG
PRDI-III (IFNβ)_rev	agctCCACTTTCACTTTCCCTTTCAGTTTCCC

2.10 Cloning and genetic constructs used in this study

2.10.1 Gene reporter and expression constructs used in this study

Wild-type (wt) and dominant negative (dn, lacking the whole DNA binding domain [DBD]) IRF3 and IRF5 constructs as well as constructs coding for all NF-κB subunits were already available in our laboratory. They are listed in Table 2.8 together with a brief description. All constructs were also cloned into pENTR4.3F and subsequently recombined into pAD/PL DEST for delivery into human primary myeloid cells. A list of luciferase gene reporter constructs used are summarised in Table 2.9.

Table 2.8: Expression constructs used in this study

Construct	Description	Vector	Tag
IRF3-C	full lenght wt	pENTR4.3	HA C-terminal
IRF3-N	full lenght wt	pENTR4.3	HA N-terminal
DN-IRF3-C	lacks DBD	pENTR4.3	HA C-terminal
DN-IRF3-N	lacks DBD	pENTR4.3	HA N-terminal
IRF5-N	full lenght wt	pENTR4.3	HA N-terminal
IRF5 Strep	full lenght wt	pENTR4.3	One-strep N-terminal, HA C-terminal
DN-IRF5-C	lacks DBD	pENTR4.3	HA C-terminal
DN-IRF5-N	lacks DBD	pENTR4.3	HA N-terminal
IRF5A68P	mutation in DBD	pENTR4.3	HA N-terminal
RelA	full lenght wt	pENTR4.3	HA C-terminal
RelB	full lenght wt	pENTR4.3	HA C-terminal
c-Rel	full lenght wt	pENTR4.3	HA C-terminal
p50	full lenght wt	pENTR4.3	HA C-terminal
p52	full lenght wt	pENTR4.3	HA C-terminal

Table 2.9: Luciferase gene reporters

Construct	Lenght	Description	Vector
IL-10 wt	195 bp 5'	wt promoter	pENTR4.3
IL-10 ISRE mut	195 bp 5'	Mutation of ISRE (AATTGAAA → gtTaGtAc)	pENTR4.3
TNF 5'wt/3'wt	1171 bp 5' 1252 bp 3'	wt promoter and 3' region	pGL3
TNF 5'mut/3'wt	1171 bp 5' 1252 bp 3'	Mutation of κ B sites in the 5' region κ B2 (GTGAATTCCC → tTGAATTCCC) κ B ζ (GTGATTTTCCAC → aTccTTTCCAC) κ B2a (GGGCTGTCCC → taGCTGTGCCC)	pGL3
TNF 5'wt/3'mut	1171 bp 5' 1252 bp 3'	Mutation of κ B sites in the 3' region κ B4 (GGGAATTTCC → cGcAATgTgC) κ B4a (GGGAATTCCA → cGcAAgTgCA)	pGL3
TNF 5'mut/3'mut	1171 bp 5' 1252 bp 3'	Mutation of all κ B sites (κ B2, κ B ζ , κ B2a, κ B4, κ B4a)	pGL3

2.10.2 PCR

PCR reactions were performed in a DYAD Peltier Thermal Cycler (Bio-Rad, UK), in 50 µl volumes. Taq polymerase and dNTPs were obtained from Roche, UK, and primers were obtained from MWG, Germany. 50 ng of template DNA was used per reaction. PCR reactions were resolved by electrophoresis in 1% agarose 1x TAE gel. Addition of either ethidium bromide or SYBR green allowed visualisation under UV light. Hyperladder IV (Bioline, UK) was used to determine the molecular weight of DNA fragments. All PCR reactions used touch-up PCR under the following thermal cycling conditions: 95°C for 10 min; 4 cycles of 95°C for 30s, 52°C for 1 min, 68°C for 3 min; 4 cycles of 95°C for 30s, 57°C for 1 min, 68°C for 3 min; 4 cycles of 95°C for 30s, 57°C for 1 min, 68°C for 3 min; 4 cycles of 95°C for 30s, 62°C for 1 min, 68°C for 3 min; 18 cycles of 95°C for 30s and 68°C for 3 min; 10 min at 68°C; 15 min at 4°C.

2.10.3 Restriction digests

Restriction digests were performed using New England Biolabs (NEB, UK) enzymes and buffers as per manufacturer's guidelines. Typically, restriction digests were performed in 20-50 µl reactions for 1-2 h in a water bath set to 37°C.

2.10.4 DNA purification

PCR or restriction digest reaction mixtures were resolved on a standard 1% agarose 1xTAE gel containing SYBR Green II. Desired DNA fragments were excised and purified using a Qiagen gel extraction kit (Qiagen, Germany) according to manufacturer's instructions.

2.10.5 DNA ligation

Purified DNA was quantified by resolution on an agarose 1x TAE gel. Ligations contained an insert to vector ratio of 3:1, where 50 ng of vector was used per ligation. The mass of insert used was calculated by the formula:

$$\text{mass of insert} = \frac{\text{mass of vector} \times \text{length of insert}}{\text{length of vector}}$$

Ligation reactions were performed in 10 μ l volumes using 1 μ l of T₄ ligase and buffer respectively, with an appropriate volume of distilled water. Reactions were carried out overnight at room temperature. Vector only ligations were performed in parallel to indicate the efficiency of self-ligation.

2.10.6 Transformation of chemically competent cells

Chemically competent DH5- α or TOP10 cells (Invitrogen, USA) were transformed with ligation products as per manufacturer's guidelines. Cells were grown on LB agar plates containing the appropriate antibiotic.

2.10.7 Amplification and isolation of plasmids

5ml aliquots of LB Broth were inoculated with individual bacterial colonies and grown overnight at 37°C in an Innovar 44 shaker (New Brunswick Scientific, USA) set to 200 rpm. Plasmids were harvested using Qiagen Mini-Prep Kit (Qiagen, Germany) as per manufacturer's instructions. The Qiagen Maxi-Prep Kit (Qiagen, Germany) was used for large scale purifications

2.10.8 Identification of positive clones

Plasmids were subsequently subjected to restriction digest to identify positive clones. Sequencing of putative positive clones was performed by MWG, Germany.

2.11 Transfection and adenoviral infection of cell cultures

In this study siRNA oligonucleotides were delivered into cell lines and primary human myeloid cells in order to knock down specific proteins. Expression constructs and luciferase gene reporter constructs (see sections 2.10.1) were delivered into cell lines by transfection. Plasmids are inefficiently transfected into primary human myeloid cells therefore the aforementioned constructs were delivered into these cells using adenoviridae. The purification of adenoviral constructs was kindly performed by Mrs Alessandra Lanfrancotti (Kennedy Institute of Rheumatology, London, United Kingdom).

2.11.1 Transfection of siRNA oligonucleotides into cell lines

siRNA knockdown in cell lines was performed using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, UK) as per the manufacturer's instructions. Briefly, cells were seeded in 96-well plates in 100 µl penicillin/streptomycin free media. The following day the desired concentration of siRNA oligonucleotide was mixed with OptiMem (Invitrogen, UK) to a final concentration of 10 µl. Then 0.2 µl of RNAiMAX was added to 10 µl of OptiMem in a separate tube. These two mixtures were incubated for 5 min, then mixed together and incubated for a further 20 min at room temperature. The 20 µl mixture was then added to the culture media and incubated at 37°C overnight. The following day the supernatants were aspirated and replaced with standard media. 72 h post-transfection the desired experimental conditions were applied.

2.11.2 Transfection of siRNA oligonucleotides into primary human cells

siRNA knockdown in MDDCs and M1 macrophages was performed in 96-well plates (in triplicate) using the transfection protocol stated below. Cells were differentiated for 4 days under appropriate conditions, harvested (supernatant was collected and stored at 4°C until further use), resuspended in fresh RPMI (10% serum, no P/S) and seeded into 96-well plates at a density of 10^5 cells/ml. Next day, cells were washed gently once with RPMI (lacking phenol red, serum, and P/S) followed by adding 30 µl of RPMI (lacking phenol red, serum, and P/S) to each well. The desired concentration of siRNA oligonucleotide was incubated with 20 µl of OptiMem as well as 0.25 µl of DharmaFECT® Transfection Reagent I and incubated for 20 min at room temperature. The mixture was then added to the cells and incubated at 37°C for 2 h. The supernatant was subsequently replaced with 100 µl media, consisting of 50 µl fresh RPMI (10% serum, no P/S) and 50 µl of the old media (collected after differentiation). 48 h post transfection, 50 µl of fresh RPMI (10% serum, no P/S) was added to each well. 72 h post-transfection the desired experimental conditions were applied.

2.11.3 Transfection of plasmids into cell lines

Transfection of plasmids into cell lines was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, UK) as per the manufacturer's instructions. Briefly, cells were seeded appropriately and the following day the desired concentration of the plasmid

was added to 100 µl of Optimem. Then 4 µl of pre-warmed Lipofectamine was added to 200 µl pre-warmed Optimem. These two mixtures were incubated for 5 min, then mixed together and incubated for a further 30 min at room temperature. The media was then aspirated from the cells and replaced with the mixture containing the plasmid. The cells were incubated at 37°C for 2 h after which the supernatant was replaced with standard media. 48 h post transfection the desired experimental conditions were applied.

For luciferase gene reporter experiments, HEK-293–TLR4-CD14/Md2 cells were seeded onto polylysine-coated 96-well plates at a density of 30,000 cells per well. The next day, cells were transfected with 10 ng of the indicated expression vector, 50 ng of TNF luciferase reporter, and 50 ng of pEAK8-Renilla using Lipofectamine2000 and the above protocol. Total amount of DNA was kept at 120 ng per well. At 48 h after transfection, the reporter activity was measured using the Dual-Glo Luciferase system (Promega, Germany) optimised for 96-well plate format according to the manufacturer's protocol (Section 2.12). Each experiment was performed in triplicate.

2.11.4 Adenoviral infection of primary human myeloid cells

Adenoviral infections of primary human myeloid cells were performed in 96-well plates in triplicate. Cells were seeded appropriately and the following evening standard media was replaced with serum free RPMI containing the desired number of viral particles (at a multiplicity of infection of 50:1 unless stated otherwise) in a final volume of 50 µl. The plates were centrifuged at 1,200rpm for 30 min then placed at 37°C overnight. The following day the supernatants were aspirated and replaced with 100 µl of standard media and the cells were allowed to recover for 24 h before the application of experimental conditions.

For luciferase gene reporter assays in primary human cells, M2 macrophages were first infected with expression constructs coding for IRF3, IRF5 or empty vector for 2 h followed by aspiration of the supernatants and replacement with 100 µl of standard media per well and a recovery phase of 6 h. After that, cells were infected with indicated IL-10 luciferase constructs for 2 h followed by aspiration of the supernatants and replacement with 100 µl of standard media per well. Cells were allowed to recover for a further 24 h before experimental assay.

2.12 Luciferase gene reporter assays

Luciferase gene reporter assays allow the transcriptional activity of specific DNA sequences to be assessed in cell cultures. This technique utilises the firefly luciferase gene: a bioluminescent enzyme that catalyses the conversion of luciferin into oxyluciferin and light. The DNA sequence of interest was cloned directly upstream of the luciferase gene and the resulting genetic construct was transfected into the cell line of choice. The cells were then subjected to the desired experimental conditions and then the transcriptional activity of the respective DNA sequence was inferred by measuring photon release after the addition of luciferin to the cell lysate. In experiments with cell lines, cells were co-transfected with a plasmid encoding Renilla luciferase, which was under the control of the tyrosine kinase promoter. This promoter was not responsive to the experimental conditions, therefore measuring Renilla activity was used to normalise for transfection efficiencies, cytotoxic effects and differences in cell number. In experiments with primary human myeloid cells, luciferase activity was normalised by presenting data as fold induction.

Luciferase assays were performed in triplicate in 96-well plates using Dual-Glo™ Luciferase Assay System reagent (Promega, Germany). After transfection of cell lines (see section 2.11.3) or infection of primary human myeloid cells (see section 2.11.4) cells were stimulated as indicated in the result text. Supernatants were then harvested and 25 µl of PBS and the Dual-Glo™ Luciferase Reagent was added to each well respectively. Cells were placed on a shaker for 20 min after which lysates were transferred into luminometer plates and luciferase activity was measured using a 1450 MicroBeta® JET (PerkinElmer). 25 µl of Dual-Glo™ Stop & Glo® Reagent was then added to each well and after 10 min Renilla activity was measured. Data were normalised by dividing luciferase activity values by renilla activity values.

2.13 Immuno-precipitation

HEK-293-TLR4-CD14/Md2 cells were transfected with an onestrep-IRF5-HA construct or corresponding empty vector. 24 h post transfection cells were fixed with 1% formaldehyde for 10 min at room temperature prior to high salt lysis and affinity purification on Strep-Tactin MacroPrep sepharose (IBA, Germany). The eluates were de-crosslinked by incubating at 65°C overnight prior to separation by SDS-PAGE. Exogenous IRF5 and

endogenous RelA were detected by immunoblotting with anti-HA-HRP (12013819001; Roche, UK) and anti-RelA (sc-372; Santa-Cruz, US). Alternatively, cells were transfected with RelA-FLAG or BAP-FLAG control protein. 24 h post transfection cells were lysed and affinity purified with anti-FLAG M2 sepharose beads (Sigma, UK). Exogenous RelA and endogenous IRF5 were detected by immunoblotting with anti-FLAG-HRP (A8592; Sigma, UK) and anti-IRF5 (ab2932; Abcam, UK). Interaction of endogenous RelA and IRF5 was detected by overnight incubation of the cell lysates with goat anti-IRF5 antibody (ab2932, Abcam, UK) or no antibody control prior to precipitation with protein G beads. IRF5 was detected by immunoblotting with mouse anti-IRF5 antibody (sc-56714; Santa-Cruz, USA) and RelA with rabbit anti-RelA antibody (sc-372; Santa-Cruz, US).

2.14 Chromatin immuno-precipitation

Chromatin immuno-precipitation (ChIP) is a technique that allows the interaction between endogenous transcription factors and endogenous promoters to be analysed in cell cultures. Cells were subjected to the desired experimental conditions then fixed by adding formaldehyde which cross-links any protein-DNA interactions. Nuclear extracts were subsequently sonicated to shear the DNA, after which the transcription factor of interest, and any interacting DNA fragments, was immuno-precipitated with specific antibodies (see Table 2.10 for a list of antibodies used for ChIP). After rigorous washing, immuno-complexes were eluted, cross-linked interactions reversed and DNA fragments were purified. The immuno-precipitated DNA fragments were then interrogated by real-time PCR. Table 2.11 shows the list of primer sequences used for TNF ChIP analysis and Table 2.12 includes primer sequences for other gene targets. A list of all the buffers and their composition used in ChIP experiments can be found in section 2.19.5.

2.14.1 Fixation and preparation of nuclear extracts

$6-7 \times 10^6$ cells were seeded in 10 cm tissue culture dishes and stimulated the following day as desired. When handling non-adherent cells, the cells were collected into 14 ml falcon tubes and treated with 1% formaldehyde (final concentration) for 5 min at room temperature. Formaldehyde was then quenched by adding 125mM Tris (final concentration) pH 7.5, rinsed with ice cold PBS and placed on ice. 3 washes were performed with ice cold

PBS to remove the formaldehyde. When handling adherent cells, formaldehyde was added directly into the tissue culture dishes for 10 min, followed by quenching and washing, before harvesting cells by scraping. Cells were then pelleted by centrifugation at 1,200 rpm at 4 °C, resuspended in 900 µl of cytoplasmic lysis buffer and placed on ice for 5 min. Cell lysates were then transferred into 1.5 ml eppendorf tubes and spun in a microcentrifuge for 5 min at 4°C to pellet the nuclei. The supernatant was aspirated and the pellet was resuspended in 600 µl nuclear lysis buffer and stored at -80°C until required.

2.14.2 Sonication

Nuclear extracts were sonicated using a Vibra CellTM.VCX130 (Sonics). Each sample was subjected to 6 times 12 second pulses at 20% amplitude. To ensure that the DNA was sheared to a suitable size i.e. 500-1,000 base pairs, a 40-50 µl aliquot was removed from each sample and analysed by electrophoresis. Briefly, the cross-linking was reversed by placing the aliquots at 65°C overnight, followed by a standard phenol chloroform extraction and ethanol precipitation. The samples were resuspended in 30 µl of water, and 10 µl was loaded onto a 1% agarose TAE gel and run at 100V until the samples were sufficiently resolved to ascertain the DNA fragment size.

2.14.3 Immuno-precipitation

Unless otherwise stated all immuno-precipitation steps were carried out at 4°C with rotation. 120 µl of nuclear extract was used per immuno-precipitation. The sample was first diluted 10 times with dilution buffer to reduce the concentration of SDS, and then pre-cleared with 80 µl Protein G SepharoseTM bead slurry (GE Healthcare, USA) for 2 h. All sepharose beads used during ChIP experiments had previously been saturated with sonicated salmon sperm (Invitrogen, UK) to reduce any non-specific binding to the beads. The sepharose beads were then pelleted by centrifugation and the supernatants transferred to fresh tubes. 2 µg of the desired antibody was added to each sample, and incubated overnight. Immuno-complexes were then collected with 30 µl of protein G sepharose beads for 30 min, and subsequently washed 3 times with wash buffer, followed by 3 washes with TE. An aliquot of unbound sample was retained for normalisation purposes (the unbound material shall be referred to as the input). The beads were then resuspended in 125 µl of extraction buffer for 20 min, after which they were pelleted and the supernatant was transferred to a fresh tube.

Table 2.10: Antibodies used for ChIP

Antibody	Source	Catalogue no.	Supplier
IgG control	Rabbit	PP64	Milipore
IRF5	Goat	ab2932	Abcam
p65/RelA	Rabbit	sc-372	Santa Cruz
Pol II	Rabbit	sc-899	Santa Cruz

2.14.4 DNA purification

Immuno-precipitated complexes and “input” fractions were heated to 65°C overnight to reverse the cross-linked protein-DNA interactions. The DNA fragments were then purified using the QIAquick PCR Purification Kit (Qiagen, Germany) as per the manufacturer’s instructions. Immuno-precipitated DNA and “input” DNA were eluted in 200 µl and 300 µl respectively and stored at -20°C until required.

2.14.5 ChIP primer design

ChIP primers were designed using the free software Primer3 (<http://frodo.wi.mit.edu/primer3/>). The guidelines listed below were applied during primer design and followed as closely as possible.

- Amplicon of 100-200bp
- Primer length of 20-27bp (optimal 22)
- GC content of 40-60% (optimal 50%)
- T_m 63-67°C (optimal 65°C)
- T_m of forward and reverse primer must not largely differ (max. 1°C)
- Pairs should not have a complementary sequence of more than 2 bases at the 3' end
- Complementary sequence of more than 3 bases should not exist within a primer
- 3' end should not be T (optimal G or C)
- No more than 2 G or C within the last 5bp of 3' end

Several of the above parameters, like amplicon length or GC content, can be pre-set in the Primer3 software whereas others (3' end should not be T) have to be corrected manually.

Each single primer and primer pair was also analysed with the free software OligoAnalyzer (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>) in addition to the above rules. This program predicts the possibility of homodimer or hairpin formation of a single primer as well as the possibility of heterodimer formation between primer pairs. All primer sets used in this study have passed the computational analysis and guideline points.

2.14.6 ChIP primer testing and optimisations

Primer pairs were ordered (Eurofins MWG, Germany) and validated on genomic DNA (gDNA) which had been fixed and sonicated beforehand. A good primer set should have a cycle threshold (C_t , the cycle at which the fluorescence from a sample crosses the threshold) below 25 cycles and follow the dilutions of gDNA (e.g. dilution of gDNA 1 in 8 = 1 in 2^3 should equate to a decrease of the C_t value by 3 cycles). All primer sets used in this study were successfully tested for their C_t value and exponential function with serial dilutions of gDNA (data not shown).

When determining the C_t values of each primer pair, melt curves were also examined for sequence specificity. The high specificity of a primer set is manifested by the presence of only one peak, usually around 85°C, occurring during melt curve analysis. Therefore, the more peaks detected during this type of analysis the more non-specific regions have been amplified. Furthermore, multiple peaks can also be a sign of primer-dimer formation resulting in false-positive signals. Examples of poor (Figure 2.1a) and good (Figure 2.1b) melt curves are shown below.

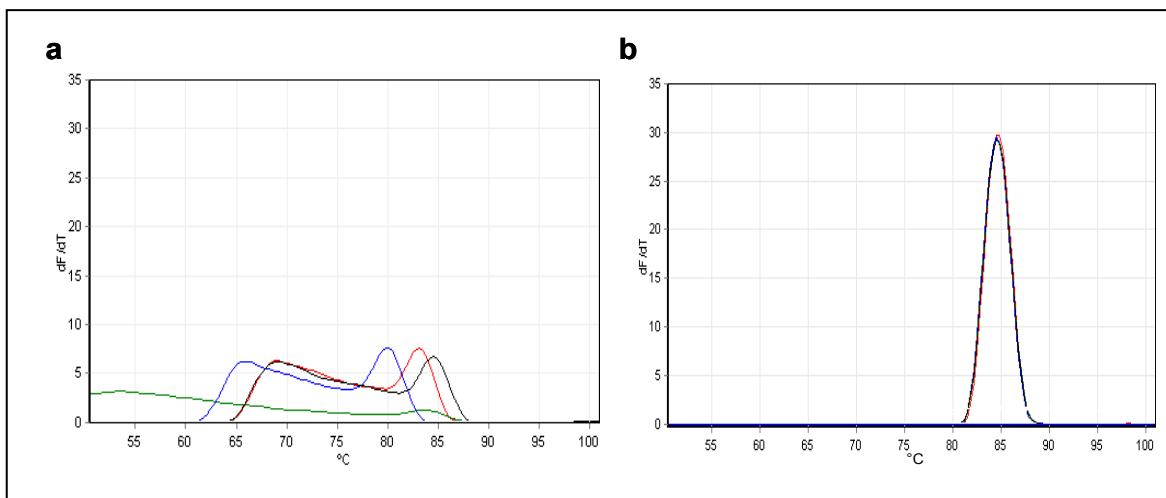


Figure 2.1 Melt curve analysis of ChIP primer pairs

(a) Melt curves of three unspecific primers (blue, red, black) and one non-working primer (green) tested on gDNA (fixed and sonicated). Primers were considered non-specific because two peaks, one around 65°C and one around 80°C, occurred indicating the amplification of two different regions.
(b) Melt curves of 4 primers (blue, red, black, green) amplifying the same regions as in **(a)**. Only one very sharp peak around 85°C is visibly after melt curve testing indicating high specificity of primers.

Another factor that can greatly influence primer behaviour and efficiency is the master mix used for ChIP RT-PCR. Two different types of SYBR Green master mixes were tested for ChIP RT-PCR, one from Applied Biosystems (SYBR[®]Green PCR master mix) and another from Takara Bio (SYBR[®]Premix Ex Taq[™] master mix II). The Takara master mix consistently out-performed the Applied Biosystems master mix for 10 primer sets that were tested and primer E is shown as a representative in Figure 2.2. There was a difference of 3 cycles between mastermixes equating to a 8 fold greater sensitivity with the Takara mastermix (shown in blue) over that from Applied Biosystems (shown in black). Therefore, all subsequent ChIP RT-PCR reactions were performed using the Takara SYBR[®]Premix Ex Taq[™] master mix II.

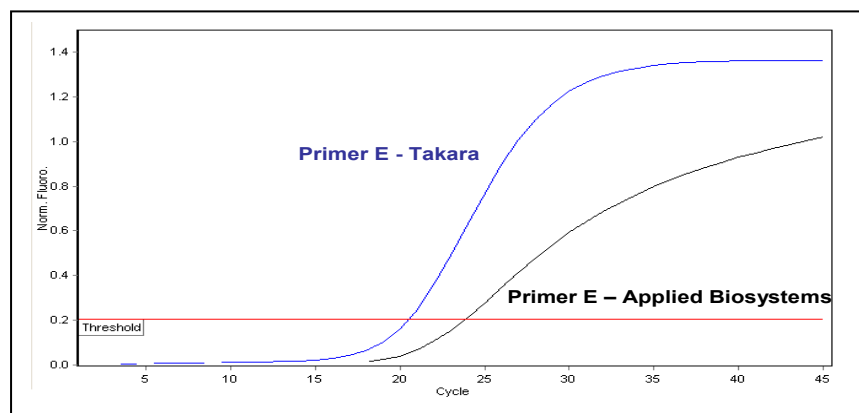


Figure 2.2 Improvement of C_t values with Takara master mix

10 different primers were run in parallel with two different master mixes. Primer E is representative and the corresponding C_t values are shown in blue (Takara, SYBR®Premix Ex Taq™ master mix II) and black (Applied Biosystems, SYBR®Green PCR master mix).

2.14.7 Real-time PCR analysis

Analysis of DNA fragments were performed in a Corbett Rotor-gene 6000 machine (Corbett Research Ltd, UK) and analysed using Corbett Rotor-Gene 6000 software. All reactions used SYBR®Premix Ex Taq™ master mix II (Takara Bio, USA), 5 µl of DNA and a final primer concentration of 0.1 µM. Primers used for TNF analysis are shown in Table 2.11 and for other gene targets in Table 2.12. The following thermal cycling conditions were used:

30 seconds at 95°C

40 cycles of: 10s at 95°C + 25s at 60°C

Table 2.11: ChIP primers used for analysing human TNF locus:

Region	Binding sites	Forward primer 5'-3'	Reverse primer 5'-3'
control	no ISRE no κB	TGTGTGTCTGGGAGTGAGAACT	TCTTCTCAGCTTCTCCTTTGCT
A	NF-κB 1	CCACAGCAATGGGTAGGAGAATGT	GAGGTCCTGGAGGCTCTTTCACT
B	ISRE 1+2 / NF-κB 2/ζ/2a	GGAAGCCAAGACTGAAACCAGCA	CCGGAATTCACAGACCCCACT
C	ISRE 3+4	TCCCTCCAACCCGTTTTCT	TAGGACCCTGGAGGCTGAAC

D	ISRE 5	AAC TTTC CAAAT CCCC GCCC	GGTGTGCCAACAACTGCCTT
E	ISRE 6+7+8 / NF- κ B 3	CAGCAAGGACAGCAGAGGAC	TCCCGGATCATGCTTTCAGT
F	ISRE 9+10	GGCAGTCAGTAAGTGTCTCCAA	TACCTACAACATGGGCTACAGG
G	ISRE 11	ACAGCTTTGATCCCTGACATCT	CTCCGTGTCTCAAGGAAGTCTG
H	no ISRE NF- κ B 4/4a/4b	ATATTCCCCATCCCCCAGGAAACA	CTGCAACAGCCGGAAATCTCACC
I	ISRE 12	GAGGACCTCACTCAGCCCTT	CGGCAGTTCGGTTCCTTGTT
J	ISRE 13	ACTGGTCTTTGTGGTGAAGGAG	GAACTAGTGGGCTCAAGTGGTC
K	ISRE 14	GCTATGATCATGCCACTGTACCC	TACCACATGGTTTTCTCCTGCC
L	ISRE 15	GCTGAAAGTCAGCCATGAAGTA	CACTTAGGGTGTCCCATTAGG

Each primer pair is specific for the designated region of the human TNF locus. The types of transcription factor binding sites present in each region are listed under column “binding sites”.

Table 2.12: Primers used for ChIP analysis:

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
IL-10	CCTGTGCCGGGAAACCTTGATTGTGGC	GTCAGGAGGACCAGGCAACAGAGCAGT
IL-12p35	TCATTTTGGGCCGAGCTGGAG	TACATCAGCTTCTCGGTGACACG
IL-12p40	TCCAGTACCAGCAACAGCAGCAGA	GTAGGGGCTTGGGAAGTGCTTACCTT
IL-23a	ACTGTGAGGCCTGAAATGGGGAGC	ACTGGATGGTCCTGGTTTCATGGGAGA

Each primer pair is specific for the designated human gene and its promoter region.

2.15 Flow cytometry (FCM) analysis

Cells were stained for surface markers with desired antibodies in FCM buffer for 30 min at 4°C. Cells were then washed twice with FCM buffer and fixed with Cytofix solution (BD Bioscience, UK) for 15 min at RT. Cells were wash again twice and either analysed immediately or stored for up to 5 days at 4°C.

For intracellular cytokine (ICC) stainin, cells were stimulated for 3-4 h with phorbol myristate acetate (PMA), ionomycin and Brefeldin A (Sigma, UK). Cells were then stained for surface markers as described above followed by fixing in Cytofix solution (BD Bioscience,

UK). Cells were washed twice with FCM buffer and permeabilized for 30 min at 4°C using PBS containing 1% FCS, 0.01% sodium azide, and 0.05% saponin. Intracellular cytokines were stained with desired antibodies in FCM permeabilization buffer for 30 min at 4°C. Cells were then washed twice with FCM permeabilization buffer followed by resuspension in FCM buffer. ICC stained cells were always analysed immediately. Samples were run on a FACS Canto II (BD Bioscience, UK) and analysed using FlowJo software (TreeStar, USA). A list of antibodies used in this study is shown in Table 2.13

Table 2.13: Antibodies used for FCM analysis

Antibody	Conjugate	Clone	Catalogue no.	Supplier
CD4	PE-Cy7	RPA-T4	25-0049	eBioscience
CD8a	PerCP-Cy5	RPA-T8	45-0088	eBioscience
CD40	APC	5C3	17-0409	eBioscience
CD163	PE	215927	FAB1607P	R&D Systems
IFN- γ	PB	S4.B3	48-7311	eBioscience
IL-17A	PE	eBio64CAP17	12-7178	eBioscience

2.16 Mixed lymphocyte reactions (MLR)

2.16.1 MLR with MDDCs as APCs

Human MDDCs were plated in 96-well, flat-bottom tissue plates at 2×10^4 cells/well. T lymphocytes were isolated from the blood of healthy MHC mismatched donors by elutriation, analysed by FCM and used if purity was >90%. T lymphocytes were added to MDDCs at 5×10^5 , such that the final MDDC to T lymphocyte ratio was 1:25. Control cultures contained medium, T lymphocytes or MDDCs alone. 10 μ g/ml of anti-TNFR1 antibody (MAB 625, R&D Systems) or IgG control antibody (MAB 002, R&D Systems) was added to the co-cultures after 6 h or 24 h were indicated. Cultures were established in duplicate and incubated at 37°C in 5% CO₂ for a total of 72 h. Following culture, supernatants were collected and stored at -20°C for later detection of cytokines.

2.16.2 MLR with macrophages as APCs

Human M2 macrophages were plated in 96-well plate as in section 2.12.1. M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) where indicated. Following infection, cells were left to recover for 24 h before addition of 5×10^5 T lymphocytes per well. Control cultures contained medium, T lymphocytes or M2 macrophages alone. After 72-96 h of co-culture supernatants were collected for detection of cytokines. In certain experiments, T lymphocytes were harvested 48 h after co-culture for mRNA analysis. For proliferation experiments, cells were pulsed with 37,000 Bp of [^3H] thymidine (Amersham Biosciences, USA) 16 h before harvest and DNA synthesis was measured by [^3H] thymidine incorporation using a Beckman beta scintillation counter (Beckman Instruments, USA). The [^3H] thymidine incorporation assays were kindly performed by Dr Saba Alzabin (Kennedy Institute of Rheumatology, London, United Kingdom).

2.17 Bioinformatic analysis

Genomic sequences were obtained using the publicly available UCSC Genome Browser website (<http://genome.ucsc.edu/>) and the Ensembl website (<http://www.ensembl.org/index.html>). Predicted transcription factor binding sites were obtained using the websites Genomatix (<http://www.genomatix.de/>) and JASPAR (<http://jaspar.cgb.ki.se/>). The website Primer 3 (<http://frodo.wi.mit.edu/>) was used for primer design in general whereas the program OligoAnalyzer (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>) was utilised for predicting the possibility of hairpin, self- or heterodimer formation of primers.

2.18 Statistical analyses

Quantitative data are presented as mean $\pm$ standard deviation or standard error as indicated. All statistical analyses were performed using GraphPad Prism 5.0 software. Experiments containing only two measurements were analysed using a T-test. Experiments containing three or more measurements were analysed using one-way ANOVA.

2.19 Buffers and Solutions

2.19.1 General buffers

PBS with pH 7.2:

4.3mM Na₂HPO₄

1.4mM KH₂CO₃

1.4mM KCl

137mM NaCl

FCM buffer

1% BSA in PBS

0.01% sodium azide

FCM permeabilization buffer

PBS

1% FCS

0.01% sodium azide

0.05% saponin

2.19.2 SDS-PAGE and Western blotting

Whole cell lysis buffer for cell lines:

20mM Tris pH 8.0

300mM NaCl

0.1% NP-40

10% glycerol

Whole cell lysis buffer for primary human myeloid cells:

20mM Tris pH 8.0

150mM NaCl

1% NP-40

10% glycerol

Cytoplasmic lysis buffer:

10mM HEPES, PH 7.9

10mM KCl

0.1mM EDTA

0.1mM EGTA

Nuclear lysis buffer:

20mM HEPES, pH 7.9

400mM NaCl

1mM EDTA

1mM EGTA

4X loading buffer:

250mM Tris, pH 6.8

6% SDS

40% Glycerol

0.04% Bromphenol Blue

20% 2-mercaptoethanol

SDS-PAGE running buffer:

25mM Tris-base

192mM Glycine

0.1% SDS

Transfer buffer:

25mM Tris-base

192mM glycine

10% methanol

Blocking buffer:

PBS

5% dried milk powder

0.1% tween

Wash buffer:

0.1% tween in PBS

2.19.3 ELISA buffers

ELISA blocking buffer

2% BSA in PBS

ELISA dilution buffer

0.5% BSA in PBS

ELISA wash buffer

0.1% Tween 20 in PBS

ELISA stop solution

940ml H₂O

60ml H₂SO₄

2.19.4 EMSA

2x Binding buffer

20mM Hepes, pH 7.9

2mM EDTA

2mM EGTA

25% glycerol

0.125-0.250 mg/ml dl-dC

2.19.5 Chromatin immuno-precipitation

Cytoplasmic lysis buffer:

50mM Tris, pH 8.0

2mM EDTA, pH 8.0

0.1% NP40

10% Glycerol

Nuclear lysis buffer:

50mM Tris pH 8.0
5mM EDTA
1 % SDS

Dilution buffer (DB):

50mM Tris, pH 8.0
5mM EDTA
200mM NaCl
0.5 % NP40

Wash Buffer (WB):

20mM Tris, pH 8.0
2mM EDTA
0.1 % SDS
1 % NP40
500 mM NaCl

Extraction Buffer (EB):

1X TE
2 % SDS

2.19.6 Molecular biology solutions

Lennox L broth base (LB)

20g LB (Invitrogen, UK)
1l H₂O
Autoclave before use

Lennox L agar (LB agar)

32g LB agar (Invitrogen, UK)
1l H₂O
Autoclaved, added antibiotics once cooled and poured into plates

Ampicillin

100 mg/ml Ampicillin (Sigma, UK)

Diluted in autoclaved H₂O and stored at -20°C

Kanamycin

30 mg/ml Kanamycine (Sigma, UK)

Diluted in autoclaved H₂O and stored at -20°C

SYBR Green II

Add 1:10 to DNA products

Store stock at -20°C

Ethidium bromide

Used at a final concentration of 0.1 µg/ml (Sigma, UK)

Stored at 4°C

3. IRF5-mediated human TNF gene expression

3.1 Introduction

As well as playing a central role in host defence against infection, TNF is a major factor in the pathogenesis of chronic inflammatory disease such as RA and IBD [282]. Its central role in the pathogenesis of RA is exemplified by the success of anti-TNF therapy developed at the Kennedy Institute of Rheumatology, which has proved to be therapeutically successful in more than 500,000 RA patients world-wide. However, the high cost, limited convenience of injections and risk of systemic side-effects are the major reason for further research into the molecular mechanisms that govern inflammatory response and identification of new targets for specific and ideally orally administered therapeutic intervention.

Regulation of transcription for many immune genes in response to TLR signalling involves a combination of NF- κ B and IRF factors [283]. IRFs appear to provide a mechanism for conferring signal specificity to a variety of target gene subsets, with IRF3 being essential for type I IFN response [284] and a suggestive role for IRF5 in the transcriptional regulation of pro-inflammatory cytokines, including TNF, IL-6 and IL-12p40 [190]. However, the molecular mechanisms underlying the role of IRF5 in regulating murine TNF gene expression were not further investigated by Takaoka and co-workers and are therefore poorly understood. Moreover, the contribution of IRF5 to the human TNF gene induction remains currently unknown.

The initial goal of this study was to clarify and establish conclusively the molecular mechanisms employed by IRF5 to regulate human TNF gene expression. In the focus of this study were primary human myeloid cells, as those are the major producers of TNF, and thus it has an immediate relevance to the human inflammatory response.

Observations made in this part of the study paved the way for the detailed investigation of the role of IRF5 in macrophage polarization and are presented in Chapter 4 and 5 of this thesis.

3.2 Results

3.2.1 IRF5 expression is high in MDDCs and controls late phase TNF secretion

Myeloid cells from *Irf5*^{-/-} mice show impaired induction of pro-inflammatory cytokines including TNF upon stimulation by different TLR ligands [190]. I therefore first examined levels of IRF5 protein following human monocyte differentiation into MDMs (in the presence of M-CSF) and MDDCs (in the presence of GM-CSF plus IL-4). No increase in the levels of IRF5 protein was observed in MDMs even after 5 days of differentiation (Figure 3.1). However, increased expression of IRF5 protein was detected following 1 day of monocyte differentiation into MDDCs and remained at an elevated level until day 7 (Figure 3.1). Significantly, whereas at least three different IRF5 isoforms were observed in human monocytes, only some of them accounted for high levels of IRF5 protein in MDDCs: one is likely to be IRF5v3/v4 [180]. Other IRF family members were not expressed in resting cells as expected (IRF7) or did not show this pattern of cell-type specific expression (IRF3 and IRF9; Figure 3.1).

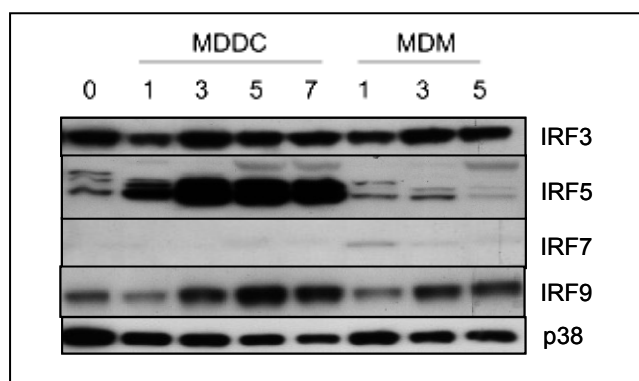


Figure 3.1 IRF5 is highly expressed in MDDCs

Cells were collected at day 0 (monocytes); day 1, 3, 5 and 7 (MDDCs) post differentiation with GM-CSF (50 ng/ml) and IL-4 (10 ng/ml); day 1, 3 and 5 post differentiation with M-CSF (50 ng/ml) (MDMs) and total protein extracts were subjected to western blot analysis with antibodies to IRF family members. p38 MAPK was used as loading control. A representative blot of 5 independent experiments each using monocytes derived from a different donor.

Next, I wanted to know if MDMs and MDDCs differ in their production of TNF in response to TLR4 stimulation. TNF protein is below the limit of detection in the supernatants of resting cells (Figure 3.2a). Following 4 h of LPS stimulation, TNF is secreted at similar levels in MDMs and MDDCs (early phase). However, a marked difference in TNF production was observed in MDMs and MDDCs stimulated with LPS for 24 h (late phase). While the level of TNF significantly decreased in MDMs, there was an increase in TNF levels in MDDCs (Figure 3.2a) in each individual blood donor (Figure 3.2b).

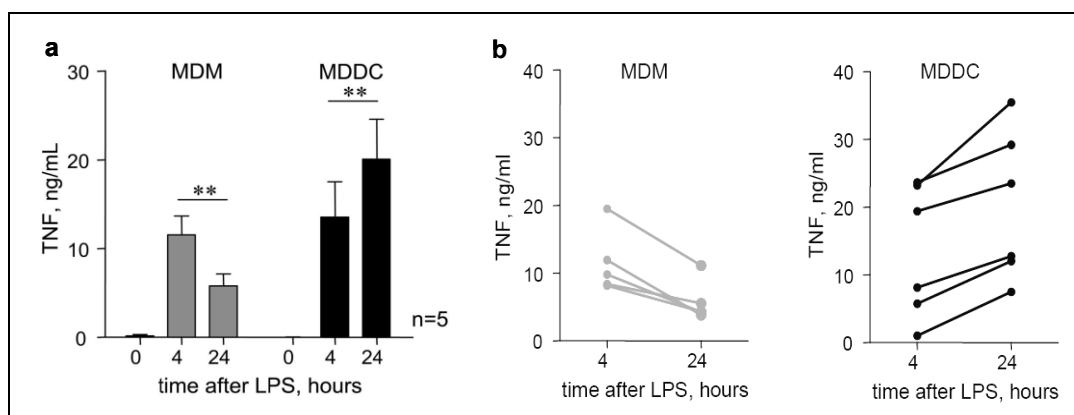


Figure 3.2 Prolonged TNF secretion in MDDCs

(a) MDDCs and MDMs were left unstimulated or stimulated with LPS (10 ng/ml) for 4 h or 24 h and secreted TNF was measured by ELISA. Data show mean $\pm$ standard error of the mean (SEM) of 5 independent experiments each using monocytes derived from a different donor: * $p < 0.05$, ** $p < 0.01$ (Student's T-test). **(b)** Data show the trend in TNF secretion in 5 (MDMs) or 6 (MDDCs) donors stimulated as in (a).

Human TNF acting through TNF receptor 1 (TNFR1, also known as p55) is involved in activation of Th1 lymphocytes, measured by the release of Interferon gamma (IFN- γ) [285]. Thus, I examined whether the late phase secretion of TNF by MDDCs is needed for IFN- γ production by T lymphocytes in a mixed lymphocyte reaction (MLR). A MLR is an *in vitro* method for assessing T lymphocyte proliferation and activation state. This assay is based on the observation that T lymphocytes will get activated and expand when they are cultured together with allogeneic (MHC mismatched haplotype) APCs. The proliferation of the lymphocyte population can be analysed by measuring the incorporation of [3 H] thymidine

(which occurs during each cell division) and the activation state can be assessed by measuring T lymphocyte-specific cytokines in the culture supernatants by ELISA.

One essential variable of the MLR that needs to be optimised is the ratio of APCs to T lymphocytes. I started by setting up MLRs with MDDCs (used as the APC population) isolated from 2 donors and T lymphocytes isolated from 2 different (MHC mismatched) donors. I used two independent donors per population in order to minimise the effect of donor variations. The MDDCs from the two donors were plated in 96-well plates at a concentration of 2×10^4 cells per well and stimulated for 2 h with LPS prior to co-culture with each T lymphocyte population at a ratio of either 1:1, 1:2, 1:5, 1:10, 1:25, 1:50 or 1:100. Cultures were set up in duplicate and the amount of secreted IFN- γ was determined after 72 h of co-culture by ELISA. Two independent donors per cell population supplied me with 4 supernatants per cell ratio helping me to determine the right culture conditions. The highest amount IFN- γ was detected in the 4 co-cultures containing a MDDC to T lymphocyte ratio of 1:25 (data not shown) and this ratio was subsequently used in MLR assays.

After establishing optimal cell culture conditions I started to examine the importance of late phase TNF secretion by MDDCs for T lymphocytes activation state. MDDCs were stimulated with LPS for 2 h and exposed to T lymphocytes from a MHC mismatched donor at a ratio of 1:25. Antibodies against TNF receptor 1 (TNFR1) or isotype IgG control were added to the reaction. T lymphocytes incubated with MDDCs treated with anti-IgG antibodies produced high levels of IFN- γ (Figure 3.3), while the control reactions (MDDCs or T lymphocytes cultured on their own) secreted no detectable IFN- γ (data not shown). Blocking TNF 6 h after co-culture start resulted in strong reduction of IFN- γ , but no effect was observed when anti-TNFR1 antibodies were added to the reaction after 24 h, suggesting that most of T lymphocytes are in activated state after the prolonged exposure to TNF (Figure 3.3). This suggests that the observed sustained expression of TNF by MDDCs is involved in establishing a robust Th1 phenotype.

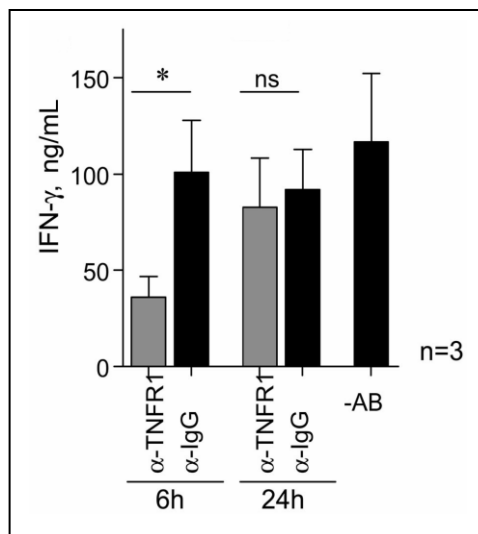


Figure 3.3 Late phase TNF secretion is required for potent T lymphocyte response

MDDCs were stimulated with LPS for 2 h and then co-cultured with T lymphocytes. Anti-TNFR1 (grey bars) or anti-IgG control antibodies (black bars) were added 6 h or 24 h after co-culture start. IFN- γ secretion was determined by ELISA after 72 h of co-culture. Data show mean $\pm$ SEM of 3 independent experiments each using cells derived from a different donor. * $p < 0.05$ (Student's T-test). -AB, a no antibody control.

Based on the above results, I hypothesised that the difference in TNF secretion profile in MDDCs and MDMs might be due to the difference in IRF5 expression in these cells. To test this hypothesis, MDMs that have low levels of endogenous IRF5 protein (Figure 3.1) were infected with adenoviral expression vector encoding IRF5, IRF3 (as a control) or an empty vector (pBent).

Adenoviruses are efficient tools for delivering transgenes to cells both *in vitro* and *in vivo*. The expression of transgenes was limited to transfection of cell lines and not possible in primary human cells prior to the discovery of adenoviruses. There are now many different delivery strategies, such as retroviral, lentiviral and adenoviral systems, that exploit the ability of viruses to transport DNA to cells. The advantage of the adenovirus system is the fact that they will infect both proliferating and quiescent cells making them suitable for targeting terminally differentiated cells such as macrophages. Disadvantages include the transient transfection of cells as viral DNA will not integrate into the host genome and the initiation of an immune response following virus administration. It is this immune response

that can be a potential problem when working with myeloid cells such as macrophages. However, I routinely analysed the secretion of endogenous IFN- λ 1 protein as well as mRNA induction of type I IFNs and observed no significant effect on resting cells 48 h or 72 h post infection. Another crucial issue is the degree of overexpression of the transgene. If a protein is overexpressed at a massive level, e.g. 100-1000-fold greater expression than the endogenous protein, resulting phenotypes could be artifacts.

IRF5 and IRF3 vectors expressed similar levels of proteins (Figure 3.4a, anti-HA blot) and blotting for endogenous IRF5 indicated an increased expression but not a swamping of the system (Figure 3.4a, anti-IRF5 blot). The overexpression of IRF5 resulted in a significant increase in TNF secretion in unstimulated cells (Figure 3.4b) which was not observed in control or IRF3 expressing cells. Consistent with previously published data, IRF3 induced IFN- λ 1 secretion, demonstrating the functionality of this construct [286], whereas ectopic IRF5 showed no effect (Figure 3.4c). Strikingly, TNF secretion in MDMs with overexpression of IRF5 remained at a steady sustained level up to 48 h post LPS stimulation (Figure 3.4d), similar to that of MDDCs with high levels of endogenous IRF5 (Figure 3.2a).

Finally, I looked at the effect of ectopic IRF5 expression in MDMs on T lymphocyte activation. MDMs were infected with adenoviral expression vector encoding IRF5 or the corresponding empty vector pBent. Exposure of T lymphocytes to MDMs with endogenous IRF5 protein resulted in an increase of IFN- γ secretion to the levels comparable to T lymphocytes exposed to MDDCs (Figure 3.4e). Taken together, these results suggest that sustained TNF secretion by MDDCs leads to a robust T lymphocytes activation and is likely to be a consequence of a high amount of IRF5 protein in these cells.

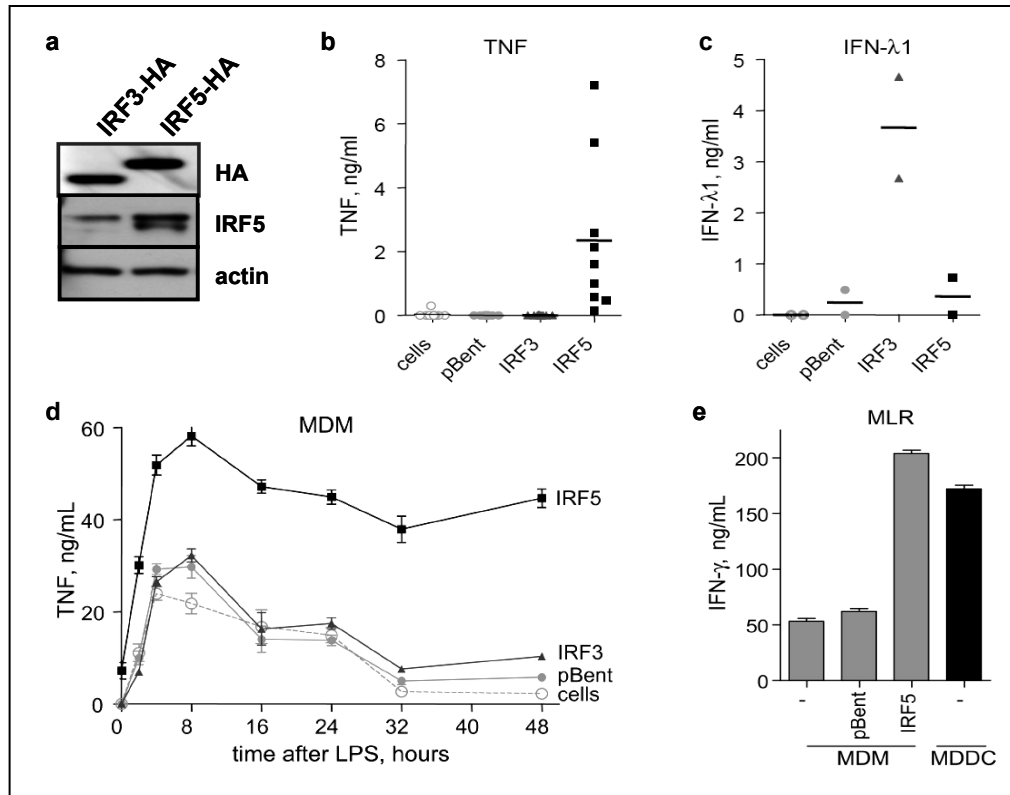


Figure 3.4 Ectopic expression of IRF5 in MDMs results in prolonged TNF secretion

(a) MDMs were infected with adenovirus encoding IRF5-HA or IRF3-HA. The expression of each construct was determined 48 h post-infection by subjecting equal amounts of whole cell protein lysates to western blot analysis and probing with anti-HA or anti-IRF5. Actin was used as a loading control and a representative blot is shown. **(b)** MDMs were left untreated (cells) or infected with adenovirus coding for IRF5, IRF3 or empty vector (pBENT) and the amount of secreted TNF protein in unstimulated cells was measured by ELISA. Data show 9 independent experiments each using MDMs derived from a different donor. **(c)** The 8 h post LPS supernatants from (b) were analysed by ELISA for IFN-λ1 secretion. Data show 2 independent experiments each using MDMs derived from a different donor. **(d)** MDMs were treated as in (a) and stimulated with LPS for the indicated times. The amount of secreted TNF protein was determined by ELISA. Data show mean $\pm$ standard deviation (SD) and are representative of 3 independent experiments each using MDMs derived from a different donor. **(e)** MDMs were left untreated (-) or infected with adenoviral vectors encoding IRF5 or empty vector pBent, stimulated with LPS for 2 h and co-cultured with T lymphocytes. IFN-γ secretion was determined by ELISA after 72 h of co-culture. Data show mean $\pm$ (SD) and are representative of 3 independent experiments each using MDMs derived from a different donor.

3.2.2 si-RNA mediated depletion of IRF5 reduces late-phase TNF secretion

Next, I wanted to know what the effect of the complementary experiment, targeting endogenous IRF5 in MDDCs by RNA-mediated interference, would have on TNF production. RNA interference (RNAi) is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid inhibits the expression of genes with complementary nucleotide sequences. A set of four individual On Target Plus short interfering RNA (siRNA) sequences targeting the human IRF5 gene were purchased from Dharmacon, UK.

I first attempted to optimise the transfection protocol in the cell lines HT1080 (data not shown) and HEK-293-TLR4-CD14/Md2 (Figure 3.5) before targeting IRF5 in MDDCs as the transfection of primary human cells is known to be difficult. A pool of 4 random non-targeting siRNA sequences (siC) was used as a control for non-sequence-specific effects. Changes in protein levels in cells treated with siC reflect a non-specific baseline of cellular response to which the levels in cells treated with target-specific siRNA can be compared. Unfortunately, neither the individual sequences nor their pool showed IRF5 knock-down at the protein level. Technical difficulties with the transfection itself were discounted as a potential problem as the siRNA pool targeting RelA worked well and equal loading of protein onto the protein gel was confirmed by the equal amounts of β -actin detected in the lysates.

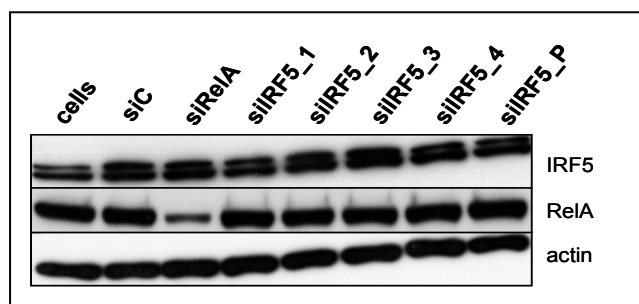


Figure 3.5 siRNA-mediated depletion of IRF5 is not possible in HEK-293-TLR4 cells

HEK-293-TLR4-CD14/Md2 cells were transfected with non-targeting siRNA (siC), individual siRNAs targeting IRF5 (siIRF5_1 to siIRF5_4), a pool consisting of these four sequences (siIRF5_P) or siRNA targeting RelA. Whole cell protein lysates were subjected to western blot analysis 72 h post transfection and IRF5 protein levels were compared to siC treated cells. Actin was used as a loading control. A

The above experiment was repeated in HEK-293-TLR4-CD14/Md2 cells in order to exclude individual errors. Furthermore, quantitative RT-PCR was performed in addition to western blot analysis to assess the knock-down of IRF5 at mRNA level as siRNA interferes with protein expression by targeting the respective mRNA for degradation. Each individual siRNA targeting IRF5 as well as the pooled sequences showed convincing depletion of IRF5 mRNA expression (data not shown) yet no effect on protein level was observed.

The following experiments were performed in order to further optimise IRF5 protein knock-down in cell lines:

1. Transfection of cells for a longer period (western blot analysis 96 h and 120 h post-transfection; data not shown)
2. The amount of siRNA was increased up to 100nM (data not shown)
3. Additional sequences targeting IRF5 were tested (data not shown)
4. Double transfection (second transfection 48 h after the first one, western blot analysis 96 h after first transfection)

None of the conditions stated above resulted in the knock-down of IRF5 protein although a decrease of IRF5 mRNA was observed. This led to the conclusion that the IRF5 protein was likely to be a very stable protein with a long half-life. The half-life of a protein can be assessed by blocking protein re-synthesis with cycloheximide followed by monitoring protein levels over time. Interestingly, no changes of IRF5 protein levels, even at later time points (8 h), were observed when cycloheximide was added to HEK-293-TLR4-CD14/Md2 cells (data not shown). I concluded that the knock-down of IRF5 in the cell lines tested was not working likely due to cell type specific reasons and decided to attempt the knock-down in MDDCs. As a rationale, these cells have at least 3 times more endogenous IRF5 protein compared to the cell lines used and are non-dividing cells where the loss of siRNA sequences during cell division should not occur. Initial experiments in MDDCs showed only a minimal depletion of IRF5 protein prompting me to further optimise the transfection protocol and the final working conditions are listed in the methods section 2.10.2.

The protein knock-down of IRF5 in MDDCs was usually between 50 and 60% (Figure 3.6a), which is comparable to other RNAi knock-downs achieved in these cells (e.g. RelA, data not shown). The expression of both protein and mRNA of other IRF family members (IRF3, IRF7 and IRF9) was not affected in si-IRF5 treated cells (data not shown). Although other potential off-target effects cannot be ruled out, this conferred some confidence as to the

sequence specificity of the si-IRF5 pool used. I also analysed the induction of type I IFNs, known to be a major obstacle for RNAi experiments, on mRNA level and found it to be minimal (3 fold induction compared to untreated cells, data not shown). However, the siRNA-mediated depletion of IRF5 in MDDCs resulted in reduction of TNF secretion at 8 and 24 h post LPS stimulation (Figure 3.6b) supporting the notion that IRF5 may be required for the late phase TNF expression.

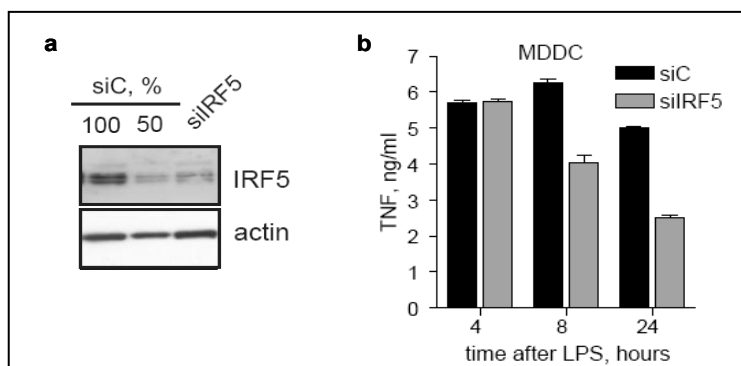


Figure 3.6 siRNA-mediated depletion of IRF5 affects late phase secretion of TNF

(a) MDDCs were transfected with siRNAs targeting IRF5 (siIRF5) or control siRNA (siC). ~50% of IRF5 protein was degraded as estimated by serial dilutions of the siC control sample analysed by western blotting. **(b)** MDDCs were transfected with siIRF5 and stimulated with LPS (10 ng/ml) for the indicated time. TNF secretion was compared to control cells transfected with siC. Data shown are the mean $\pm$ SD and are representative of 2 independent experiments each using MDDCs derived from a different donor.

3.2.3 IRF5 is involved in transcriptional regulation of TNF

I next sought to investigate whether IRF5 is involved in transcriptional regulation of TNF gene expression. In human MDDCs stimulation with LPS resulted in a rapid up-regulation of TNF mRNA expression, which reached the peak between 1 and 2 h but remained at a steady level until 8 h post stimulation (Figure 3.7a). Consistent with the observed differences in protein secretion, TNF mRNA expression in MDMs was characterised by more transient kinetics (Figure 3.7a). The siRNA-mediated depletion of IRF5 in MDDCs reduced TNF mRNA expression (Figure 3.7b) and the observed inhibition was statistically significant when analysed in multiple blood donors (Figure 3.7c). In the same cells siRNA-mediated inhibition of NF- κ B RelA, a transcription factor previously shown to be important for an efficient TNF production by human MDDCs [287], resulted in reduction of TNF mRNA

expression at the initial phase of gene induction (1-2 h post LPS stimulation) (Figure 3.7b). Within this time window, depletion of both IRF5 and RelA had the strongest effect on mRNA expression (Figure 3.7b), indicating that RelA and IRF5 may cooperate in controlling transcription of the TNF gene.

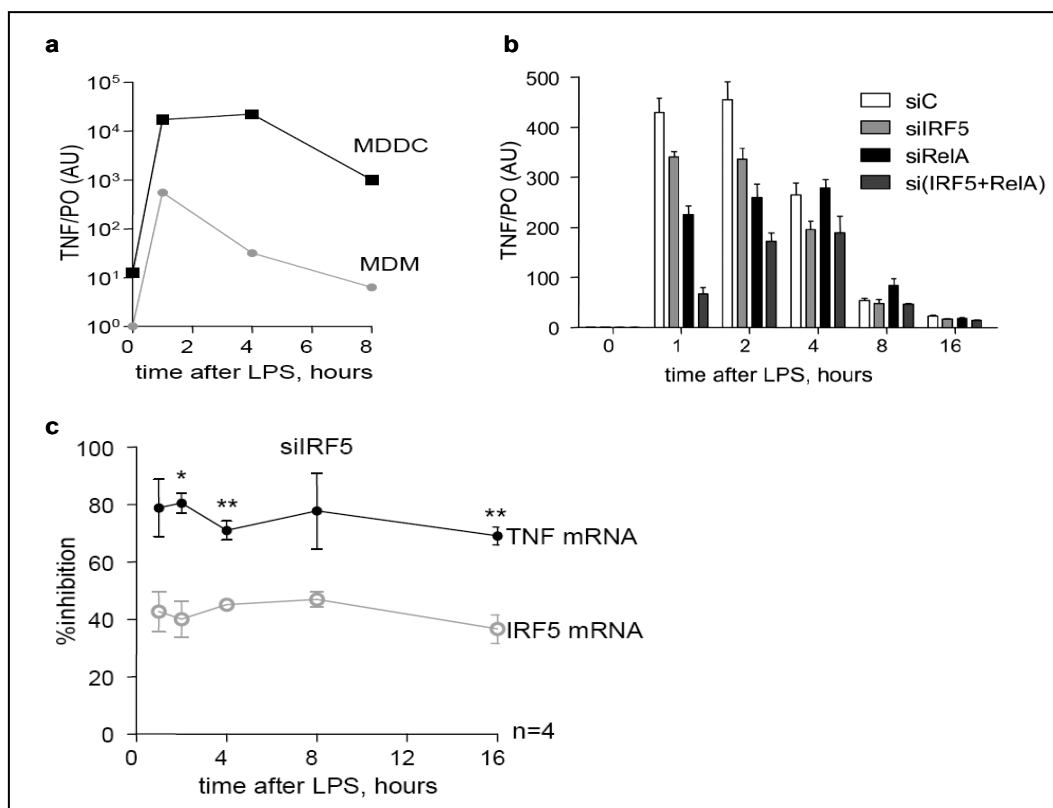


Figure 3.7 LPS-induced expression of TNF mRNA is IRF5 dependent

(a) MDDCs and MDMs from the same donor were stimulated with LPS for the indicated time and TNF mRNA expression was determined by 2-standard curve RT-PCR. Data shown are from a representative experiment. (b) MDDCs were transfected with siRNAs targeting IRF5 (siIRF5), RelA (siRelA) or both (si(IRF5+RelA)) and stimulated with LPS (10 ng/ml) for the indicated time. TNF mRNA expression was compared to control cells transfected with non-targeting siRNA (siC). Data shown are the mean $\pm$ SD and are representative of 4 independent experiments each using MDDCs derived from a different donor. (c) MDDCs were transfected with siRNAs targeting IRF5 (siIRF5) or control siRNA (siC). ~60% of IRF5 mRNA was degraded and affected LPS-induced TNF mRNA expression in MDDCs. Data shown are the mean $\pm$ SEM of 4 independent experiments presented as a % of reduction in TNF mRNA levels by siIRF5: * $p < 0.05$, ** $p < 0.01$ (Student's T-test).

To investigate whether IRF5 can directly modulate transcription of the TNF gene, I used a gene-reporter plasmid in which the luciferase gene was flanked with 1171 bp 5' upstream and 1252 bp 3' downstream of the human TNF gene (TNF 5'wt/3'wt). This construct encompassed all evolutionary conserved sequences in the region and contained known κ B sites [288, 289]. It was generated by combining a luciferase construct containing the 1171 bp 5' upstream region of the TNF gene (pGL3 TNF 5'wt) and a construct containing the 1252 bp 3' downstream region (TOPO TA TNF 3'wt, kindly provided by Dr. Tim Smallie, Kennedy Institute of Rheumatology, London, United Kingdom). The 3' downstream region was excised from the TOPO TA vector with XbaI/Sall and sub-cloned into the pGL3 TNF 5'wt luciferase construct using the same restriction enzymes. Positive clones were identified by restriction digest and sequencing.

The TNF 5'wt/3'wt was co-expressed with constructs coding for IRF5, IRF3 and NF- κ B subunits in HEK-293-TLR4-CD14/Md2 cells, and luciferase activities were compared to empty vector pBent. RelA and IRF5 transfected cells showed a significant increase in luciferase activity (Figure 3.8a). Other NF- κ B subunits or IRF3 had little or no effect (Figure 3.8a and b). Of interest, a deletion of the IRF5 DNA-binding domain (IRF5 Δ DBD) or a point alanine to proline mutation in it (IRF5 A68P) previously shown to act as dominant negative mutant of IRF5 [189, 290], resulted either in a slight inhibition (IRF5 Δ DBD) of luciferase activity or had no effect (IRF5 A68P) as compared to control transfected cells (Figure 3.8c). Preliminary experiments using ectopic expression of IRF5 Δ DBD in MDDCs showed a decrease in TNF mRNA expression (4 h and 8 h post LPS stimulation, data not shown) as well as a diminished secretion of TNF protein (24 h post LPS stimulation, data not shown) further highlighting the importance of this domain.

I concluded that IRF5 along with RelA is likely to be directly involved in the transcriptional regulation of the human TNF gene. While the initial phase of TNF induction depends on both factors, only IRF5 appears to be crucial for maintaining prolonged TNF transcription in MDDCs. Moreover, the DNA-binding domain of IRF5 is required for the optimal level of TNF gene induction.

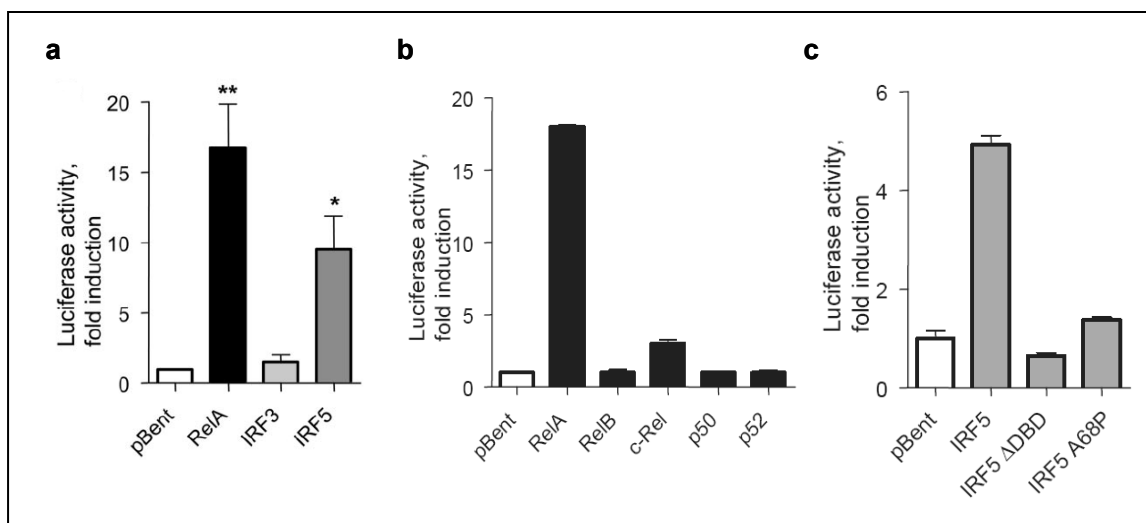


Figure 3.8 IRF5 is involved in transcriptional regulation of TNF

(a) HEK-293-TLR4-CD14/Md2 cells were co-transfected with the TNF 5'wt/3'wt reporter plasmid and equal amounts of expression plasmids encoding for human IRF5, RelA, IRF3 or empty vector (pBent). 48 h post-transfection cells were harvested and luciferase activity was measured as described. Data are presented as a fold over pBent $\pm$ SEM from 4 independent experiments: * $p < 0.05$, ** $p < 0.01$ (One-way ANOVA). **(b)** HEK-293-TLR4-CD14/Md2 cells were co-transfected with the TNF 5'wt/3'wt reporter plasmid and equal amounts of expression plasmids encoding NF- κ B subunits or empty vector (pBent). **(c)** HEK-293-TLR4-CD14/Md2 cells were co-transfected with the TNF 5'wt/3'wt reporter plasmid and equal amounts of expression plasmids encoding IRF5, IRF5 Δ DBD, IRF5 A68P or empty vector (pBent). (b,c) Data are presented as a fold over pBent $\pm$ SD from a representative experiment.

3.2.4 Computational analysis of the human TNF locus

The fact that IRF5 is involved in the transcriptional regulation of TNF (Figure 3.7b and c) and that its DBD is required for this effect (Figure 3.8c) prompted me to investigate IRF5 recruitment to the human TNF locus *in vivo* using chromatin immuno-precipitation (ChIP) analysis.

The principle of this assay is that DNA-binding proteins (e.g. transcription factors) can be cross-linked to chromatin in living cells by formaldehyde fixation. Following fixation, the cells are lysed and the chromatin is broken down to 0.5 - 1 kb in length by sonication. Once the proteins are immobilized on the chromatin and the chromatin is fragmented, whole protein-DNA complexes can be immunoprecipitated by using specific antibodies against the protein/transcription factor of interest. The DNA from the isolated protein/DNA fraction can

then be purified. The identity of the DNA fragments isolated in complex with the protein of interest can then be determined by quantitative RT-PCR using primers specific for the DNA regions under investigation. The DBD of IRF factors is well-conserved and recognises a class of DNA sequences termed IFN-stimulated response element (ISRE). I therefore analysed the human TNF locus for the presence of putative ISREs.

The genomic sequence of human TNF gene was obtained from the publicly available Ensembl website (<http://www.ensembl.org/index.html>). The region chosen for further analysis is spanning from -1,420bp to 2,222bp relative to the transcription start site or in other words, the whole region between Lymphotoxin α and Lymphotoxin β . The prediction of transcription factor binding sites was conducted using the websites Genomatix (<http://www.genomatix.de/>) and JASPAR (<http://jaspar.cgb.ki.se/>). The combination of these two websites allowed me to identify 15 putative ISREs within the TNF locus which are shown schematically in Figure 3.9. Only ISREs with a perfect match or no more than two alterations to the binding sequence GAAANNGAAACT were considered as putative ISREs. Interestingly, some of the putative ISREs were located in a close proximity to the four known NF- κ B binding regions, namely NF- κ B1, NF- κ B2/ ζ /2a, NF- κ B3 and NF- κ B4/4a/4b. Thus, I decided to include these regions in the final list of transcription factor binding sites of interest.

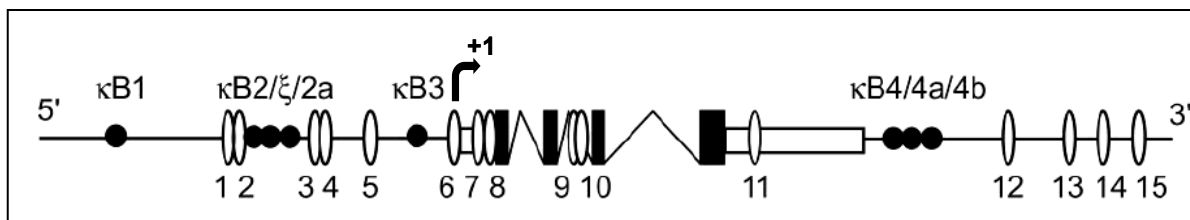


Figure 3.9 Schematic of the human TNF locus

The region shown is spanning from -1,420 bp to 2,222 bp relative to the TSS (indicated as +1) of the human TNF gene. Protein coding and non-coding exons are shown in black and white, respectively. Putative ISREs are allocated as white ovals and κ B sites as black circles.

3.2.5 Design of ChIP primers

The specificity of *in vivo* transcription factor binding detection greatly depends on the selection of primers used in the final step of the RT-PCR analysis. Therefore, I paid special attention to the design and optimisation of my primer pairs.

The analysed TNF locus was first of all segmented into smaller regions, each containing either ISREs or NF- κ B binding sites, or both. This resulted in a total of 12 regions named region A to region L. A further region, termed control region (CO), containing no ISRE or NF- κ B site was chosen as a negative control (Figure 3.10a). Altogether 13 primer pairs, named primer A to primer L and primer CO, were designed and ordered from Eurofins MWG, Germany. More details and guidelines about primer design can be found in chapter 2, section 2.12.5. The primer sets were analysed for their C_t values and melt curves (see chapter 2, section 2.12.6) on genomic DNA (gDNA) which had been fixed and sonicated beforehand. In summary, I tested 53 primer pairs for their C_t values and melt curves in order to get the 13 (12 regions plus one control region) working sets needed for ChIP experiments.

3.2.6 IRF5 is recruited to a 5' upstream and 3' downstream region of TNF

Once the primer design was completed I started to perform ChIP experiments in HEK-293-TLR4-CD14/Md2 cells in order to investigate the effect of LPS stimulation on recruitment of IRF5 to the TNF locus. A schematic diagram of the TNF locus illustrating primer amplicons and binding regions of interest is shown in Figure 3.12 a.

I observed an increased occupancy of IRF5 at regions A, B, C, G and H 4 h post LPS stimulation followed by a decrease after 24 h (Figure 3.10b). Taking into consideration the average ChIP fragment size of around 500 bp and the close proximity of the sequences amplified, some degree of overlap in regions A-C was inevitable and might have accounted for the observed symmetrical distribution of enrichment at regions A, B and C. While the enrichment of IRF5 signal at region B was expected due to the presence of putative ISRE 1 and 2, it was surprising to observe the recruitment of IRF5 at region H since this region contains no putative ISREs (Figure 3.10a). Neighbouring regions showed either a weaker (region G) or no (region I) signal compared to region H indicating that the binding of IRF5 was occurring in region H. It is worth noting, that formaldehyde cross-linker used in these

experiments can cross-link proteins which are bound directly to DNA but also proteins interacting with proteins already bound to DNA. Therefore, it is possible to detect protein recruitment to DNA even if there is no actual binding site for the protein in question.

Thus, one potential interpretation of these results was that the recruitment of IRF5 to region H was indirect and dependent on other co-factors. The presence of κ B sites made NF- κ B family members strong candidates. Therefore, I also investigated the recruitment of NF- κ B RelA to the TNF locus and observed LPS-induced binding of RelA to regions B, E and H (Figure 3.10c), which correlated with the distribution of multiple NF- κ B binding sites in these regions.

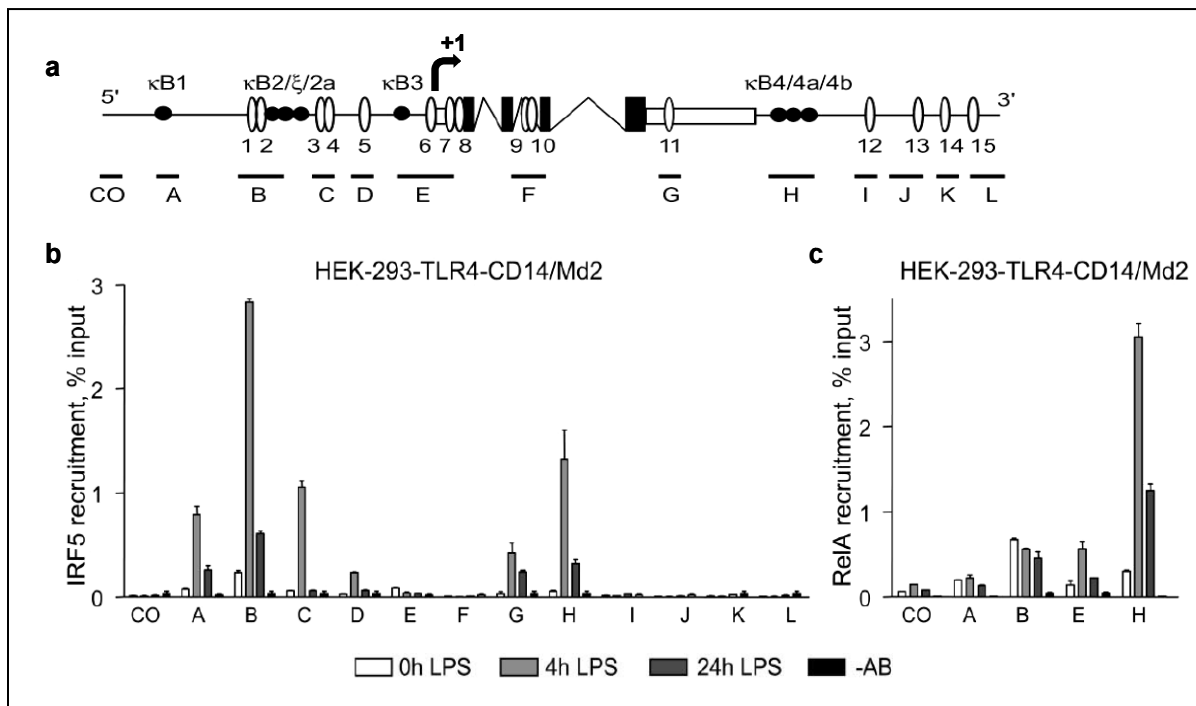


Figure 3.10 Recruitment of IRF5 to 5' upstream and 3' downstream region of TNF

(a) Schematic diagram of the TNF locus. Protein coding and non-coding exons are shown in black and white; the TSS is indicated as +1. Putative ISREs are allocated as white ovals; κ B sites as black circles. The approximate amplicon size of primer sets spanning the TNF locus (A to L) are indicated by black lines. CO – a control primer set containing neither an ISRE nor a κ B site. (b, c) HEK-293-TLR4-Md2/CD14 cells were left unstimulated or stimulated with LPS (1 μ g/ml) for 4 and 24 h and analysed by ChIP with antibodies specific to IRF5 (b) or RelA (c). Data show mean % input relative to genomic DNA (gDNA) $\pm$ SD of a representative experiment. –AB, a no antibody control.

In order to further investigate the observed IRF5 binding pattern I performed EMSA experiments. An EMSA is a common *in vitro* affinity electrophoresis technique which can be used to study protein-DNA interactions. This procedure can determine if a protein or mixture of proteins is capable of binding to a given DNA sequence and can sometimes indicate if more than one protein is involved in the binding complex. The principle of an EMSA is based on the fact that the speed at which different molecules move through the gel is determined by their size and charge, and to a lesser extent, their shape. The binding of a protein to a given DNA sequence will result in a less mobile (slower moving) complex which is 'shifted' up on the gel compared to DNA on its own. An antibody that recognizes a candidate protein can be added to the mixture to create an even larger complex that migrates even slower. This method is referred to as supershift assay and is used to identify a protein present in the protein-DNA complex. The DNA sequence usually contains a radioactive or fluorescent label for visualization purposes.

The EMSA experiments were performed with radio-labeled probes corresponding to selected ISREs from the TNF promoter region (ISRE1, ISRE2 and ISRE5) as well as κ B sites from the 3' downstream region H (κ B4, κ B4a and κ B4b). A sequence identical to the positive regulatory domain (PRD) I to III of the human IFN β promoter served as a positive control. Recombinant full-length IRF5 protein was not available therefore, recombinant DBD of IRF5 (bacterially expressed and purified), kindly provided by Dr Daniel Wong (Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom), was used. EMSA experiments were kindly performed by Dr Irina Udalova and Mrs Alessandra Lanfrancotti (both Kennedy Institute of Rheumatology, London, United Kingdom).

As shown in Figure 3.11, IRF5 can bind to oligonucleotides encompassing ISRE1 and ISRE5 and to a lesser extent to ISRE2 *in vitro*. A slower migrating complex observed in the ISRE1, ISRE5 and PRDI-III lanes could represent the simultaneous binding of two IRF5 DBD proteins (indicated as IRF5DBD:IRF5DBD) to the oligonucleotide probes. No IRF5-DNA binding to oligonucleotides corresponding to the κ B binding sites in region H (κ B4, 4a and 4b) or at additional non-consensus control sites, called 'ISRE'16 and 'ISRE'17, in the vicinity of region H were observed (Figure 3.11). This further supports the hypothesis that IRF5 is recruited indirectly to region H, likely via yet unknown co-factors.

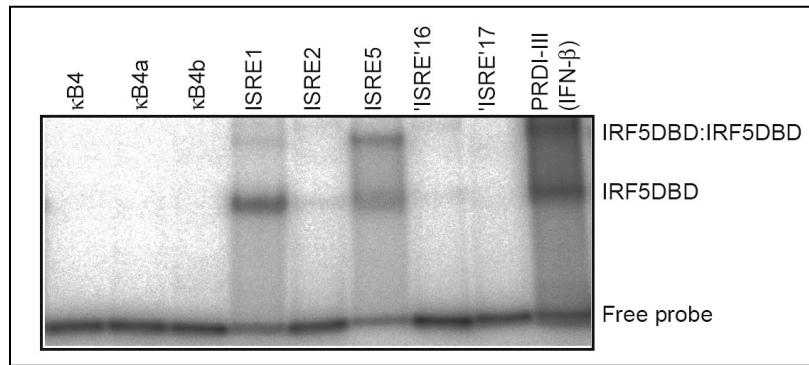


Figure 3.11 *In vitro* binding of IRF5 to 5' upstream and 3' downstream regions of TNF

Recombinant purified IRF5 DBD was used in an EMSA with radioactive probes corresponding to selected ISREs and κ B sites. PRDI-III from the IFN- β promoter was used as a positive control and non-consensus ISRE16 and ISRE17 served as negative controls. Probes bound by single (IRF5DBD) or multiple IRF5 DBD proteins (IRF5DBD:IRF5DBD) are indicated.

Next, I validated the pattern of IRF5 and RelA recruitment to the TNF locus in MDDCs stimulated with LPS for 0, 1 and 4 h. Enrichment of both IRF5 (Figure 3.12a) and RelA (Figure 3.12b) was observed at regions B and H. This was reproducible and statistically significant when analysed in cells derived from multiple blood donors.

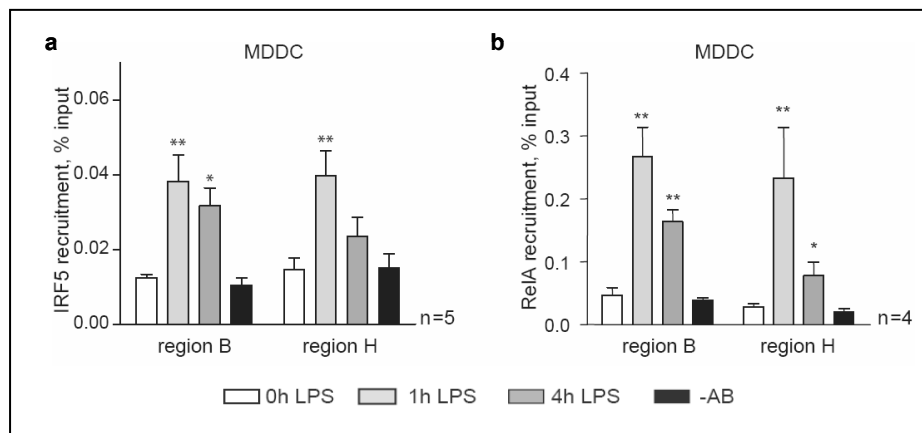


Figure 3.12 IRF5 and RelA are recruited to 5' upstream and 3' downstream regions of the TNF gene

(a, b) MDDCs were left unstimulated or stimulated with LPS (10 ng/ml) for 1 h or 4 h followed by ChIP with antibodies specific to IRF5 (a) or RelA (b). Data are shown as the mean % input relative to gDNA $\pm$ SEM of 5 (IRF5) or 4 (RelA) independent experiments each using MDDCs derived from a different donor: * $p < 0.05$, ** $p < 0.01$ (One-way ANOVA). –AB, a no antibody control.

In summary, IRF5 along with RelA is efficiently recruited to the 5' upstream region B and 3' downstream region H of the human TNF gene in response to LPS stimulation. Significantly, the lack of putative ISRE binding sites in the 3' downstream region of the TNF gene strongly suggested that recruitment of IRF5 to this region may be mediated via interactions with other transcription factors, like NF- κ B RelA, or accessory proteins.

3.2.7 IRF5 forms specific physical interactions with RelA

The experiments described in this section were performed either by or with great help from Dr David Saliba (Kennedy Institute of Rheumatology, London, United Kingdom). In order to acknowledge his input results are discussed in plural.

To tease out whether IRF5 recruitment to region H was indeed mediated via its interactions with RelA, we conducted sequential ChIP analysis of region H. We first performed a ChIP with anti-RelA antibodies and analysed the pulldown efficiency which was comparable between experimental groups (Figure 3.13a). We then assessed the co-recruitment of IRF5 by performing a ChIP with IRF5-specific antibodies on the same samples and found that IRF5 binding to region H was co-dependent on RelA following LPS stimulation (Figure 3.13b).

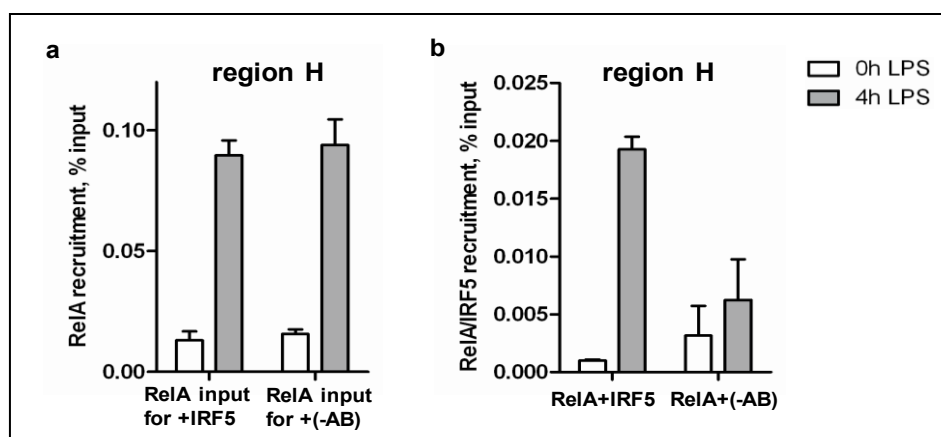


Figure 3.13 Co-recruitment of RelA and IRF5 to region H

HEK-293-TLR4-CD14/Md2 cells were left unstimulated or stimulated with LPS (1 μ g/ml) for 4 h. **(a)** RelA binding to region H was analysed using anti-RelA antibodies. **(b)** The co-recruitment of RelA and IRF5 to region H was assessed by re-ChIP of the samples from (a) with IRF5-specific antibodies or a no antibody control (-AB). **(a, b)** Data show mean % input relative to gDNA $\pm$ SD of a representative experiment.

This finding prompted us to investigate whether IRF5 and RelA interact physically. IRF5 with an N-terminal one-strep tag and a C-terminal HA tag was expressed in HEK-293-TLR4-CD14/Md2 cells. Figure 3.14a shows that in conditions similar to ChIP analysis (i.e. *in-vivo* crosslinking with formaldehyde) ectopically expressed IRF5 efficiently pulls down endogenous RelA (Figure 3.14a, compare lanes 3 and 4). To determine whether this interaction was specific, we immunoblotted for other NF- κ B family members: Rel-B, c-Rel, p50 and p52, or a control protein tubulin. None of these resulted in a positive interaction (Figure 3.14a). Furthermore we conducted a complementary experiment, in which human RelA containing C-terminal FLAG tag was expressed in HEK-293-TLR4-CD14/Md2 cells and immunoprecipitated in the absence of a cross-linking agents on anti-FLAG sepharose. Specific interactions between ectopically expressed RelA and endogenous IRF5 were observed. No interaction was detected between a control FLAG-tagged protein, bacterial alkaline phosphatase (BAP), and IRF5 (Figure 3.14b, compare lanes 1 and 2).

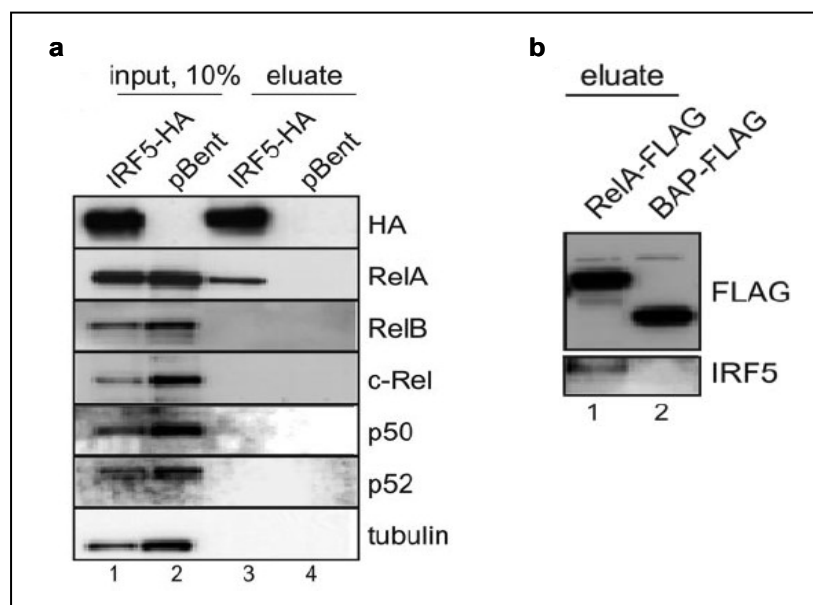


Figure 3.14 Ectopic IRF5 specifically interacts with RelA in HEK-293 cells

(a) HEK-293-TLR4-Md2/CD14 cells were transfected with human IRF5 tagged with onestrep tag (N-terminus) and HA tag (C-terminus) (lanes 1 and 3) or an empty vector pBent (lanes 2 and 4) and fixed with formaldehyde. Cell lysates were immunoprecipitated using streptavidin columns followed by reversion of the crosslink and immunoblotting for bait IRF5 (anti-HA antibodies), or NF- κ B subunits as well as tubulin. (b) HEK-293-TLR4-Md2/CD14 cells were transfected with RelA-FLAG (lane 1) or BAP-FLAG (lane 2). Cell lysates were immunoprecipitated with M2 anti-FLAG sepharose and immunoblotted for bait RelA (anti-FLAG antibodies) or IRF5.

Next, we examined whether an interaction between endogenous RelA and IRF5 could be detected in MDDCs and if this interaction may be inducible upon LPS stimulation. IRF5 was immunoprecipitated from the cells stimulated with LPS for 0 or 1 h using anti-IRF5 antibodies. The western blot for RelA revealed a specific interaction with IRF5 (Figure 3.15a). A densitometry analysis of quantities of the bait and target proteins indicated that the quantity of RelA bound to IRF5 was somewhat higher in LPS stimulated cells (Figure 3.15a, lane 4) compared to unstimulated cells. Finally, we asked the question whether the observed RelA-IRF5 interactions are dependent on the simultaneous binding of both TFs to DNA, i.e. RelA and IRF5 interact only when bound to corresponding κ B and ISRE binding sites in close proximity to each other. To address this, we extracted nuclei from HEK-293-TLR4-CD14/Md2 cells stimulated with LPS for 0, 1, 4 and 8 h and subjected the chromatin to DNase I digestion. Subsequent precipitation of endogenous immune complexes with anti-IRF5 antibodies revealed that RelA interacted with IRF5 even in the absence of DNA bridging (Figure 3.15b, lanes 5-8). Once again, the number of RelA-IRF5 complexes increased with LPS stimulation, corresponding to the rise in nuclear RelA (Figure 3.15b, lanes 1-4).

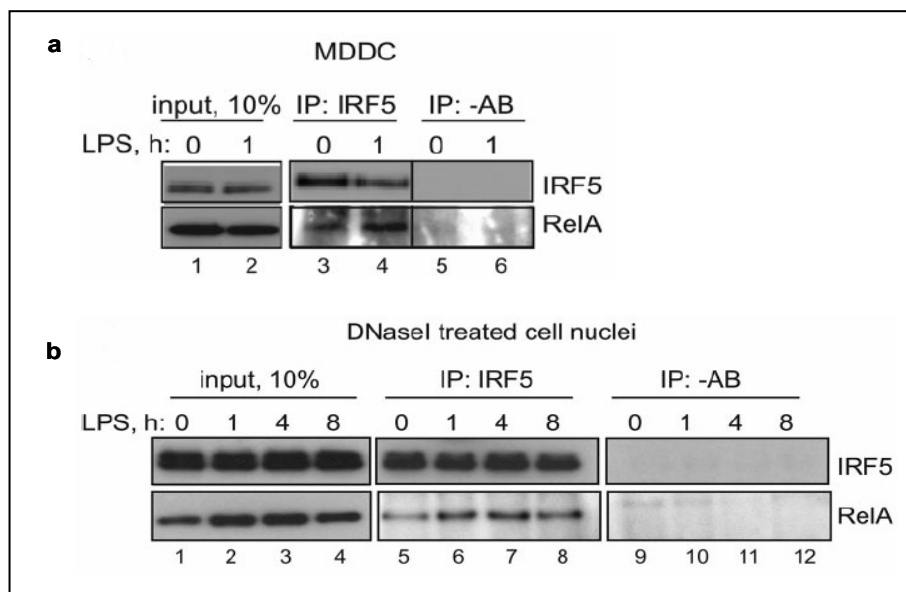


Figure 3.15 Endogenous IRF5 interacts with RelA in MDDCs

(a) MDDCs were left untreated or stimulated with LPS for 1 h. The endogenous interaction between RelA and IRF5 was examined by IP with anti-IRF5 antibody and blotting with anti-RelA antibody. -AB – a mock IP. **(b)** Nuclear pellet from triton extracted HEK-293-TLR4-Md2/CD14 cells was solubilised with DNase I and endogenous interaction between RelA and IRF5 was examined after IP as in (b).

In summary, IRF5 can specifically interact with RelA but not with any of the other NF- κ B family members. The interaction is not dependent on IRF5 binding to DNA and the quantity of IRF5/RelA complexes is increased in response to LPS stimulation. Thus, we hypothesised that IRF5 recruitment to the 3' downstream region of the TNF gene lacking putative ISRE sites is a consequence of direct physical interactions between DNA-bound RelA and IRF5.

3.2.8 RelA is required for IRF5-dependent transactivation of the TNF gene

To test the above hypothesis, I first analysed IRF5 recruitment to the TNF locus in the cells in which the levels of RelA protein were significantly reduced. In HEK-293-TLR4-CD14/Md2 cells, siRNA-mediated depletion of RelA mRNA resulted in approximately 75% reduction in RelA protein (Figure 3.16a) and about a 10-fold decline in its recruitment to region H following 4 h of LPS stimulation (Figure 3.16b). As predicted, the IRF5 recruitment to the same region was prevented (Figure 3.16c) further supporting a RelA-dependent recruitment of IRF5 to region H.

Next, I examined the effect of site-specific mutations in the κ B sites on the ability of IRF5 to activate the TNF gene. A panel of four gene-reporter constructs was used in this analysis:

- (1) 5'wt/3'wt (as in Figure 3.8)
- (2) 5'mut/3'wt (mutated κ B2/2 ξ /2a sites in the TNF 5' upstream region)
- (3) 5'wt/3'mut (mutated κ B4/4a sites in the 3' TNF downstream region)
- (4) 5'mut/3'mut (all κ B sites described above mutated)

The reporter constructs were co-expressed with IRF5 and RelA in HEK-293-TLR4-Md2/CD14 cells and luciferase activities were compared to empty vector pBent. As expected, removal of either 5' upstream or 3' downstream κ B sites diminished the ability of RelA to drive the gene-reporter activity (Figure 3.17). However, the transactivation of the reporter constructs by IRF5 (supported by ectopically expressed Myd88) appeared to be largely unaffected by mutations in the 5' upstream κ B sites, suggesting that IRF5 does not utilise κ B2/2 ξ /2a sites for its binding to the TNF 5' upstream and its likely to involve the identified ISRE1 and ISRE 2 sites. However, the transactivation of the reporter construct

with mutations in κ B4/4a sites by IRF5 was significantly reduced, suggesting that IRF5 activity depends on NF- κ B binding to this region (Figure 3.17).

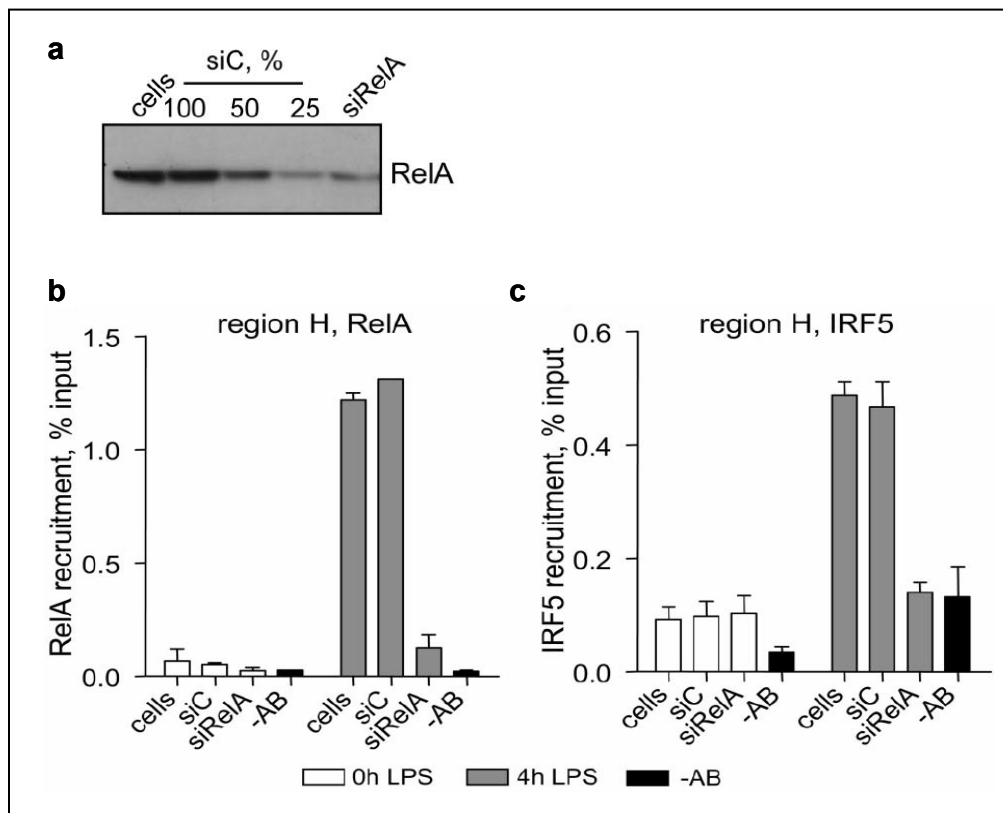


Figure 3.16 RelA is required for IRF5-binding to region H of the TNF gene

(a-c) HEK-293-TLR4-Md2/CD14 cells were transfected with siRNA against RelA (siRelA) or with non-targeting siRNA (siC) and used in ChIP analysis of RelA and IRF5 recruitment. Data indicate mean % input relative to gDNA $\pm$ SD of a representative experiment. -AB, a no antibody control. **(a)** 75% of RelA protein was degraded as estimated by serial dilutions of the siC control sample analysed by western blotting. **(b, c)** Reduction in LPS-induced RelA (b) and IRF5 (c) recruitment to region H in siRelA treated cells.

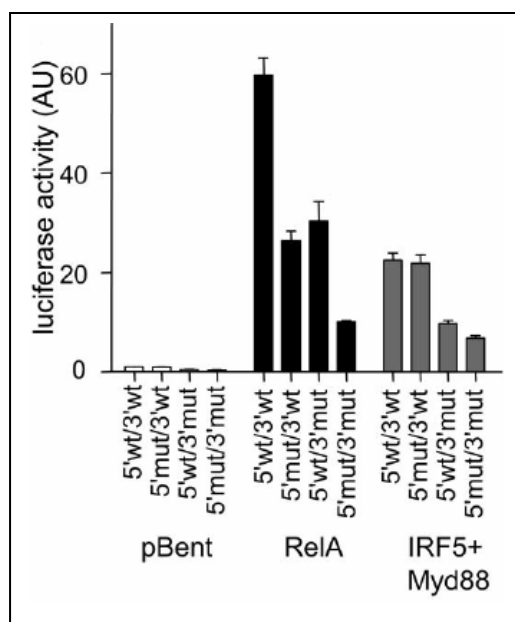


Figure 3.17 RelA is required for IRF5-mediated activation of TNF

HEK-293-TLR4-Md2/CD14 cells were transfected with RelA, IRF5 and MyD88 expression constructs together with the TNF 5' upstream/luciferase/TNF 3' downstream reporter plasmids: 5'wt/3'wt - wild type construct, 5'mut/3'wt - mutated κ B2/ κ B β / κ B2a sites in TNF 5' upstream; 5'wt/3'mut - mutated κ B4/ κ B4a sites in TNF 3' downstream; 5'mut/3'mut - all κ B sites mutated. Data show means $\pm$ SD and are representative of 3 independent experiments, each performed in triplicate.

Thus, IRF5 recruitment to the TNF 3' downstream region is mediated by way of a complex assembly with RelA and does not involve a direct contact to DNA. Importantly, another mode of function of IRF5 in TNF regulation is a direct recruitment to the TNF gene 5' upstream. The two functional modes also imply the possibility of a higher order enhancer structure at the TNF locus which might involve IRF5/RelA mediated intrachromosomal looping.

3.3 Conclusion

Production of the key immune modulator TNF is both cell-type and stimulus-specific with myeloid cells among the major producers of TNF in response to TLR4 stimulation [291]. Consequently, a tight control of the amount and duration of TNF expression by these cells is critical for a self-limited immune response. In this chapter, I aimed to understand the molecular bases of differential TNF expression in primary human dendritic cells and macrophages. I demonstrate that IRF5 appears to be a defining factor in maintaining TNF gene transcription in monocyte-derived dendritic cells and unravel a complex molecular mechanism employed by IRF5 to control the human TNF gene expression: two spatially separated regulatory regions and two independent modes of actions are involved.

IRF5 is highly expressed in MDDCs (Figure 3.1) which acquire a particular phenotype during differentiation, characterized amongst other markers by higher levels of late-phase TNF secretion compared to MDMs (Figure 3.2). Interestingly, forced expression of IRF5 in MDMs led to prolonged TNF secretion (Figure 3.4), while depletion of IRF5 in MDDCs resulted in reduction of TNF expression, particularly at later time (4 h) post LPS stimulation (Figures 3.6 and 3.7). One of the main problems associated with RNAi technology is the non-specific effect on other transcripts than the target sequence. However, if results obtained with overexpression show the opposite phenotype to siRNA, it is less unlikely that results using siRNA-mediated depletion are due to off-target effects. Furthermore, I can not formally rule out other factors that might feed into the TNF expression system at a later time but the ability of IRF5 to activate the TNF gene-reporter construct (Figure 3.8) and its efficient recruitment to the TNF locus (Figures 3.10 and 3.12) strongly suggest a direct role for IRF5 in TNF gene induction in response to TLR4 stimulation.

The recruitment of IRF5 to the human TNF locus was examined by ChIP experiments conducted in the cell line HEK-293-TLR4-CD14/Md2 (Figure 3.10) and MDDCs (Figure 3.12). The data indicate that IRF5 is recruited to two specific regions, region B and region H, within the TNF locus (Figures 3.10 and 3.12). Of particular interest was region H because it contains binding sites for NF- κ B family members (NF- κ B 4/4a/4b) but not putative ISRE (Figure 3.9). Additional ChIP experiments conducted in HEK-293-TLR4-CD14/Md2 with depleted RelA protein demonstrated that IRF5 recruitment to region H is RelA dependent whereas its recruitment to region B occurred RelA-independent, probably via direct binding of IRF5 to identified ISRE sites (Figure 3.11). The importance of RelA-

binding to NF- κ B sites in the 3' region and subsequent recruitment of IRF5 was confirmed in re-ChIP experiments (Figure 3.13) and *in vitro* using coexpression of IRF5 with sets of TNF luciferase constructs containing mutations in NF- κ B 4/4a/4b sites (Figure 3.17).

A subsequent analysis of protein-protein interactions demonstrated an interaction between RelA and IRF5 (Figure 3.14). This interaction is induced following stimulation of MDDCs with LPS, while no other NF- κ B family member appears to form complexes with IRF5 (Figures 3.15). Considering that the Rel homology domain is a highly conserved domain, present in all NF- κ B proteins, the exclusiveness of IRF5 interactions with RelA is somewhat surprising. The precise interface of the IRF5/RelA interaction is currently being mapped in the laboratory of Dr Irina Udalova.

Overall, this suggests IRF5-induced TNF expression involves two spatially separated regulatory regions (region B and H) and two independent modes of actions (direct binding to DNA and co-recruitment mediated via protein-protein interactions with RelA) (Figure 3.18). Another member of the IRF family, IRF3 was shown to interact with RelA [292, 293] and to specifically activate the expression of selected NF- κ B target genes like *Scyb9* or *Clic4* [293]. Similarly, IRF5 might interact with RelA to ensure a specific activation of another yet to be defined subset of genes.

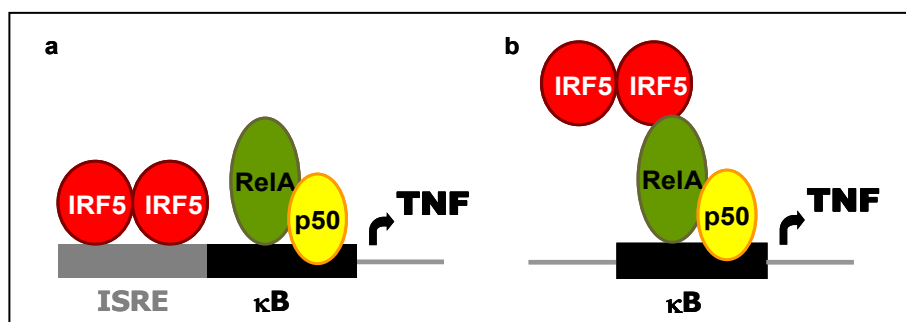


Figure 3.18 Modes of IRF5 recruitment to regions of the human TNF locus

(a) Binding of IRF5 to TNF at region B. After cell activation IRF5 dimerises and translocates to the nucleus where it binds to the identified ISREs present in region B. IRF5 binding, which occurs independently of RelA, induces TNF gene expression. **(b)** Binding of IRF5 to TNF at region H. IRF5 activation leads to its translocation into the nucleus where it interacts with RelA. The interaction is essential for RelA-mediated recruitment of IRF5 to region H which is necessary for the full induction of TNF gene expression.

Why is there a functional advantage for maintaining TNF secretion for longer in MDDCs compared to MDMs (Figure 3.2)? Dendritic cells are professional antigen-presenting cells that are crucial for both innate and adaptive responses to infection. They sense invading pathogens and respond by secreting various cytokines as well as by upregulating the expression of MHC II and costimulatory molecules, essential for efficient antigen presentation to T lymphocytes [294]. The mature dendritic cells migrate to the draining lymph nodes, where they initiate Th1 differentiation. TNF acting through TNF receptor is involved in DC maturation from bone marrow progenitors [295, 296] and a recent study demonstrated that TNF blockade impaired DC survival and function in RA [297]. My data showing that TNF produced by MDDCs is a key factor in human Th1 activation support this study. Moreover, it is the late phase TNF secretion that is needed to achieve the full activation potential of T lymphocytes (Figure 3.3). Macrophages, on the other hand, do not migrate to the draining lymph nodes but accumulate in large numbers at a site of inflammation, secrete inflammatory cytokines and attract other immune cells via chemotaxis [298]. Thus, a mechanism which would restrain the degree and duration of TNF secretion by macrophages would be important for ensuring resolution of acute inflammatory response thereby limiting tissue damage.

However, macrophages are a heterogeneous cell population that can be classified as either pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages [43]. Although both human macrophages subsets are somewhat different to MDDCs, it is the M1 phenotype that has a higher phenotypic resemblance to MDDCs. Similar to MDDCs, M1 macrophages upregulate the expression of HLA molecules and co-stimulatory receptors such as CD40, CD80 and CD86 upon TLR4 stimulation making them good antigen presenters. In addition to surface receptor expression, the cytokine profile of M1 macrophages is in many ways comparable to that of MDDCs [64, 67]. Here I used monocyte-derived macrophages differentiated in the presence of M-CSF which leads to the generation of the anti-inflammatory M2 subset. When I generated macrophages with an M1 differentiation protocol (treatment of monocytes with GM-CSF) I observed a similar TNF secretion profile to MDDCs after LPS stimulation, that is, prolonged and sustained (data not shown). It is reasonable to speculate that the different TNF production by M1 macrophages compared to M2 macrophages is also due to variations in IRF5 protein levels. However, the role of IRF5 in macrophage subsets is yet to be determined. I therefore set out to investigate the expression and function of IRF5 in macrophage populations in more detail and results are presented in chapter 4 and 5 of this thesis.

Regulation of IRF5 activity is an important issue since the excessive activation of this protein may lead to pathology. Interestingly, another member of the IRF family, IRF4, was shown to act as a negative regulator of TLR signalling by inhibiting the production of selected IRF5-dependent genes, including TNF, via direct competition with IRF5 for interactions with Myd88 [299]. This could indicate that a self-controlled IRF5-IRF4 regulatory system might have developed to finely modulate TLR signalling pathways and production of IRF5-dependent inflammatory cytokines. The rivalry between IRF5 and IRF4 will also be discussed later in light of their different roles in shaping different macrophage phenotypes.

In summary, my data suggest that sustained TNF secretion in human MDDCs is mediated by cooperative action of IRF5 and RelA at the 5' upstream and 3' downstream regions of the human TNF gene. TLR4 stimulation induces protein-protein interactions between RelA and IRF5 and allows for DNA-independent recruitment of IRF5 to the TNF 3' downstream region. Based on the resistance of *Irf5*^{-/-} mice to lethal endotoxic shock and impaired production of pro-inflammatory cytokines, IRF5 was proposed as a target for therapeutic interventions [190]. Here I define RelA-IRF5 interactions as a putative target for cell-specific modulation of TNF expression, and possible other selected inflammatory mediators.

4. IRF5 promotes inflammatory macrophage polarization

4.1 Introduction

Macrophages are a heterogeneous population of immune cells that are essential for the initiation and resolution of pathogen- or tissue damage-induced inflammation [42]. They demonstrate remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype and physiology in response to cytokines and microbial stimuli [43]. These changes can give rise to populations of macrophages with distinct functions, which are phenotypically characterised by production of pro-inflammatory and anti-inflammatory cytokines [46]. Based on the Th1/Th2 polarization concept [44] these cells can roughly be classified as M1 (classical) macrophages, that produce pro-inflammatory cytokines (IL-12^{high}, IL-23^{high}, IL-10^{low}) and mediate resistance to pathogens and tissue destruction and M2 (alternative) macrophages, that produce anti-inflammatory cytokines (IL-12^{low}, IL-23^{low}, IL-10^{high}) and promote tissue repair and remodelling as well as tumour progression [45, 46].

The activation of a subset-defining transcription factor is characteristic of a particular T lymphocyte lineage commitment: T-bet is associated with Th1, GATA3 with Th2, FOXP3 with Treg lymphocytes and ROR γ T with Th17 lymphocytes [99, 101, 109, 124]. Dendritic cells also employ subset-selective expression of IRF4 and IRF8 for their commitment, discussed in Chapter 1.3.3. However, transcription factors underlying macrophage polarization remain largely undefined. In mouse models, activation of NF- κ B p50 has been previously associated with inhibition of M1 polarizing genes [300], whereas CREB mediated induction of C/EBP β has been shown to upregulate M2-specific genes [301]. More recent evidence suggests that, again in mice, IRF4 may control M2 macrophage polarization by stimulating the expression of selected M2 macrophage markers [302].

The observed low expression of IRF5 in monocyte-derived macrophages (Chapter 3.2.1) which were differentiated in M2 polarizing conditions prompted me to investigate IRF5 levels under M1 conditions. The overall phenotype of M1 macrophages is quite similar to monocyte-derived DCs and therefore it was expected that M1 macrophages would express a high level of IRF5 protein. I speculated that IRF5 might be employed by M1 macrophages

to fine-tune (like late-phase TNF secretion which was only seen in MDDCs with high IRF5 levels) or even define different macrophage phenotypes. If IRF5 is indeed a transcription factor that influences macrophage polarization the following questions were of particular interest:

- Which environmental signals can induce IRF5 expression in human macrophages?
- Is IRF5 expression static or can changes occur dependent on different experimental conditions?
- Are any subset-defining cytokines under the transcriptional control of IRF5?
- What is the effect of overexpression or siRNA-mediated depletion of IRF5 on macrophage phenotype and cytokine profile?

The identification of a master regulator of macrophage phenotype and function would have ramifications for all inflammatory diseases characterised by excessive type 1 cytokines and would provide a potential new target for immune intervention.

4.2 Results

4.2.1 IRF5 expression is high in human M1 macrophages

Macrophages evolve within a specific microenvironment out of monocytes [30] and the differentiation of monocytes depends on lineage-determining cytokines, with M-CSF and GM-CSF among the most important ones [64]. The M1 macrophage phenotype is induced by IFN- γ followed by stimulation with bacterial products like LPS or by treatment of monocytes with GM-CSF. [63-67]. Conversely, the M2 macrophage phenotype manifests when monocytes are cultured in the presence of M-CSF [63-65, 67]. Before analysing IRF5 levels in macrophage subsets I had to be sure that these culture methods are actually working in my hands and therefore compared the cytokine profile of M1 and M2 differentiated human macrophages (Figure 4.1). Consistent with previously published data [64, 67], human monocytes cultured in the presence of GM-CSF and stimulated with LPS displayed an M1 phenotype with high expression of IL-12p70 and IL-23 but low expression of IL-10 whereas differentiation with M-CSF led to the opposite phenotype (Figure 4.1).

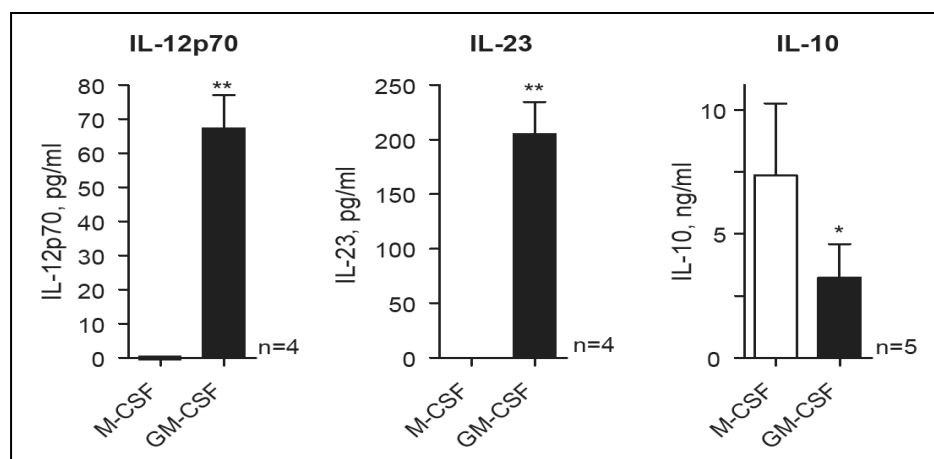


Figure 4.1 The cytokine environment determines macrophage phenotype

Monocytes from the same donor were differentiated with M-CSF (100 ng/ml) or GM-CSF (50 ng/ml) for 5 days. Cells were stimulated with LPS (10 ng/ml) for 24 h and the secretion of IL-12p70, IL-23 and IL-10 was determined by ELISA. Data shown are the mean $\pm$ SEM from 4 to 5 independent experiments each using macrophages derived from a different donor: * $p < 0.05$ and ** $p < 0.01$ (One-way ANOVA).

After gaining confidence in the differentiation protocols I examined the levels of IRF5 expression in primary human monocytes or in macrophage subsets and I observed an increase in IRF5 protein expression in the population differentiated with GM-CSF compared with that differentiated in the presence of M-CSF (Figure 4.2a and b). Furthermore, treatment of monocytes with GM-CSF resulted in upregulation of IRF5 mRNA expression within 2 h of stimulation but treatment with M-CSF did not (Figure 4.2c). Surprisingly, the expression of IRF4 protein, which was previously shown to control polarization towards the M2 phenotype [302], was equally induced during monocyte differentiation into M1 or M2 macrophages (Figure 4.2a). The expression of IRF3 protein, another member of the IRF family central to the innate immune response, was not affected by differentiation into macrophage subtypes (Fig 4.2a). In addition, I observed no significant difference between M1 and M2 macrophages in the basal or LPS-induced levels of NF- κ B p50 protein, previously implicated in macrophage polarization towards M2 phenotype [300] (data not shown). I also examined the effect of LPS on IRF3, IRF4 and IRF5 protein expression. I observed no changes in the protein levels of IRF3 and IRF4 upon TLR4 activation but a weak upregulation of IRF5 protein in all cell types tested (Figure 4.2a). To confirm that

stimulation of the cells was successful membranes were routinely re-probed for IRF7, a transcription factor that is strongly induced by LPS (data not shown).

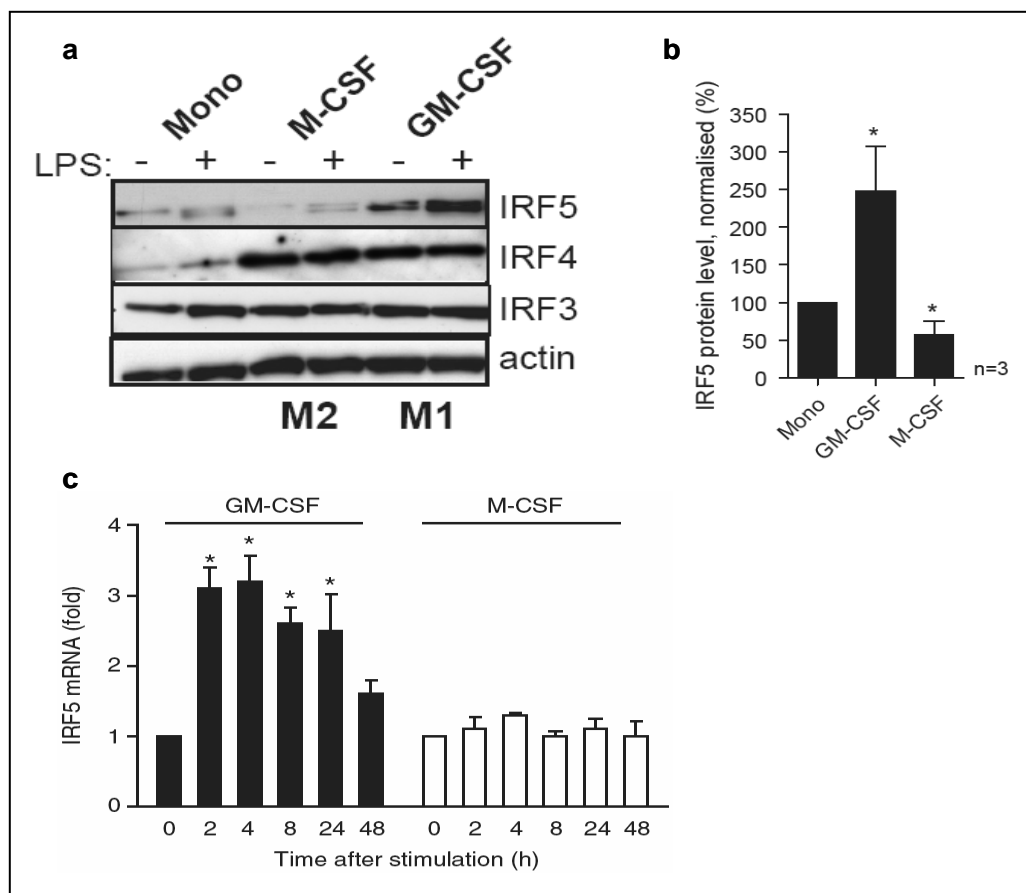


Figure 4.2 High expression of IRF5 in M1 macrophages and upregulation by GM-CSF

(a) Western blot analysis of total protein extracts from monocytes collected at day 0 (Mono) or differentiated for 5 d into M1 macrophages with GM-CSF (50 ng/ml) or into M2 macrophages with M-CSF (100 ng/ml), then left untreated (-) or stimulated for 24 h with 10 ng/ml LPS (+). Actin serves as a loading control. A representative blot of at least 4 independent experiments, each using cells derived from a different donor is shown. **(b)** IRF5 protein expression was analysed in total cell lysates of cells differentiated as in (a). Densitometric analysis was performed using Quantity One software and data were normalised to actin. Shown are the mean ± SEM from 3 independent experiments presented as % of increase in IRF5 protein levels relative to monocytes. * $p < 0.05$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test). **(c)** RT-PCR analysis of IRF5 mRNA in monocytes left untreated (0) or stimulated for 2, 4, 8, 24 or 48 h with GM-CSF (50 ng/ml) or M-CSF (100 ng/ml) Results are the mean ± SEM from 5 independent experiments each using monocytes derived from a different donor presented relative to those of untreated monocytes, set as 1. * $p < 0.001$ (two-way ANOVA).

To account for possible differences in macrophage *in vitro* differentiation protocols, I analysed the level of IRF5 in macrophages treated with either IFN- γ alone or in combination with LPS for 24 h and found that IRF5 protein levels were similar to the ones in GM-CSF treated cells (Figure 4.3).

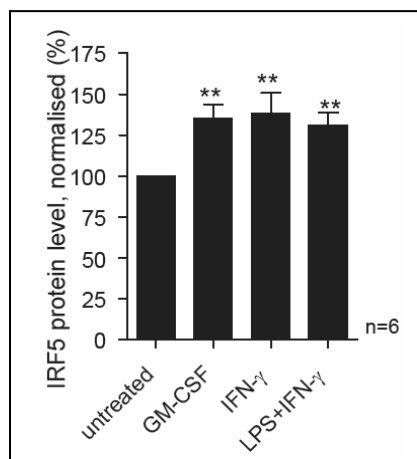


Figure 4.3 IRF5 expression is induced by M1 macrophage maturation protocols

M2 macrophages were left untreated or treated with GM-CSF (50 ng/ml), IFN- γ (50 ng/ml), or LPS (10 ng/ml) plus IFN- γ for 24 h and total protein extracts were subjected to Western blot analysis followed by densitometry. Data shown are the mean $\pm$ SEM from 6 independent experiments presented as % increase in IRF5 protein levels relative to untreated cells. ** $p < 0.01$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

Thus, I concluded that IRF5 is induced in pro-inflammatory M1 macrophages independently of the *in vitro* differentiation protocol, whereas the levels of IRF4 and IRF3 are comparable between the macrophage populations.

4.2.2 IRF5 expression is plastic in human macrophages

Macrophages are able to respond to environmental changes and cytokines by adopting their phenotype as well as their physiology. To examine whether IRF5 contributes to the plasticity of macrophage polarization, I attempted to convert one population into another by culturing M2 macrophages with GM-CSF and M1 macrophages with M-CSF. Following LPS stimulation GM-CSF treated M2 macrophages were now capable of producing the M1

phenotypic markers IL-12p70 and IL-23 whereas the levels of IL-10 decreased (M2 to M1; Figure 4.4a). Conversely, treatment of M1 macrophages with M-CSF led to higher LPS-induced levels of the M2 marker IL-10 but a diminished production of IL-12p70 and IL-23 (M1 to M2; Figure 4.4b).

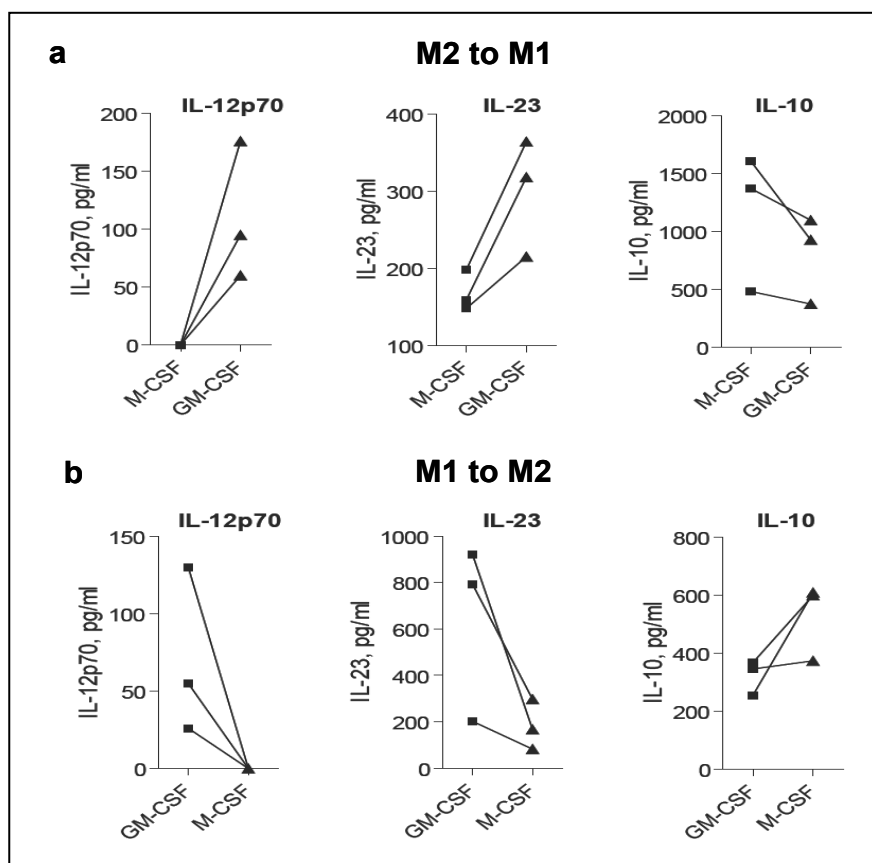


Figure 4.4 Plasticity of macrophages in response to environmental changes

(a) For M2 to M1 cytokine profiles, M-CSF-derived M2 macrophages at day 5 were either left in M-CSF containing medium or washed with PBS and exchanged for GM-CSF (100 ng/ml) containing medium and after 24 h subjected to LPS stimulation (10 ng/ml). (b) For M1 to M2 cytokine profiles, GM-CSF derived M1 macrophages at day 5 were either left in GM-CSF containing medium or washed with PBS and exchanged for M-CSF (100 ng/ml) containing medium and after 24 h subjected to LPS stimulation (10 ng/ml). (a, b) Data shown are the changes in secretion of IL-12p70, IL-23 and IL-10 determined by ELISA from 3 independent experiments each using macrophages derived from a different donor.

More strikingly, western blot analysis demonstrated that M2 to M1 conversion enhanced the protein levels of IRF5 whereas M1 to M2 macrophages showed the opposite result (Figure 4.5a and b). The changes seen in IRF5 protein levels were reproducible and statistically significant when analysed in multiple independent blood donors (Figure 4.5c and d). Once again, the expression of IRF4 and IRF3 proteins was unchanged (Figure 4.5a and b).

These results indicate that IRF5 expression rapidly responds to environmental stimuli and suggest that it participates in establishing macrophage plasticity.

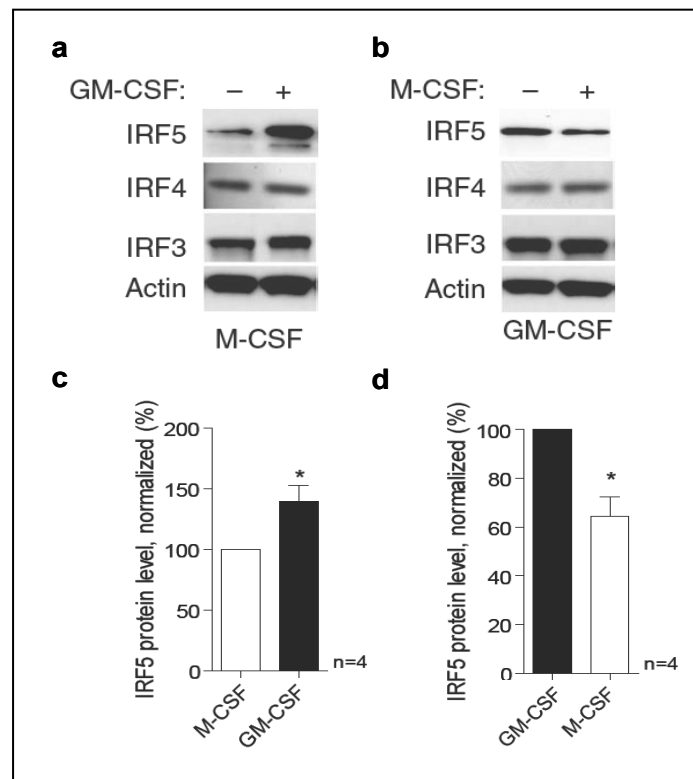


Figure 4.5 Plastic expression of IRF5 in macrophages

(a, b) Western blot analysis of total protein extracts from (a) M2 macrophages left untreated (-) or treated (+) for 24 h with GM-CSF (50 ng/ml) for M2 to M1 polarization and from (b) M1 macrophages left untreated (-) or treated (+) for 24 h with M-CSF (100 ng/ml) for M1 to M2 polarization. A representative blot of 4 independent experiments, each using macrophages derived from a different donor is shown. (c, d) The change in IRF5 protein expression of cells treated as in (a) and (b) was analysed by western blotting followed by densitometric analysis using Quantity One software. The IRF5 measurements were normalised to actin. Shown are the mean $\pm$ SEM from 4 independent experiments presented as % of increase (c) or decrease (d) in IRF5 protein levels relative to the initial condition: * $p < 0.05$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

4.2.3 IRF5 influences the polarization of human macrophages

Based on the above results, I hypothesised that the phenotypic differences between M1 and M2 macrophages might be due to differential IRF5 protein expression in these populations (Figure 4.2). To test this hypothesis, M2 macrophages were infected with adenoviruses containing expression constructs encoding IRF5, IRF3 (as a control) or an empty vector (pENTR) and the production of IL-12p70, IL-23 and IL-10 protein was analysed. I detected IL-12p70 and IL-23 in M2 macrophages infected with IRF5 expressing construct but only minimal amounts after infection with a construct encoding IRF3 or empty vector (Figure 4.6a). The secretion of both IL-12p70 and IL-23 induced by IRF5 overexpression peaked at 24 h after stimulation with LPS and remained sustained up to at least 48 h (Figure 4.6b). IRF5 overexpression also induced a vast increase in secretion of the p40 subunit which is shared by the two cytokines (Figure 4.6a).

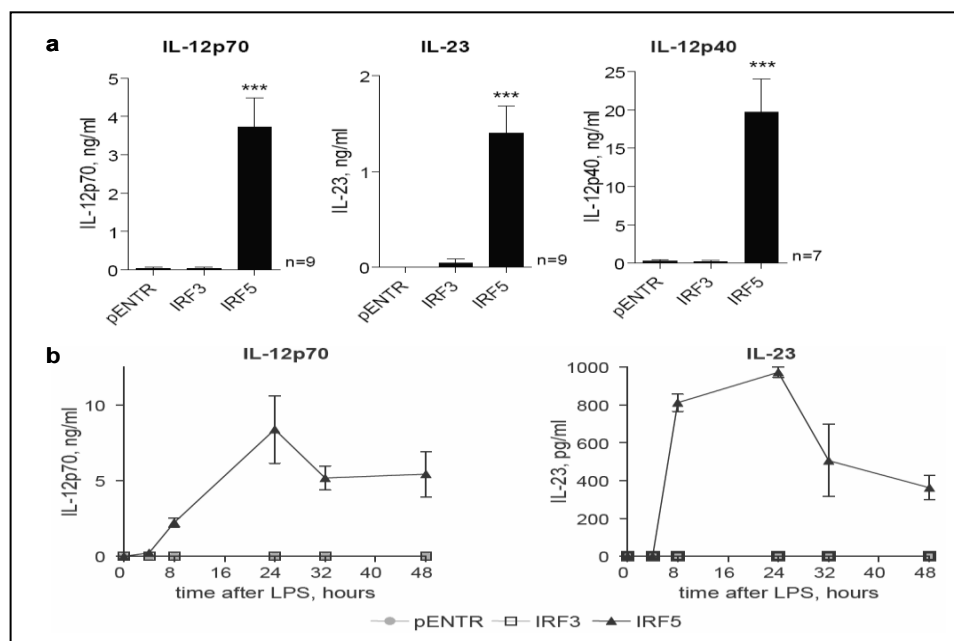


Figure 4.6 Ectopic IRF5 influences the production of IL-12p70 and IL-23

(a) ELISA of IL-12p70, IL-23 and IL-12p40 protein secretion in M2 macrophages infected with adenoviral vector encoding IRF5, IRF3 or empty vector (pENTR) and stimulated for 24 h with LPS. Data are mean $\pm$ SEM of 7 to 9 independent experiments, each with macrophages derived from a different donor. *** $p < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test). **(b)** M2 macrophages were infected as in **a** and left unstimulated or stimulated with LPS (10 ng/ml) for 4, 8, 24, 32 or 48 h. The amount of secreted IL-12p70 and IL-23 protein was determined by ELISA. Data shown are the mean $\pm$ SD and are representative of 3 independent experiments each using macrophages derived from a different donor.

I also observed higher production of other key pro-inflammatory cytokines, such as IL-1 β and TNF, by IRF5-expressing M2 macrophages (Figure 4.7). The effect of ectopic IRF5 on TNF expression is supported by my previous findings in Chapter 3 where I describe in detail the IRF5-mediated induction of TNF.

In line with the previously reported low expression of IL-10 in M1 macrophages, the expression of this cytokine was reduced significantly after LPS stimulation in macrophages overexpressing IRF5 (Figure 4.7). I also observed a reduction of IL-10 protein secretion in cells overexpressing IRF3 (Figure 4.7), which might represent a negative feedback regulation of IL-10 expression via dual specificity protein phosphatase 1 (DUSP1) [303], since the main direct target of IRF3, IFN- β , induces IL-10 [304].

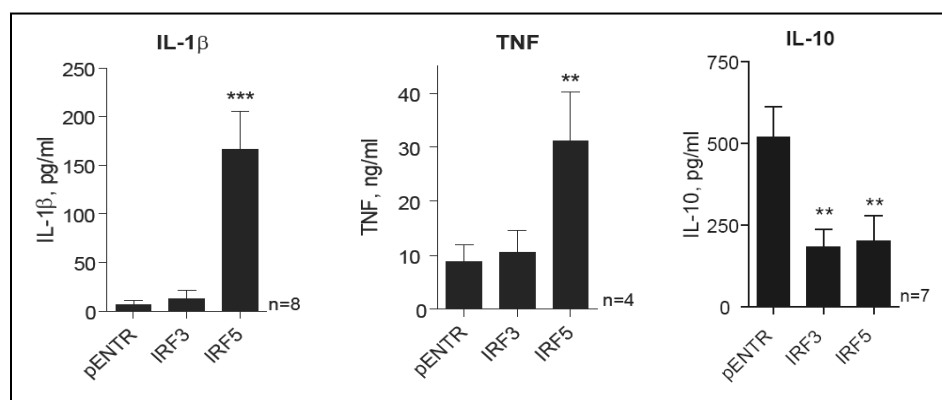


Figure 4.7 Ectopic IRF5 induces IL-1 β and TNF but inhibits IL-10 secretion

M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) and stimulated with LPS for 24 h. The amount of secreted IL-1 β , TNF and IL-10 protein was determined by ELISA. Data show the mean $\pm$ SEM of 4 to 8 independent experiments each using M2 macrophages derived from a different donor: ** $p < 0.01$, *** $p < 0.001$, (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

Next, I performed the complementary experiment of targeting endogenous IRF5 in M1 macrophages by RNA-mediated interference (Figure 4.7). I used the same protocol as previously optimised (Methods section 2.11.2) which usually resulted in 50 to 60% knock-down of IRF5 protein in M1 macrophages estimated by serial dilutions of the control siRNA treated cells (Figure 4.8). The delivery of nucleic acids into macrophages runs the risk of mimicking viral infection, associated with induction of type I IFN genes, thus potentially

creating an artefact of the experimental system. As mentioned previously, I routinely analysed the mRNA induction of type I IFNs and the secretion of IP-10 (a known type I IFN-inducible gene) which were found to be minimal.

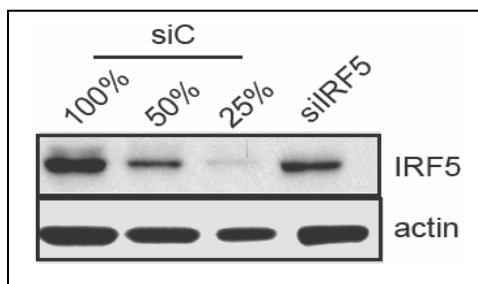


Figure 4.8 siRNA-mediated knock-down of IRF5 in M1 macrophages

M1 macrophages were transfected with siRNA pool targeting IRF5 (siIRF5) or control siRNA (siC) and total protein extracts were analysed by Western blot. Actin is used as a loading control. Blots shown are from a representative experiment.

The response to TLR4 stimulation and subsequently cytokine production of siRNA transfected M1 macrophages was generally lower compared to untreated cells from the same donor (data not shown). I therefore opted for a costimulation of LPS (10 ng/ml) plus IFN- γ (50 ng/ml) in order to provoke a more robust and reproducible response after siRNA transfection.

The siRNA-mediated knock-down of IRF5 in M1 macrophages resulted in significant inhibition of LPS plus IFN- γ costimulation-induced production of both IL-12p70 and IL-23 (Figure 4.9). Secretion of IL-12p40 was also lower in these cells (Figure 4.9), consistent with data obtained with *Irf5*-deficient mouse myeloid cells [190]. However, the production of IL-10 was higher in IRF5-depleted macrophages compared to siC transfected cells mirroring the data from the overexpression experiments (Figure 4.7).

Together these results suggest that IRF5 influences M1 macrophage polarization by equipping the cells with an IL-12^{hi}, IL-23^{hi} and IL-10^{low} cytokine profile.

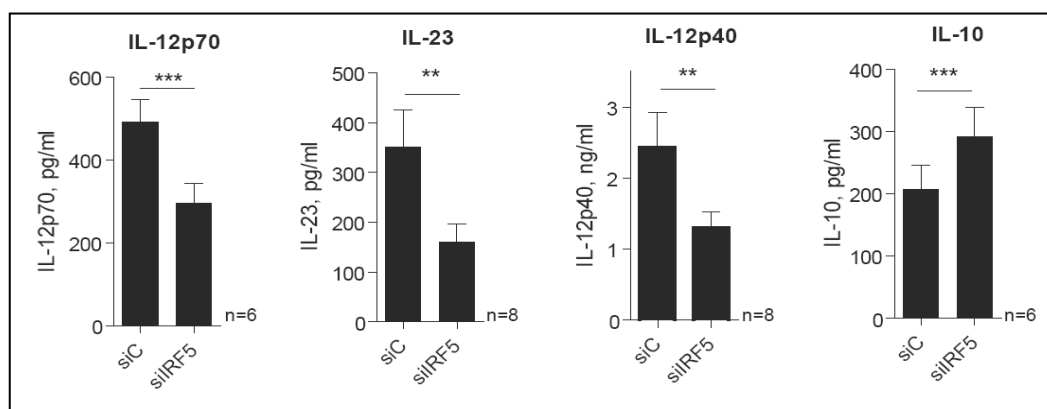


Figure 4.9 Depletion of IRF5 influences the production of lineage-specific cytokines

ELISA of the secretion of IL-12p70, IL-23, IL-12p40 and IL-10 by M1 macrophages transfected with siRNA targeting IRF5 (siIRF5) or nontargeting control siRNA (siC) and stimulated for 24 h with LPS (10 ng/ml) plus IFN- γ (50 ng/ml). Data are the mean $\pm$ SEM of 6 to 8 independent experiments, each with macrophages derived from a different donor. ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test).

4.2.4 IRF5 induces the mRNA expression of macrophage lineage-specific cytokines

IRF5 is a transcription factor that can bind to the regulatory regions of target genes and modulate their expression. I therefore thought to investigate whether the role of IRF5 in the differential regulation of the secretion of IL-12p70, IL-23 and IL-10 cytokines was a direct consequence of its function as a transcription factor. The expression of mRNA for IL-12p40, IL-12p35 and IL-23p19 was considerably induced in resting M2 macrophages infected with an adenoviral vector construct encoding IRF5 but not in those infected with a construct encoding IRF3 or with empty vector (Figure 4.10). Consistent with the protein-secretion data, the expression of IL-10 mRNA was inhibited by ectopic IRF5 (Figure 4.10). However, the expression of IL-10 mRNA was not altered by IRF3 (Figure 4.10), which suggests the lack of a direct role for IRF3 in transcription of the gene encoding IL-10.

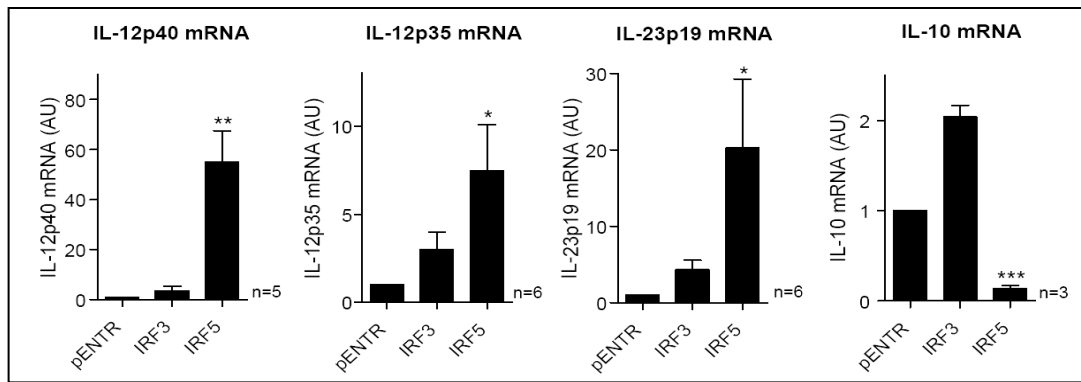


Figure 4.10 IRF5 induces the mRNA expression of lineage-specific cytokines

Quantitative RT-PCR analysis of mRNA for IL-12p40, IL-12p35, IL-23p19 and IL-10 in M2 macrophages infected with adenoviral vector encoding IRF5 or IRF3; basal expression is presented relative to that of control cells infected with empty vector. Data are the mean $\pm$ SEM of 3 to 6 independent experiments, each with macrophages derived from a different donor. * p <0.05, ** p <0.01 and *** p <0.001 (one-way ANOVA with Dunnett's multiple-comparison post-test).

Depletion of endogenous IRF5 in M1 macrophages via RNA-mediated interference resulted in lower expression of mRNA for IL-12p40, p35 and IL23p19 at 8 h after stimulation with LPS (Figure 4.11). Conversely, the expression of IL-10 mRNA was higher in M1 macrophages with suppressed expression of IRF5 protein (Figure 4.11).

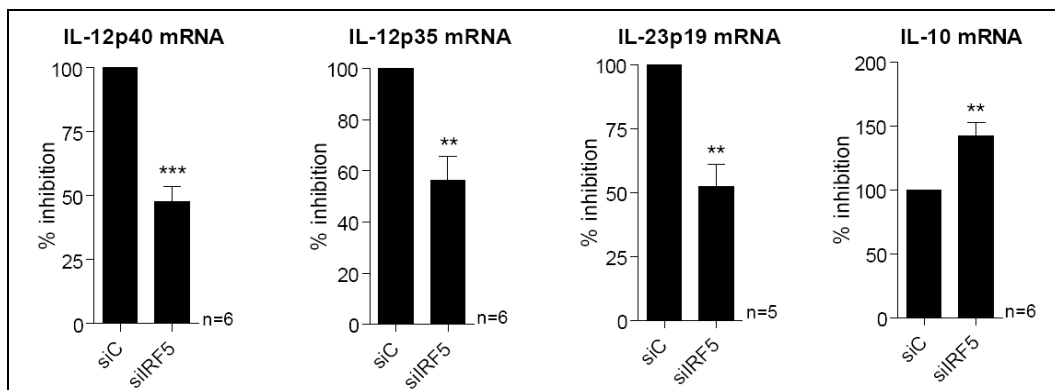


Figure 4.11 IRF5 depletion influences mRNA expression of lineage-specific cytokines

Quantitative RT-PCR analysis of mRNA expression of IL-12p40, IL-12p35, IL-23p19 and IL-10 in M1 macrophages transfected with siRNA targeting IRF5 and left untreated or stimulated for 8 h with LPS (10 ng/ml). Results are presented as percent inhibition relative to those of control cells transfected with nontargeting control siRNA. Data are the mean $\pm$ SEM of 5 to 6 independent experiments, each with macrophages derived from a different donor. ** p <0.01 and *** p <0.001 (Student's *t*-test).

To formally define the global expression profile induced by IRF5, I performed genome-wide expression analysis in which I compared M2 macrophages overexpressing IRF5 with previously defined human M1 and M2 macrophage subset [67, 305]. Gene expression data were obtained by hybridising RNA from a total of 8 samples from two experimental groups to Illumina HumanHT-12 Expression BeadChips. The experimental groups consisted of RNA obtained from macrophages infected with empty vector pENTR and macrophages infected with vector encoding IRF5 (n=4 per group as the macrophages used were derived from monocytes of 4 independent blood donors). Similar to previous experiments the RNA extraction was performed 48 h post infection. An aliquot of each RNA sample was first reverse transcribed to obtain cDNA and subsequently analysed for the induction of genes and transcription factors associated with cell activation. The quality control of the RNA samples (which did not reveal any outlier samples) and the hybridisation reaction was carried out by Dr Dilair Baban at the Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom.

Raw signal intensities were background corrected based on background intensity of negative control probes on the array. Next, the dataset was filtered to remove probes not detected (detection score <0.95) in any of the samples, resulting in a final dataset of 25,620 probes. The raw p-values were then corrected for multiple testing and adjusted p-values below 0.01 were considered significant. The resulting probe lists were annotated and used for further biological investigation. The Micro array analysis was kindly performed by Mrs Helen Lockstone and Miss Natasha Sahgal (both Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom).

The mRNA expression of NF- κ B family members, IRF3, IRF4 and IRF7 as well as type I IFNs was not affected 48 h post infection in IRF5 or empty vector treated cells. Therefore, changes in gene expression observed are likely due to the presence and activity of ectopic IRF5 although other potential secondary effects cannot be entirely ruled out. Overall, the expression of about 90% of known human polarization-specific markers was affected by ectopic IRF5 (Figure 4.12). Overexpression of IRF5 induced 20 M1-specific genes and inhibited 19 M2-specific genes encoding cytokines, chemokines, costimulatory molecules and surface receptors (Figure 4.12) in resting cells. Moreover, by using this genome-wide approach I was able to identify many previously unknown IRF5-regulated genes that probably contribute to the main functional features of macrophage subsets, such as phagocytosis and antigen presentation (Table 4.1).

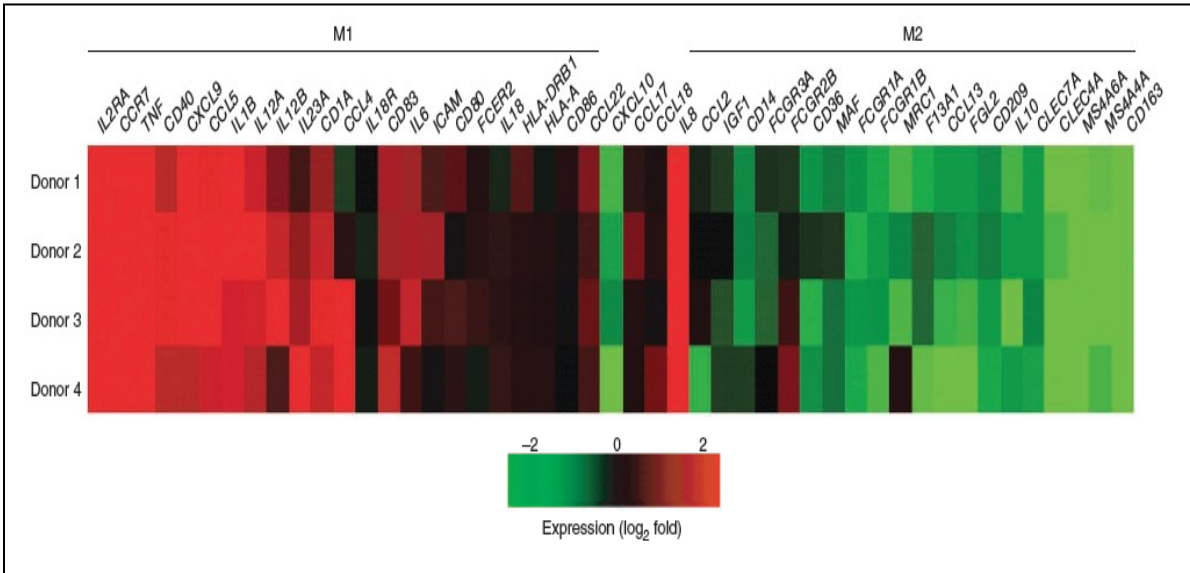


Figure 4.12 IRF5 influences the global expression profile of macrophage subsets

Global mRNA expression (for sets of M1- and M2-specific genes [67, 305]; above plot) by unstimulated M2 macrophages infected with adenoviral vector encoding IRF5, presented relative to that of unstimulated M2 macrophages infected with empty vector. Results are presented as higher (red) or lower (green) expression (log₂ fold change) after infection with vector encoding IRF5. Data are from a representative experiment with four different donors.

M1-specific genes that were induced by exogenous IRF5 include IL12A, IL12B and IL23A (coding for IL-12p35, IL-12p40 and IL-23p19 respectively) further supporting the dependence of these genes on IRF5. Interestingly, Epstein-Barr virus induced 3 (EBI3), also belonging to the IL-12 gene family, was highly upregulated in IRF5 expressing cells as well (Table 4.1). In line with my previous results, TNF was another gene whose expression was induced in the presence of IRF5 (Chapter 3). Genes coding for costimulatory molecules that were induced by IRF5 include CD40, CD80, CD83 as well as members of the tumor necrosis factor (ligand) superfamily (TNFSF, Table 4.1). CCR7, CXCR3 CXCR4 CXCR5 and CXCR7 were amongst the M1-specific surface receptors upregulated in IRF5-expressing macrophages. On the contrary, genes coding for surface receptors associated with M2 macrophage polarization (CD36, CD163, FCGR1A, FCGR1B and MRC1) were decreased. Furthermore, ectopic expression of IRF5 in M2 macrophages reduces mRNA expression of IL-10 and other cytokine markers of M2 macrophages, such as CCL2 and CCL13.

Table 4.1 Newly identified IRF5 target genes

Genes up-regulated by ectopic IRF5		
<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Fold change</i>
CXCR3	2883	2.9
CXCR4	7852	3.8
CXCR5	643	9.4
CXCR7	57007	4.1
EBI3	10148	38.2
TNFSF4	7292	17.9
TNFSF7	970	25.5
TNFSF9	8744	15.0
LTA	4049	13.1
LTB	4050	8.9
IFN- γ	3458	2.5
CCL1	6346	18.6
CCL3	6348	8.1
CXCL5	6374	7.4
IL-19	29949	4.2
IL-32	9235	4.2

Genes down-regulated by ectopic IRF5		
<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Fold change</i>
CSF1R	1436	0.28
IL-1R2	7850	0.28
IL-1RA	3557	0.38
TGF β 1	7045	0.42

Global mRNA expression was analysed as in Figure 4.12. Results are presented for selected genes and are presented as fold change in IRF5 expressing macrophages relative to that of unstimulated M2 macrophages infected with empty vector. Data are from a representative experiment with four different donors.

The data from the genome-wide mRNA expression analysis corresponded well with the protein data of the cytokines investigated so far in this chapter (IL-12p70, IL-23, TNF, IL-1 β and IL-10). In order to improve on the validity of the micro array data set, I decided to confirm additional genes by performing ELISAs as well as FCM analysis using M2 macrophages with exogenous IRF5. As expected, these experiments resulted in an increased production of M1-specific proteins (Figure 4.13a) but decreased production of M2-associated proteins (Figure 4.13b). Thus, IRF5 influences the expression of most cytokines that define human macrophage lineage.

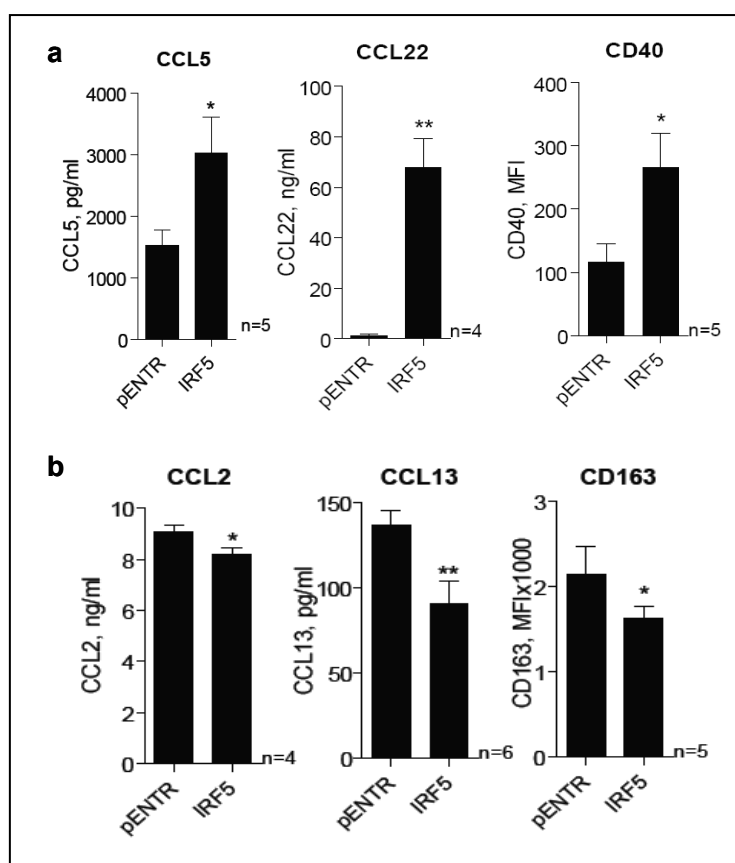


Figure 4.13 IRF5 drives the production of selected lineage specific cytokines

M2 macrophages were infected with adenoviral vectors encoding IRF5 or empty vector (pENTR) and stimulated with LPS for 24 h. The amount of secreted CCL5, CCL22 (**a**) or CCL2, CCL13 (**b**) protein was determined by ELISA. The amount of CD40 (**a**) or CD163 (**b**) surface expression was determined by FCM and expressed as MFI. Data are shown as the mean $\pm$ SEM of 4 to 6 independent experiments each using M2 macrophages derived from a different donor: * $p < 0.05$ and ** $p < 0.01$, (Student's t-test).

4.2.5 IRF5 directly regulates mRNA expression of lineage-specific cytokines

IRF5 overexpression in resting M2 macrophages resulted in a profound change in basal levels of mRNA expression of lineage-specific genes. This suggested that IRF5 might be directly involved in the transcriptional regulation of lineage-specific genes and I therefore initiated the investigation into the LPS-induced recruitment of IRF5 to the promoter loci of selected genes.

All IRF family members share a well-conserved amino-terminal DNA-binding domain that recognises ISREs binding sites. Computational analysis of the regions 2,000 bp 5' upstream and 2,000 bp downstream of the transcription start sites of *IL12A* (IL-12p35), *IL12B* (IL-12p40), *IL23A* (IL-23p19), *IL10* and other IRF5-regulated genes led to the identification of several ISREs (Table 4.2).

Table 4.2 putative IRF5 binding sites in -2000/+2000 bp relative to the TSSs of selected genes

Genes up-regulated by ectopic IRF5				
<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Number of ISREs</i>	<i>Sequence</i>	<i>Strand</i>
IL12A	3592	9	gctctcaTTTCttttctttc	-
			atgtaaattaGAAActgtgtc	+
			gcgaacaTTTCgctttcattt	-
			atttcgcTTTCattttgggcc	-
			atccGAAAgcgccgcaagccc	+
			gaaggagacaGAAAgcaagag	+
			tcgtagaggaGAAActgaggc	+
			cacctggtctgggTTTCcctg	-
			tgtctccagaGAAAgcaagag	+
IL12B	3593	8	gttacagTTTTtttttttaa	-
			cccgggtTTCCcatttccccc	-
			gagggtaTTTCactttctgct	-
			aagtcagTTTCtagttaagt	-
			tttctagttaagTTTCcatc	-
			gttacagTGTCcattttaaaa	-
			gttaaaaaatGAAAgctatt	+
			actgGAATctgaaattgtatg	+

<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Number of ISREs</i>	<i>Sequence</i>	<i>Strand</i>
IL23A	51561	6	accaggaagtGAAAc aaagag	+
			gggtagaTTTCcattttttt	-
			gtgatgaaatcggTGTCagtg	-
			ccatGAAAccaggaccatcca	+
			ctgaGAAAaagaagcccgttt	+
			ttgggaaagaGAAAtcgatgg	+
TNFSF4	7292	9	aatgtactttacaTTTCccac	-
			cacaaacTTTCtctttaagt	-
			tgctcaTTTCcattttttct	-
			agatcttTTTCttttcttg	-
			agaccagTTCCactttcccat	-
			taaaataTTTCcatttttctt	-
			attttcTTTCactttattct	-
			attatttttcttATTCagta	-
			cacctccaatGAAAccagaat	+
EBi3	10148	9	ctctgtgTTTCtctttctgtt	-
			gtttctcTTTCgtttccatc	-
			gtatctcTGTCactttctctg	-
			ctgtcacTTTCtctgtcatct	-
			ccttggTTTCtttttggtt	-
			ttttggtttgtTTTTtgag	-
			ccagGAATtcgagaccagcct	+
			gcaacatagtGAAAccggacc	+
			tagtGAAAccggacccacca	+
TNFSF7	970	14	ctgcctcattcagTTTCtggt	-
			cattcagTTTCtggttctggt	-
			gtttctgTTTCgttttcaca	-
			agggGAATaggaagattgaat	+
			catgGAAAtggaagatgactc	+
			ccagGAAAacgattcgggaaa	+
			aaaataaaatGAAAtaaaatc	+
			gagggaaacgGAGAgggggag	+
			agaaGAAGgggaaagaaagaa	+
			cggagaaagaGAAAaaagaca	+
			aagaagaaagGAAAagaaaaa	+
			aaaagaaaaaGAAAgaaagga	+
			aaaggaaaaaGAAAgaaagaa	+
			agaagaaagaGAAAaaaagaa	+
TNFSF9	8744	7	tcaacacTGTCcctttcttgc	-
			gagacaaagaGAGActaaaga	+
			cagaGATAacggagccagaga	+
			cagaGATAacggagccagaga	+
			agggagaaagGAACctggagc	+
			gccggaaacgGAAAggagagc	+
			gtaccccTCTCctttcaaga	-

<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Number of ISREs</i>	<i>Sequence</i>	<i>Strand</i>
CCL1	6346	6	ttcatgaTTTCaatgcctaga	-
			aacaaaaaggGAAAttcccc	+
			aatagaaatgGCAAatatcta	+
			gtgtGAATatgaattgggta	+
			actctacTTTCtctatcagt	-
			actggaaagaGCAAgggaacc	+
CCL3	6348	12	gctttcaTTTCttttctact	-
			caaagaaatgGGAAatcaaga	+
			ccattgaacaGAAActtcagc	+
			ttcagaaaaaGAAAAaaataa	+
			ctcatgcTTTCtattcctcca	-
			ccccagattccaTTTCccca	-
			gcccccaagaGAAAgagagaac	+
			cttggtcTTTCtcttaagac	-
			cagagaaacaGAGAaccact	+
			agaggaaaggGACAggaagaa	+
			aattattttcgaTTTCacag	-
			agtttggtttgtTTTCctgg	-
IL-2Ra	3559	5	ggaggggTTTCttttgtaa	-
			aattGAACttgaaaaaaaaa	+
			caatgaatttcctTTTAttct	-
			tgcaaattttaaTTTCattc	+
			ccaaGAACaggaaaatctga	+
CCR7	1236	9	tcaagaaagtGAAAgatgat	+
			aaaaaaaaaGAAaaaagaaa	+
			tcaacaaTTTCacttctaggt	-
			agctaaaaggGAAAcagccc	+
			ttcaGAATaggaaaatctata	+
			aaagGAAAggaagggagggg	+
			acccagactaggTTTAgggg	-
			gggagggTTTCgttacacaa	-
			tacacaaaatGAAActccca	+
CXCR5	643	8	ttggtgaTTTCactttttt	-
			ttttttTTTCtttagagac	-
			aagttgaTTTCattttgtct	-
			tgagGAAAtgaagggttgga	+
			gtggtggTTTCattacaagtt	-
			gaaaGAAGctgaaatgctga	+
			caaaaaacaGAAAgaccca	+
			aatgcaaaatGAAAcatggg	+

Genes down-regulated by ectopic IRF5				
<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Number of ISREs</i>	<i>Sequence</i>	<i>Strand</i>
IL-10	3586	7	ctgttatttcaaCTTCttcc	-
			acaactaaaaGAAActctaag	+
			acgcGAATgagaaccacagc	+
			tgcaaaaattGAAActaagt	+
			caggGAAAtttaaattgcctc	+
			cttctgcTTTCcctcaaaat	-
			ttgctcaTTTCtcttgagca	-
CD163	9332	10	ggatgaaactGGAAccatca	+
			ttgctaattttgTTTCacca	-
			gtatGAAAtggaacctcagct	+
			gtagcctTTTCattttcatga	-
			tcatGAAAgagaagtatttt	+
			gatgttgTTTCcattttccag	-
			gccctctTTTCttttcacag	-
			caaaggaggaGAAActcaga	+
			agataagTTTCagtctagcgt	-
			ctagtcttttcatCTTCataa	-
MS4A6A	64231	12	aaagacaagaGAAAggagaat	+
			agccaaaatgGAAAaaaaaag	+
			cgctGAGAactaatccagcct	+
			tgactggctctggTTTCcttg	-
			tgggGAATtagaaaagcaaga	+
			ttagaaaactGAAGcttcaag	+
			aatttaactGAAActccttg	+
			gaaggagTATCgtttttaac	-
			ccgtGAAAgggatccaagct	+
			tccatacTATCagtttcttc	-
			ctatcagTTTCtttctaat	-
			gactgagTTACTgttttgga	-
CXCL10	3627	11	caactaaaatAAAActgtcac	+
			ttgcctTTCCggttccac	-
			ccaacactttttTTTCtttt	-
			ctttttTTTCttttcttg	-
			caacctgTTTCccttctgtct	-
			atgatgtTTTCattcaggac	-
			tataagacgtGAAActtggtt	+
			ttggaaagtGAAAcctaatt	+
			aagtGAAAcctaattcactat	+
			catgcagagtGAAActtaaat	+
			ttaggaaacgGCAAtcttggg	+

<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Number of ISREs</i>	<i>Sequence</i>	<i>Strand</i>
CLEC4A	50856	5	gacttggtgtgggTTTCagaa	-
			gaaagacaatGAAAgcagggt	+
			gagaGAAAtccactccagttc	+
			tagagtacaaGAAActatggg	+
			actatagTTACgctttctaaa	-
IL-1R2 isoform1	7850	13	ggcatggttttgcTTCCtctc	-
			ccaaatatttcacCTTCtaat	-
			gtaaGAAAatgaagatctgca	+
			ctctGAAAacaaaacaaaaca	+
			gaaaaataggGAAActtatgc	+
			cagagaaacaGAGAcagaaaag	+
			cagagaaacaGACAgagatag	+
			gacaGAGAcagagaccaagac	+
			gctctcgggtggTTTCtggg	-
			ctcagggTCTCcatttcacc	-
			ctctctgTCTCgtttctctc	-
			ctctatgTCTCgtttctccc	-
			taattgcattcccTTTTgggg	-
IL-1R2 isoform2	7850	11	ttcactcTTCCagtttctcac	-
			tttgctcTCTCccttctctgg	-
			gaacaaaattTAAActgttct	+
			acgatggcttcacTTACatgg	-
			ttataagacaGAAAgcaaaat	+
			gacaGAAAgcaaaattgttt	+
			tttaGAAActgaagctgtatc	+
			tgaacactttctTTTTGcagc	-
			gggaGAATttgaagcctgtgg	+
			ttgaatgagcGAAAacatgag	+
			ccatctgTATCagtttctgcc	-
IL-1RA isoform1	3557		ggaaGAAAtccaatctatttc	+
			aatctagtttctgATTCtta	-
			agagGAAAttgaaggccctta	+
			attctgaTTTCattatatata	-
			ctctaatttaagTTTCtaat	-
			tttaagTTTCtaatttaaaa	-
			taaataaaaatGAAAtaaaata	+
			agaggaaatgGATAtagagag	+
			actcggactgGAAActggaag	+
			actggaaactGGAagggtgag	+

<i>Gene symbol</i>	<i>Entrez Gene ID</i>	<i>Number of ISREs</i>	<i>Sequence</i>	<i>Strand</i>
IL-1RA isoform4	3557	11	tacaaaaaatGAAAatgaact	+
			aaatGAAAatgaactaaactt	+
			cacacagTTTGaattcctggg	-
			tggaactGAATctcaaaa	+
			ctcaGAAAaggaagctgggtt	+
			ggaggaaaatGCAAattgaaa	+
			aatgcaaattGAAAagttgct	+
			ccttgctTTTCccttgaatg	-
			aagagggaataGGAActgcacc	+
			cctcttcctcagTTTCagct	-
			ccttcagTTTCagcttgaata	-

Putative ISREs of selected genes were identified using the bioinformatic programs Genomatix and JASPAR. Capital letters in the binding sequence indicate the core binding sequence of ISREs (perfect match equals GAAA on + strand and TTTC on – strand)

I designed primers encompassing the ISREs of *IL12A* (IL-12p35), *IL12B* (IL-12p40), *IL23A* (IL-23p19) and *IL10* and used them in quantitative ChIP experiments with M1 macrophages stimulated with LPS for 0, 1, 2, 4, 8 or 24 h. RNA polymerase II ChIP was used in combination with IRF5 ChIP to provide an estimate of active transcription of the genes of interest upon LPS-stimulation. I observed LPS-induced enrichment of IRF5 at the promoter regions of *IL12A*, *IL12B* and *IL23A* up to 8 h after stimulation which matched the kinetics of the recruitment of RNA polymerase II to the genes (Figure 4.14 a-c).

In contrast to the positively regulated genes, at the *IL10* promoter region, LPS-induced recruitment of IRF5 took place between 1 h and 4 h after stimulation, whereas RNA polymerase II bound to the region only 8 h after stimulation (Figure 4.14d). This suggests a previously unknown inhibitory role for IRF5 in the transcriptional regulation of selected genes.

Together these results suggest that in M1 Macrophages, IRF5 directly regulates the transcription of genes encoding IL-12p40, IL-12p35, IL-23p19 and IL-10 through its recruitment to their promoter regions. Furthermore, it shifts the global expression profile of M2 macrophages to a more pro-inflammatory “M1-like” phenotype.

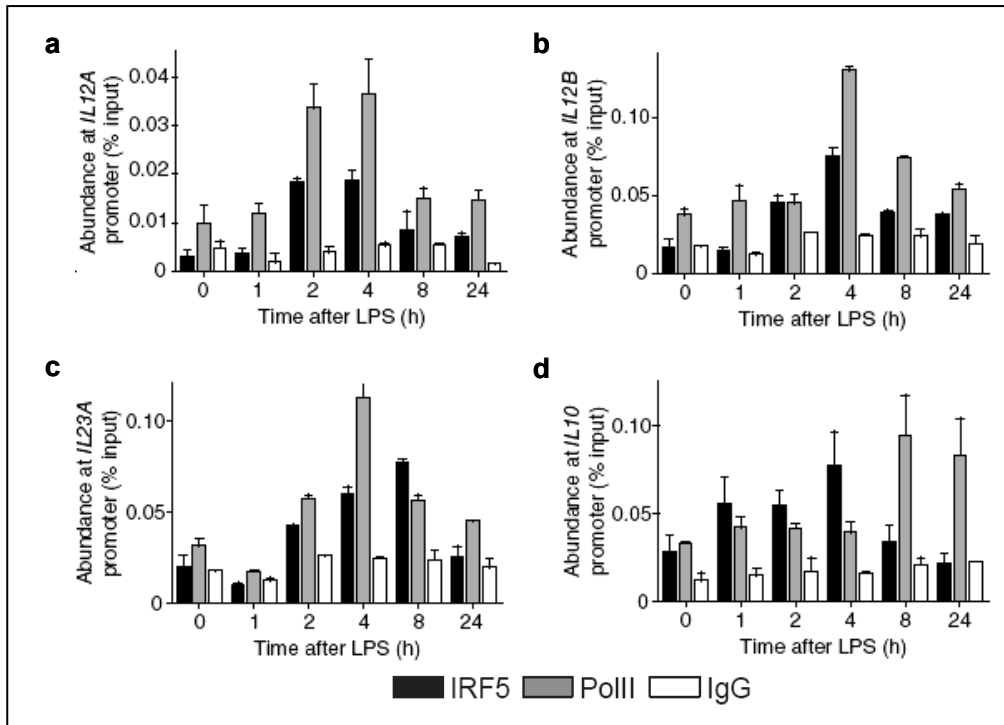


Figure 4.14 LPS-induced recruitment of IRF5 to promoter regions of lineage-specific cytokines

Recruitment of proteins to the promoters of genes encoding IL-12p35 (*IL12A*; **a**), IL-12p40 (*IL12B*; **b**), IL-23p19 (*IL23A*; **c**) or IL-10 (**d**) in M1 macrophages left unstimulated (0) or stimulated for 1, 2, 4, 8 or 24 h with LPS (10 ng/ml), assessed by ChIP with anti-IRF5, anti-RNA polymerase II (Pol II) or immunoglobulin G (IgG; control); results are presented relative to those obtained with genomic DNA (input). Data show the mean $\pm$ SD from one experiment representative of three.

4.2.6 IRF5 inhibits the transcription of the human IL-10 gene

The observed effect of IRF5 on IL-10 gene expression led me to investigate whether it can directly repress the transcription of the gene encoding IL-10. To test this, I used an adenovirus construct with a gene reporter in which the luciferase reporter construct was flanked with 195 bp 5' upstream of the human IL-10 gene [306]. I coinfect M2 macrophages with that construct plus a vector encoding IRF5 or empty vector and quantified luciferase activity. IRF5-expressing cells showed significantly less luciferase activity, both in resting cells and at 4 h after stimulation with LPS (Figure 4.15a). To confirm the importance of the binding of IRF5 to the IL10 promoter, I used a mutant of IRF5 lacking the DNA-binding domain IRF5 (IRF5 Δ DBD). This IRF5 mutant was not able to inhibit the wild-type IL-10 luciferase reporter construct (Figure 4.15a). To further explore the molecular

mechanism of IRF5-mediated suppression of IL10 transcription, I introduced point mutations into the ISRE identified (positions –182 to –172 relative to the transcription start site) and coinfecting M2 macrophages with this mutated luciferase reporter construct and vector encoding IRF5 or empty vector. The luciferase reporter construct with a mutated ISRE produced a different response than did the wild-type construct in that ectopic IRF5 was no longer able to suppress luciferase activity (Figure 4.15b), which suggested that IRF5 inhibits IL-10 by direct binding to the ISRE of the IL10 promoter. This was opposite to the positive regulatory activity of IRF5 at the TNF promoter (Chapter 3).

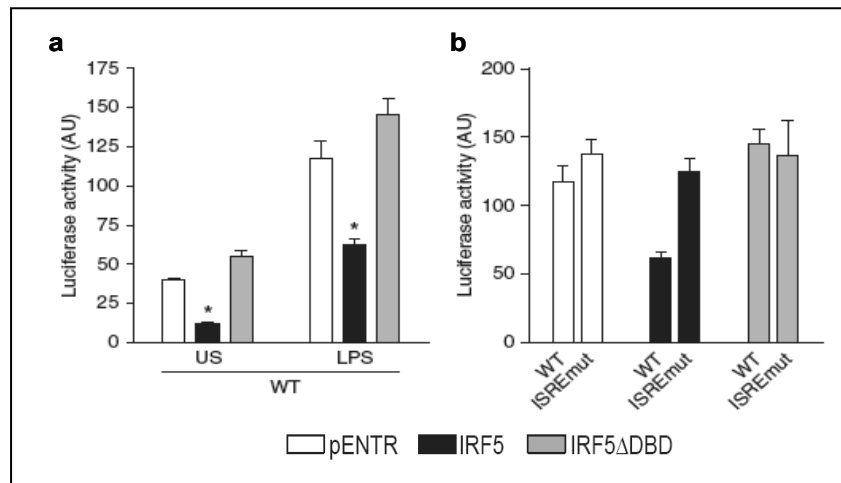


Figure 4.15 IRF5 inhibits the transcriptional activation of the human IL-10 gene

(a) Luciferase activity of M2 macrophages infected for 24 h with a wild-type IL10 luciferase reporter plasmid (WT), plus a construct encoding IRF5 or an IRF5 mutant lacking the DNA-binding domain (IRF5ΔDBD) or empty vector, then left unstimulated (US) or stimulated for 4 h with LPS (10 ng/ml). **(b)** Luciferase activity of M2 macrophages infected for 24 h with the wild-type reporter in (a) or an IL10 luciferase reporter plasmid with site-specific mutations in the ISRE site at positions –180 to –173 (ISREmut), plus the constructs in (a) and stimulated for 4 h with LPS (10 ng/ml). Data are the mean ± SEM from three independent experiments, each with macrophages derived from a different donor. * $p < 0.01$ (one-way ANOVA with Dunnett's multiple-comparison post-test).

Therefore, IRF5 can act not only as a transcriptional activator but also as a suppressor of certain target genes, in this case the gene encoding the anti-inflammatory mediator IL-10. The mode of inhibition is mediated by direct binding of IRF5 to the promoter region of IL10 and may involve engagement of as-yet-unidentified cofactors.

4.3 Conclusion

Macrophages are key mediators of the immune response during inflammation. Plasticity and functional polarization are hallmarks of the macrophage system resulting in phenotypic diversity of macrophage lineage populations [61]. Here I investigated whether IRF5 is involved in macrophage polarization. I demonstrate that IRF5 is indeed a major factor defining macrophage lineage commitment: it is highly expressed in M1 macrophages and induces a characteristic gene expression and cytokine secretion profile. I also unravel a new regulatory role for IRF5 as an inhibitor of M2 macrophage marker expression. Finally, IRF5 contributes to the macrophage system plasticity, i.e. modulation of its levels leads to the conversion of one macrophage subset's phenotype into the other.

IRF5 protein levels are induced in M1 macrophages differentiated with GM-CSF (Figure 4.2) or M2 macrophages stimulated with IFN- γ plus LPS (Figure 4.3). In priming experiments, a 24 h treatment of M2 macrophages with GM-CSF significantly upregulates IRF5 protein levels suggesting that it is likely to be a direct consequence of GM-CSF treatment (Figure 4.5). This was further supported by the kinetics of IRF5 mRNA expression in monocytes stimulated with GM-CSF, which showed rapid upregulation of mRNA 2 h post stimulation (Figure 4.2c). IFN- γ stimulation of monocytes also induced IRF5 expression, with the magnitude and kinetic comparable to that of GM-CSF treatment (data not shown). Altogether, this strongly supports the conclusion that IRF5 is induced by either *in vitro* M1 macrophage differentiation protocol.

The rapid and potent transcriptional response developed by macrophages encountering microbial stimuli, such as LPS, or subsequently cytokines, is orchestrated by many TFs. Among them are class III TFs, such as PU.1, C/EBP β , RUNX1 and IRF8, which are lineage-specific transcriptional regulators turned on during macrophage differentiation [307]. The combinatorial expression of these proteins specifies the macrophage phenotype via constitutive activation or repression of genes and chromatin remodelling at inducible loci. For instance, PU.1 is required for maintaining H3K4me1 enhancer marks at macrophage-specific enhancers [308]. But only a small proportion of the macrophage transcriptome is altered by cell polarization [305] and among the genes differentially expression between the M1 and M2 subsets are those regulated by IRF5, such as IL-12p40, IL-12p35, IL-23p19, IL-1 β , TNF, macrophage inflammatory protein 1a, Rantes, CD1a, CD40, CD86, CCR7 (Figure 4.12). Another member of the IRF family, IRF4, known to inhibit IRF5 activation by

competing for interaction with Myd88 [299], has been recently reported to control the expression of prototypical mouse M2 macrophage markers [302]. Of interest, the expression of IRF4 is equally induced by M-CSF or GM-CSF differentiation (Figure 4.2) and is further enhanced by exposure to IL-4 [309]. IRF5 expression, on the other hand, is specifically induced by GM-CSF or IFN- γ (Figures 4.2 and 4.3), but is unresponsive to IL-4 (data not shown). Thus, IRF5 and IRF4 may be classified as class III TFs but with the difference that they define specific macrophage subsets rather than the global macrophage lineage.

Consistent with published studies [213, 215], I found no expression of the most widely used prototypical mouse M2 markers (Arg1, Ym1 and Fizz 1) in human macrophages (data not shown) – after all, man is not a mouse. On that note, a number of molecules not involved in macrophage polarization in the murine system have been shown to be markers for human macrophage alternative activation, including fibrinolytic (F13A1), fibrinogen-like 2 (FGL2) and insulin-like growth factor 1 (IGF1) [305], all of which were decreased in the presence of endogenous IRF5 (Figure 4.12). Furthermore, expression of some chemokines defined as M1 markers (CXCL10) or M2 markers (CCL17, CCL18 and CCL22) in mouse macrophages did not follow the expected pattern of IRF5 dependence (that is, induction for M1 and inhibition for M2), which possibly reflects again species-specific gene repertoires [214, 310]. Although human M1 macrophages but not M2 macrophages have been shown to secrete large amounts of CCL22 upon LPS stimulation [67], there is some controversy in the literature about whether CXCL10 is a marker of the M1 or M2 macrophage phenotype [67, 304, 305]; my data would classify CXCL10 as an M2 macrophage marker. Therefore, investigators have to be cautious against direct mouse-to-human translation of polarization markers and a direct comparison based on expression profiling results will be required to fully describe interspecies variability.

I used adenoviral delivery of an IRF5 expression construct to further explore the effect of IRF5 on macrophage polarization. It is known that the initiation of an immune response following virus administration can be a potential problem when working with myeloid cells such as macrophages. However, I routinely analysed the secretion of endogenous IFN- λ 1 protein as well as mRNA induction of type I IFNs and observed no significant effect on resting macrophages 48 h or 72 h post infection. Similarly, the mRNA expression of transcription factors associated with an immune response (NF- κ B family members, IRF3 and IRF7) was not affected in adenoviral treated cells (data not shown). Although other

potential effects cannot be entirely ruled out, this conferred some confidence as to the suitability of this technique.

Ectopic expression of IRF5 in M2 macrophages resulted in the upregulation of both mRNA and protein levels of IL-12p70 and IL-23 (Figures 4.6 and 4.10). Conversely, siRNA-mediated inhibition of IRF5 in M1 macrophages showed a reduction of these cytokines upon LPS stimulation (Figures 4.8 and 4.9). In order to clarify the type of involvement of IRF5 in regulation of IL-12p40, IL-12p35 and IL-23p19, I employed a ChIP approach and detected LPS-induced recruitment of IRF5 to all promoter regions in M1 macrophages (Figure 4.14). The binding of IRF5 to the aforementioned gene promoters was accompanied by the recruitment of Pol II which suggests active transcription of the genes. Although more work is required to thoroughly dissect the contribution of other bioinformatically predicted ISRE sites in IRF5 binding, since no significant IRF5 recruitment was observed before LPS induction, I concluded that IRF5 was needed to induce the subset-specific gene expression rather than to alter the chromatin state.

The role of IRF5 in the inhibition of IL-10 gene transcription is novel and important in view of its well documented immunosuppressive activity. *Il-10*^{-/-} mice develop spontaneous autoimmune diseases and show increased resistance to infection [311-313]. IL-10 represses immune responses by down-regulating inflammatory cytokines like TNF [314, 315] and major producers of IL-10 include M2 macrophages, B cells and T lymphocytes [316], whereas M1 macrophages and monocyte-derived DCs are only weak producers [67]. Ectopic expression of IRF5 in M2 macrophages reduces IL-10 secretion upon LPS stimulation (Figure 4.7) and also affects mRNA expression of IL-10 (Figure 4.10) as well as a number of other markers of human M2 macrophage phenotype, such as mannose receptor C type I, insulin-like growth factor 1, CCL2, CCL13, CD163, MCSF receptor and macrophage scavenger receptor 1 (Figure 4.12).

I used IL-10 luciferase constructs to further characterise IRF5-mediated inhibition of IL-10 and found that direct DNA binding of IRF5 was necessary, as a DNA-binding mutant of IRF5 (IRF5 Δ DBD) was not able to inhibit the reporter gene (Figure 4.15a). Moreover, I showed that the previously identified ISRE site, involved in IFN- α but not LPS-induced IL-10 secretion [306], was absolutely required for the inhibitory effects of IRF5 as the mutation of this site abolished the IRF5-mediated inhibition (Figure 4.15b). The recruitment of IRF5 to the promoter ISRE of IL-10 occurred between 1 and 4 h post stimulation in counter-phase

with Pol II recruitment which was only detectable 8 h after activation (Figure 4.14d), suggesting that binding of IRF5 inhibited the recruitment of Pol II presumably via interactions with yet unknown co-factors.

In summary, a distinct systemic role of IRF5 in macrophages is the orchestration of transcriptional activation of pro-inflammatory cytokines, chemokines and co-stimulatory molecules associated with the M1 phenotype. I suggest that IRF5 should be considered as the transcription factor that defines the M1 macrophage phenotype and IRF5 participates, together with other established factors of the M2 macrophage phenotype (C/EBP β , IRF4 and NF- κ B p50), in regulating macrophage polarization (Figure 4.16). Taken into account that IRF5 and IRF4 seem to have opposing function, it is possible that relative concentrations of IRF5 and IRF4 are important for defining a particular macrophage phenotype. This would suggest a new paradigm for macrophage polarization and designates the IRF5-IRF4 regulatory axis as a new target for therapeutic intervention: inhibition of IRF5 activity would specifically affect the pro-inflammatory M1 macrophage phenotype and dampen innate immune responses.

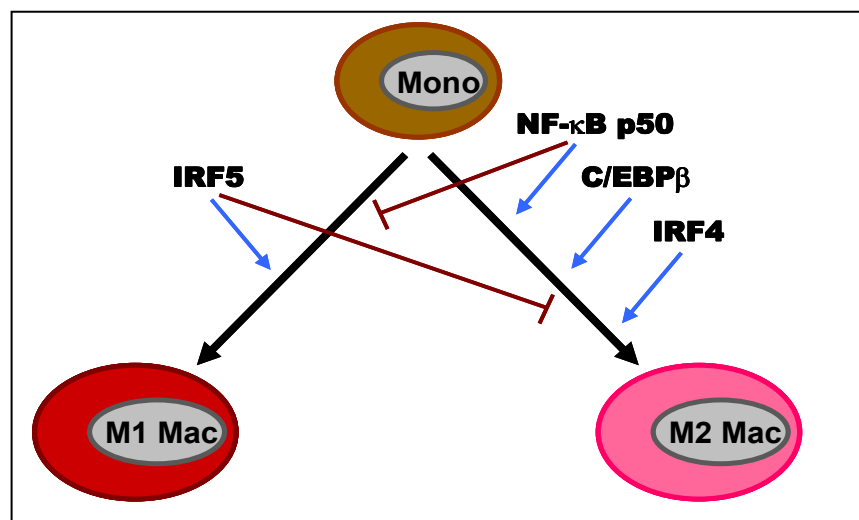


Figure 4.16 Transcription factors underlying macrophage polarization

C/EBP β and IRF4 both regulate M2-specific genes with apparently no effect on M1-specific genes [301, 302]. NF- κ B p50 too, regulates the M2 phenotype but is also able to inhibit certain M1-specific genes [300]. This thesis defines IRF5 as the transcriptional activator of M1-specific genes with additional inhibitory properties regarding M2-specific genes. Mono - Monocyte, M1 Mac - M1 macrophage, M2 Mac - M2 Macrophage

5. IRF5 promotes Th1/Th17 responses and its importance for *in vivo* inflammation

5.1 Introduction

The differences in cytokine production between M1 and M2 macrophages (Chapter 4) have implications for other immune cells, especially T lymphocytes. One of the hallmarks of M1 macrophage polarization is the acquisition of antigen-presenting features, which leads to efficient T lymphocyte responses [63, 64]. IL-12p70 drives polarization of naïve T lymphocytes towards Th1 cell development and induces the predominant release of IFN- γ [106]. IFN- γ mainly serves to activate macrophages at the site of inflammation resulting in increased production of IL-12p70 and other M1-associated cytokines and therefore promotes a positive feedback loop. IL-23 has recently been identified as an IL-12-like cytokine [106] which affects T lymphocyte proliferation and Th17 lineage commitment [317]. IL-10, on the other hand, can repress immune responses by down-regulating inflammatory cytokines like TNF [314, 315] and is important for generation of regulatory T lymphocytes that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens [318, 319]. In this Chapter, I specifically wanted to know what effect forced expression of IRF5 in M2 macrophages would have on the development and activation state of T lymphocytes.

Furthermore, as our group obtained *Irf5*^{-/-} animals during the final year of my PhD, I also thought it would be important to gather more information on the *in vivo* role of IRF5 during inflammatory responses. The use of experimental animals was essential to address this question because these responses cannot be dissected *in vivo* in humans and *in vitro* culture techniques cannot entirely replicate the dynamics or complexity of what occurs *in vivo*. In addition, the use of animals would allow me to gauge the contribution of IRF5-expressing macrophages during inflammation and to have the opportunity to influence and manipulate the immunological system. In order to investigate the function of IRF5 in pathological conditions, I used a mouse model of M1 inflammation and compared the outcome in wild-type and *Irf5*^{-/-} animals.

5.2 Results

5.2.1 IRF5 promotes human T lymphocyte proliferation and Th1 responses

To determine whether IRF5 aided the polarization of T lymphocyte proliferation, fate or activation state I intended to perform MLR experiments. I previously optimised the ratio of APC to T lymphocyte in the MLRs performed in chapter 3 and expected that a similar ratio should work in MLRs using macrophages as APCs. However, I first needed to make sure that adenoviral delivery of constructs in the reduced number of macrophages (2×10^4 cells/well for MLR compared to the usual 1×10^5 cells/well) would still be efficient and not toxic to the cells. I titrated the number of human M2 macrophages (1×10^5 , 5×10^4 and 2×10^5 cells/well), infected them with expression constructs for IRF5, IRF3 or empty vector and measured the expression of GFP which was proportional to the cell number and equal between groups (data not shown). I also determined cell viability in these optimisation experiments using the colorimetric MTT assay and found that the metabolic activity of the cells expressing IRF5, IRF3 or empty vector was again proportional to the cell number and not different between groups (data not shown). I concluded that I could reduce the number of macrophages per well to 2×10^4 in MLR experiments without compromising construct expression or cell viability. Next, I determined the ratio of M2 macrophages to T lymphocytes extracted and purified from the peripheral blood of MHC mismatched donors and performed initial experiments using the ratios 1:10, 1:25 and 1:50. Similar to the MLRs performed in chapter 3, the best ratio of macrophages to T lymphocytes was 1:25, assessed by secretion of IFN- γ (as a measure of T lymphocyte activation) and T lymphocyte proliferation. Therefore, I used 2×10^4 macrophages per well and cultured them with T lymphocytes in a ratio of 1:25 in all following MLR experiment.

Next, I infected human M2 macrophages with viral expression constructs coding for IRF5 or IRF3 or empty vector and exposed the cells to human T lymphocytes in a MLR using the parameters described above. The proliferation of T lymphocytes was assessed 3 days after co-culture by [^3H] thymidine incorporation and was considerably greater when they were cultured together with IRF5-expressing macrophages (Figure 5.1) compared to control or IRF3 expressing cells. The [^3H] thymidine incorporation assays were kindly performed by Dr Saba Alzabin (Kennedy Institute of Rheumatology, London, United Kingdom).

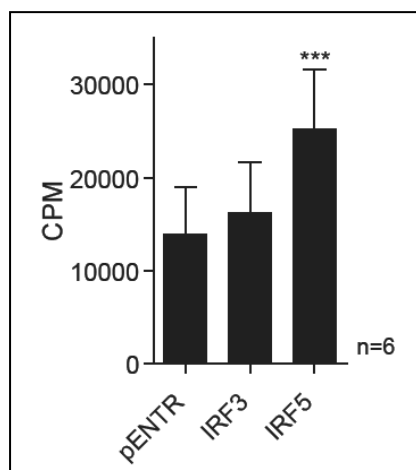


Figure 5.1 IRF5 induced T lymphocyte proliferation

M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) and cultured in triplicate for 72 h with T lymphocytes from MHC mismatched donors. Cultures were pulsed with [^3H] thymidine for the last 16 h to measure DNA synthesis. Control cultures contained macrophages or T lymphocytes alone. Results are expressed as counts per minute (CPM) minus proliferation of macrophage-only cultures. Data are shown as the mean $\pm$ SEM of 6 independent experiments each using macrophages derived from a different donor: *** $p < 0.001$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

Next I analysed the activation of specific T lymphocyte subsets by flow cytometry analysis. Flow cytometry (FCM) allows the simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of cells per second. The detectable parameters of FCM are constantly expanding and include amongst other things cell surface antigens, intracellular antigens such as cytokines and transcription factors as well as cell cycle analysis. There are a wide range of fluorophores available for FCM with each having a characteristic peak excitation and emission wavelength. However, the combination of labels which can be used depends on the wavelength of the laser(s) used to excite the fluorophores and on the detectors available as the emission spectra of the labels often overlap.

Here, I wanted to know what the effect of ectopic expression of IRF5 in M2 macrophages would have on T lymphocyte activation state and fate. I focused on the T helper lymphocyte population which is characterised by the surface expression of CD4 and can be further divided into Th1 (identified by intracellular IFN- γ), Th17 (identified by intracellular IL-17) and Th2 (identified by intracellular IL-4) populations. The MLR optimization experiments

revealed only very low or no intracellular expression of IL-4 in all experimental groups (data not shown) and thus was not analysed in further experiments. Upon analysis of the T lymphocytes I found that only IRF5-expressing macrophages provided the cytokine environment necessary for the population expansion and activation of Th1 lymphocytes, indicated by an increase in $CD4^+/IFN-\gamma^+$ T lymphocytes (Figure 5.2a) which was reproducible in multiple donors (Figure 5.2b).

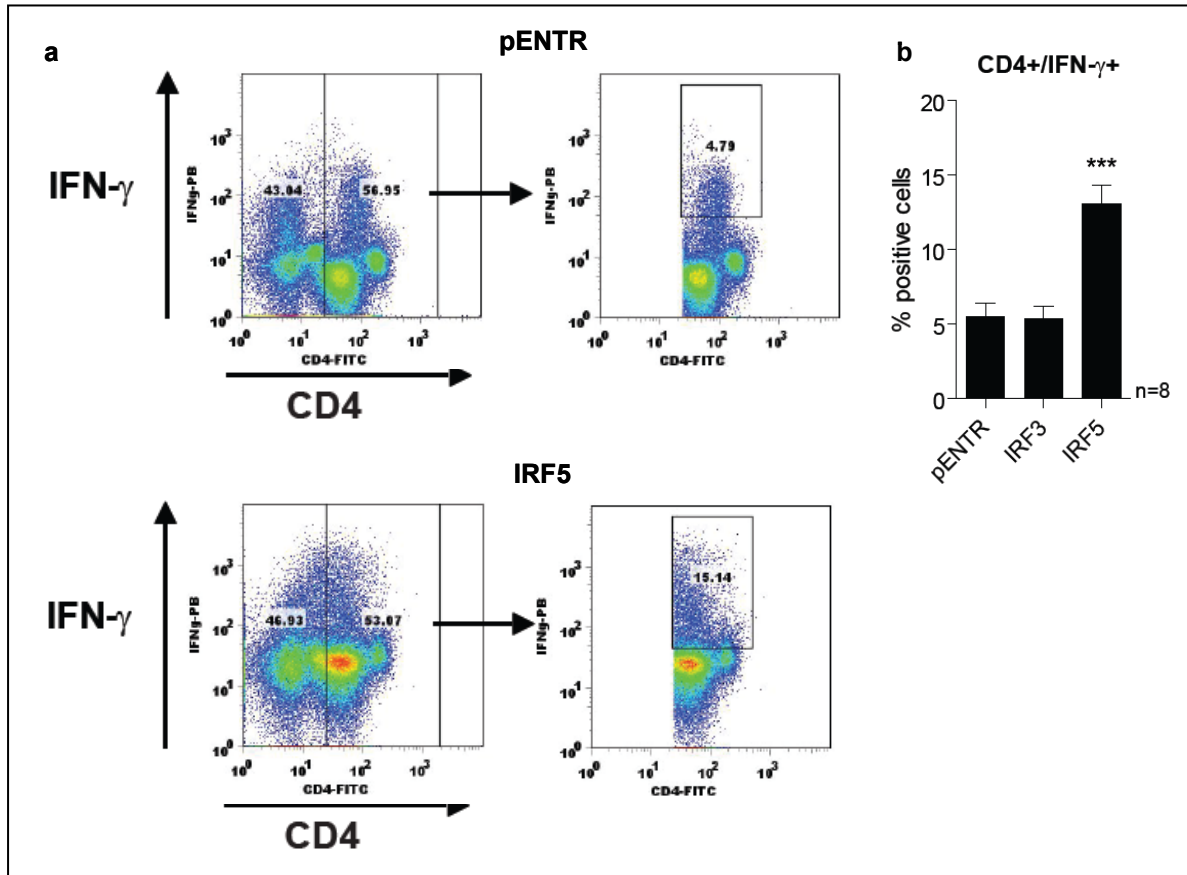


Figure 5.2 IRF5-expressing macrophages induce Th1 activation

(a) M2 macrophages were infected with adenoviral vectors encoding IRF5 or empty vector (pENTR) and cultured with T lymphocytes from mismatched donors. After 4 days, cells were stimulated for 3 h with PMA/ionomycin/Brefeldin A and stained for CD4 and IFN- γ . Representative FCM plots are shown. **(b)** The percentage of $CD4^+/IFN-\gamma^+$ cells was determined by ICC staining in cells treated as in a. Data are shown as the percentage of $CD4^+/IFN-\gamma^+$ cells and are the mean $\pm$ SEM of 8 independent experiments each using cells derived from a different donor. $p < 0.001$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

The increase in CD4⁺/IFN- γ ⁺ lymphocytes was accompanied by higher expression of IFN- γ mRNA in the total T lymphocyte population (Figure 5.3a) as well as increased production and secretion of IFN- γ protein (Figure 5.3b and c). Furthermore, there was significant induction of expression of mRNA for the Th1-specifying transcription factor T-bet (TBX21) in T lymphocytes cultured together with IRF5-expressing macrophages but not control or IRF3 expressing cells (Figure 5.3d).

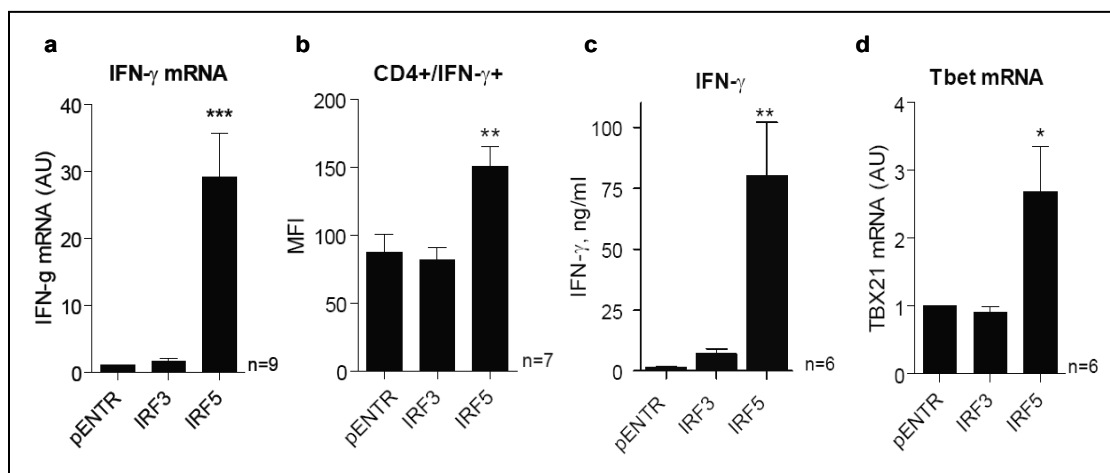


Figure 5.3 IRF5-expressing macrophages induce Th1 development

(a-d) M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) and cultured with T lymphocytes from MHC mismatched donors. **(a)** Expression of IFN- γ mRNA in T lymphocytes after 2 days of co-culture presented in arbitrary units (AU) relative to those obtained with empty vector control. **(b)** Immunocytochemical staining of IFN- γ in T lymphocytes after 2 days of co-culture and then stimulated for 3 h with PMA/ionomycin/Brefeldin A. Results are presented as mean fluorescence intensity (MFI). **(c)** IFN- γ in supernatants of the cells in (b) determined by ELISA. **(d)** Expression of TBX21 (Tbet) mRNA in T lymphocytes from (a) presented as in (a). Data are shown as the mean $\pm$ SEM of 6 to 9 independent experiments each using cells derived from a different donor. * p <0.05, ** p <0.01 and *** p <0.001 (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

5.2.2 IRF5 promotes human Th17 responses

Next I analysed the activation of Th17 lymphocytes by FCM analysis and found that again only IRF5-expressing macrophages provided the cytokine environment necessary for the population expansion and activation of Th17 lymphocytes. I observed an increase in CD4⁺/IL-17⁺ T lymphocytes (Figure 5.4a) which was reproducible and significant when

analysed in multiple donors (Figure 5.4b). In line with studies demonstrating that IL-23 enhances the emergence of an IL-17⁺/IFN- γ ⁺ population of T lymphocytes [228], about 25% of IL-17⁺ lymphocytes were also IFN- γ ⁺ (data not shown), which supported the idea of a close developmental relationship between human Th17 and Th1 lymphocytes [320].

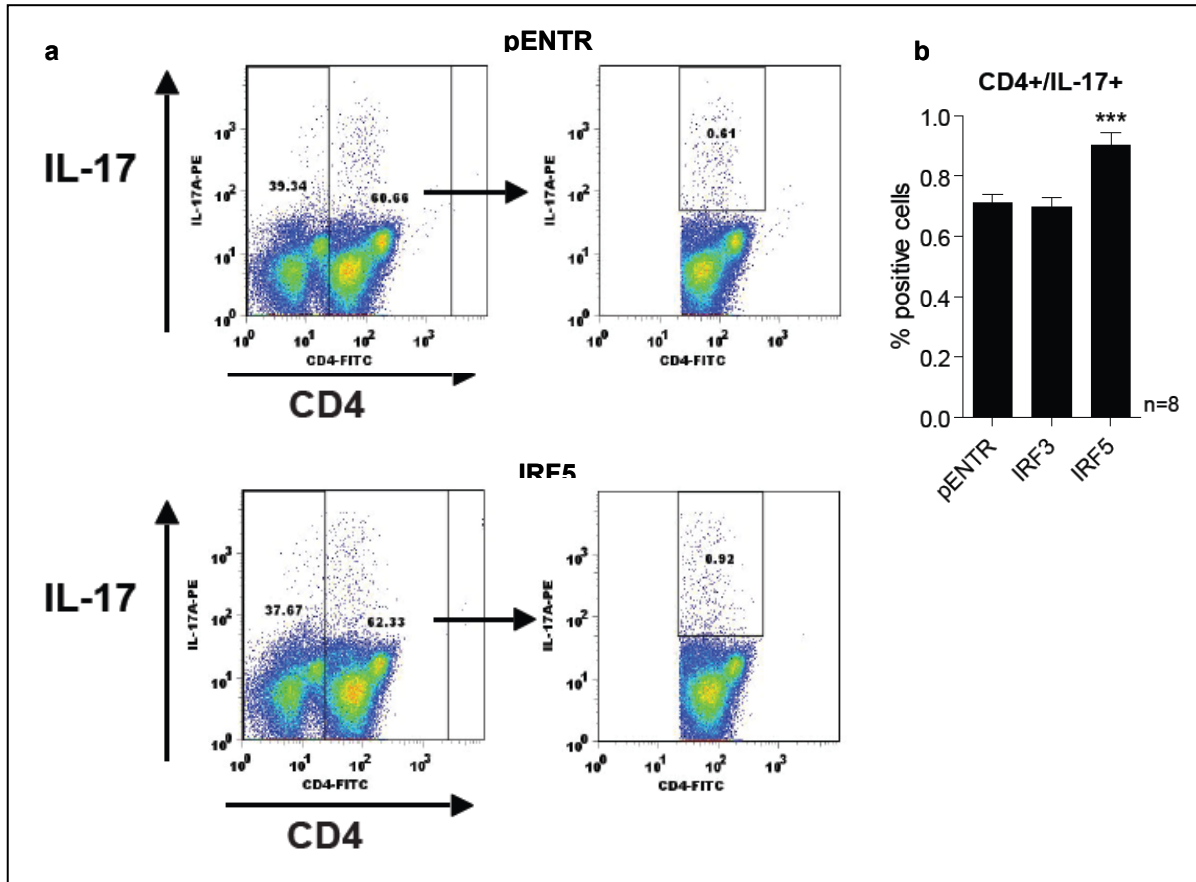


Figure 5.4 IRF5-expressing macrophages induce Th17 activation

(a) M2 macrophages were infected with adenoviral vectors encoding IRF5 or empty vector (pENTR) and cultured with T lymphocytes from MHC mismatched donors. After 4 days, cells were stimulated for 3 h with PMA/ionomycin/Brefeldin A and stained for CD4 and IL-17A. Representative FCM plots are shown. **(b)** The percentage of CD4⁺/IL-17A⁺ cells was determined by ICC staining in cells treated as in (a). Data are shown as the percentage of CD4⁺/IL-17A⁺ cells and are the mean $\pm$ SEM of 8 independent experiments each using cells derived from a different donor. *** $p < 0.001$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

Similar to before, the increase in IL-17-producing CD4⁺ lymphocytes was accompanied by higher expression of IL-17A mRNA in the total T lymphocyte population (Figure 5.5a) as well as increased production and secretion of IL-17A protein (Figure 5.5b and c). As expected, there was significant induction of expression of mRNA for the Th17-specifying transcription factor ROR γ T (RORC2) in T lymphocytes cultured together with IRF5-expressing macrophages but not control or IRF3 expressing cells (Figure 5.5d).

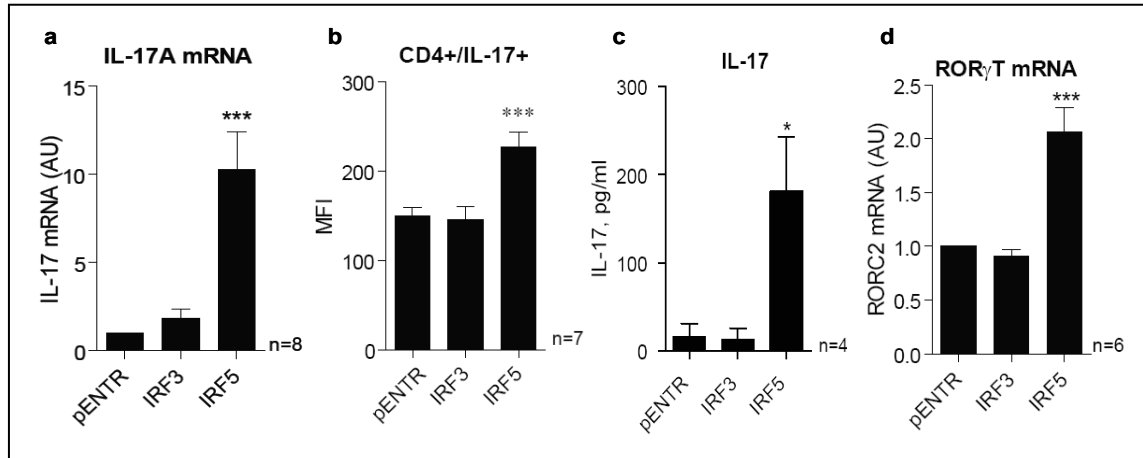


Figure 5.5 IRF5-expressing macrophages induce Th17 development

(a-d) M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) and cultured with T lymphocytes from MHC mismatched donors. **(a)** Expression of IL-17A mRNA in T lymphocytes after 2 days of co-culture presented in arbitrary units (AU) relative to those obtained with empty vector control. **(b)** Immunocytochemical staining of IL-17A in T lymphocytes after 2 days of co-culture and then stimulated for 3 h with PMA/ionomycin/Brefeldin A. Results are presented as MFI. **(c)** IL-17A in supernatants of the cells in (b) determined by ELISA. **(d)** Expression of RORC2 (ROR γ T) mRNA in T lymphocytes from (a) presented as in (a). Data are shown as the mean $\pm$ SEM of 4 to 8 independent experiments each using cells derived from a different donor. * $p < 0.05$ and *** $p < 0.001$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

Deciphering the precise cytokine profile of Th-17 lymphocytes has been the focus of many recent studies [111, 321]. It is now clear that there are many other phenotypic markers of these cells in addition to the expression of ROR γ T and the subsequent IL-17A production. I therefore analysed the expression of mRNA coding for IL-17F, IL-21, IL-22, IL-26 and IL-23 receptor (IL-23R) in MLR experiments. I found that all of the above genes were upregulated in T lymphocytes co-cultured with IRF5-expressing macrophages whereas control or IRF3-expressing cells showed no effect (Figure 5.6).

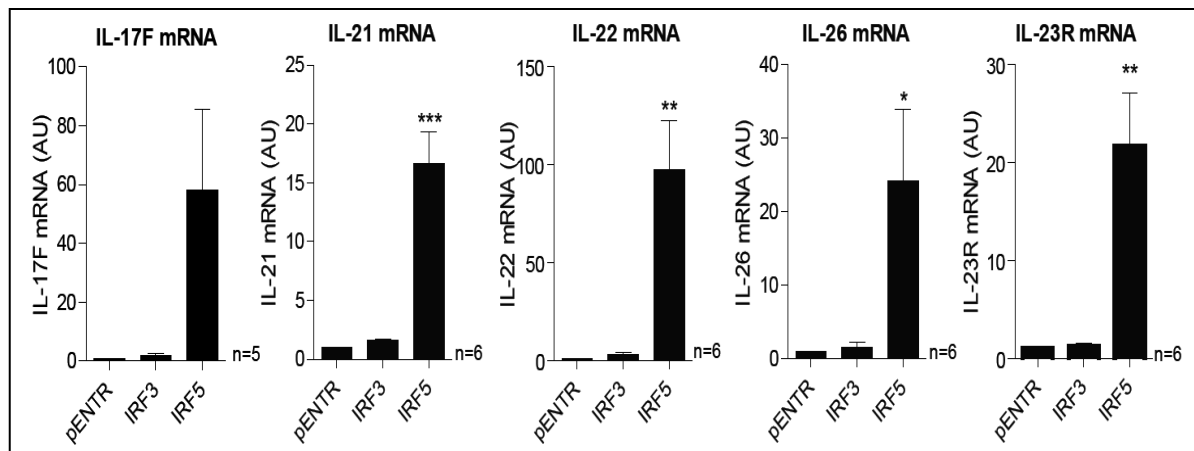


Figure 5.6 IRF5-expressing macrophages induce Th17 cytokine profile

M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) and cultured with T lymphocytes from MHC mismatched donors. The Expression of IL-17F, IL-21, IL-22, IL-26 and IL-23R mRNA in T lymphocytes after 2 days of co-culture was determined by RT-PCR and is presented as arbitrary units (AU) relative to those obtained with empty vector control. Data are shown as the mean $\pm$ SEM of 5 to 6 independent experiments each using cells derived from a different donor: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

Of interest, expression of GATA-3 (essential for Th2 lineage commitment) and Foxp3 mRNA (essential for Treg lineage commitment) was lower in the presence of IRF5-expressing macrophages (Figure 5.7). Signature cytokines of the Th2 lymphocyte lineage include IL-4 as well as IL-13 and the lack of Th2 development is further supported by the fact that IL-4 protein levels were below the limit of detection in all MLR experiments (data not shown)

Hence, IRF5-expressing macrophages promote the proliferation of T lymphocytes and provide the cytokine environment for the activation of the Th1 and Th17 lineage but do not induce the Th2 or Treg lineage. This further highlights the importance of IRF5 in establishing the M1 cytokine profile (IL-12^{hi}IL-23^{hi}IL-10^{low}) which is required for a robust Th1/Th17 response.

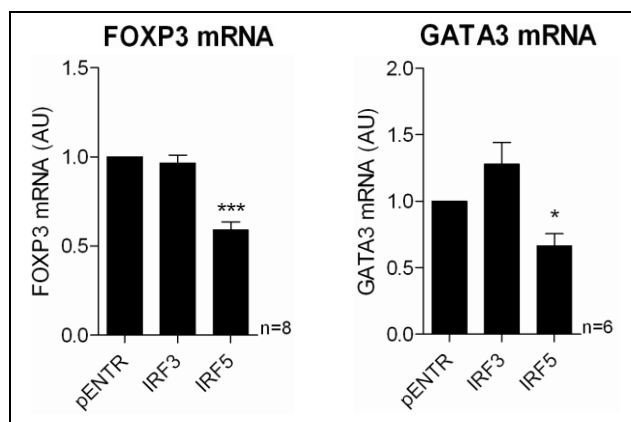


Figure 5.7 IRF5-expressing macrophages do not induce the Treg or Th2 lineage

M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or empty vector (pENTR) and cultured with T lymphocytes from MHC mismatched donors. The expression of FOXP3 and GATA-3 mRNA in T lymphocytes after 4 days of co-culture was determined by RT-PCR and is presented as arbitrary units (AU) relative to those obtained with empty vector control. Data are shown as the mean $\pm$ SEM of 8 independent experiments each using cells derived from a different donor: * $p < 0.05$ and *** $p < 0.001$ (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

5.2.3 IRF5 regulates the phenotype of mouse macrophages

In the last part of my detailed study into the diverse roles of IRF5 in regulating immune responses I wanted to investigate its role during a mouse model of M1 inflammation. As all my previous experiments were exclusively performed in primary human cells or where appropriate in human cell lines I first had to establish that the role of IRF5 is similar in human and mouse. Therefore, I analysed IRF5 protein levels in mouse bone marrow-derived macrophages (BMDMs) differentiated either with GM-CSF or M-CSF. Similar to their human counterparts, GM-CSF differentiated BMDMs had higher expression of IRF5 protein than M-CSF-derived BMDMs (Figure 5.8). Simultaneously, I analysed IRF5 protein expression in BMDMs obtained from IRF5-deficient animals and found it to be undetectable as expected.

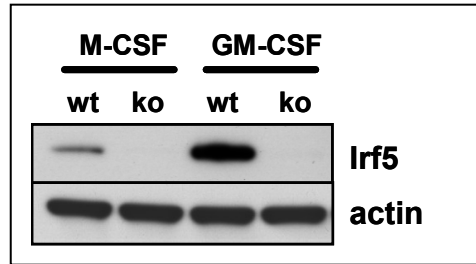


Figure 5.8 IRF5 expression is high in GM-CSF differentiated BMDMs

Bone marrow cells were obtained from a wild-type (wt) and IRF5^{-/-} (ko) mouse and differentiated for 8 days into M1 macrophages with GM-CSF (20 ng/ml) or M2 macrophages with M-CSF (100 ng/ml). Total protein extracts were subjected to western blot analysis with antibodies to IRF5 and actin as a loading control. A representative blot of three independent experiments is shown.

Next, I analysed the cytokine profile of GM-CSF or M-CSF differentiated BMDMs. GM-CSF-derived BMDMs but not M-CSF-derived BMDMs were capable of secreting IL-12p70 and IL-23 upon LPS stimulation (data not shown) which is in line with previously published data [63-67]. However, BMDMs obtained from *Irf5*^{-/-} mice and differentiated with GM-CSF secreted significantly less IL-12p70 and IL-23 but produced higher amounts of IL-10 in response to stimulation with LPS than did wild-type cells (Figure 5.9). This is an independent validation of the results obtained using siRNA targeting IRF5 in human cells and adds to the mounting evidence that IRF5 regulates M1-specific cytokines.

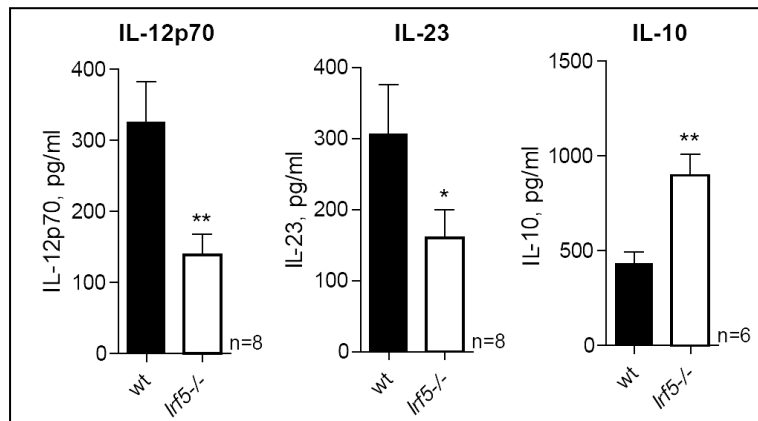


Figure 5.9 Impaired induction of M1 cytokines in BMDMs of IRF5^{-/-} mice

ELISA of IL-12p70, IL-23 and IL-10 secreted by M1 macrophages obtained from *Irf5*^{-/-} or littermate C57BL/6 wt mice and stimulated for 24 h with LPS (100 ng/ml). Data shown are the mean ± SEM of 6 to 8 BMDM cultures from three independent experiments. **p*<0.05 and ***p*<0.01 (Student's *t*-test).

5.2.4 Importance of IRF5 in a mouse model of M1 inflammation

To investigate the function of IRF5 in pathologic conditions associated with M1 polarized inflammation, I conducted an intra peritoneal challenge of *Irf5*^{-/-} mice with bacterial derived LPS. I would like to thank Miss Katrina Blazkova (Kennedy Institute of Rheumatology, London, United Kingdom) who was instrumental in establishing and optimising these *in vivo* experiments.

I needed to optimise this model first as it has not been performed before at the Kennedy Institute and started with injecting PBS on its own into wild-type C57BL/6 mice followed by collecting serum after 3 h. I could not detect cytokines, such as TNF, IL-23 and IL-10, by ELISA in any of the PBS treated animals (data not shown) indicating that the injection does not induce measurable cellular responses. Next, I injected wild-type C57BL/6 mice with 1 µg of LPS or PBS (as a control) and collected serum 3 h later. Analysis of cytokines in the serum by ELISA and CBA revealed very low levels of TNF and IL-6 but IL-23 and IL-10 were not detectable (data not shown) suggesting that a higher dose of LPS is needed for a better induction of these cytokines. I performed additional experiments using 10 µg and 20 µg of LPS for 3 h as well as 6 h and found that 20 µg was sufficient to induce detectable amounts of cytokines (TNF, IL-23, IL-6 and IL-10, data not shown) while not harming the mice. This was important as our animal licence only allows me to conduct an intra peritoneal challenge with a sub-lethal dose of LPS and for a short time. Levels of the cytokines mentioned above were higher at 3 h compared to 6 h therefore all following intra peritoneal challenges were terminated after 3 h.

I injected IRF5-deficient and wild-type littermate control mice with 20 µg of LPS or PBS (as a control) for 3 h followed by collecting serum, peritoneal cells, lymph nodes and spleens. I used a total of 10 mice (5 males and 5 females), ranging from 8 to 14 weeks old, per experimental condition. Within 3 h there was a significant difference between wild-type and *Irf5*^{-/-} mice in the serum concentrations of selected macrophage-subset specific cytokines. The responses were consistent with both the human and mouse data; that is, *Irf5*^{-/-} mice had lower serum concentrations of IL-12p40 and IL-23 but expressed increased amounts of IL-10 (Figure 5.10). In addition, the expression of IL-1β, TNF as well as IL-6 was considerably lower in *Irf5*^{-/-} mice compared to their wild-type littermate controls (Figure 5.10). Thus, confirming the *in vitro* results obtained with human si-IRF5 depleted macrophages or mouse BMDMs derived from IRF5-deficient mice.

The expression of IL-12p70 was below the limit of detection in the experimental conditions described above (data not shown). Control animals injected with PBS secreted undetectable levels of the cytokines measured (data not shown).

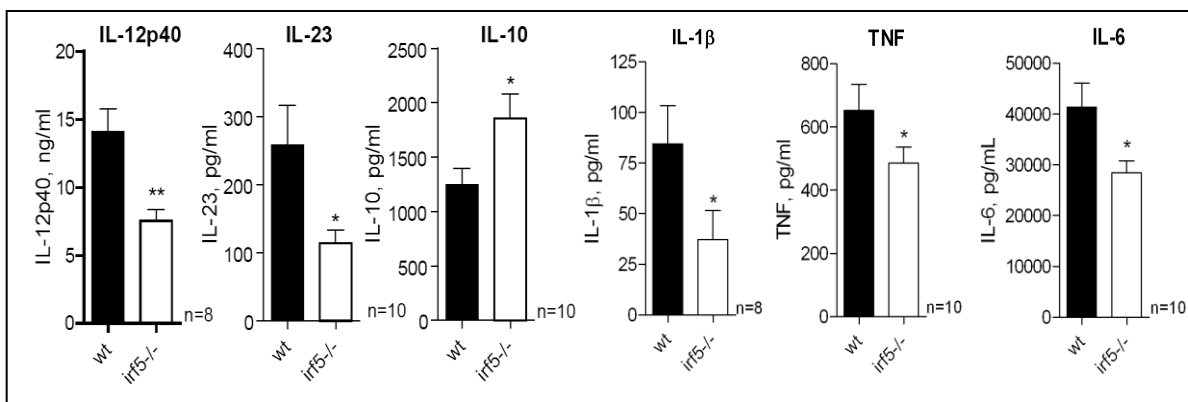


Figure 5.10 Impaired induction of M1 cytokines in LPS challenged *Irf5*^{-/-} mice

ELISA (IL-12p40 and IL-23,) or cytometric bead assay (IL-10, IL-1β, TNF and IL-6) of the serum concentrations of cytokines in *Irf5*^{-/-} mice ($n = 10$) and their wild-type littermates ($n = 10$) injected intraperitoneally with LPS (20 μg), assessed 3 h later. Data shown are the mean ± SEM of 8 to 10 serum samples from three independent experiments. * $p < 0.05$ and ** $p < 0.01$ (Student's *t*-test).

Overall, this data supported the idea that IRF5 is important for inflammatory responses in an animal model of inflammation. The *in vivo* model provided an additional line of evidence for the central role of IRF5 in the induction of M1-specific cytokines and validated both human and mouse *in vitro* data. However, measuring the concentration of cytokines in the serum did not give me further information on which cells or organs were in particular dependent on IRF5 protein expression. I therefore analysed the responses in specific organs, like peritoneum, lymph node and spleen, which were collected during the inflammation model.

I focused first on the peritoneum, as macrophages should be readily recruited into the peritoneal cavity during the intra peritoneal LPS challenge. I performed a peritoneal lavage and found that the number of macrophages recruited into the peritoneal cavity of LPS-challenged mice was similar for wild-type and *Irf5*^{-/-} mice (data not shown). However, RT-PCR analysis of these cells showed an impaired induction of genes coding for M1 macrophage markers IL-12p35, IL-12p40, IL-23p19, IL-1β, TNF and IL-6 in IRF5-deficient animals (Figure 5.11). The expression of genes coding for M2-specific markers in *Irf5*^{-/-}

mice was either significantly higher (IL-10, Retnla (Fizz1), Arg1, Figure 5.11) or showed a positive trend (MRC1 and Chi3l3 (Ym1), data not shown).

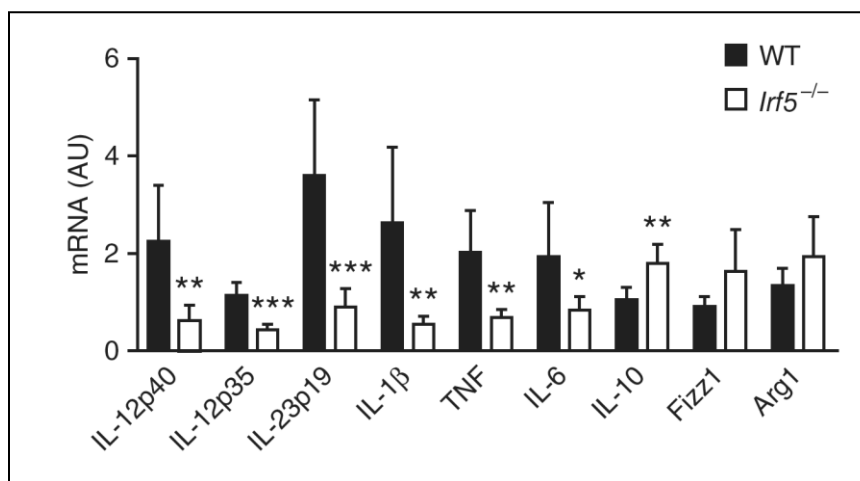


Figure 5.11 Impaired mRNA induction of M1-specific cytokines in peritoneal cells from *Irf5*^{-/-} mice

mRNA expression of M1 and M2 markers (horizontal axis) in peritoneal cells obtained from the LPS-injected mice in Figure 5.2.10. mRNA expression of the genes analysed is presented relative to that of the wild-type sample with the lowest relative expression, set as 1. Data shown are the mean $\pm$ SEM of 10 wild-type and *Irf5*^{-/-} samples from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

Next, I analysed the mesenteric lymph nodes (lymph nodes of the large intestine and lower abdomen) from the LPS challenged animals. Again, there was no difference in size or cell number between wild-type and *Irf5*^{-/-} mice (data not shown). Furthermore, mRNA expression of T lymphocyte-specific cytokines (IFN- γ and IL-17A) was also similar between experimental groups, probably due to the short time of exposure of the animals to the inflammatory agent.

The last organ I collected and focused on in more detail was the spleen. I extracted splenocytes from LPS challenged *Irf5*^{-/-} and wild-type littermate controls and cultured them *ex vivo* for an additional 48 h in the presence of anti-CD3 antibodies. Here I observed significantly less secretion of IFN- γ and IL-17 in the cultures obtained from *Irf5*^{-/-} mice

(Figure 5.12) further supporting the importance of IRF5 for Th1/Th17 responses which is in accordance with the data obtained with human cells (Figures 5.2 to 5.6).

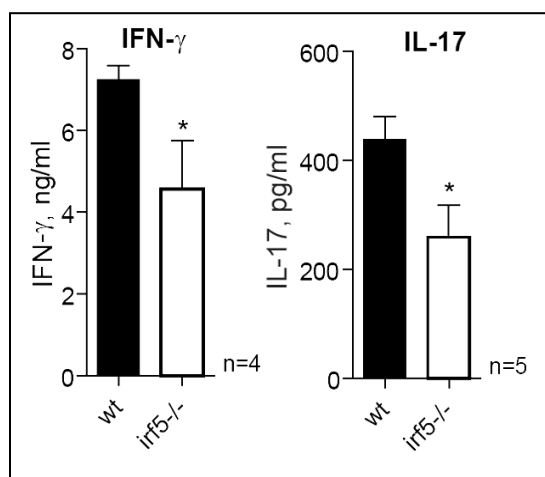


Figure 5.12 Reduction in IFN- γ and IL-17 production in ex vivo splenocyte cultures

ELISA of IFN- γ and IL-17A in spleen cells obtained from the LPS-injected mice in Figure 5.2.10 and cultured for 48 h in the presence of antibody to CD3. Data shown are the mean $\pm$ SEM of 4 to 5 spleen cultures from two independent experiments * p <0.05 and ** p <0.01 (Student's *t*-test).

In summary the data presented here supports the idea that IRF5 is important in establishing a pro-inflammatory macrophage phenotype in animal models of M1-polarizing inflammation. In addition, IRF5 is essential for a robust Th1/Th17 response under these experimental conditions which is in accordance with the data obtained in human MLR experiments (Figures 5.2 to 5.6).

5.3 Conclusion

One of the hallmarks of macrophage polarization is the acquired ability to promote adaptive immune responses. The type of adaptive immune response is shaped by the cytokine environment provided by macrophage subsets, with M1 macrophages setting up the environment for Th1/Th17 responses and M2 macrophages favouring Th2 responses [63, 64]. Here I investigated whether forced expression of IRF5 in M2 macrophages would alter their ability to control adaptive immunity and examined the *in vivo* role of IRF5 during polarizing inflammation. I demonstrate that IRF5-expressing macrophages induce T lymphocyte proliferation and are essential for a robust Th1/Th17 response. I also show that IRF5 expression is similar in man and mouse, that is, high in M1 and low in M2 macrophages. Consequently, IRF5-deficient mice produced significantly less M1-specific cytokines compared to wild-type littermate controls during an *in vivo* model of polarizing inflammation.

IRF5-expressing macrophages were better in promoting T lymphocyte proliferation compared to control or IRF3-expressing macrophages (Figure 5.1). Macrophages with exogenous IRF5 initiated a potent Th1 response (Figure 5.2) measured by induction and secretion of significant IFN- γ protein levels (Figure 5.3). The effect of IRF5 on Th1 development was further highlighted by an increase in T-bet (Tbx21) mRNA expression (5.3d), which is the established master regulator of Th1 development, in co-cultures containing IRF5-expressing macrophages. This priming effect is most likely due to the secretion of IL-12 [106] and the upregulation of co-stimulatory molecules such as CD40, CD80 and CD83 (Figure 4.12) by IRF5-expressing macrophages.

Of particular interest was the increased production of IL-17 from T lymphocytes co-cultured with IRF5-expressing macrophages (Figure 5.5). Th17 lymphocytes are characterized by the expression of the subset-defining transcription factor RORC2 (ROR γ T) as well as IL-23R, IL-17A/F, IL-21, IL-22 and IL-26 all of which are up-regulated in the presence of IRF5-expressing macrophages (Figure 5.6). Human Th17 lymphocytes seem to exhibit different features from murine Th17 lymphocytes: while murine Th17 originate from a progenitor common to Treg lymphocytes when IL-6 is produced in combination with TGF- β , human Th17 lymphocytes originate from CD161⁺CD4⁺ progenitors in the presence of IL-23 and IL-1 β , with little involvement of IL-6 and indirect role for TGF- β [114]. In line with that, human IRF5-expressing macrophages produce significant amounts of IL-23 and IL-1 β

(Figure 4.6 and 4.7) but perhaps not unexpectedly, the dependence of IL-6 expression on IRF5 is much greater in mouse macrophages (Figures 5.10 and 5.11 for mouse IL-6, data not shown for human IL-6). Both Th1 and Th17 lymphocytes promote cellular immune function and have the capacity to cause inflammation and autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis [112, 113].

Recently, two independent laboratories reported that GM-CSF is crucial for the proinflammatory properties of Th17 lymphocytes [322, 323]. Using the EAE mouse model of multiple sclerosis, both groups found that IL-23 promotes the production of GM-CSF in Th17 lymphocytes and that GM-CSF is essential for the ability of Th17 lymphocytes to drive inflammation in the central nervous system. One group suggested that GM-CSF stimulates the production of IL-23 as well as other proinflammatory cytokines and this would promote both, continued activation of Th17 lymphocytes and the *de novo* generation of Th17 effector lymphocytes [323]. In light of my data, I propose a potential positive feedback loop whereby IRF5-expressing M1 macrophages initially provide the cytokine environment for Th17 lineage commitment. The secretion of IL-23 would induce the production of GM-CSF by Th17 lymphocytes which in turn would increase or maintain IRF5 protein expression in M1 macrophages. This would enhance their ability to produce proinflammatory cytokines and further sustaining a Th17 response (Figure 5.13). If not tightly controlled, this IRF5/IL-23/GM-CSF axis could lead to the prolongation of an inflammatory response and ultimately result in chronic inflammation.

Similar to the situation in human (Chapter 4), mouse macrophage subsets display differences in IRF5 protein levels when differentiated *in vitro* using M-CSF and GM-CSF respectively [65]. High expression of IRF5 was again characteristic of M1 macrophages (Figure 5.8) and consequently the production of M1-signature cytokines was reduced in IRF5 deficient animals upon LPS stimulation whereas an increase in IL-10 production was observed (Figure 5.9). In order to investigate the function of IRF5 in pathologic conditions associated with M1 polarized inflammation, I conducted an intra-peritoneal challenge of *Irf5*^{-/-} mice with a sublethal dose of LPS. Compared to wild-type littermate controls, the serum levels of IL-12p40, IL-23, TNF, IL-1 β and IL-6 were all reduced in *Irf5*^{-/-} mice (Figure 5.10). In general, I would expect a bigger effect under more extreme conditions but as mentioned previously, we are somewhat limited by our animal licence which only allows us to conduct an intra peritoneal challenge with a sub-lethal dose of LPS and for a short time. Nevertheless, all effects observed here *in vivo* are statistically significant and confirmed

results obtained *in vitro* using either human macrophages treated with siRNA targeting IRF5 (Figure 4.9 and 4.11) or bone-marrow derived macrophages from IRF5-deficient animals (Figure 5.9).

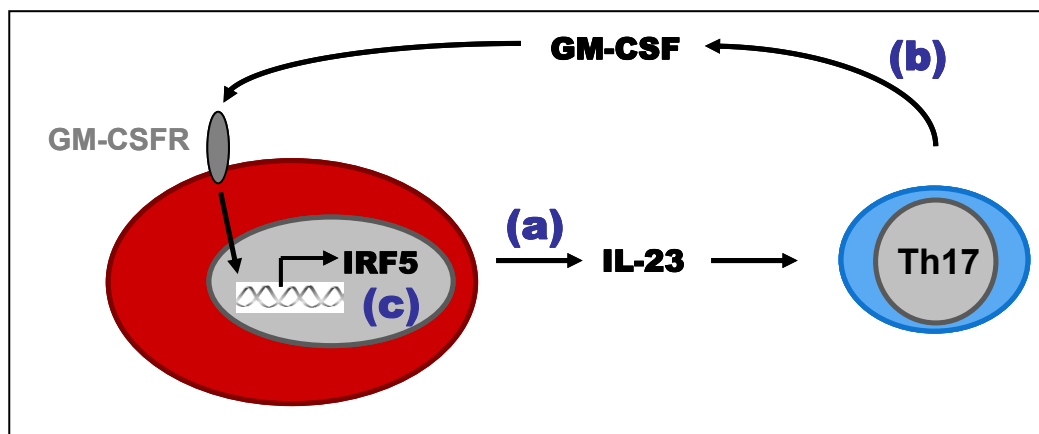


Figure 5.13 Potential feedback loop involving IRF5, IL-23 and GM-CSF

(a) M1 macrophages (shown in red) secrete IL-23 which is needed for the maintenance of Th17 lymphocytes (Th17, shown in blue) but also for GM-CSF production by them (b). GM-CSF will signal via GM-CSFR expressed on macrophages and induce the transcription of downstream target genes, including IRF5 (c). Increased IRF5 protein in turn promotes further Th17 activation leading to an increase in GM-CSF production and sustained inflammation.

I also analysed the response of organ-specific cells after LPS-challenge and found an impaired induction of M1-specific cytokines in peritoneal macrophages derived from IRF5 deficient animals (Figure 5.11). Interestingly, I observed a slight increase in the expression of mouse-specific M2-signature genes such as *Fizz1* and *Arg1* in *Irf5*^{-/-} mice compared to wild-type controls (Figure 5.11) further supporting a shift from M1 to M2 macrophage polarization in the absence of IRF5. Lastly, IRF5-deficient mice showed an impaired production of Th1/Th17 cytokines in *ex vivo* splenocyte cultures (Figure 5.12) confirming the role of IRF5-expressing macrophages in the orchestration of immune responses.

In summary, IRF5-expressing M1 macrophages are essential for a robust Th1 response and furthermore provide the cytokine environment for Th17 lineage activation. Using an

independent approach, such as IRF5-deficient animals, I was able to demonstrate that IRF5 induces M1-specific but represses M2-specific genes validating my previous data obtained with human cells. Overall, these results strongly suggest that IRF5 is a defining transcription factor of both human and mouse macrophage polarization. The data presented here make IRF5 a new target for therapeutic intervention as its inhibition would potentially affect pro-inflammatory cytokine expression and decrease the number of effector T lymphocytes.

6. Discussion

IRF5 was mostly known for its ability to regulate the expression of type I IFNs in response to virus infection such as NDV, HSV-1 and VSV [186, 192]. However, the generation of *Irf5*^{-/-} mice revealed an unexpected anti-inflammatory phenotype. Myeloid cells of these animals showed a significant decrease in the expression of pro-inflammatory cytokines, especially TNF, IL-6 and IL-12p40 upon TLR stimulation [190]. Consequently, *Irf5*^{-/-} mice survived lethal shock induced by CpG-B and were resistant to LPS-induced endotoxic shock. However, the molecular mechanism of IRF5-mediated induction of pro-inflammatory cytokines had not been investigated and the role of IRF5 in human myeloid cells was to date unclear. My study provides an insight into the regulation of IRF5 expression in primary human myeloid cells as well as its involvement in the gene induction of human TNF downstream of TLR4 signalling. Furthermore, I establish IRF5 as a subset-defining transcription factor of macrophage polarization and describe a mechanism of IRF5-mediated inhibition of IL-10 expression.

In Chapter three, I demonstrated that MDDCs acquire a particular phenotype during differentiation, characterised by higher levels of IRF5 protein and higher levels of late-phase TNF secretion compared to M2 macrophages. I have found that ectopic expression of IRF5 in M2 macrophages restored their potential to secrete TNF for a longer period whereas depletion of IRF5 in MDDCs resulted in reduction of TNF expression, especially at later time points post stimulation. The molecular mechanism of IRF5-induced TNF expression was examined using luciferase reporter assays and ChIP experiments. Results obtained with ChIP indicated that IRF5 is recruited to two specific regions, region B and region H, within the TNF locus. I could demonstrate that IRF5 recruitment to region H is dependent on the interaction with RelA whereas recruitment to region B seems to be RelA-independent. The importance of RelA-binding to NF- κ B sites in the 3' region H and subsequent recruitment of IRF5 were confirmed in re-ChIP experiments and also by performing IRF5-specific ChIP in RelA depleted cells. A subsequent analysis of protein-protein interactions demonstrated an interaction between RelA and IRF5 but not other NF- κ B family members. I concluded that IRF5-induced TNF expression involves two spatially separated regulatory regions (region B and H) and two independent modes of actions (direct binding to DNA and co-recruitment mediated via protein-protein interactions with RelA).

Regions B and H are characterized by high levels of sequence conservation [288, 324] and both contain cell type-specific DNase I hypersensitivity sites [324, 325]. Moreover, these regions have been shown to physically interact in T lymphocytes *in vitro* leading to the formation of an intrachromosomal loop [324]. Tsytyskova and co-workers proposed that this loop formation would promote the re-initiation of transcription. This model could be of relevance to TNF expression by MDDCs, in which a co-operative action of RelA and IRF5 at both the 5' upstream and 3' downstream region appears to be essential for maintaining TNF gene transcription over a prolonged period of time. Therefore, a third mode of action could involve a locus circularization in MDDCs directed via the newly unraveled protein-protein interactions between RelA and IRF5.

The precise mechanism of transmitting the activation signal from the 3' end of the TNF gene to Pol II and the possibility of intrachromosomal looping requires further investigation. However, the two independent modes of IRF5 recruitment to the gene promoter/enhancer described in this thesis raises 2 important questions:

1. Are there other genes with a similar recruitment profile (5' upstream and 3' downstream)?
2. Are there other genes that show RelA-mediated recruitment of IRF5?

In order to answer these questions I suggest making use of novel genomic technologies, such as ChIP followed by deep-sequencing (ChIP-Seq). Using ChIP-Seq, it is possible to systematically identify the recruitment of a specific transcription factor throughout the entire genome. A good yield of chromatin material is necessary for ChIP-Seq and consequently a large number of cells per time point is required. The number of cells obtained from one single human blood donor is not sufficient to performing a ChIP-Seq that includes a binding kinetic profile of the protein of interest following stimulation. Therefore, most ChIP-Seq experiments are currently performed using mouse bone marrow-derived cells.

It would be interesting to perform a ChIP-Seq experiment in LPS-stimulated bone marrow-derived M1 macrophages using anti-IRF5, anti-RelA and anti-Pol II antibodies. Bioinformatic analysis of several positively regulated IRF5 target genes (e.g. IL-12 family member genes, TNFSF4, TNFSF7 and CCR7) revealed putative ISREs within the respective promoters as well as 3' downstream regions (data not shown). I therefore hypothesise that the recruitment of IRF5 to 5' upstream and 3' downstream regions of target genes would be a

common feature. As RelA ChIP-Seq would be performed in parallel, it would also be possible to bioinformatically identify genes with overlapping recruitment of IRF5 and RelA. At genes that show recruitment of both IRF5 and RelA to the same region, the corresponding binding sequence of IRF5 peaks could be further analysed to single out peaks that contain no ISRE sites. By using this approach, it should be possible to identify genes that are potentially dependent on RelA-mediated recruitment of IRF5 and therefore determine the gene subset that is regulated in an IRF5/RelA-dependent manner. The addition of Pol II ChIP-Seq into the experimental setup would give further information on which genes of the IRF5/RelA-dependent subset are actively transcribed upon cell activation. Furthermore, it would be desirable to map the IRF5/RelA interface and once identified, try to disrupt the interaction, e.g. using a peptide-based approach. As a functional readout, I would suggest to first use TNF gene expression and later other genes identified by ChIP-Seq as being IRF5/RelA-dependent.

The expression of IRF5 protein analysed in primary human myeloid cells demonstrated that IRF5 is highly expressed in MDDCs as well as M1 macrophages and to a far lesser extent in monocytes and M2 macrophages. A number of different IRF5 isoforms were observed: one isoform (of higher molecular mass) was present in all unstimulated myeloid cell types tested and another (of lower molecular mass) was present in unstimulated MDDCs and M1 macrophages. The different IRF5 isoforms are likely a product of alternative splicing of exon 6 [180]. Splicing can give rise to an IRF5 protein with 498 amino acids (sometimes referred to as variant 3/4), whereas alternative splicing can result in an isoform with a length of 514 amino acids (sometimes referred to as variant 5). The IRF5 expression constructs used in this study to ectopically express IRF5 in primary human cells and cell lines is the 498 amino acids (aa) isoform. It has been shown that the 498aa isoform formed homodimers as well as heterodimers with IRF3 in NDV infected cells [191] but the 514aa isoform failed to do so [193]. One explanation could be that the 514aa isoform is not activated by NDV or that it might be a self inhibiting protein.

In this respect, it would be very interesting to further investigate and dissect the role of the nine IRF5 isoforms (also known as variants 1 to 9) in a cell-type specific manner and to determine whether the trans-activation potential is similar between isoforms. Considering that the IRF5 variant 7 lacks the DBD, variant 8 has deficiencies in the IAD and variant 9 lacks the whole C-terminus, one would expect differences in their ability to induce gene transcription. Interestingly, IRF5 variants 7, 8 and 9 were found to be expressed in

haematological malignancies [180] which could suggest that these isoforms may play an important role in establishing the tumor environment by acting as dominant-negative mutants to endogenous IRF5. Furthermore, it would also be important to investigate whether all isoforms are able to interact with RelA. Similar to above, TNF gene expression could be used as a functional readout. Expression constructs coding for human IRF5 isoforms are available and the Udalova laboratory is currently trying to obtain them for further characterisation.

In Chapter four I described a new role for IRF5 in inhibiting gene transcription of human IL-10. Overexpression of IRF5 in M2 macrophages affected their ability to secrete IL-10 upon LPS stimulation by actively inhibiting IL-10 mRNA expression and direct DNA binding of IRF5 was necessary for this, as a DNA-binding mutant of IRF5 (IRF5 Δ DBD) was not able to inhibit IL-10 expression. Using ChIP, I showed that IRF5 binds to the promoter region of IL-10 but in contrast to IRF5 positively-regulated genes, Pol II recruitment was only detectable upon dissociation of IRF5 from the IL-10 promoter. Furthermore, a previously identified ISRE site within the IL-10 promoter [306] was required for the inhibitory effects of IRF5 as mutation of this site abolished the IRF5-mediated inhibition of IL-10. This suggests that IRF5 binding to the promoter ISRE inhibits the recruitment of Pol II and subsequent IL-10 mRNA expression presumably via interactions with yet unknown co-factors.

It is known that different IRF heterodimers can either repress or activate gene transcription. For instance, the IRF8/IRF1 complex generally acts as a transcriptional repressor on ISREs whereas the IRF8/PU.1 complex is associated with gene induction [326]. However, the co-factors that enable IRF5 to distinguish between induction and inhibition of gene expression remain mostly unknown. A recent study suggested a model whereby IRF5 switches from a silencing to an activating complex depending on the interaction with either histone acetyltransferases (HATs) or histone deacetylases (HDACs) [327]. Feng *et al* demonstrated that IRF5 binds to the IFN- α promoter in unstimulated cells but the transcription is silenced through the binding of co-repressor proteins such as Sin3a and silencing mediator for retinoid or thyroid-hormone receptor (SMRT) to the N-terminus of IRF5. Virus infection leads to the nuclear export of Sin3a and SMRT resulting in IRF5-mediated recruitment of the co-activator proteins p300/CBP and induction of gene transcription. However, I did not detect IRF5 recruitment to the IL-10 promoter in unstimulated cells suggesting that the

mode of IL-10 inhibition is different to IRF5-mediated inhibition of IFN- α transcription. Furthermore, IRF5 participates in both the repression as well as induction of IFN- α in the mechanism described by Feng *et al* whereas I only observed a role for IRF5 in the transcriptional silencing of IL-10. It would therefore be interesting to identify IRF5 interaction partners, for example by using IRF5 overexpression followed by immuno-precipitation and mass spectrometry analysis. The characterisation of novel IRF5 binding partners would possibly help to further dissect the IRF5-mediated inhibition of IL-10 expression and allow the postulation of an IRF5-mediated transcriptional repression model.

In Chapter four, I also investigated the role of IRF5 in regulating macrophage lineage commitment. I showed that IRF5 protein expression is a hallmark of M1 macrophages in which it is involved in the induction of the characteristic M1 gene expression and cytokine secretion profile (IL-12^{high}, IL-23^{high}, IL-10^{low}). IRF5 mRNA expression was rapidly induced in monocytes treated with GM-CSF or IFN- γ and similar to MDDCs but a distinct IRF5 isoform expression pattern was observed. Stimulation of monocytes with GM-CSF (M1 differentiation) induced the protein expression of an isoform of lower molecular mass which was not detected in monocytes differentiated in the presence of M-CSF (M2 differentiation). Preliminary experiments using IRF5 variant-specific TaqMan RT-PCR probes indicate that treatment of monocytes with GM-CSF or IFN- γ induces variants 1, 2, 3/4 and 5 in a similar way. However, the specificity of these probes is under question and the design of our own variant-specific primer sets might result in a better discrimination between variants. Furthermore, a practical and valuable tool for studying IRF5 isoforms in a cellular context would be the generation of isoform-specific antibodies.

Signalling via the GM-CSF and IFN- γ receptor leads to the downstream activation of the STAT pathway [328, 329]. Using oligonucleotide binding assays, it has been shown previously that STATs can bind to IRF5 promoter sequences upon IFN- α stimulation [180] which suggests a contribution of STATs to the activation of IRF5 gene expression. It would be interesting to further investigate which STAT proteins are involved in regulating IRF5 expression and if IRF5 isoform expression is dependent on the binding of different STAT homo- or heterodimer complexes. A recent publication also suggests that IRF4 participates in the regulation of IRF5 expression [330]. Xu *et al* show that IRF5 expression is inhibited both *in vitro* and *in vivo* by IRF4 in Epstein-Barr virus transformed B lymphocytes. In addition to this direct effect on IRF5 expression, it is known that IRF4 inhibits IRF5 function by competing for interaction with Myd88 [299] and IRF4 has been recently reported to

control the expression of prototypical mouse M2 macrophage markers [302]. The regulation of these M2 macrophage markers is in stark contrast to the role of IRF5 in defining the M1 macrophage phenotype described in this thesis. Consistent with its role as a negative regulator of TLR signalling, LPS-induced proinflammatory cytokine expression is enhanced in *Irf4*^{-/-} macrophages and *Irf4*^{-/-} mice are highly sensitive to endotoxic shock induced by CpG [299, 331]. This further supports the idea that IRF5 and IRF4 have opposing roles in regulating immune responses and their function is controlled by negative feedback regulation and by antagonizing each other.

Another question that remains is how IRF5 is activated by TLR4 signalling. Takaoka *et al* demonstrated that ectopically expressed IRF5 translocates to the cell nuclei in response to LPS, and that this translocation is dependent on the presence of Myd88 [190]. I observed endogenous nuclear IRF5 even in resting MDDCs and M1 macrophages and its level was not increased after LPS stimulation (data not shown). It is possible that an active nuclear export-import mechanism, induced by phosphorylation at previously described serine residues [193], enables IRF5 to shuttle in these cells. However, I was not able to test this hypothesis due to the lack of phospho-specific antibodies to IRF5 but the recent acquisition of IRF5-deficient animals will provide new approaches for studying IRF5 activation. I suggest experiments where the phenotype of IRF5-deficient cells is rescued with either IRF5 wild-type protein or IRF5 protein with mutations in previously described serine/leucine residues supposedly important for activation or translocation [187, 188, 193].

In Chapter five I examined the relationship of IRF5-expressing macrophages and T lymphocytes as the type of adaptive immune response is shaped by the cytokine environment provided by macrophage subsets. I demonstrated that IRF5-expressing macrophages set up the environment for Th1/Th17 responses both *in vitro* and *in vivo*. Genetic polymorphisms in the IRF5 gene, leading to expression of several unique isoforms of IRF5 or increased expression of IRF5 mRNA, are associated with a number of autoimmune diseases. The remarkable consistency of IRF5 detection in genome-wide association studies of autoimmune diseases is likely to relate to their common inflammatory origin. Many autoimmune diseases are characterised by the predominant presence of Th1 and Th17 lymphocytes as well as macrophages with an M1 phenotype (IL-12^{high}, IL-23^{high}, IL-10^{low}) which can be characterized by high expression of IRF5 protein. As described in this thesis, the expression of IRF5 is induced upon stimulation of cells with GM-CSF.

M-CSF is constitutively produced by several cell types, including fibroblasts, endothelial cells, stromal cells and osteoblasts. It is likely that this steady state production of M-CSF polarizes macrophages towards the M2 phenotype. By contrast, GM-CSF production by the same cell types requires stimulation and occurs usually at a site of inflammation or infection. In this respect, it has been shown that *in vitro* cultured RA synovial macrophages spontaneously produce GM-CSF [261]. Furthermore, in the collagen-induced arthritis (CIA) mouse model of RA, GM-CSF depletion has therapeutic benefits [332, 333]. In these studies, GM-CSF deficient mice failed to develop arthritis despite making a normal humoral immune response to the arthritogenic stimulus [332]. Furthermore, the blockade of GM-CSF in wild-type mice controlled disease activity and decreased the levels of pro-inflammatory mediators, including TNF, in the joints during CIA [333]. One possible explanation for the positive effects of GM-CSF blockade on inflammation and autoimmunity is the link between its expression and its potential to control differentiation of macrophage subpopulations by inducing the gene expression of IRF5. Therefore, if therapeutic targeting of IRF5 is not possible, for example due to its cellular distribution, the next best approach would be to target IRF5 inducing stimuli such as GM-CSF.

New emerging data on the role of GM-CSF in autoimmunity suggest a positive feedback loop involving IL-23 and Th17 lymphocytes [322, 323]. Th17 lymphocytes produce many pro-inflammatory cytokines, including IL-17A/F and IL-22, and are characterized by the expression of the IL-23R [110]. The blockade of Th17-mediated disease, such as IBD and CIA, has proven to be only partially effective when neutralizing antibodies to IL-17A/F or IL-22 were used, which suggests that additional factors may be more important for Th17 function. One consistent finding is that neutralizing IL-23 effectively abrogates the effector response of Th17 lymphocytes in most models tested [110]. A link between IL-23 and Th17 lymphocytes was established when investigators showed that IL-23 promotes the production of IL-17 by activated T lymphocytes [334] and that IL-23-expanded T lymphocytes are able to transfer CIA [112]. Support for the importance of IL-23 signalling comes from genome-wide association studies demonstrating that SNPs in the human IL-23R confer an increased risk to Crohn's disease [230], ulcerative colitis [231] and psoriasis [335].

A common feature of IL-23-producing cells, including MDSCs and M1 macrophages, is the high expression of IRF5 protein. I demonstrated that IRF5 is involved in the transcriptional regulation of IL-23 expression in M1 macrophages and that IRF5-expressing cells provide

the cytokine environment for Th17 activation. The fact that Th17 lymphocytes are able to produce GM-CSF in response to IL-23 indicates an even tighter relationship between IL-23-producing cells and Th17 responses than previously expected. In fact, GM-CSF secreted by Th17 lymphocytes will not only stimulate cells already present at the site of inflammation but also affect newly recruited monocytes/macrophages (Figure 6.1). As demonstrated, IRF5 expression is GM-CSF dependent and infiltrating monocytes/macrophages would rapidly induce IRF5 expression and subsequently adopt the M1 macrophage phenotype. This suggests that the IRF5/IL-23/GM-CSF feedback loop could potentially be a new target for therapeutic intervention. Considering the data described in this thesis, IRF5 could be regarded as the initiation factor of this feedback loop and therefore targeting IRF5 function might be beneficial when interfering with this vicious cycle.

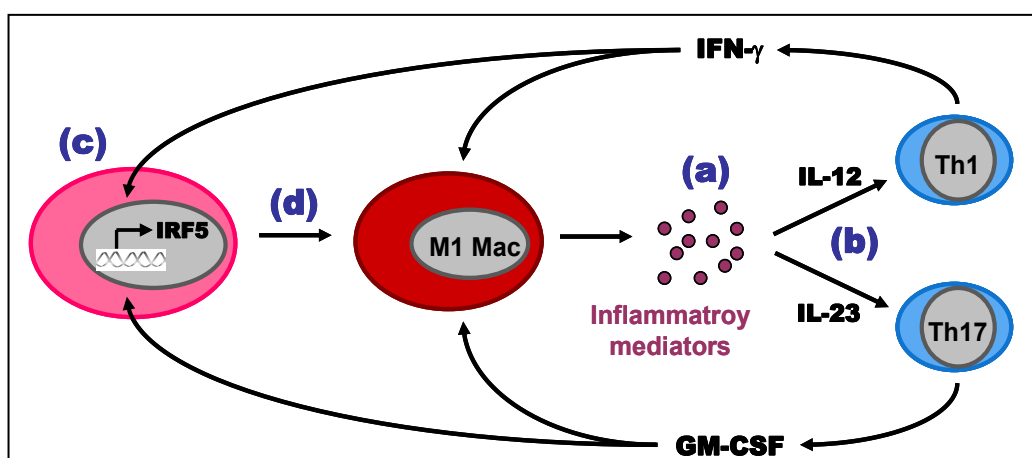


Figure 6.1 Interplay between M1 macrophages, T lymphocytes and infiltrating cells

(a) IRF5-expressing M1 macrophages (M1 Mac) secrete inflammatory mediators, including IL-12 and IL-23, which promote Th1 and Th17 lineage commitment (b). Once activated, Th1 and Th17 lymphocytes will produce IFN- γ and GM-CSF respectively, which will act on M1 macrophages present at the site of inflammation as well as infiltrating monocytes/macrophages (shown in pink). Stimulation of newly recruited monocytes/macrophages with IFN- γ or GM-CSF induces the expression of IRF5 (c) which will in turn promote polarization towards the M1 phenotype (d) resulting in prolonged inflammation.

We are only at the beginning of understanding how IRF5 regulates immune responses and further investigations using mouse models of inflammatory diseases associated with the M1 macrophage phenotype, such as CIA and colitis models, will be crucial to further dissect the full functional properties of this transcription factor.

Recent discoveries into the role of IRF5 in SLE demonstrated its importance in B lymphocyte development and consequently autoantibody production [213-215]. Macrophages and DCs were minimally analysed in these studies, although Tada *et al* noted that splenic DCs from IRF5-deficient animals produced lower levels of inflammatory cytokines *in vitro* following TLR7 or TLR9 stimulation [215].

In order to determine the role of IRF5 in IBD, colonic inflammation could be induced in wild-type and IRF5-deficient mice, by using either the dextran sodium sulphate (DSS) or the *Helicobacter hepaticus*-induced IBD model. DSS causes acute T lymphocyte-independent colitis which provides a useful model of acute intestinal pathology [336]. Conversely, *Helicobacter hepaticus* induces chronic IBD-like inflammation in C57BL/6 mice when administered in combination with a blocking antibody to the IL-10 receptor [337, 338]. Both models could be performed in parallel allowing the characterisation of chronic inflammation in *Helicobacter hepaticus*-infected mice and comparison of the results with those obtained from acute inflammation evoked by DSS. The phenotype of colonic macrophages isolated during various phases of disease pathology could be assessed using a combination of flow cytometry, ELISA and RT-PCR. It has been shown previously that switching of macrophages from an M1 toward an alternatively activated M2 phenotype is an essential step for resolution of other inflammatory diseases, such as peritonitis [339]. I would therefore expect that IRF5-deficient mice display a protective phenotype in both IBD models as the quantity of M2 macrophages should be significantly increased in these animals. Furthermore, it is generally accepted that IL-23 and its effect on Th17 lymphocytes is crucial in mouse models of IBD [228, 234]. Considering the data presented in this thesis, the amount of IL-23 should be reduced in IRF5-deficient mice resulting in a diminished Th17 response and consequently protection in mouse models of IBD.

The involvement of IRF5 in RA pathogenesis could be determined using the collagen-induced arthritis (CIA) model. CIA is an animal model of RA that is widely used to characterise the pathogenesis of immune cells and to validate therapeutic targets. Arthritis is normally induced in mice by immunisation with autologous or heterologous type II

collagen in adjuvant. CIA is considered to be a good model for human RA because of the observed similarities with respect to synovial inflammation, cartilage/bone destruction as well as a robust T and B lymphocyte response to the inducing agent [340, 341]. Similar to above, CIA could be induced in wild-type and IRF5-deficient mice and macrophage phenotype analysed by flow cytometry, ELISA and RT-PCR. T lymphocyte responses could be characterised using the same techniques and additionally, *in vitro* proliferation and type II collagen re-call experiments could be performed. Again, I expect that IRF5-deficient animals would be protected from CIA due to a shift from M1 toward M2 macrophage phenotype.

A completely different approach of investigating IRF5 function would be to focus on chronic inflammatory diseases associated with a shift toward M2 macrophage polarization, with asthma being a prime example. Murine models of asthma demonstrated the presence of M2 macrophages and enhanced expression of Th2 cytokines in the airway tissue [68, 86, 87]. Rather than being protected, IRF5-deficient animals should be more susceptible to asthma models. The strategy here would be to boost IRF5 expression in affected animals and therefore provoke a shift from Th2 to Th1 lymphocyte responses. A possible experiment would be the transfer of either *in vitro* differentiated M1 macrophages, M2 macrophages or M2 macrophages with exogenous IRF5 expression into *Irf5*^{-/-} mice. I would expect that the transfer of both M1 macrophages and M2 macrophages with exogenous IRF5 protein are beneficial for the recipient animals whereas M2 macrophages with endogenous levels of IRF5 protein will be detrimental to the animal.

In summary, I intended to provide new insights into the regulation and function of the transcription factor IRF5 in human myeloid cells. I demonstrated that GM-CSF and IFN- γ are potent inducers of IRF5 expression which is a hallmark of M1 macrophages. In these cells, IRF5 transcriptionally regulates M1-specific cytokines, chemokines and co-stimulatory molecules but represses genes associated with the M2 phenotype. As shown for IL-10, IRF5-mediated inhibition involves direct binding to the promoter and subsequent prevention of Pol II recruitment and is likely dependent on yet to be identified co-factors. Conversely, IRF5-induced gene activation of TNF involves two spatially separated regulatory regions (5' upstream and 3' downstream of the gene) and two independent modes of actions (direct DNA binding and formation of IRF5/RelA complex). As a consequence of IRF5 function,

IRF5-expressing M1 macrophages are essential for a robust Th1 response and furthermore provide the cytokine environment for Th17 lineage commitment.

Although the full mechanism underlying IRF5-mediated responses remains to be elucidated in more detail *in vivo*, the data presented here make IRF5 a new target for therapeutic intervention as its inhibition would affect pro-inflammatory cytokine expression and decrease the number of effector T lymphocytes.

7. Abbreviations

Amino acids	aa
Antigen presenting cell	APC
Anti-nuclear antibodies	ANA
Arbitrary units	AU
B cell-activating factor	BAFF
Bacterial alkaline phosphatase	BAP
Base pair	bp
Bone marrow media	BM media
Bone marrow-derived macrophages	BMDM
Bovine Serum Albumin	BSA
Chemokine receptor	CCR
Chromatin immuno-precipitation	ChIP
Cluster of differentiation	CD
Collagen induced arthritis	CIA
Common myeloid progenitor	CMP
Conventional DC	cDC
Counts per minute	CPM
CREB binding protein	CBP
Cycle thresholds	CT
Cytometric bead assay	CBA
Cytotoxic T-lymphocyte	CTL
Danger-associated molecular patterns	DAMPs
Dendritic cell	DC
Dextran sodium sulphate	DSS
Dimethylsulfoxide	DMSO
DNA binding domain	DBD
Dominant negative	DN
Double negative	DN
Dual specificity protein phosphatase 1	DUSP1
Dulbecco's Modified Eagle's Medium	DMEM
Encephalomyocarditis virus	EMCV
Enzyme linked immunosorbent assay	ELISA
Epstein-Barr virus induced 3	EBI3

Experimental autoimmune encephalomyelitis	EAE
Flow cytometry	FCM
Foetal calf serum	FCS
Genome-wide association studies	GWAS
Genomic DNA	gDNA
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Hank's Balanced Salt Solution	HBSS
Herpes simplex virus-1	HSV-1
Histone acetyltransferases	HATs
Histone deacetylases	HDACs
Human embryonic kidney	HEK
Human leukocyte antigen	HLA
IFN-stimulated regulatory element	ISRE
IL-23 receptor	IL-23R
Inflammatory bowel disease	IBD
Inhibitor of NF- κ B kinase- α	IKK α
Interferon gamma	IFN- γ
Interferon regulatory factor 3	IRF3
Interferon regulatory factor 4	IRF4
Interferon regulatory factor 5	IRF5
Interferon regulatory factor 8	IRF8
Interferon regulatory factor	IRF
Interleukin 1 receptor-associated kinase 1	IRAK1
Interleukin	IL
Intestinal epithelial cells	IEC
Intracellular cytokine staining	ICC
IRF association domain	IAD
Lennox L agar	LB agar
Lennox L broth base	LB
Leucin-rich repeat-containing receptors	NLRs
Lipopolysaccharide	LPS
Macrophage-colony stimulating factor receptor I	M-CSF-R
Macrophage-colony stimulating factor	M-CSF
Major histocompatibility complex	MHC
Major histocompatibility complex class I	MHC I

Major histocompatibility complex class II	MHC II
Mean fluorescence intensity	MFI
Mixed lymphocyte reaction	MLR
Monocyte-derived dendritic cell	MDDC
Monocyte-derived macrophage	MDM
Mononuclear phagocyte system	MPS
Mouse embryonic fibroblasts	MEF
MyD88-adaptor-like	Mal
Myeloid differentiation primary-response protein 88	MyD88
Natural killer	NK
Newcastle disease virus	NDV
Non-targeting siRNA	siC
Nuclear export signal	NES
Nuclear factor κ B	NF- κ B
Nuclear localisation signals	NLS
Pathogen-associated molecular pattern	PAMP
Pattern recognition receptor	PRR
Peripheral blood mononuclear cells	PBMC
Phorbol myristate acetate	PMA
Phosphate buffered saline	PBS
plasmacytoid DC	pDC
Polyinosinic Polycytidylic Acid	pl:C
Polyvinylidene difluoride	PVDF
Protein tyrosine phosphatase nonreceptor type 22	PTPN22
Quantitative real-time polymerase chain reaction	RT-PCR
Regulatory T lymphocyte	Treg
Rel homology domain	RHD
Retinoic acid-inducible gene I	RIG-I
Revolutions per minute	RPM
Rheumatoid arthritis	RA
RIG-I-like receptors	RLRs
RNA Polymerase II	Pol II
Roswell Park Memorial Institute media	RPMI
Short interfering RNA	siRNA
Signal and transducer and activator of transcription	STAT

Silencing mediator for retinoid or thyroid-hormone receptor	SMRT
Single-nucleotide polymorphism	SNP
Single-stranded RNA	ssRNA
siRNAs targeting IRF5	siIRF5
Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis	SDS-PAGE
Standard deviation	SD
Standard error of the mean	SEM
Systemic lupus erythematosus	SLE
TANK binding kinase 1	TBK1
T-cell receptor	TCR
T-helper	Th
TIR domain-containing adapter inducing IFN β	TRIF
TNF receptor 1	TNFR1
Toll/IL-1R	TIR
Toll-like receptor	TLR
Transcription start site	TSS
Transforming growth factor beta	TGF- β
TRIF-related adaptor molecule	TRAM
Tumor necrosis factor	TNF
Tumor necrosis factor (ligand) superfamily	TNFSF
Tumor necrosis factor receptor-associated factor 6	TRAF6
Tumor-associate macrophages	TAMs
Type I Interferon	IFN
Untranslated region	UTR
Vesicular stomatitis virus	VSV
Virus responsive elements	VRE
Wild-type	WT

8. References

1. Medzhitov, R. and C.A. Janeway, Jr., *Decoding the patterns of self and nonself by the innate immune system*. Science, 2002. **296**(5566): p. 298-300.
2. Medzhitov, R. and C.A. Janeway, Jr., *Innate immunity: impact on the adaptive immune response*. Curr Opin Immunol, 1997. **9**(1): p. 4-9.
3. Matzinger, P., *The danger model: a renewed sense of self*. Science, 2002. **296**(5566): p. 301-5.
4. Medzhitov, R., *Origin and physiological roles of inflammation*. Nature, 2008. **454**(7203): p. 428-35.
5. Piccinini, A.M. and K.S. Midwood, *DAMPening inflammation by modulating TLR signalling*. Mediators Inflamm, 2010. **2010**.
6. Iwasaki, A. and R. Medzhitov, *Regulation of adaptive immunity by the innate immune system*. Science, 2011. **327**(5963): p. 291-5.
7. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity*. Int Immunol, 2005. **17**(1): p. 1-14.
8. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses*. Nat Immunol, 2004. **5**(10): p. 987-95.
9. Drummond, R.A., et al., *The role of Syk/CARD9 coupled C-type lectins in antifungal immunity*. Eur J Immunol, 2011. **41**(2): p. 276-81.
10. Pichlmair, A. and C. Reis e Sousa, *Innate recognition of viruses*. Immunity, 2007. **27**(3): p. 370-83.
11. Martinon, F., A. Mayor, and J. Tschopp, *The inflammasomes: guardians of the body*. Annu Rev Immunol, 2009. **27**: p. 229-65.
12. Segal, A.W., *How neutrophils kill microbes*. Annu Rev Immunol, 2005. **23**: p. 197-223.
13. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells*. Annu Rev Immunol, 2009. **27**: p. 669-92.
14. Dai, X.M., et al., *Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects*. Blood, 2002. **99**(1): p. 111-20.

15. Cecchini, M.G., et al., *Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse*. Development, 1994. **120**(6): p. 1357-72.
16. Sasmono, R.T., et al., *A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse*. Blood, 2003. **101**(3): p. 1155-63.
17. Kawasaki, E.S., et al., *Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1)*. Science, 1985. **230**(4723): p. 291-6.
18. Lin, H., et al., *Discovery of a cytokine and its receptor by functional screening of the extracellular proteome*. Science, 2008. **320**(5877): p. 807-11.
19. Weber, C., et al., *Differential chemokine receptor expression and function in human monocyte subpopulations*. J Leukoc Biol, 2000. **67**(5): p. 699-704.
20. Ziegler-Heitbrock, H.W., et al., *Differential expression of cytokines in human blood monocyte subpopulations*. Blood, 1992. **79**(2): p. 503-11.
21. Passlick, B., D. Flieger, and H.W. Ziegler-Heitbrock, *Identification and characterization of a novel monocyte subpopulation in human peripheral blood*. Blood, 1989. **74**(7): p. 2527-34.
22. Belge, K.U., et al., *The proinflammatory CD14⁺CD16⁺DR⁺⁺ monocytes are a major source of TNF*. J Immunol, 2002. **168**(7): p. 3536-42.
23. Grage-Griebenow, E., et al., *Identification of a novel dendritic cell-like subset of CD64(+) / CD16(+) blood monocytes*. Eur J Immunol, 2001. **31**(1): p. 48-56.
24. Mizuno, K., et al., *Selective expansion of CD16^{high}CCR2⁻ subpopulation of circulating monocytes with preferential production of haem oxygenase (HO)-1 in response to acute inflammation*. Clin Exp Immunol, 2005. **142**(3): p. 461-70.
25. Cros, J., et al., *Human CD14^{dim} monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors*. Immunity, 2010. **33**(3): p. 375-86.
26. Quah, B.J. and H.C. O'Neill, *Maturation of function in dendritic cells for tolerance and immunity*. J Cell Mol Med, 2005. **9**(3): p. 643-54.
27. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development*. Nat Rev Immunol, 2007. **7**(1): p. 19-30.
28. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells*. Science, 2010. **327**(5966): p. 656-61.

29. Shortman, K. and Y.J. Liu, *Mouse and human dendritic cell subtypes*. Nat Rev Immunol, 2002. **2**(3): p. 151-61.
30. Volkman, A. and J.L. Gowans, *THE ORIGIN OF MACROPHAGES FROM BONE MARROW IN THE RAT*. Br J Exp Pathol, 1965. **46**: p. 62-70.
31. Nerlov, C. and T. Graf, *PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors*. Genes Dev, 1998. **12**(15): p. 2403-12.
32. Dakic, A., et al., *PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis*. J Exp Med, 2005. **201**(9): p. 1487-502.
33. Bakri, Y., et al., *Balance of MafB and PU.1 specifies alternative macrophage or dendritic cell fate*. Blood, 2005. **105**(7): p. 2707-16.
34. Reddy, M.A., et al., *Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes*. J Exp Med, 1994. **180**(6): p. 2309-19.
35. DeKoter, R.P., J.C. Walsh, and H. Singh, *PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors*. EMBO J, 1998. **17**(15): p. 4456-68.
36. Holtschke, T., et al., *Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene*. Cell, 1996. **87**(2): p. 307-17.
37. Scheller, M., et al., *Altered development and cytokine responses of myeloid progenitors in the absence of transcription factor, interferon consensus sequence binding protein*. Blood, 1999. **94**(11): p. 3764-71.
38. Tamura, T., et al., *ICSBP directs bipotential myeloid progenitor cells to differentiate into mature macrophages*. Immunity, 2000. **13**(2): p. 155-65.
39. Tsujimura, H., et al., *IFN consensus sequence binding protein/IFN regulatory factor-8 guides bone marrow progenitor cells toward the macrophage lineage*. J Immunol, 2002. **169**(3): p. 1261-9.
40. Kallies, A., et al., *Accumulation of c-Cbl and rapid termination of colony-stimulating factor 1 receptor signaling in interferon consensus sequence binding protein-deficient bone marrow-derived macrophages*. Blood, 2002. **99**(9): p. 3213-9.
41. Meraro, D., et al., *Protein-protein and DNA-protein interactions affect the activity of lymphoid-specific IFN regulatory factors*. J Immunol, 1999. **163**(12): p. 6468-78.

42. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity*. Nat Rev Immunol, 2005. **5**(12): p. 953-64.
43. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation*. Nat Rev Immunol, 2008. **8**(12): p. 958-69.
44. Romagnani, P., et al., *Th1/Th2 cells, their associated molecules and role in pathophysiology*. Eur Cytokine Netw, 2000. **11**(3): p. 510-1.
45. Martinez, F.O., et al., *Macrophage activation and polarization*. Front Biosci, 2008. **13**: p. 453-61.
46. Gordon, S., *Alternative activation of macrophages*. Nat Rev Immunol, 2003. **3**(1): p. 23-35.
47. Nathan, C.F., et al., *Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity*. J Exp Med, 1983. **158**(3): p. 670-89.
48. Stein, M., et al., *Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation*. J Exp Med, 1992. **176**(1): p. 287-92.
49. Loke, P., et al., *IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype*. BMC Immunol, 2002. **3**: p. 7.
50. Doyle, A.G., et al., *Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma*. Eur J Immunol, 1994. **24**(6): p. 1441-5.
51. Goerdts, S. and C.E. Orfanos, *Other functions, other genes: alternative activation of antigen-presenting cells*. Immunity, 1999. **10**(2): p. 137-42.
52. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization*. Trends Immunol, 2004. **25**(12): p. 677-86.
53. Hagemann, T., et al., *Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB*. Blood, 2009. **113**(14): p. 3139-46.
54. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes*. Trends Immunol, 2002. **23**(11): p. 549-55.
55. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions*. Immunity, 2010. **32**(5): p. 593-604.

56. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm*. Nat Immunol, 2010. **11**(10): p. 889-96.
57. D'Andrea, A., et al., *Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production*. J Exp Med, 1995. **181**(2): p. 537-46.
58. El Chartouni, C. and M. Rehli, *Comprehensive analysis of TLR4-induced transcriptional responses in interleukin 4-primed mouse macrophages*. Immunobiology, 2010. **215**(9-10): p. 780-7.
59. Stout, R.D., et al., *Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences*. J Immunol, 2005. **175**(1): p. 342-9.
60. Major, J., J.E. Fletcher, and T.A. Hamilton, *IL-4 pretreatment selectively enhances cytokine and chemokine production in lipopolysaccharide-stimulated mouse peritoneal macrophages*. J Immunol, 2002. **168**(5): p. 2456-63.
61. Hamilton, J.A., *Colony-stimulating factors in inflammation and autoimmunity*. Nat Rev Immunol, 2008. **8**(7): p. 533-44.
62. Stanley, E., et al., *Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology*. Proc Natl Acad Sci U S A, 1994. **91**(12): p. 5592-6.
63. Hoeve, M.A., et al., *Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells*. Eur J Immunol, 2006. **36**(3): p. 661-70.
64. Verreck, F.A., et al., *Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria*. Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4560-5.
65. Fleetwood, A.J., et al., *Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation*. J Immunol, 2007. **178**(8): p. 5245-52.
66. Lari, R., et al., *Macrophage lineage phenotypes and osteoclastogenesis--complexity in the control by GM-CSF and TGF-beta*. Bone, 2007. **40**(2): p. 323-36.
67. Verreck, F.A., et al., *Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation*. J Leukoc Biol, 2006. **79**(2): p. 285-93.

68. Martinez, F.O., L. Helming, and S. Gordon, *Alternative activation of macrophages: an immunologic functional perspective*. Annu Rev Immunol, 2009. **27**: p. 451-83.
69. Van Ginderachter, J.A., et al., *Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion*. Immunobiology, 2006. **211**(6-8): p. 487-501.
70. Jouanguy, E., et al., *IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men*. Curr Opin Immunol, 1999. **11**(3): p. 346-51.
71. Shaughnessy, L.M. and J.A. Swanson, *The role of the activated macrophage in clearing Listeria monocytogenes infection*. Front Biosci, 2007. **12**: p. 2683-92.
72. Benoit, M., B. Desnues, and J.L. Mege, *Macrophage polarization in bacterial infections*. J Immunol, 2008. **181**(6): p. 3733-9.
73. Ehrt, S., et al., *Reprogramming of the macrophage transcriptome in response to interferon-gamma and Mycobacterium tuberculosis: signaling roles of nitric oxide synthase-2 and phagocyte oxidase*. J Exp Med, 2001. **194**(8): p. 1123-40.
74. O'Reilly, M., D.E. Newcomb, and D. Remick, *Endotoxin, sepsis, and the primrose path*. Shock, 1999. **12**(6): p. 411-20.
75. Bozza, F.A., et al., *Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis*. Crit Care, 2007. **11**(2): p. R49.
76. Lopez-Bojorquez, L.N., A.Z. Dehesa, and G. Reyes-Teran, *Molecular mechanisms involved in the pathogenesis of septic shock*. Arch Med Res, 2004. **35**(6): p. 465-79.
77. Smith, M.W., et al., *Induction of pro- and anti-inflammatory molecules in a mouse model of pneumococcal pneumonia after influenza*. Comp Med, 2007. **57**(1): p. 82-9.
78. Fairweather, D. and D. Cihakova, *Alternatively activated macrophages in infection and autoimmunity*. J Autoimmun, 2009. **33**(3-4): p. 222-30.
79. Reece, J.J., M.C. Siracusa, and A.L. Scott, *Innate immune responses to lung-stage helminth infection induce alternatively activated alveolar macrophages*. Infect Immun, 2006. **74**(9): p. 4970-81.
80. Urban, J.F., Jr., et al., *IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis*. Immunity, 1998. **8**(2): p. 255-64.
81. Mountford, A.P., et al., *Signaling via interleukin-4 receptor alpha chain is required for successful vaccination against schistosomiasis in BALB/c mice*. Infect Immun, 2001. **69**(1): p. 228-36.

82. Herbert, D.R., et al., *Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology*. Immunity, 2004. **20**(5): p. 623-35.
83. Silveira-Lemos, D., et al., *Eosinophil activation status, cytokines and liver fibrosis in Schistosoma mansoni infected patients*. Acta Trop, 2008. **108**(2-3): p. 150-9.
84. Schneider, B.S., et al., *Aedes aegypti salivary gland extracts modulate anti-viral and TH1/TH2 cytokine responses to sindbis virus infection*. Viral Immunol, 2004. **17**(4): p. 565-73.
85. Miller, J.L., et al., *The mannose receptor mediates dengue virus infection of macrophages*. PLoS Pathog, 2008. **4**(2): p. e17.
86. Grunig, G., et al., *Requirement for IL-13 independently of IL-4 in experimental asthma*. Science, 1998. **282**(5397): p. 2261-3.
87. Song, H.M., et al., *Ym1 and Ym2 expression in a mouse model exposed to diesel exhaust particles*. Environ Toxicol, 2008. **23**(1): p. 110-6.
88. Vivier, E., et al., *Innate or adaptive immunity? The example of natural killer cells*. Science, 2011. **331**(6013): p. 44-9.
89. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets*. Trends Immunol, 2001. **22**(11): p. 633-40.
90. Raulet, D.H. and N. Guerra, *Oncogenic stress sensed by the immune system: role of natural killer cell receptors*. Nat Rev Immunol, 2009. **9**(8): p. 568-80.
91. Haskins, K., J. Kappler, and P. Marrack, *The major histocompatibility complex-restricted antigen receptor on T cells*. Annu Rev Immunol, 1984. **2**: p. 51-66.
92. Gellert, M., *V(D)J recombination: RAG proteins, repair factors, and regulation*. Annu Rev Biochem, 2002. **71**: p. 101-32.
93. Germain, R.N., I. Stefanova, and J. Dorfman, *Self-recognition and the regulation of CD4+ T cell survival*. Adv Exp Med Biol, 2002. **512**: p. 97-105.
94. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision*. Nat Rev Immunol, 2002. **2**(5): p. 309-22.
95. Slavik, J.M., J.E. Hutchcroft, and B.E. Bierer, *CD28/CTLA-4 and CD80/CD86 families: signaling and function*. Immunol Res, 1999. **19**(1): p. 1-24.
96. Jager, A. and V.K. Kuchroo, *Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation*. Scand J Immunol, 2010. **72**(3): p. 173-84.

97. Santana, M.A. and Y. Rosenstein, *What it takes to become an effector T cell: the process, the cells involved, and the mechanisms*. J Cell Physiol, 2003. **195**(3): p. 392-401.
98. Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages*. Science, 1993. **260**(5107): p. 547-9.
99. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment*. Cell, 2000. **100**(6): p. 655-69.
100. Bettelli, E., et al., *Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis*. J Exp Med, 2004. **200**(1): p. 79-87.
101. Zheng, W. and R.A. Flavell, *The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells*. Cell, 1997. **89**(4): p. 587-96.
102. Murphy, K.M. and S.L. Reiner, *The lineage decisions of helper T cells*. Nat Rev Immunol, 2002. **2**(12): p. 933-44.
103. Ferber, I.A., et al., *Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE)*. J Immunol, 1996. **156**(1): p. 5-7.
104. Matthys, P., et al., *Anti-IL-12 antibody prevents the development and progression of collagen-induced arthritis in IFN-gamma receptor-deficient mice*. Eur J Immunol, 1998. **28**(7): p. 2143-51.
105. Becher, B., B.G. Durell, and R.J. Noelle, *Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12*. J Clin Invest, 2002. **110**(4): p. 493-7.
106. Oppmann, B., et al., *Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12*. Immunity, 2000. **13**(5): p. 715-25.
107. Cua, D.J., et al., *Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain*. Nature, 2003. **421**(6924): p. 744-8.
108. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation*. J Exp Med, 2005. **201**(2): p. 233-40.
109. Ivanov, I.I., et al., *The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells*. Cell, 2006. **126**(6): p. 1121-33.
110. Korn, T., et al., *IL-17 and Th17 Cells*. Annu Rev Immunol, 2009. **27**: p. 485-517.

111. Wilson, N.J., et al., *Development, cytokine profile and function of human interleukin 17-producing helper T cells*. Nat Immunol, 2007. **8**(9): p. 950-7.
112. Murphy, C.A., et al., *Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation*. J Exp Med, 2003. **198**(12): p. 1951-7.
113. Yen, D., et al., *IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6*. J Clin Invest, 2006. **116**(5): p. 1310-6.
114. Romagnani, S., et al., *Properties and origin of human Th17 cells*. Mol Immunol, 2009. **47**(1): p. 3-7.
115. Bettelli, E., et al., *Induction and effector functions of T(H)17 cells*. Nature, 2008. **453**(7198): p. 1051-7.
116. Matusevicius, D., et al., *Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis*. Mult Scler, 1999. **5**(2): p. 101-4.
117. Chabaud, M., et al., *Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium*. Arthritis Rheum, 1999. **42**(5): p. 963-70.
118. Kotake, S., et al., *IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis*. J Clin Invest, 1999. **103**(9): p. 1345-52.
119. Nakae, S., et al., *Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice*. J Immunol, 2003. **171**(11): p. 6173-7.
120. Hofstetter, H.H., et al., *Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis*. Cell Immunol, 2005. **237**(2): p. 123-30.
121. Dardalhon, V., et al., *IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells*. Nat Immunol, 2008. **9**(12): p. 1347-55.
122. Veldhoen, M., et al., *Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset*. Nat Immunol, 2008. **9**(12): p. 1341-6.
123. Staudt, V., et al., *Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells*. Immunity, 2010. **33**(2): p. 192-202.
124. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3*. Science, 2003. **299**(5609): p. 1057-61.

125. Cui, W. and S.M. Kaech, *Generation of effector CD8⁺ T cells and their conversion to memory T cells*. Immunol Rev, 2010. **236**: p. 151-66.
126. Zozulya, A.L. and H. Wiendl, *The role of CD8 suppressors versus destructors in autoimmune central nervous system inflammation*. Hum Immunol, 2008. **69**(11): p. 797-804.
127. Heyman, B., *Regulation of antibody responses via antibodies, complement, and Fc receptors*. Annu Rev Immunol, 2000. **18**: p. 709-37.
128. Esser, C. and A. Radbruch, *Immunoglobulin class switching: molecular and cellular analysis*. Annu Rev Immunol, 1990. **8**: p. 717-35.
129. Taniguchi, T., et al., *IRF family of transcription factors as regulators of host defense*. Annu Rev Immunol, 2001. **19**: p. 623-55.
130. Mamane, Y., et al., *Interferon regulatory factors: the next generation*. Gene, 1999. **237**(1): p. 1-14.
131. Nehyba, J., et al., *A novel interferon regulatory factor (IRF), IRF-10, has a unique role in immune defense and is induced by the v-Rel oncoprotein*. Mol Cell Biol, 2002. **22**(11): p. 3942-57.
132. Escalante, C.R., et al., *Structure of IRF-1 with bound DNA reveals determinants of interferon regulation*. Nature, 1998. **391**(6662): p. 103-6.
133. Fujita, T., et al., *Delimitation and properties of DNA sequences required for the regulated expression of human interferon-beta gene*. Cell, 1985. **41**(2): p. 489-96.
134. Goodbourn, S. and T. Maniatis, *Overlapping positive and negative regulatory domains of the human beta-interferon gene*. Proc Natl Acad Sci U S A, 1988. **85**(5): p. 1447-51.
135. Raj, N.B., et al., *Virus infection and interferon can activate gene expression through a single synthetic element, but endogenous genes show distinct regulation*. J Biol Chem, 1989. **264**(28): p. 16658-66.
136. Takaoka, A., T. Tamura, and T. Taniguchi, *Interferon regulatory factor family of transcription factors and regulation of oncogenesis*. Cancer Sci, 2008. **99**(3): p. 467-78.
137. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. **124**(4): p. 783-801.
138. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway*. Science, 2003. **301**(5633): p. 640-3.

139. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling*. Nat Rev Immunol, 2007. **7**(5): p. 353-64.
140. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling*. Cell, 2008. **132**(3): p. 344-62.
141. Hoffmann, A., G. Natoli, and G. Ghosh, *Transcriptional regulation via the NF-kappaB signaling module*. Oncogene, 2006. **25**(51): p. 6706-16.
142. Udalova, I.A., et al., *Functional consequences of a polymorphism affecting NF-kappaB p50-p50 binding to the TNF promoter region*. Mol Cell Biol, 2000. **20**(24): p. 9113-9.
143. Vallabhapurapu, S. and M. Karin, *Regulation and function of NF-kappaB transcription factors in the immune system*. Annu Rev Immunol, 2009. **27**: p. 693-733.
144. Liou, H.C., et al., *The NF-kappa B p50 precursor, p105, contains an internal I kappa B-like inhibitor that preferentially inhibits p50*. EMBO J, 1992. **11**(8): p. 3003-9.
145. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation*. Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.
146. Pomerantz, J.L. and D. Baltimore, *Two pathways to NF-kappaB*. Mol Cell, 2002. **10**(4): p. 693-5.
147. Xiao, G., E.W. Harhaj, and S.C. Sun, *NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100*. Mol Cell, 2001. **7**(2): p. 401-9.
148. Senftleben, U., et al., *Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway*. Science, 2001. **293**(5534): p. 1495-9.
149. Sato, M., et al., *Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction*. Immunity, 2000. **13**(4): p. 539-48.
150. Erlandsson, L., et al., *Interferon-beta is required for interferon-alpha production in mouse fibroblasts*. Curr Biol, 1998. **8**(4): p. 223-6.
151. Honda, K., et al., *IRF-7 is the master regulator of type-I interferon-dependent immune responses*. Nature, 2005. **434**(7034): p. 772-7.
152. Asselin-Paturel, C., et al., *Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology*. Nat Immunol, 2001. **2**(12): p. 1144-50.

153. Krug, A., et al., *Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells*. Eur J Immunol, 2001. **31**(7): p. 2154-63.
154. Nakano, H., M. Yanagita, and M.D. Gunn, *CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells*. J Exp Med, 2001. **194**(8): p. 1171-8.
155. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA*. Science, 2004. **303**(5663): p. 1529-31.
156. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8*. Science, 2004. **303**(5663): p. 1526-9.
157. Krug, A., et al., *Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9*. Blood, 2004. **103**(4): p. 1433-7.
158. Kawai, T., et al., *Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6*. Nat Immunol, 2004. **5**(10): p. 1061-8.
159. Hoshino, K., et al., *IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9*. Nature, 2006. **440**(7086): p. 949-53.
160. Izaguirre, A., et al., *Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells*. J Leukoc Biol, 2003. **74**(6): p. 1125-38.
161. Honda, K., et al., *Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction*. Nature, 2005. **434**(7036): p. 1035-40.
162. Yasuda, K., et al., *CpG motif-independent activation of TLR9 upon endosomal translocation of "natural" phosphodiester DNA*. Eur J Immunol, 2006. **36**(2): p. 431-6.
163. Mittrucker, H.W., et al., *Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function*. Science, 1997. **275**(5299): p. 540-3.
164. Aliberti, J., et al., *Essential role for ICSPB in the in vivo development of murine CD8alpha + dendritic cells*. Blood, 2003. **101**(1): p. 305-10.
165. Schiavoni, G., et al., *ICSPB is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells*. J Exp Med, 2002. **196**(11): p. 1415-25.

166. Tsujimura, H., T. Tamura, and K. Ozato, *Cutting edge: IFN consensus sequence binding protein/IFN regulatory factor 8 drives the development of type I IFN-producing plasmacytoid dendritic cells*. J Immunol, 2003. **170**(3): p. 1131-5.
167. Tamura, T., et al., *IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity*. J Immunol, 2005. **174**(5): p. 2573-81.
168. Schiavoni, G., et al., *ICSBP is critically involved in the normal development and trafficking of Langerhans cells and dermal dendritic cells*. Blood, 2004. **103**(6): p. 2221-8.
169. Sigurdsson, S., et al., *Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus*. Am J Hum Genet, 2005. **76**(3): p. 528-37.
170. Graham, R.R., et al., *A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus*. Nat Genet, 2006. **38**(5): p. 550-5.
171. Lee, Y.H. and G.G. Song, *Association between the rs2004640 functional polymorphism of interferon regulatory factor 5 and systemic lupus erythematosus: a meta-analysis*. Rheumatol Int, 2008.
172. Dieguez-Gonzalez, R., et al., *Association of interferon regulatory factor 5 haplotypes, similar to that found in systemic lupus erythematosus, in a large subgroup of patients with rheumatoid arthritis*. Arthritis Rheum, 2008. **58**(5): p. 1264-74.
173. Kim, Y.J., et al., *Putative role of functional interferon regulatory factor 5 (IRF5) polymorphism in rheumatoid arthritis in a Korean population*. J Rheumatol, 2008. **35**(11): p. 2106-18.
174. Maalej, A., et al., *Association of IRF5 gene polymorphisms with rheumatoid arthritis in a Tunisian population*. Scand J Rheumatol, 2008. **37**(6): p. 414-8.
175. Shimane, K., et al., *A single nucleotide polymorphism in the IRF5 promoter region is associated with susceptibility to rheumatoid arthritis in the Japanese patients*. Ann Rheum Dis, 2008.
176. Anderson, C.A., et al., *Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47*. Nat Genet, 2011. **43**(3): p. 246-52.
177. Parkes, M., et al., *Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility*. Nat Genet, 2007. **39**(7): p. 830-2.

178. Dideberg, V., et al., *An insertion-deletion polymorphism in the interferon regulatory Factor 5 (IRF5) gene confers risk of inflammatory bowel diseases*. Hum Mol Genet, 2007. **16**(24): p. 3008-16.
179. Kozyrev, S.V., et al., *Structural insertion/deletion variation in IRF5 is associated with a risk haplotype and defines the precise IRF5 isoforms expressed in systemic lupus erythematosus*. Arthritis Rheum, 2007. **56**(4): p. 1234-41.
180. Mancl, M.E., et al., *Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms. Multiple isoforms with distinct cell type-specific expression, localization, regulation, and function*. J Biol Chem, 2005. **280**(22): p. 21078-90.
181. Kozyrev, S.V. and M.E. Alarcon-Riquelme, *The genetics and biology of Irf5-mediated signaling in lupus*. Autoimmunity, 2007. **40**(8): p. 591-601.
182. Rechsteiner, M. and S.W. Rogers, *PEST sequences and regulation by proteolysis*. Trends Biochem Sci, 1996. **21**(7): p. 267-71.
183. Cunninghame Graham, D.S., et al., *Association of IRF5 in UK SLE families identifies a variant involved in polyadenylation*. Hum Mol Genet, 2007. **16**(6): p. 579-91.
184. Graham, R.R., et al., *Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus*. Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6758-63.
185. Karpova, A.Y., P.M. Howley, and L.V. Ronco, *Dual utilization of an acceptor/donor splice site governs the alternative splicing of the IRF-3 gene*. Genes Dev, 2000. **14**(22): p. 2813-8.
186. Barnes, B.J., et al., *Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes*. Mol Cell Biol, 2002. **22**(16): p. 5721-40.
187. Lin, R., et al., *A CRM1-dependent nuclear export pathway is involved in the regulation of IRF-5 subcellular localization*. J Biol Chem, 2005. **280**(4): p. 3088-95.
188. Chen, W., et al., *Insights into interferon regulatory factor activation from the crystal structure of dimeric IRF5*. Nat Struct Mol Biol, 2008. **15**(11): p. 1213-20.
189. Schoenemeyer, A., et al., *The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling*. J Biol Chem, 2005. **280**(17): p. 17005-12.
190. Takaoka, A., et al., *Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors*. Nature, 2005. **434**(7030): p. 243-9.

191. Barnes, B.J., A.E. Field, and P.M. Pitha-Rowe, *Virus-induced heterodimer formation between IRF-5 and IRF-7 modulates assembly of the IFNA enhanceosome in vivo and transcriptional activity of IFNA genes*. J Biol Chem, 2003. **278**(19): p. 16630-41.
192. Barnes, B.J., P.A. Moore, and P.M. Pitha, *Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes*. J Biol Chem, 2001. **276**(26): p. 23382-90.
193. Cheng, T.F., et al., *Differential activation of IFN regulatory factor (IRF)-3 and IRF-5 transcription factors during viral infection*. J Immunol, 2006. **176**(12): p. 7462-70.
194. Barnes, B.J., et al., *Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection*. J Biol Chem, 2004. **279**(43): p. 45194-207.
195. Yanai, H., et al., *Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression*. Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3402-7.
196. Paun, A., et al., *Functional characterization of murine interferon regulatory factor 5 (IRF-5) and its role in the innate antiviral response*. J Biol Chem, 2008. **283**(21): p. 14295-308.
197. Kaiser, R. and L.A. Criswell, *Genetics research in systemic lupus erythematosus for clinicians: methodology, progress, and controversies*. Curr Opin Rheumatol, 2010. **22**(2): p. 119-25.
198. Isenberg, D.A., et al., *Fifty years of anti-ds DNA antibodies: are we approaching journey's end?* Rheumatology (Oxford), 2007. **46**(7): p. 1052-6.
199. Rahman, A. and D.A. Isenberg, *Systemic lupus erythematosus*. N Engl J Med, 2008. **358**(9): p. 929-39.
200. Berden, J.H., et al., *Role of nucleosomes for induction and glomerular binding of autoantibodies in lupus nephritis*. Curr Opin Nephrol Hypertens, 1999. **8**(3): p. 299-306.
201. Shiina, T., H. Inoko, and J.K. Kulski, *An update of the HLA genomic region, locus information and disease associations: 2004*. Tissue Antigens, 2004. **64**(6): p. 631-49.
202. Kyogoku, C., et al., *Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE*. Am J Hum Genet, 2004. **75**(3): p. 504-7.
203. Remmers, E.F., et al., *STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus*. N Engl J Med, 2007. **357**(10): p. 977-86.

204. Kozyrev, S.V., et al., *Functional variants in the B-cell gene BANK1 are associated with systemic lupus erythematosus*. Nat Genet, 2008. **40**(2): p. 211-6.
205. Hom, G., et al., *Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX*. N Engl J Med, 2008. **358**(9): p. 900-9.
206. Harley, J.B., et al., *Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PTK, KIAA1542 and other loci*. Nat Genet, 2008. **40**(2): p. 204-10.
207. Graham, R.R., et al., *Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus*. Nat Genet, 2008. **40**(9): p. 1059-61.
208. Siu, H.O., et al., *Association of a haplotype of IRF5 gene with systemic lupus erythematosus in Chinese*. J Rheumatol, 2008. **35**(2): p. 360-2.
209. Niewold, T.B., et al., *Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients*. Arthritis Rheum, 2008. **58**(8): p. 2481-7.
210. Kawasaki, A., et al., *Association of IRF5 polymorphisms with systemic lupus erythematosus in a Japanese population: support for a crucial role of intron 1 polymorphisms*. Arthritis Rheum, 2008. **58**(3): p. 826-34.
211. Rullo, O.J., et al., *Association of IRF5 polymorphisms with activation of the interferon alpha pathway*. Ann Rheum Dis, 2010. **69**(3): p. 611-7.
212. Feng, D., et al., *Genetic variants and disease-associated factors contribute to enhanced interferon regulatory factor 5 expression in blood cells of patients with systemic lupus erythematosus*. Arthritis Rheum, 2010. **62**(2): p. 562-73.
213. Richez, C., et al., *IFN regulatory factor 5 is required for disease development in the FcgammaRIIB^{-/-}Yaa and FcgammaRIIB^{-/-} mouse models of systemic lupus erythematosus*. J Immunol, 2010. **184**(2): p. 796-806.
214. Savitsky, D.A., et al., *Contribution of IRF5 in B cells to the development of murine SLE-like disease through its transcriptional control of the IgG2a locus*. Proc Natl Acad Sci U S A, 2010. **107**(22): p. 10154-9.
215. Tada, Y., et al., *Interferon regulatory factor 5 is critical for the development of lupus in MRL/lpr mice*. Arthritis Rheum, 2011. **63**(3): p. 738-48.
216. Loftus, E.V., Jr., *Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences*. Gastroenterology, 2004. **126**(6): p. 1504-17.

217. Saleh, M. and G. Trinchieri, *Innate immune mechanisms of colitis and colitis-associated colorectal cancer*. Nat Rev Immunol, 2011. **11**(1): p. 9-20.
218. Van Limbergen, J., D.C. Wilson, and J. Satsangi, *The genetics of Crohn's disease*. Annu Rev Genomics Hum Genet, 2009. **10**: p. 89-116.
219. Vereecke, L., et al., *Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis*. J Exp Med, 2010. **207**(7): p. 1513-23.
220. Steinbrecher, K.A., et al., *Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation*. J Immunol, 2008. **180**(4): p. 2588-99.
221. Van den Brande, J.M., et al., *Prediction of antitumour necrosis factor clinical efficacy by real-time visualisation of apoptosis in patients with Crohn's disease*. Gut, 2007. **56**(4): p. 509-17.
222. Zeissig, S., et al., *Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor alpha antibody treatment*. Gut, 2004. **53**(9): p. 1295-302.
223. Marini, M., et al., *TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis*. Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8366-71.
224. Hanauer, S.B., et al., *Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial*. Lancet, 2002. **359**(9317): p. 1541-9.
225. De Jager, P.L., et al., *The role of the Toll receptor pathway in susceptibility to inflammatory bowel diseases*. Genes Immun, 2007. **8**(5): p. 387-97.
226. Hugot, J.P., et al., *Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease*. Nature, 2001. **411**(6837): p. 599-603.
227. Pierik, M., et al., *Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases*. Inflamm Bowel Dis, 2006. **12**(1): p. 1-8.
228. Ahern, P.P., et al., *Interleukin-23 drives intestinal inflammation through direct activity on T cells*. Immunity, 2010. **33**(2): p. 279-88.
229. Ahern, P.P., et al., *The interleukin-23 axis in intestinal inflammation*. Immunol Rev, 2008. **226**: p. 147-59.
230. Duerr, R.H., et al., *A genome-wide association study identifies IL23R as an inflammatory bowel disease gene*. Science, 2006. **314**(5804): p. 1461-3.

231. Burton, P.R., et al., *Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants*. Nat Genet, 2007. **39**(11): p. 1329-37.
232. Elson, C.O., et al., *Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice*. Gastroenterology, 2007. **132**(7): p. 2359-70.
233. Hue, S., et al., *Interleukin-23 drives innate and T cell-mediated intestinal inflammation*. J Exp Med, 2006. **203**(11): p. 2473-83.
234. Izcue, A., et al., *Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis*. Immunity, 2008. **28**(4): p. 559-70.
235. Landre-Beauvais, A.J., *The first description of rheumatoid arthritis. Unabridged text of the doctoral dissertation presented in 1800*. Joint Bone Spine, 2001. **68**(2): p. 130-43.
236. Smolen, J. and D. Aletaha, *The burden of rheumatoid arthritis and access to treatment: a medical overview*. Eur J Health Econ, 2008. **8 Suppl 2**: p. S39-47.
237. Gravallese, E.M., *Bone destruction in arthritis*. Ann Rheum Dis, 2002. **61 Suppl 2**: p. ii84-6.
238. MacKay, K., et al., *Whole-genome linkage analysis of rheumatoid arthritis susceptibility loci in 252 affected sibling pairs in the United Kingdom*. Arthritis Rheum, 2002. **46**(3): p. 632-9.
239. Bellamy, N., et al., *Rheumatoid arthritis in twins: a study of aetiopathogenesis based on the Australian Twin Registry*. Ann Rheum Dis, 1992. **51**(5): p. 588-93.
240. Silman, A.J., et al., *Twin concordance rates for rheumatoid arthritis: results from a nationwide study*. Br J Rheumatol, 1993. **32**(10): p. 903-7.
241. Gregersen, P.K., J. Silver, and R.J. Winchester, *The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis*. Arthritis Rheum, 1987. **30**(11): p. 1205-13.
242. Cornelis, F., et al., *New susceptibility locus for rheumatoid arthritis suggested by a genome-wide linkage study*. Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10746-50.
243. Shiozawa, S., et al., *Identification of the gene loci that predispose to rheumatoid arthritis*. Int Immunol, 1998. **10**(12): p. 1891-5.
244. Stahl, E.A., et al., *Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci*. Nat Genet, 2010. **42**(6): p. 508-14.

245. Rueda, B., et al., *Analysis of IRF5 gene functional polymorphisms in rheumatoid arthritis*. Arthritis Rheum, 2006. **54**(12): p. 3815-9.
246. Garnier, S., et al., *IRF5 rs2004640-T allele, the new genetic factor for systemic lupus erythematosus, is not associated with rheumatoid arthritis*. Ann Rheum Dis, 2007. **66**(6): p. 828-31.
247. Han, S.W., et al., *Association of polymorphisms in interferon regulatory factor 5 gene with rheumatoid arthritis: a metaanalysis*. J Rheumatol, 2009. **36**(4): p. 693-7.
248. Sigurdsson, S., et al., *Association of a haplotype in the promoter region of the interferon regulatory factor 5 gene with rheumatoid arthritis*. Arthritis Rheum, 2007. **56**(7): p. 2202-10.
249. Gabriel, S.E., *The epidemiology of rheumatoid arthritis*. Rheum Dis Clin North Am, 2001. **27**(2): p. 269-81.
250. Jansson, L. and R. Holmdahl, *Estrogen-mediated immunosuppression in autoimmune diseases*. Inflamm Res, 1998. **47**(7): p. 290-301.
251. Klareskog, L., et al., *A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination*. Arthritis Rheum, 2006. **54**(1): p. 38-46.
252. Mahdi, H., et al., *Specific interaction between genotype, smoking and autoimmunity to citrullinated alpha-enolase in the etiology of rheumatoid arthritis*. Nat Genet, 2009. **41**(12): p. 1319-24.
253. Carty, S.M., N. Snowden, and A.J. Silman, *Should infection still be considered as the most likely triggering factor for rheumatoid arthritis?* Ann Rheum Dis, 2004. **63 Suppl 2**: p. ii46-ii49.
254. van Lent, P.L., et al., *Toll-like receptor 4 induced FcgammaR expression potentiates early onset of joint inflammation and cartilage destruction during immune complex arthritis: Toll-like receptor 4 largely regulates FcgammaR expression by interleukin 10*. Ann Rheum Dis, 2007. **66**(3): p. 334-40.
255. Abdollahi-Roodsaz, S., et al., *Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis*. Arthritis Rheum, 2007. **56**(9): p. 2957-67.
256. Sacre, S.M., et al., *Inhibitors of TLR8 reduce TNF production from human rheumatoid synovial membrane cultures*. J Immunol, 2008. **181**(11): p. 8002-9.
257. Klareskog, L., et al., *Appearance of anti-HLA-DR-reactive cells in normal and rheumatoid synovial tissue*. Scand J Immunol, 1981. **14**(2): p. 183-92.

- 258. Van Boxel, J.A. and S.A. Paget, *Predominantly T-cell infiltrate in rheumatoid synovial membranes*. N Engl J Med, 1975. **293**(11): p. 517-20.
- 259. Buchan, G., et al., *Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha*. Clin Exp Immunol, 1988. **73**(3): p. 449-55.
- 260. Field, M., et al., *Interleukin-6 localisation in the synovial membrane in rheumatoid arthritis*. Rheumatol Int, 1991. **11**(2): p. 45-50.
- 261. Alvaro-Gracia, J.M., et al., *Cytokines in chronic inflammatory arthritis. VI. Analysis of the synovial cells involved in granulocyte-macrophage colony-stimulating factor production and gene expression in rheumatoid arthritis and its regulation by IL-1 and tumor necrosis factor-alpha*. J Immunol, 1991. **146**(10): p. 3365-71.
- 262. Brennan, F.M., et al., *Cytokine production in culture by cells isolated from the synovial membrane*. J Autoimmun, 1989. **2 Suppl**: p. 177-86.
- 263. Katsikis, P.D., et al., *Immunoregulatory role of interleukin 10 in rheumatoid arthritis*. J Exp Med, 1994. **179**(5): p. 1517-27.
- 264. Haringman, J.J., et al., *Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis*. Ann Rheum Dis, 2005. **64**(6): p. 834-8.
- 265. Gerlag, D.M., et al., *Effects of oral prednisolone on biomarkers in synovial tissue and clinical improvement in rheumatoid arthritis*. Arthritis Rheum, 2004. **50**(12): p. 3783-91.
- 266. Strand, V., R. Kimberly, and J.D. Isaacs, *Biologic therapies in rheumatology: lessons learned, future directions*. Nat Rev Drug Discov, 2007. **6**(1): p. 75-92.
- 267. Baeten, D., et al., *Macrophages expressing the scavenger receptor CD163: a link between immune alterations of the gut and synovial inflammation in spondyloarthritis*. J Pathol, 2002. **196**(3): p. 343-50.
- 268. Baeten, D., et al., *Association of CD163+ macrophages and local production of soluble CD163 with decreased lymphocyte activation in spondylarthropathy synovitis*. Arthritis Rheum, 2004. **50**(5): p. 1611-23.
- 269. Vandooren, B., et al., *Absence of a classically activated macrophage cytokine signature in peripheral spondylarthritis, including psoriatic arthritis*. Arthritis Rheum, 2009. **60**(4): p. 966-75.
- 270. Brennan, F.M., et al., *Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis*. Lancet, 1989. **2**(8657): p. 244-7.

271. Butler, D.M., et al., *Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist*. Eur Cytokine Netw, 1995. **6**(4): p. 225-30.
272. Williams, R.O., M. Feldmann, and R.N. Maini, *Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis*. Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9784-8.
273. Elliott, M.J., et al., *Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha*. Arthritis Rheum, 1993. **36**(12): p. 1681-90.
274. Elliott, M.J., et al., *Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis*. Lancet, 1994. **344**(8930): p. 1105-10.
275. Maini, R.N., et al., *Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis*. Arthritis Rheum, 1998. **41**(9): p. 1552-63.
276. Maini, R., et al., *Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial*. ATTRACT Study Group. Lancet, 1999. **354**(9194): p. 1932-9.
277. Team, R.D.C., *R: A language and environment for statistical computing*, in *R Foundation for Statistical Computing*. 2010.
278. Gentleman, R.C., et al., *Bioconductor: open software development for computational biology and bioinformatics*. Genome Biol, 2004. **5**(10): p. R80.
279. Huber, W., et al., *Variance stabilization applied to microarray data calibration and to the quantification of differential expression*. Bioinformatics, 2002. **18 Suppl 1**: p. S96-104.
280. Smyth, G.K., et al., *Limma: linear models for microarray data*, in *Bioinformatics and Computational Biology Solutions using R and Bioconductor*. 2005, Springer, New York. p. 397-420.
281. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing*. Journal of the Royal Statistical Society. Series B (Methodological), 1995. **57**(1): p. 289-300.
282. Feldmann, M., et al., *Anti-TNF therapy: where have we got to in 2005?* J Autoimmun, 2005. **25 Suppl**: p. 26-8.

283. Honda, K. and T. Taniguchi, *Toll-like receptor signaling and IRF transcription factors*. IUBMB Life, 2006. **58**(5-6): p. 290-5.
284. Sakaguchi, S., et al., *Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock*. Biochem Biophys Res Commun, 2003. **306**(4): p. 860-6.
285. Aspalter, R.M., M.M. Eibl, and H.M. Wolf, *Regulation of TCR-mediated T cell activation by TNF-RII*. J Leukoc Biol, 2003. **74**(4): p. 572-82.
286. Thomson, S.J., et al., *The role of transposable elements in the regulation of IFN-lambda1 gene expression*. Proc Natl Acad Sci U S A, 2009. **106**(28): p. 11564-9.
287. Yoshimura, S., et al., *Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines*. Int Immunol, 2001. **13**(5): p. 675-83.
288. Kuprash, D.V., et al., *Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide*. J Immunol, 1999. **162**(7): p. 4045-52.
289. Kuprash, D.V., et al., *Conserved kappa B element located downstream of the tumor necrosis factor alpha gene: distinct NF-kappa B binding pattern and enhancer activity in LPS activated murine macrophages*. Oncogene, 1995. **11**(1): p. 97-106.
290. Yang, L., et al., *Functional analysis of a dominant negative mutation of interferon regulatory factor 5*. PLoS One, 2009. **4**(5): p. e5500.
291. Grivennikov, S.I., et al., *Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects*. Immunity, 2005. **22**(1): p. 93-104.
292. Leung, T.H., A. Hoffmann, and D. Baltimore, *One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers*. Cell, 2004. **118**(4): p. 453-64.
293. Ogawa, S., et al., *Molecular determinants of crosstalk between nuclear receptors and toll-like receptors*. Cell, 2005. **122**(5): p. 707-21.
294. Ueno, H., et al., *Dendritic cell subsets in health and disease*. Immunol Rev, 2007. **219**: p. 118-42.
295. Abe, K., et al., *Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo*. Blood, 2003. **101**(4): p. 1477-83.

296. Ritter, U., et al., *Analysis of the maturation process of dendritic cells deficient for TNF and lymphotoxin-alpha reveals an essential role for TNF*. J Leukoc Biol, 2003. **74**(2): p. 216-22.
297. Baldwin, H.M., et al., *TNF{alpha} blockade impairs dendritic cell survival and function in rheumatoid arthritis*. Ann Rheum Dis, 2009.
298. Glaros, T., M. Larsen, and L. Li, *Macrophages and fibroblasts during inflammation, tissue damage and organ injury*. Front Biosci, 2009. **14**: p. 3988-93.
299. Negishi, H., et al., *Negative regulation of Toll-like-receptor signaling by IRF-4*. Proc Natl Acad Sci U S A, 2005. **102**(44): p. 15989-94.
300. Porta, C., et al., *Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB*. Proc Natl Acad Sci U S A, 2009. **106**(35): p. 14978-83.
301. Ruffell, D., et al., *A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair*. Proc Natl Acad Sci U S A, 2009. **106**(41): p. 17475-80.
302. Satoh, T., et al., *The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection*. Nat Immunol, 2010. **11**(10): p. 936-44.
303. Hammer, M., et al., *Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock*. J Exp Med, 2006. **203**(1): p. 15-20.
304. Fleetwood, A.J., et al., *GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling*. J Leukoc Biol, 2009. **86**(2): p. 411-21.
305. Martinez, F.O., et al., *Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression*. J Immunol, 2006. **177**(10): p. 7303-11.
306. Ziegler-Heitbrock, L., et al., *IFN-alpha induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3*. J Immunol, 2003. **171**(1): p. 285-90.
307. Medzhitov, R. and T. Horng, *Transcriptional control of the inflammatory response*. Nat Rev Immunol, 2009. **9**(10): p. 692-703.
308. Ghisletti, S., et al., *Identification and Characterization of Enhancers Controlling the Inflammatory Gene Expression Program in Macrophages*. Immunity, 2010.

309. El Chartouni, C., L. Schwarzfischer, and M. Rehli, *Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming*. Immunobiology, 2010. **215**(9-10): p. 821-5.
310. Mestas, J. and C.C. Hughes, *Of mice and not men: differences between mouse and human immunology*. J Immunol, 2004. **172**(5): p. 2731-8.
311. Berg, D.J., et al., *Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Schwartzman reaction but not endotoxin tolerance*. J Clin Invest, 1995. **96**(5): p. 2339-47.
312. Bettelli, E., et al., *IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice*. J Immunol, 1998. **161**(7): p. 3299-306.
313. Hunter, C.A., et al., *IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi*. J Immunol, 1997. **158**(7): p. 3311-6.
314. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages*. J Immunol, 1991. **147**(11): p. 3815-22.
315. Smallie, T., et al., *IL-10 inhibits transcription elongation of the human TNF gene in primary macrophages*. J Exp Med, 2010. **207**(10): p. 2081-8.
316. Mosser, D.M. and X. Zhang, *Interleukin-10: new perspectives on an old cytokine*. Immunol Rev, 2008. **226**: p. 205-18.
317. Lyakh, L., et al., *Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans*. Immunol Rev, 2008. **226**: p. 112-31.
318. Toubi, E., *The role of CD4+CD25+ T regulatory cells in autoimmune diseases*. Clin Rev Allergy Immunol, 2008. **34**(3): p. 338-44.
319. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance*. Cell, 2000. **101**(5): p. 455-8.
320. Nistala, K., et al., *Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment*. Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14751-6.
321. Nurieva, R., et al., *Essential autocrine regulation by IL-21 in the generation of inflammatory T cells*. Nature, 2007. **448**(7152): p. 480-3.
322. Codarri, L., et al., *RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation*. Nat Immunol, 2011. **12**(6): p. 560-7.

323. El-Behi, M., et al., *The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF*. Nat Immunol, 2011. **12**(6): p. 568-75.
324. Tsytsykova, A.V., et al., *Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers*. Proc Natl Acad Sci U S A, 2007. **104**(43): p. 16850-5.
325. Taylor, J.M., et al., *Chromatin profiling across the human tumour necrosis factor gene locus reveals a complex, cell type-specific landscape with novel regulatory elements*. Nucleic Acids Res, 2008. **36**(15): p. 4845-62.
326. Tamura, T., et al., *The IRF family transcription factors in immunity and oncogenesis*. Annu Rev Immunol, 2008. **26**: p. 535-84.
327. Feng, D., et al., *Differential requirement of histone acetylase and deacetylase activities for IRF5-mediated proinflammatory cytokine expression*. J Immunol, 2010. **185**(10): p. 6003-12.
328. Dunn, G.P., C.M. Koebel, and R.D. Schreiber, *Interferons, immunity and cancer immunoediting*. Nat Rev Immunol, 2006. **6**(11): p. 836-48.
329. Hamilton, J.A., *GM-CSF in inflammation and autoimmunity*. Trends Immunol, 2002. **23**(8): p. 403-8.
330. Xu, D., et al., *Interferon Regulatory Factor 4 (IRF-4) Targets IRF-5 to Regulate Epstein-Barr Virus Transformation*. J Biol Chem, 2011. **286**(20): p. 18261-7.
331. Honma, K., et al., *Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS*. Proc Natl Acad Sci U S A, 2005. **102**(44): p. 16001-6.
332. Campbell, I.K., et al., *Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice*. J Immunol, 1998. **161**(7): p. 3639-44.
333. Cook, A.D., et al., *Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage colony-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease*. Arthritis Res, 2001. **3**(5): p. 293-8.
334. Aggarwal, S., et al., *Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17*. J Biol Chem, 2003. **278**(3): p. 1910-4.

- 335. Cargill, M., et al., *A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes*. Am J Hum Genet, 2007. **80**(2): p. 273-90.
- 336. Cooper, H.S., et al., *Clinicopathologic study of dextran sulfate sodium experimental murine colitis*. Lab Invest, 1993. **69**(2): p. 238-49.
- 337. Kullberg, M.C., et al., *IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis*. J Exp Med, 2006. **203**(11): p. 2485-94.
- 338. Kullberg, M.C., et al., *Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism*. Infect Immun, 1998. **66**(11): p. 5157-66.
- 339. Bystrom, J., et al., *Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP*. Blood, 2008. **112**(10): p. 4117-27.
- 340. Myers, L.K., et al., *Collagen-induced arthritis, an animal model of autoimmunity*. Life Sci, 1997. **61**(19): p. 1861-78.
- 341. Williams, R.O., *Collagen-induced arthritis as a model for rheumatoid arthritis*. Methods Mol Med, 2004. **98**: p. 207-16.

9. Supplementary material - Publications

The data described in this thesis led to the publication of the following manuscripts which can be found at the end of this thesis as well as online:

Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, Hussell T, Feldmann M, Udalova IA (2011) IRF5 promotes inflammatory macrophage polarization and T(H)1-T(H)17 responses. *Nat Immunol*, 2011. **12**(3): p. 231-8.

Krausgruber T, Saliba D, Ryzhakov G, Lanfrancotti A, Blazek K, Udalova IA (2010) IRF5 is required for late-phase TNF secretion by human dendritic cells. *Blood* **115**(22): 4421-4430.

In addition, I contributed (mainly ChIP and overexpression experiments) to the following manuscripts which can be found online:

Goh FG, Thomson SJ, **Krausgruber T**, Lanfrancotti A, Copley RR, Udalova IA (2010) Beyond the enhanceosome: cluster of novel kappaB sites downstream of the human IFN-beta gene is essential for lipopolysaccharide-induced gene activation. *Blood* **116**(25): 5580-5588.

Goh FG, Piccinini AM, **Krausgruber T**, Udalova IA, Midwood KS (2010) Transcriptional regulation of the endogenous danger signal tenascin-C: a novel autocrine loop in inflammation. *J Immunol* **184**(5): 2655-2662.

Thomson SJ, Goh FG, Banks H, **Krausgruber T**, Kotenko SV, Foxwell BM, Udalova IA (2009) The role of transposable elements in the regulation of IFN-lambda1 gene expression. *Proc Natl Acad Sci U S A* **106**(28): 11564-11569.

IRF5 promotes inflammatory macrophage polarization and T_H1-T_H17 responses

Thomas Krausgruber¹, Katrina Blazek¹, Tim Smallie¹, Saba Alzabin¹, Helen Lockstone², Natasha Sahgal², Tracy Hussell³, Marc Feldmann¹ & Irina A Udalova¹

Polymorphisms in the gene encoding the transcription factor IRF5 that lead to higher mRNA expression are associated with many autoimmune diseases. Here we show that IRF5 expression in macrophages was reversibly induced by inflammatory stimuli and contributed to the plasticity of macrophage polarization. High expression of IRF5 was characteristic of M1 macrophages, in which it directly activated transcription of the genes encoding interleukin 12 subunit p40 (IL-12p40), IL-12p35 and IL-23p19 and repressed the gene encoding IL-10. Consequently, those macrophages set up the environment for a potent T helper type 1 (T_H1)-T_H17 response. Global gene expression analysis demonstrated that exogenous IRF5 upregulated or downregulated expression of established phenotypic markers of M1 or M2 macrophages, respectively. Our data suggest a critical role for IRF5 in M1 macrophage polarization and define a previously unknown function for IRF5 as a transcriptional repressor.

Macrophages are a heterogeneous population of immune cells that are essential for the initiation and resolution of pathogen- or tissue damage-induced inflammation¹. They demonstrate considerable plasticity that allows them to respond efficiently to environmental signals and change their phenotype and physiology in response to cytokines and microbial signals². These changes can give rise to populations of cells with distinct functions that are phenotypically characterized by the production of proinflammatory and anti-inflammatory cytokines³. On the basis of the T helper type 1 (T_H1) and T_H2 polarization idea⁴, these cells are now referred to as M1 (classic) macrophages, which produce proinflammatory cytokines and mediate resistance to pathogens and contribute to tissue destruction, and M2 (alternative) macrophages, which produce anti-inflammatory cytokines and promote tissue repair and remodeling as well as tumor progression^{3,5}.

The activation of a subset-defining transcription factor is characteristic of commitment to a particular T cell lineage: T-bet is associated with the T_H1 subset; GATA-3 is associated with the T_H2 subset; Foxp3 is associated with regulatory T cells; and RORγt is associated with the T_H17 subset⁶. Dendritic cells (DCs) also use subset-selective expression of the transcription factors IRF4 and IRF8 for their commitment. CD4⁺ DCs have high expression of IRF4, but plasmacytoid DCs have low expression of IRF4. As a consequence, the CD4⁺ DC population is absent from *Irfa*^{-/-} mice. Conversely, plasmacytoid DCs and CD8⁺ DCs have high expression of IRF8; thus, *Irf8*^{-/-} mice are largely devoid of these DC subsets⁷. However, the transcription factors that underlie macrophage polarization remain largely undefined. Activation of the transcription factor NF-κB subunit p50 has been associated with the inhibition of M1-polarizing genes⁸, whereas transcription factor CREB-mediated induction of the transcription factor C/EBPβ has

been shown to upregulate M2-specific genes⁹. Subsequent evidence has suggested that in mice, IRF4 controls M2 macrophage polarization by stimulating the expression of specific M2 macrophage markers¹⁰.

IRF5, another member of the interferon-regulatory factor (IRF) family, has diverse activities, such as the activation of genes encoding type I interferon, inflammatory cytokines (including tumor necrosis factor (TNF), IL-6, IL-12 and IL-23) and tumor suppressors¹¹. Consequently, *Irf5*-deficient mice are resistant to lethal endotoxic shock¹². Human IRF5 is expressed in many splice variants with distinct cell type-specific expression, cellular localization, differences in regulation and functions¹³. Moreover, genetic polymorphisms in human *IRF5* that lead to the expression of various unique isoforms or higher expression of IRF5 mRNA have been linked to autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, multiple sclerosis and inflammatory bowel disease^{14–18}. Here we demonstrate a role for IRF5 in determining commitment to the M1 macrophage lineage. M1 macrophages were characterized by large amounts of IRF5, whose expression was induced during their differentiation. Forced expression of IRF5 in M2 macrophages drove global expression of M1-specific cytokines, chemokines and costimulatory molecules and led to a potent T_H1-T_H17 response. Conversely, the induction of M1-specific cytokines was impaired in human M1 macrophages with knockdown of IRF5 expression mediated by small interfering RNA (siRNA) and in the peritoneal macrophages of *Irf5*^{-/-} mice. Our data suggest that activation of IRF5 expression defines commitment to the macrophage lineage by driving M1 macrophage polarization and, together with published results demonstrating a role for IRF4 in controlling M2 macrophage markers¹⁰, establish a new paradigm for

¹Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, London, UK. ²Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK. ³National Heart and Lung Institute, Faculty of Medicine, Imperial College of Science, Technology and Medicine, South Kensington Campus, London, UK. Correspondence should be addressed to I.A.U. (i.udalova@imperial.ac.uk).

Received 2 November 2010; accepted 20 December 2010; published online 16 January 2011; doi:10.1038/ni.1990

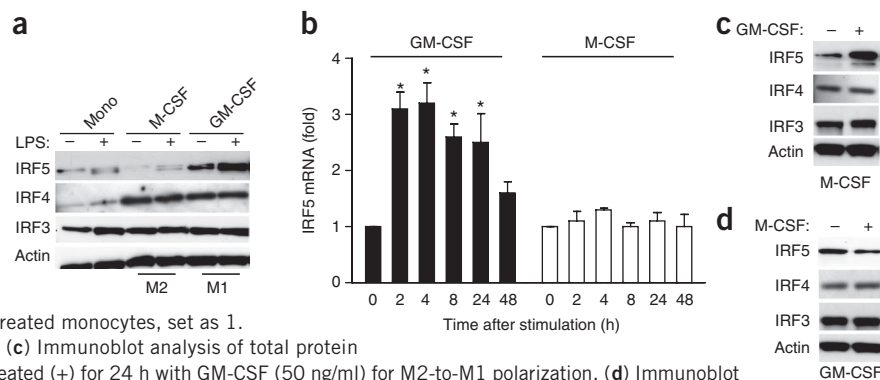
Figure 1 High expression of IRF5 in M1 macrophages and upregulation by GM-CSF.

(a) Immunoblot analysis of total protein extracts from monocytes collected at day 0 (Mono) or differentiated for 5 d into M1 macrophages with GM-CSF (50 ng/ml) or into M2 macrophages with M-CSF (100 ng/ml), then left untreated (–) or stimulated for 24 h with LPS (+). Actin serves as a loading control throughout. (b) RT-PCR analysis of IRF5 mRNA in monocytes left untreated (0) or stimulated for 2, 4, 8, 24 or 48 h with GM-CSF (50 ng/ml) or M-CSF (100 ng/ml); results are presented relative to those of untreated monocytes, set as 1.

* $P < 0.001$ (two-way analysis of variance (ANOVA)). (c) Immunoblot analysis of total protein

extracts from M2 macrophages left untreated (–) or treated (+) for 24 h with GM-CSF (50 ng/ml) for M2-to-M1 polarization. (d) Immunoblot analysis of total protein extracts from M1 macrophages left untreated or treated for 24 h with M-CSF (100 ng/ml) for M1-to-M2 polarization.

Results are from at least four independent experiments, each with cells derived from a different donor (a,c,d), or are from five independent experiments, each with cells derived from a different donor (b; mean and s.e.m.).



macrophage polarization and highlight the potential for therapeutic interventions via modulation of the IRF5-IRF4 balance.

RESULTS

High expression of IRF5 in human M1 macrophages

The M1 macrophage phenotype is induced by interferon- γ (IFN- γ), followed by stimulation with bacterial products such as lipopolysaccharide (LPS) or by treatment of monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF)^{19–21} (Supplementary Fig. 1a). IRF5 mRNA expression was higher in primary human monocytes and in monocyte-derived macrophages differentiated with GM-CSF than in those differentiated with M-CSF (Fig. 1a and Supplementary Fig. 1b). Treatment of monocytes with GM-CSF resulted in upregulation of IRF5 mRNA expression within 2 h of stimulation, but treatment with macrophage colony stimulating factor (M-CSF), an M2-differentiating factor, did not (Fig. 1b). To account for possible differences in protocols for the *in vitro* differentiation of macrophages, we analyzed the amount of IRF5 in macrophages treated for 24 h with IFN- γ alone or in combination with LPS and found that these were similar to those in GM-CSF-treated cells (Supplementary Fig. 1c). The expression of IRF4, which controls M2 polarization in mice¹⁰, was induced equally during the differentiation of monocytes into M1 or M2 macrophages (Fig. 1a). The expression of IRF3, another member of the IRF family central to the innate immune response, was not affected by differentiation into macrophage subtypes (Fig. 1a). Thus, IRF5 is induced in proinflammatory M1 macrophages independently of the *in vitro* differentiation protocol, whereas the abundance of IRF4 and IRF3 protein is similar in the macrophage populations. There was no substantial difference between M1 and M2 macrophages in their basal or LPS-induced NF- κ B p50 protein (Supplementary Fig. 1d,e), which has been linked before to the polarization of macrophages toward the M2 phenotype⁸.

Plastic expression of IRF5 in human macrophages

To examine whether IRF5 contributes to the plasticity of macrophage polarization, we attempted to convert one population into another by culturing M2 macrophages with GM-CSF and M1 macrophages with M-CSF. As expected, treatment of M2 macrophages with GM-CSF or IFN- γ led to the production of M1 phenotypic markers after stimulation with LPS (M2-to-M1; Supplementary Fig. 2a). Conversely, treatment of M1 macrophages with M-CSF led to the production of M2 phenotypic markers after stimulation with LPS (M1-to-M2; Supplementary Fig. 2b). M2-to-M1 conversion of macrophages resulted in more IRF5 protein (Fig. 1c and Supplementary

Fig. 2c), whereas M1-to-M2 macrophages had less IRF5 (Fig. 1d and Supplementary Fig. 2d). Once again, the quantities of IRF4 and IRF3 were unchanged (Fig. 1c,d). These results demonstrate that IRF5 expression is quickly adapted to environmental stimuli and suggest that it participates in establishing macrophage plasticity.

IRF5 influences polarization of human M1 macrophages

We next investigated whether IRF5 would directly induce the expression of M1 macrophage phenotypic markers. We detected bioactive IL-12p70 and IL-23 in M2 macrophages infected with an adenoviral expression construct encoding human IRF5 (variant 3/4)¹³ but detected minimal amounts after infection with a construct encoding IRF3 or an empty vector²² (Fig. 2a). The upregulation of IL-12p70 and IL-23 was reflected by much more secretion of the p40 subunit shared by the two cytokines (Fig. 2a). The secretion of both IL-12p70 and IL-23 peaked at 24 h after stimulation with LPS and remained sustained up to at least 48 h (Supplementary Fig. 3a). We also observed much more production of other key proinflammatory cytokines, such as IL-1 β and TNF, by IRF5-expressing macrophages (Supplementary Fig. 3b). Notably, IL-10 production was much lower in the IRF5-expressing cells (Fig. 2a). We also observed inhibition of IL-10 protein in cells overexpressing IRF3 (Fig. 2a), which might represent a negative feedback regulation of IL-10 expression²³, as the main direct target of IRF3, IFN- β , induces IL-10 (ref. 24). The complementary experiment targeting endogenous IRF5 in M1 macrophages by RNA-mediated interference (Supplementary Fig. 3c) resulted in significant inhibition of IL-12p70 and IL-23 and more IL-10 (Fig. 2b). Secretion of IL-12p40 was also lower in these cells (Fig. 2b), consistent with data obtained with *Irf5*-deficient mouse myeloid cells¹². Together these results indicate that IRF5 influences M1 macrophage polarization by equipping the cells with an IL-12^{hi}IL-23^{hi}IL-10^{lo} cytokine profile.

IRF5 promotes human T_H1-T_H17 responses

One of the hallmarks of M1 macrophage polarization is the acquisition of antigen-presenting features, which leads to efficient T_H1 responses^{20,21}. To determine whether IRF5 aided the polarization of the T lymphocyte proliferation, fate or activation state, we infected human M2 macrophages with viral expression constructs for IRF5 or IRF3 or empty vector and exposed the cells to human T lymphocytes extracted and purified from the peripheral blood of major histocompatibility complex-mismatched donors in a mixed-lymphocyte reaction. We assessed total T lymphocyte proliferation 3 d after infection and analyzed activation of specific T cell subsets by flow cytometry (Supplementary Fig. 4a). The proliferation of

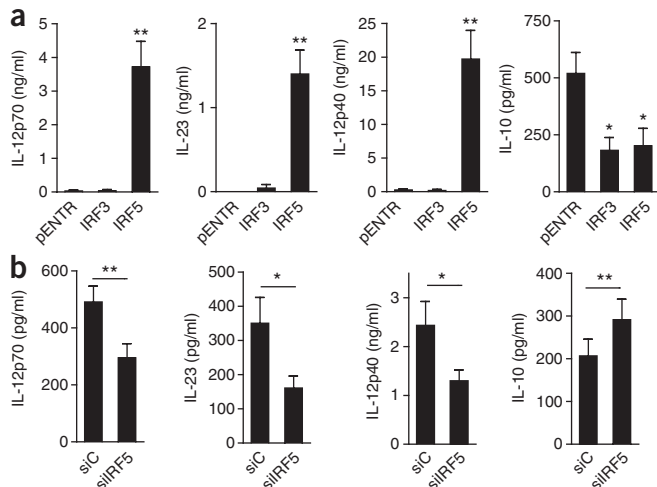


Figure 2 IRF5 influences the production of macrophage lineage-specific cytokines. (a) Enzyme-linked immunosorbent assay (ELISA) of the secretion of IL-12p70, IL-23, IL-12p40 and IL-10 by M2 macrophages infected with adenoviral vector encoding IRF5 or IRF3 or empty vector (pENTR) and stimulated for 24 h with LPS. * $P < 0.01$ and ** $P < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test). (b) ELISA of the secretion of IL-12p70, IL-23, IL-12p40 and IL-10 by M1 macrophages transfected with siRNA targeting IRF5 (siIRF5) or nontargeting (control) siRNA (siC) and stimulated for 24 h with LPS (10 ng/ml) plus IFN- γ (50 ng/ml). * $P < 0.01$ and ** $P < 0.001$ (Student's *t*-test). Data are representative of seven to nine (a) or six to eight (b) independent experiments, each with cells derived from a different donor (mean and s.e.m.).

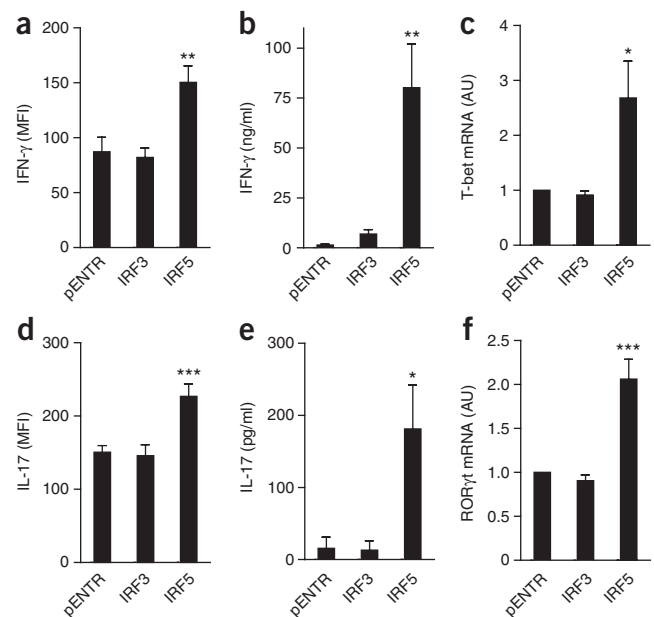
IRF5 directly induces transcription of human M1 genes

IRF5 is a transcription factor that can bind to the regulatory regions of target genes and modulate their expression. We next determined whether the role of IRF5 in the differential regulation of the secretion of IL-12p70, IL-23 and IL-10 cytokines was a direct consequence of its function as a transcription factor. The expression of mRNA for IL-12p40, IL-12p35 and IL-23p19 was considerably induced in M2 macrophages infected with an adenoviral vector construct encoding IRF5 but not in those infected with a construct encoding IRF3 or with empty vector (Fig. 4a). Moreover, the IRF5-driven expression of IL-12p40 mRNA was sustained until at least 16 h after stimulation with LPS (Supplementary Fig. 5a). Consistent with the protein-secretion data, the expression of IL-10 mRNA was inhibited by IRF5 (Fig. 4a). However, the expression of IL-10 mRNA was not altered by IRF3, which suggests the lack of a direct role for IRF3 in transcription of the gene encoding IL-10. Inhibition of endogenous IRF5 in M1 macrophages via RNA-mediated interference resulted in lower expression of mRNA for IL-12p40, p35 and IL23p19 at 8 h after stimulation with LPS (Fig. 4b). IL-12p40 was considerably inhibited throughout the time course analyzed, even 16 h after stimulation with LPS (Supplementary Fig. 5b). The expression of IL-10 mRNA was higher in the cells with knocked-down expression of IRF5 (Fig. 4b).

To formally define the global expression profile induced by IRF5, we did genome-wide expression analysis in which we compared M2 macrophages transduced with ectopic IRF5 with previously defined human M1 and M2 macrophage subsets^{21,27}. We found that the expression of about 90% of known human polarization-specific

T lymphocytes (Supplementary Fig. 4b) was considerably greater when they were cultured together with IRF5-expressing macrophages. Furthermore, only IRF5-expressing macrophages provided the cytokine environment necessary for the population expansion and activation of T_H1 cells, indicated by more IFN- γ -producing CD4⁺ cells (Fig. 3a and Supplementary Fig. 4c) and higher expression of IFN- γ mRNA (Supplementary Fig. 4d) and IFN- γ protein (Fig. 3b). There was significant induction of expression of mRNA for the T_H1-specifying transcription factor T-bet in T cells cultured together with IRF5-expressing macrophages (Fig. 3c). In these cultures we also observed population expansion and activation of T_H17 cells, indicated by more IL-17-producing CD4⁺ cells (Fig. 3d and Supplementary Fig. 4e), secretion of IL-17A (Fig. 3e) and expression of mRNA for IL-17A, IL-17F, IL-21, IL-22, IL-26 and IL-23R (Supplementary Fig. 4f). In line with studies demonstrating that IL-23 enhances the emergence of an IL-17⁺IFN- γ ⁺ population of T cells²⁵, about 25% of IL-17⁺ cells were also IFN- γ ⁺ (data not shown), which supported the idea of a close developmental relationship between human T_H17 cells and T_H1 cells²⁶. There was significant induction of expression of mRNA for the T_H17-specifying transcription factor ROR γ t in T cells cultured together with IRF5-expressing macrophages (Fig. 3f). Of interest, expression of GATA-3 mRNA and Foxp3 mRNA was lower in the presence of IRF5-expressing macrophages (data not shown). Hence, IRF5 promotes the proliferation of T lymphocytes and activation of the T_H1 and T_H17 lineages but does not induce the T_H2 lineage or regulatory T cell lineage.

Figure 3 IRF5 promotes lymphocyte proliferation and T_H1-T_H17 responses. (a) Immunocytochemical staining of IFN- γ in T lymphocytes cultured for 4 d together with M2 macrophages obtained from mismatched donors and infected with adenoviral vector encoding IRF5 or IRF3 or empty vector, then stimulated for 3 h with PMA, ionomycin and brefeldin A; results are presented as mean fluorescence intensity (MFI). (b) IFN- γ in supernatants of the cells in a after 4 d of coculture. (c) Expression of T-bet mRNA in T lymphocytes cultured for 2 d together with M2 macrophages obtained from mismatched donors and infected with adenoviral vectors as in a; results are presented in arbitrary units (AU) relative to those obtained with empty vector control. (d) Immunocytochemical staining of IL-17A in the T lymphocytes in a. (e) IL-17A in supernatants of the cells in a after 4 d of coculture. (f) Expression of ROR γ t mRNA, assessed and presented as in c. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test). Data are representative of seven (a,d), six (b,c,f) or four (e) independent experiments, each with cells derived from a different donor (mean and s.e.m.).



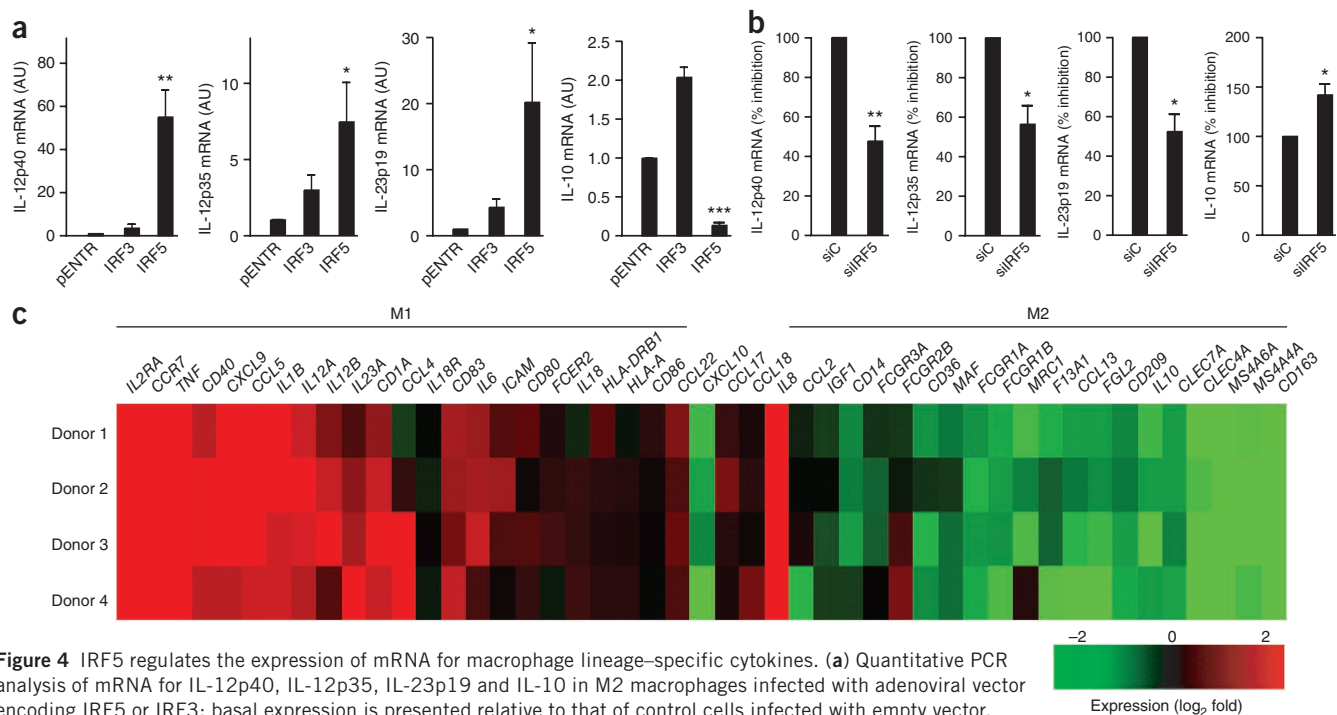


Figure 4 IRF5 regulates the expression of mRNA for macrophage lineage-specific cytokines. **(a)** Quantitative PCR analysis of mRNA for IL-12p40, IL-12p35, IL-23p19 and IL-10 in M2 macrophages infected with adenoviral vector encoding IRF5 or IRF3; basal expression is presented relative to that of control cells infected with empty vector. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test). **(b)** Expression of mRNA for IL-12p40, IL-12p35, IL-23p19 and IL-10 in M1 macrophages transfected with siRNA targeting IRF5 and left untreated or stimulated for 8 h with LPS (10 ng/ml); results are presented (as percent inhibition) relative to those of control cells transfected with non-targeting control siRNA. * $P < 0.01$ and ** $P < 0.001$ (Student's t -test). **(c)** Global mRNA expression (for sets of M1- and M2-specific genes^{21,27}; above plot) by unstimulated M2 macrophages infected with adenoviral vector encoding IRF5, presented relative to that of unstimulated M2 macrophages infected with empty vector. Results are presented as higher (red) or lower (green) expression after infection with vector encoding IRF5 (key (below), log₂ fold change). Data are from three to six independent experiments, each with cells derived from a different donor (**a**; mean and s.e.m.), are from five to six independent experiments (**b**; mean and s.e.m.) or are representative of one experiment with four different donors (**c**).

markers was driven by IRF5 (**Fig. 4c**). IRF5 induced 20 M1-specific genes and inhibited 19 M2-specific genes encoding cytokines, chemokines, costimulatory molecules and surface receptors (**Fig. 4c**), which resulted in more or less production of the corresponding proteins, respectively (**Supplementary Fig. 5c–e**). Moreover, we identified many previously unknown IRF5-regulated genes that probably contribute to the main functional features of macrophage subsets, such as phagocytosis and antigen presentation (**Supplementary Table 1**).

Next we investigated the LPS-induced recruitment of IRF5 to the promoter loci of the genes noted above. All IRF family members share a well-conserved amino-terminal DNA-binding domain that recognizes interferon-stimulated response elements (ISREs).

Computational analysis of the regions 2,000 nucleotides 5' upstream and 1,000 nucleotides downstream of the transcription start sites of *IL12A* (IL-12p35), *IL12B* (IL-12p40), *IL23A* (IL-23p19), *IL10* and other IRF5-regulated genes (**Fig. 5** and **Supplementary Table 1**) led to the identification of several ISREs (**Supplementary Table 2**). We designed primers encompassing these ISREs and used them in quantitative chromatin-immunoprecipitation experiments with M1 macrophages stimulated for 0, 1, 2, 4, 8 or 24 h with LPS. We observed LPS-induced enrichment of IRF5 at the promoter regions of *IL12A*, *IL12B* and *IL23A* up to 8 h after stimulation, which matched the kinetics of the recruitment of RNA polymerase II to the genes (**Fig. 5a–c**). In contrast, at the *IL10* promoter region, LPS-induced recruitment of IRF5 took place between 1 h and 4 h after stimulation,

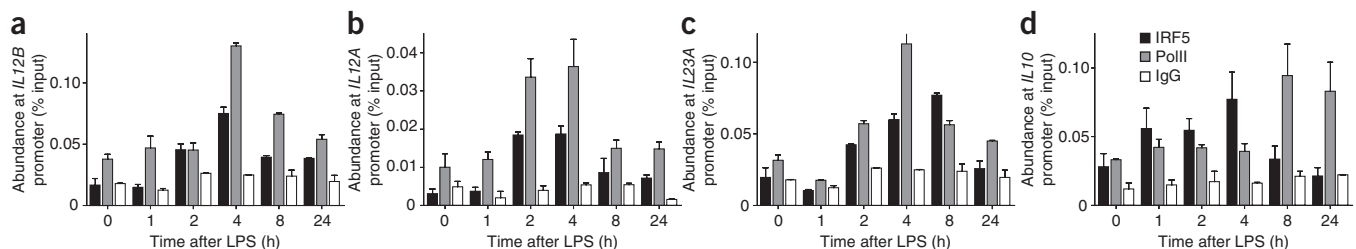
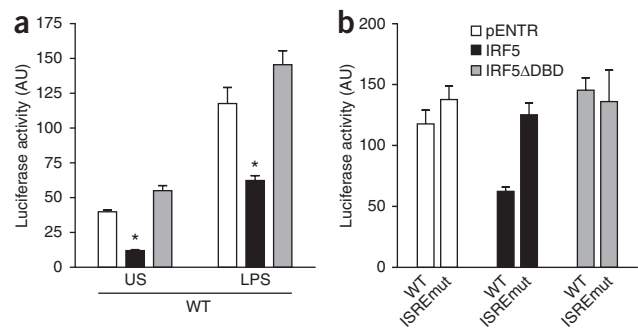


Figure 5 IRF5 is directly involved in the transcriptional regulation of lineage-specific cytokines. Recruitment of proteins to the promoters of genes encoding IL-12p40 (*IL12B*; **a**), IL-12p35 (*IL12A*; **b**), IL-23p19 (*IL23A*; **c**) or IL-10 (**d**) in M1 macrophages left unstimulated (0) or stimulated for 1, 2, 4, 8 or 24 h with LPS (10 ng/ml), assessed by chromatin immunoprecipitation with antibody to IRF5, antibody to RNA polymerase II (PoIII) or immunoglobulin G (IgG; control); results are presented relative to those obtained with genomic DNA (input). Data are from one experiment representative of three (error bars, s.d.).

Figure 6 IRF5 inhibits the transcriptional activation of human *IL10*. (a) Luciferase activity of M2 macrophages infected for 24 h with a wild-type *IL10* luciferase reporter plasmid (WT), plus a construct encoding IRF5 or an IRF5 mutant lacking the DNA-binding domain (IRF5 Δ DBD) or empty vector, then left unstimulated (US) or stimulated for 4 h with LPS (10 ng/ml). (b) Luciferase activity of M2 macrophages infected for 24 h with the wild-type reporter in a or an *IL10* luciferase reporter plasmid with site-specific mutations in the ISRE site at positions -180 to -173 (ISREmut), plus the IRF5 constructs in a or empty vector, then stimulated with LPS as in a. * $P < 0.01$ (one-way ANOVA with Dunnett's multiple-comparison post-test). Data are from three independent experiments, each with cells derived from a different donor (mean and s.e.m.).



whereas RNA polymerase II bound to the region only 8 h after stimulation (Fig. 5d), which suggests a previously unknown inhibitory role for IRF5 in the transcriptional regulation of selected genes. Together these results indicate that IRF5 regulates the transcription of genes encoding IL-12p40, IL-12p35, IL-23p19 and IL-10 through its recruitment to their promoter regions. It also influences the expression of most cytokines that define human lineage.

IRF5 inhibits the transcription of human *IL10*

To investigate whether IRF5 can directly repress transcription of the gene encoding IL-10, we used an adenovirus construct with a gene reporter in which a luciferase reporter construct was flanked by 195 nucleotides 5' upstream of the human gene encoding IL-10 (ref. 28). We coinfecting M2 macrophages with that construct plus a vector encoding IRF5 or empty vector and quantified luciferase activity. IRF5-expressing cells had significantly less luciferase activity, both in the unstimulated condition and 4 h after stimulation with LPS (Fig. 6a). To confirm the importance of the binding of IRF5 to the *IL10* promoter, we generated a mutant of IRF5 lacking the DNA-binding domain. This IRF5 mutant was not able to inhibit the wild-type IL-10 luciferase reporter construct (Fig. 6a). To further explore the molecular mechanism of IRF5-mediated suppression of *IL10* transcription, we introduced point mutations into the ISRE identified (positions -182 to -172 relative to the transcription start site) and coinfecting M2 macrophages with this mutated luciferase reporter construct and vector encoding IRF5 or empty vector. The luciferase reporter construct with a mutated ISRE produced a different response

than did the wild-type construct in that ectopic IRF5 was no longer able to suppress luciferase activity (Fig. 6b), which suggested that IRF5 inhibits IL-10 by direct binding to the ISRE of the *IL10* promoter. This was opposite to the positive regulatory activity of IRF5 at the *TNF* promoter²² and *IL12A* promoter (Supplementary Fig. 6). Therefore, IRF5 can act not only as a transcriptional activator but also as a suppressor of certain target genes, in this case the gene encoding the anti-inflammatory mediator IL-10. The mode of inhibition is mediated by direct binding of IRF5 to the promoter region of *IL10* and probably by engagement of as-yet-unidentified cofactors.

Importance of IRF5 in a mouse model of M1 inflammation

Similar to their human counterparts, mouse bone marrow-derived macrophages (BMDMs) differentiated with GM-CSF had higher expression of IRF5 protein than did M-CSF-derived BMDMs (Fig. 7a) and were the only cells secreting IL-12p70 and IL-23 (data not shown). Consequently, BMDMs obtained from *Irf5*^{-/-} mice and differentiated with GM-CSF secreted significantly less IL-12p70 and IL-23 or more IL-10 in response to stimulation with LPS than did wild-type cells (Fig. 7b). We observed no difference between wild-type and *Irf5*^{-/-} M-CSF-derived BMDMs in their IL-10 secretion (data not shown). To investigate the function of IRF5 in an *in vivo* model of M1-polarized inflammation, we challenged *Irf5*^{-/-} mice with a sublethal dose of LPS injected intraperitoneally. Within 3 h there was a significant difference between wild-type and *Irf5*^{-/-} mice in the serum concentrations of certain cytokines. Responses were consistent with the human data; that is, *Irf5*^{-/-} mice had lower

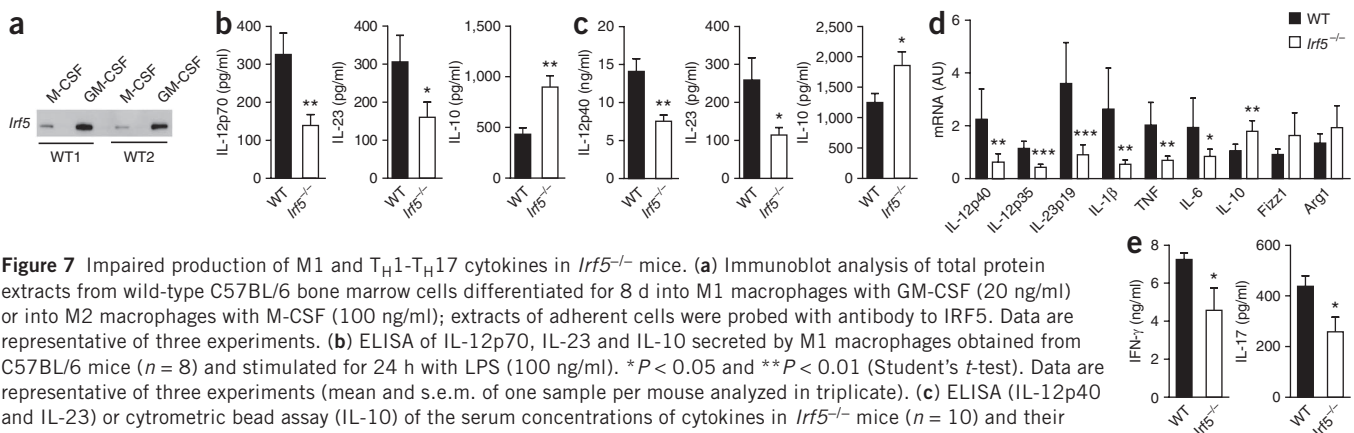


Figure 7 Impaired production of M1 and T_H1-T_H17 cytokines in *Irf5*^{-/-} mice. (a) Immunoblot analysis of total protein extracts from wild-type C57BL/6 bone marrow cells differentiated for 8 d into M1 macrophages with GM-CSF (20 ng/ml) or into M2 macrophages with M-CSF (100 ng/ml); extracts of adherent cells were probed with antibody to IRF5. Data are representative of three experiments. (b) ELISA of IL-12p70, IL-23 and IL-10 secreted by M1 macrophages obtained from C57BL/6 mice ($n = 8$) and stimulated for 24 h with LPS (100 ng/ml). * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are representative of three experiments (mean and s.e.m. of one sample per mouse analyzed in triplicate). (c) ELISA (IL-12p40 and IL-23) or cytometric bead assay (IL-10) of the serum concentrations of cytokines in *Irf5*^{-/-} mice ($n = 10$) and their wild-type littermates ($n = 10$) injected intraperitoneally with LPS (20 μ g), assessed 3 h later. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are from three independent experiments (mean and s.e.m. of eight to ten serum samples). (d) Expression of mRNA for M1 and M2 markers (horizontal axis) in peritoneal cells from the LPS-injected mice in c. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (Student's *t*-test). Data are from three independent experiments (mean and s.e.m. of ten samples). (e) ELISA of IFN- γ and IL-17 in spleen cells obtained from the LPS-injected mice in c and cultured for 48 h in the presence of antibody to CD3. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are from two independent experiments (mean and s.e.m. of four to five spleen cultures).

serum concentrations of IL-12p40, IL-23 (Fig. 7c) and TNF, as well as IL-6 (Supplementary Fig. 7) but had more IL-10 (Fig. 7c). Mice injected with PBS secreted no cytokines. The number of macrophages recruited into the peritoneal cavity of LPS-challenged mice was similar for wild-type and *Irf5*^{-/-} mice (data not shown), but the expression of genes encoding M1 macrophage markers (*Il12a*, *Il12b*, *Il23a*, *Il1b*, *Tnf* and *Il6*) was significantly impaired in these cells (Fig. 7d). The expression of genes encoding M2 markers in *Irf5*^{-/-} mice (*Il10*, *Arg1*, *Retnla* (Fizz1) and *Chi3l3* (Ym1)) was either significantly higher or showed a positive trend (Fig. 7d and data not shown). In addition, in splenocytes from LPS-challenged *Irf5*^{-/-} mice cultured *ex vivo* for an additional 48 h, we observed significantly less production of IFN- γ and IL-17 (Fig. 7e). In summary, our data here, together with the reported role of IRF5 in LPS-induced lethal endotoxic shock¹², support of the idea that IRF5 is important in establishing a proinflammatory macrophage phenotype in animal models of M1-polarizing inflammation.

DISCUSSION

Macrophages are key mediators of the immune response during inflammation. Plasticity and functional polarization are hallmarks of macrophages that result in the phenotypic diversity of macrophage populations²⁹. Given that deficiency in IRF5 in mice leads to less production of IL-12p40 and IL-23p19 (refs. 11,12), which are universal markers of M1 macrophage subsets, we investigated whether IRF5 is involved in macrophage polarization. We found that IRF5 was indeed a major factor in defining macrophage polarization: it had high expression in M1 macrophages and induced a characteristic gene-expression and cytokine-secretion profile and promoted robust T_H1-T_H17 responses. We also identified a previously unknown regulatory role for IRF5 as an inhibitor of M2 macrophage marker expression. Finally, IRF5 contributed to macrophage plasticity; that is, modulation of its expression led to the conversion of one macrophage subset phenotype into the other.

The rapid and potent transcriptional response developed by macrophages encountering microbial stimuli (such as LPS) or, subsequently, cytokines is orchestrated by many transcription factors. Among these are class III transcription factors, such as PU.1, C/EBP β , Runx1 and IRF8, which are lineage-specific transcriptional regulators turned on during macrophage differentiation³⁰. The combinatorial expression of these proteins specifies macrophage phenotype through the constitutive activation or repression of genes and chromatin remodeling at inducible loci. For example, PU.1 is required for maintaining monomethylated histone 3 Lys4 enhancer marks at macrophage-specific enhancers³¹. However, only a small proportion of the macrophage transcriptome is altered by cell polarization²⁷, and among the genes with differences in expression in the M1 and M2 subsets are those regulated by IRF5, including *Il12A*, *Il12B*, *Il23A*, *Il1B*, *Tnf*, *CCL3* (encoding MIP-1 α), *RANTES*, *CD1A*, *CD40*, *CD86* and *CCR7*. Another member of the IRF family, IRF4, known to inhibit IRF5 activation by competing for interaction with the adaptor Myd88 (ref. 32), is reported to control the expression of prototypical mouse M2 macrophage markers¹⁰. We found that in human cells, IRF4 expression was induced equally well by differentiation with M-CSF or GM-CSF and was further enhanced by exposure to IL-4 (ref. 33). IRF5 expression, in contrast, was induced specifically by GM-CSF or IFN- γ but was unresponsive to IL-4. Thus, IRF5 and IRF4 may be classified as class III transcription factors with the caveat that they define specific macrophage subsets rather than the global macrophage lineage. NF- κ B proteins, in particular c-Rel and RelA, are important for the expression of M1-specific

cytokines^{34,35}. IRF5 and RelA act together in inducing the gene encoding TNF²². We speculate that the genes encoding IL-12, IL-23 subunits and other M1-specific markers might be under similar joint transcriptional control. Thus, IRF5 may participate in combinatorial assembly with macrophage-specific transcription factors such as PU.1 and environmentally induced NF- κ B³¹ to define the activity of specific M1 enhancers.

The role of IRF5 in inhibiting the transcription of the gene encoding IL-10 that we have identified here is important given its well-documented immunosuppressive activity. *Il10*^{-/-} mice develop spontaneous autoimmune diseases and show greater resistance to infection³⁶. IL-10 represses immune responses by downregulating inflammatory cytokines such as TNF³⁷ and is important for the generation of regulatory T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self antigens³⁸. The main producers of IL-10 include M2 macrophages, B cells and T cells³⁹, whereas M1 macrophages and DCs are only weak producers²¹. Ectopic expression of IRF5 in M2 macrophages results in less IL-10 secretion after stimulation with LPS and also affects the expression of mRNA for IL-10 and many other markers of the human M2 macrophage phenotype, such as MRC1, IGF-1, CCL2, CCL13, CD163, M-CSFR and MSR1. Consistent with published studies⁴⁰, we found no expression of the most widely used prototypical mouse M2 markers (Arg1, Ym1 and Fizz 1) in human macrophages (data not shown), whereas their expression in LPS-elicited mouse peritoneal macrophages showed a positive trend in the absence of IRF5. Expression of some chemokines defined as M1 markers (CXCL10) or M2 markers (CCL17, CCL18 and CCL22) in mouse macrophages did not follow the expected pattern of IRF5 dependence (that is, induction for M1 and inhibition for M2), which possibly reflects species-specific gene repertoires⁴¹. Although human M1 macrophages but not M2 macrophages have been shown to secrete large amounts of CCL22 (ref. 21), there is some controversy in the literature about whether CXCL10 is a marker of the M1 or M2 macrophage phenotype^{21,24,27}; our data would classify CXCL10 as an M2 macrophage marker.

The swift modulation of IRF5 expression and cytokine production by colony-stimulating factors can help to explain the considerable plasticity of macrophages in adjusting their phenotype in response to environmental signals². M-CSF is constitutively produced by several cell types, including fibroblasts, endothelial cells, stromal cells and osteoblasts. This steady-state production of M-CSF probably polarizes macrophages toward the M2 phenotype by keeping IRF5 expression low. In contrast, GM-CSF production by the same cell types requires stimulation and usually occurs at a site of inflammation or infection, which is also characterized by large amounts of IFN- γ . The resolution of inflammation may once again coincide with the predominance of M-CSF and a switch in IRF5-driven cytokine production, as treatment of M2-to-M1 macrophages with M-CSF restored the original M2 phenotype (M2-to-M1-to-M2). The activation of receptors for both GM-CSF and IFN- γ stimulates the Jak kinase-STAT transcription factor pathway²⁹, and an ISRE in the *Irf5* promoter can bind STAT1 and STAT2 (ref. 13), which suggests a possible mechanism for IRF5 expression induced by GM-CSF and IFN- γ . Consequently, high expression of IRF5 results in polarization of the macrophage phenotype toward M1. IRF5 induces expression of IFN- γ mRNA, which indicates an autocrine loop in macrophage polarization.

We found that IRF5-expressing macrophages promoted the proliferation and activation of T lymphocytes and drove them toward T_H1 or T_H17 phenotype through IL-12 secretion⁴² or secretion of IL-23 and/or IL-1 β ⁴³, respectively. T_H1 cells constitutively express

IFN- γ and T-bet, whereas T_H17 cells express ROR γ t, IL-23R, IL-17A–IL-17F, IL-21, IL-22 and IL-26. All these T_H1 and T_H17 markers are upregulated in the presence of IRF5-expressing macrophages. Human T_H17 cells and mouse T_H17 cells seem to have different characteristics: whereas mouse T_H17 cells originate from a precursor common to regulatory T cells when IL-6 is produced in combination with TGF- β , human T_H17 cells originate from CD161⁺CD4⁺ precursors in the presence of IL-23 and IL-1 β , with little involvement of IL-6 and an indirect role for TGF- β ⁴³. Perhaps not unexpectedly, the dependence of IL-6 expression on IRF5 is much greater in mouse macrophages.

Both the T_H1 subset and the T_H17 subset promote cellular immune function and can cause inflammation and autoimmune diseases, such as inflammatory bowel disease and collagen-induced arthritis^{44,45}. Notably, more IRF5 mRNA has been found in splenic cells from certain autoimmune-prone mouse strains than in non-autoimmune mice⁴⁶, whereas IRF5-deficient mice show impaired production of T_H1 or T_H17 cytokines. This indicates a possible broad effect of therapies targeting the induction of IRF5 expression by macrophages, such as by targeting IRF5-inducing stimuli. Related to this, GM-CSF-deficient mice fail to develop arthritis despite having a normal humoral immune response to the arthritogenic stimulus⁴⁷, and blockade of GM-CSF in wild-type mice controls disease activity and the concentration of proinflammatory mediators in the joints⁴⁸. In summary, a distinct systemic role for IRF5 in macrophages is the orchestration of transcriptional activation of proinflammatory cytokines, chemokines and costimulatory molecules that leads to efficient effector T cell responses, rather than induction of a type I interferon-induced transcriptional network⁴⁹. Our data have established a new paradigm for macrophage polarization and have designated the IRF5–IRF4 regulatory axis as a new target for therapeutic intervention in which inhibition of IRF5 activity would specifically affect the expression of proinflammatory cytokines and would result in fewer effector T cells.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank M. Cavanach for assistance with cell-characterization experiments; D. Barban for microarray hybridizations; F.G. Goh, D.G. Saliba and S. Thomson for advice and suggestions on RNA-mediated interference and chromatin immunoprecipitation; X. Ma (Cornell University) for luciferase constructs driven by the IL12A promoter; and C. Monaco and M.E. Goddard for support with animal experiments. Supported by the Medical Research Council (82189 to I.A.U.), the European Community Seventh Framework Programme FP7/2007–2013 (222008) and Arthritis Research UK.

AUTHOR CONTRIBUTIONS

T.K., T.S., K.B. and S.A. did research; T.K., H.L., N.S. and I.A.U. designed research and analyzed data; and T.K., M.F., T.H. and I.A.U. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at <http://www.nature.com/natureimmunology/>.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>.

- Gordon, S. & Taylor, P.R. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5**, 953–964 (2005).
- Mosser, D.M. & Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**, 958–969 (2008).

- Gordon, S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**, 23–35 (2003).
- Romagnani, P., Annunziato, F., Piccinni, M.P., Maggi, E. & Romagnani, S. T_H1/T_H2 cells, their associated molecules and role in pathophysiology. *Eur. Cytokine Netw.* **11**, 510–511 (2000).
- Martinez, F.O., Sica, A., Mantovani, A. & Locati, M. Macrophage activation and polarization. *Front. Biosci.* **13**, 453–461 (2008).
- Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K. IL-17 and T_H17 Cells. *Annu. Rev. Immunol.* **27**, 485–517 (2009).
- Tamura, T. et al. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J. Immunol.* **174**, 2573–2581 (2005).
- Porta, C. et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κ B. *Proc. Natl. Acad. Sci. USA* **106**, 14978–14983 (2009).
- Ruffell, D. et al. A CREB/C/EBP β cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc. Natl. Acad. Sci. USA* **106**, 17475–17480 (2009).
- Satoh, T. et al. The Jmjd3–Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat. Immunol.* **11**, 936–944 (2010).
- Ouyang, X. et al. Cooperation between MyD88 and TRIF pathways in TLR synergy via IRF5 activation. *Biochem. Biophys. Res. Commun.* **354**, 1045–1051 (2007).
- Takaoka, A. et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**, 243–249 (2005).
- Mancil, M.E. et al. Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms. Multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. *J. Biol. Chem.* **280**, 21078–21090 (2005).
- Dideberg, V. et al. An insertion-deletion polymorphism in the interferon regulatory factor 5 (IRF5) gene confers risk of inflammatory bowel diseases. *Hum. Mol. Genet.* **16**, 3008–3016 (2007).
- Dieguez-Gonzalez, R. et al. Association of interferon regulatory factor 5 haplotypes, similar to that found in systemic lupus erythematosus, in a large subgroup of patients with rheumatoid arthritis. *Arthritis Rheum.* **58**, 1264–1274 (2008).
- Graham, R.R. et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat. Genet.* **38**, 550–555 (2006).
- Kristjansdottir, G. et al. Interferon regulatory factor 5 (IRF5) gene variants are associated with multiple sclerosis in three distinct populations. *J. Med. Genet.* **45**, 362–369 (2008).
- Miceli-Richard, C. et al. Association of an IRF5 gene functional polymorphism with Sjogren's syndrome. *Arthritis Rheum.* **56**, 3989–3994 (2007).
- Fleetwood, A.J., Lawrence, T., Hamilton, J.A. & Cook, A.D. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J. Immunol.* **178**, 5245–5252 (2007).
- Hoeve, M.A. et al. Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. *Eur. J. Immunol.* **36**, 661–670 (2006).
- Verreck, F.A., de Boer, T., Langenberg, D.M., van der Zanden, L. & Ottenhoff, T.H. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN- γ and CD40L-mediated costimulation. *J. Leukoc. Biol.* **79**, 285–293 (2006).
- Krausgruber, T. et al. IRF5 is required for late-phase TNF secretion by human dendritic cells. *Blood* **115**, 4421–4430 (2010).
- Hammer, M. et al. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *J. Exp. Med.* **203**, 15–20 (2006).
- Fleetwood, A.J., Dinh, H., Cook, A.D., Hertzog, P.J. & Hamilton, J.A. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J. Leukoc. Biol.* **86**, 411–421 (2009).
- Ahern, P.P. et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* **33**, 279–288 (2010).
- Nistala, K. et al. T_H17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc. Natl. Acad. Sci. USA* **107**, 14751–14756 (2010).
- Martinez, F.O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol.* **177**, 7303–7311 (2006).
- Ziegler-Heitbrock, L. et al. IFN- α induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3. *J. Immunol.* **171**, 285–290 (2003).
- Hamilton, J.A. Colony-stimulating factors in inflammation and autoimmunity. *Nat. Rev. Immunol.* **8**, 533–544 (2008).
- Medzhitov, R. & Horng, T. Transcriptional control of the inflammatory response. *Nat. Rev. Immunol.* **9**, 692–703 (2009).
- Ghisletti, S. et al. Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity* **32**, 317–328 (2010).
- Negishi, H. et al. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc. Natl. Acad. Sci. USA* **102**, 15989–15994 (2005).

33. El Chartouni, C., Schwarzfischer, L. & Rehli, M. Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming. *Immunobiology* **215**, 821–825 (2010).
34. Sanjabi, S., Hoffmann, A., Liou, H.C., Baltimore, D. & Smale, S.T. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc. Natl. Acad. Sci. USA* **97**, 12705–12710 (2000).
35. Mise-Omata, S. *et al.* A proximal κ B site in the IL-23 p19 promoter is responsible for RelA- and c-Rel-dependent transcription. *J. Immunol.* **179**, 6596–6603 (2007).
36. Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* **10**, 170–181 (2010).
37. Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M. & O'Garra, A. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147**, 3815–3822 (1991).
38. Wing, K. & Sakaguchi, S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat. Immunol.* **11**, 7–13 (2010).
39. Mosser, D.M. & Zhang, X. Interleukin-10: new perspectives on an old cytokine. *Immunol. Rev.* **226**, 205–218 (2008).
40. Schneemann, M. & Schoeden, G. Macrophage biology and immunology: man is not a mouse. *J. Leukoc. Biol.* **81**, 579 (2007).
41. Ponting, C.P. The functional repertoires of metazoan genomes. *Nat. Rev. Genet.* **9**, 689–698 (2008).
42. Oppmann, B. *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**, 715–725 (2000).
43. Romagnani, S., Maggi, E., Liotta, F., Cosmi, L. & Annunziato, F. Properties and origin of human T_H17 cells. *Mol. Immunol.* **47**, 3–7 (2009).
44. Murphy, C.A. *et al.* Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* **198**, 1951–1957 (2003).
45. Yen, D. *et al.* IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* **116**, 1310–1316 (2006).
46. Shen, H. *et al.* Gender-dependent expression of murine Irf5 gene: implications for sex bias in autoimmunity. *J. Mol. Cell Biol.* **2**, 284–290 (2010).
47. Campbell, I.K. *et al.* Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. *J. Immunol.* **161**, 3639–3644 (1998).
48. Cook, A.D., Braine, E.L., Campbell, I.K., Rich, M.J. & Hamilton, J.A. Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage colony-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease. *Arthritis Res.* **3**, 293–298 (2001).
49. Lacaze, P. *et al.* Combined genome-wide expression profiling and targeted RNA interference in primary mouse macrophages reveals perturbation of transcriptional networks associated with interferon signalling. *BMC Genomics* **10**, 372 (2009).

ONLINE METHODS

Plasmids. Expression constructs encoding full-length human IRF3, IRF5 variant 3/4 and the IRF5 mutant lacking the DNA-binding domain have been described²². Vectors encoding IRF5 and IRF3 expressed similar amounts of protein but only IRF5 resulted in much more TNF secretion, whereas only IRF3 induced type III interferons²². The *IL10* promoter-driven luciferase reporter constructs have been described²⁸. The constructs with wild-type and mutated ISREs in the *IL12A* promoter⁵⁰ were a gift from X. Ma. Sequences and restriction maps are available on request.

Mice. The generation of *Irf5*^{-/-} mice has been described¹². For the generation of BMDMs differentiated with GM-CSF, bone marrow of wild type or *Irf5*^{-/-} was cultured in RPMI-1640 medium (PAA Laboratories) supplemented with recombinant mouse GM-CSF (20 ng/ml; Peprotech) or recombinant mouse M-CSF (100 ng/ml; Peprotech). After 8 d, adherent cells were washed with PBS and replated, then stimulated with LPS (100 ng/ml; Alexis Biochemicals). For *in vivo* experiments, *Irf5*^{-/-} mice and their wild-type littermates were injected intraperitoneally with 20 µg LPS in 200 µl sterile PBS. Mice were killed after 3 h and serum was collected. Spleens were removed and cultured for 48 h in DMEM supplemented with antibody to CD3 (anti-CD3; 10 ng/ml; 145-2C11; BD Bioscience). The experimental protocol was approved by the UK Home Office.

Cell culture. Enriched populations of human monocytes were obtained from the blood of healthy donors by elutriation as described²². M1 and M2 macrophages were obtained after 5 d of culture of human monocytes in RPMI-1640 medium (PAA Laboratories) supplemented with GM-CSF (50 ng/ml) or M-CSF (100 ng/ml; Peprotech). Cells were stimulated with LPS alone (10 ng/ml; Alexis Biochemicals) or with LPS (10 ng/ml) plus IFN-γ (50 µg/ml; Peprotech). For 'priming' experiments, M1 macrophages at day 5 were stimulated for 24 h with M-CSF (100 ng/ml). Similarly, M2 macrophages at day 5 were stimulated for 24 h with GM-CSF (50 ng/ml), with IFN-γ alone (50 ng/ml) or with LPS (10 ng/ml) plus IFN-γ (50 ng/ml).

Measurement of cytokine production. Cytokine secretion was quantified by ELISA specific for human IL-12p40, IL-12p70, IL-10, IFN-γ, TNF, CXCL10 or IL-1β (BD Bioscience); IL-23 or CCL2 (eBioscience); CCL5, CCL13 or CCL22 (R&D Systems); or IL-17A or IL-4 (Insight Biotechnology). Mouse cytokine secretion was quantified with ELISA specific for IL-12p70, IL-23 or IL-10 (eBioscience) or for IFN-γ or IL-17A (BD Bioscience), and serum concentrations of mouse IL-1β, TNF, IL-6 and IL-10 were determined by cytometric bead assay on a FACSCanto II (BD Bioscience).

Mixed-lymphocyte reaction. Human macrophages were plated in 96-well plates at a density of 2×10^4 cells per well. T lymphocytes were isolated from the blood of healthy donors by elutriation, analyzed by flow cytometry and used when their purity was >90%. T lymphocytes were added to macrophages at a density of 5×10^5 cells per well. Control cultures contained medium only or T lymphocytes or M2 macrophages alone. After 72–96 h of coculture, supernatants were collected for analysis of cytokines. For proliferation experiments, cells were pulsed with 37,000 Bq of [³H]thymidine (Amersham Biosciences) 16 h before being collected, and DNA synthesis was measured by [³H]thymidine incorporation with a β-scintillation counter (Beckman Instruments).

RNA-mediated interference. The ON-TARGETplus SMARTpool reagent (Dharmacon) designed to target human IRF5 was used for siRNA-mediated knockdown. DharmaFECT 1 was used as the siRNA transfection reagent according to the manufacturer's instructions (Dharmacon).

Adenoviral infection. M2 macrophages were infected as described²².

RNA extraction and quantitative real-time RT-PCR. Total RNA was extracted from cells with an RNeasy Mini kit according to the manufacturer's instructions (Qiagen), then cDNA was synthesized from total RNA with

SuperScript III reverse transcriptase (Invitrogen) and 18-residue oligo(dT) (Eurofins MWG Operon). Gene expression was measured by the change-in-threshold ($\Delta\Delta C_T$) method based on quantitative real-time PCR in an ABI 7900HT (Applied Biosystems) with TaqMan primer sets for human IL-12p35, IL-12p40, IL-23p19, IL-10, IFN-γ, IL-17A-IL-17F, IL-21, IL-22, IL-26, IL-23R, TBX21 (for T-bet), Mrc1, Arg1, Rentr1 (for Fizz1) and PO (Applied Biosystems). RORγt was detected by SybrGreen with a primer set for human *RORC2* (*RORC2_F1*, TGAGAAGGACAGGGAGCCAA; and *RORC2_R1*: CCACAGATTTTGAAGGGATCA).

Luciferase reporter assay. M2 macrophages were infected in 96-well plates in triplicate at a multiplicity of infection of 50:1. Cells were seeded in serum-free, antibiotic-free RPMI medium containing the desired number of viral particles in a final volume of 50 µl. Cells were infected for 6 h with expression construct encoding IRF5 or the IRF5 mutant lacking the DNA-binding domain or empty vector, followed by infection with the *IL10* luciferase constructs. Cells were allowed to recover for 24 h before experimental assay. Human embryonic kidney (HEK293) cells expressing Toll-like receptor 4 and its coreceptor MD2, plus the LPS receptor CD14, were cotransfected with the constructs with wild-type and mutated ISREs of the promoter of the gene encoding IL-12p35 as described²².

Total protein extracts and immunoblot analysis. Total protein extracts were prepared as described²². Equal amounts of protein were resolved by SDS-PAGE and analyzed with anti-IRF5 (ab2932 or ab21689; Abcam); anti-IRF3 (sc-9082x), anti-IRF4 (sc-28696), anti-p50 (sc-114x) or anti-RelA (sc-372x; all from Santa Cruz); or anti-actin (A5541; Sigma).

Flow cytometry. For staining of T cell surfaces, cells were stained for 30 min at 4 °C with fluorescein isothiocyanate-conjugated anti-CD4 (RPA-T4; BD Bioscience) and peridinin chlorophyll protein-indodicarbocyanine-conjugated anti-CD8 (RPA-T8; BD Bioscience). For intracellular cytokine staining, cells were stimulated for 3–4 h with phorbol myristate acetate, ionomycin and brefeldin A (Sigma-Aldrich). Cells were stained for cell surface markers, were fixed in Cytofix (BD Bioscience) and then were made permeable with PBS containing 1% (vol/vol) FCS, 0.01% (vol/vol) sodium azide, and 0.05% (vol/vol) saponin and were stained with Pacific blue-conjugated anti-IFN-γ (4S.B3; eBioscience) and phycoerythrin-conjugated anti-IL-17 (eBIO-64CAP17; eBioscience). For surface staining, macrophages were incubated for 30 min at 4 °C with allophycocyanin-conjugated anti-CD40 (5C3; eBioscience) and phycoerythrin-conjugated anti-CD163 (215927; R&D Systems). Samples were analyzed on a FACSCanto II (BD Bioscience) and data were analyzed with FlowJo software (TreeStar).

Chromatin immunoprecipitation. Chromatin-immunoprecipitation assays were done essentially as described²² with anti-IRF5 (ab2932; Abcam), antibody to RNA polymerase II (sc-899; Santa Cruz) or immunoglobulin G control (PP64; Milipore). The immunoprecipitated DNA fragments were then analyzed by real-time PCR with SYBR Premix Ex Taq II master mix (Takara Bio) and the following primers: locus encoding IL-12p35, 5'-TCATTTTGGGCGAGCTGGAG-3' and 5'-TACATCAGCTTCTCGGTGACACG-3'; locus encoding IL-12p40, 5'-TCCAGTACCAGCAACAGCAGCAGA-3' and 5'-GTAGGGGCTTGGG AAGTGCTTACCTT-3'; locus encoding IL-23p19, 5'-ACTGTGAGGCCTG AAATGGGGAGC-3' and 5'-ACTGGATGGTCTCGGTTTCATGGGAGA-3'; and locus encoding IL-10, 5'-CCTGTGCCGGGAAACCTTGATTGTGGC-3' and 5'-GTCAGGAGGACCAGGCAACAGAGCAGT-3'. Data were analyzed with ABI 7900HT software (Applied Biosystems).

Microarray analysis, statistics and bioinformatics. Microarrays, statistics and bioinformatic analyses are described in the **Supplementary Methods**.

50. Liu, J., Cao, S., Herman, L.M. & Ma, X. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-γ primed IL-12 production by IFN regulatory factor 1. *J. Exp. Med.* **198**, 1265–1276 (2003).

IRF5 is required for late-phase TNF secretion by human dendritic cells

*Thomas Krausgruber,¹ *David Saliba,¹ Grigory Ryzhakov,¹ Alessandra Lanfrancotti,¹ Katrina Blazek,¹ and Irina A. Udalova¹

¹Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, London, United Kingdom

Spatially and temporally controlled expression of inflammatory mediators is critical for an appropriate immune response. In this study, we define the role for interferon regulatory factor 5 (IRF5) in secretion of tumor necrosis factor (TNF) by human dendritic cells (DCs). We demonstrate that DCs but not macrophages have high levels of IRF5 protein, and that IRF5 is responsible for the late-phase

expression of TNF, which is absent in macrophages. Sustained TNF secretion is essential for robust T-cell activation by DCs. Systematic bioinformatic and biochemical analyses of the *TNF* gene locus map 2 sites of IRF5 recruitment: 5' upstream and 3' downstream of the *TNF* gene. Remarkably, while IRF5 can directly bind to DNA in the upstream region, its recruitment to the downstream region

depends on the protein-protein interactions with NF- κ B RelA. This study provides new insights into diverse molecular mechanisms employed by IRF5 to regulate gene expression and implicates RelA-IRF5 interactions as a putative target for cell-specific modulation of TNF expression. (*Blood*. 2010;115(22):4421-4430)

Introduction

Tumor necrosis factor (TNF) is one of the major cytokines responsible for effector immune functions. As well as playing a central role in host defense against infection, TNF is a major factor in the pathogenesis of chronic inflammatory disease such as rheumatoid arthritis (RA). Consequently, tightly controlled regulation of its expression is critical for an appropriate immune response. This occurs at the transcriptional and posttranscriptional levels, with transcriptional regulation showing specificity for both stimulus and cell type.¹ The NF- κ B family of transcription factors (TFs) plays a major role in transcriptional up-regulation of the *TNF* gene by lipopolysaccharide (LPS) in both mouse and human myeloid cells.²⁻⁵

Regulation of transcription for many immune genes in response to Toll-like receptor (TLR) signaling involves a combination of NF- κ B and interferon regulatory factor (IRF) factors.⁶ IRFs appear to provide a mechanism for conferring signal specificity to a variety of target gene subsets, with IRF3 being essential for type I interferon (IFN) response,⁷ and IRF5 playing a key role in induction of proinflammatory cytokines, including TNF, IL-6, and IL-12.⁸ Consequently, *IRF5*^{-/-} mice show resistance to lethal shock induced by CpG-B or LPS.⁸

Unlike other IRF family members, IRF5 contains 2 nuclear localization signals (NLSs), 1 in the N-terminus and the other in the C-terminus of the protein. This results in low levels of nuclear translocation and therefore weak *trans*-activation activity of IRF5, even in unstimulated cells.⁹ The molecular pathways leading to IRF5 activation are not well understood, but it was shown that TLR signaling induces the formation of MyD88-IRF5-TRAF6 complexes,⁸ and is probably followed by phosphorylation of specific sites within the IRF5 C-terminal autoinhibitory domain.¹⁰

Human *IRF5* is expressed as multiple spliced variants with distinct cell type-specific expression, cellular localization, differential regulation, and dissimilar functions.¹¹ Moreover, genetic poly-

morphisms in the *IRF5* gene leading to expression of several unique isoforms have been implicated in autoimmune diseases, including systemic lupus erythematosus (SLE), RA and Sjogren syndrome.¹²⁻¹⁵ *IRF5* mRNA expression has been detected in B cells, dendritic cells (DCs), monocytes, and natural killer (NK) cells but not in T cells,¹¹ yet little is known about the *IRF5* protein expression in these cells.

Here, we demonstrate that human monocytes acquire high levels of IRF5 protein during differentiation into monocyte-derived DCs (MDDCs) but not monocyte-derived macrophages (MDMs). This leads to a sustained secretion of TNF by MDDCs compared with MDMs and efficient activation of T cells. IRF5 is recruited to both upstream and downstream regions of the gene after LPS induction, and its cooperative action with NF- κ B RelA is important for maintaining the *TNF* gene transcription. Remarkably, IRF5 displays 2 independent modes of transcriptional activity: direct binding to DNA and indirect recruitment via the formation of a protein complex with RelA. Our results provide novel insights into the molecular basis for cell specificity in TNF production by human immune cells and highlight RelA-IRF5 interactions as a novel target for cell-specific modulation of TNF expression.

Methods

Plasmids

Expression constructs encoding full-length human IRF3, IRF5v3/v4, and NF- κ B subunits tagged with HA-tag in modified pENTR vector (pBent) were described in Thomson et al.¹⁶ IRF5 Δ DBD, IRF5A68P mutants were generated. The constructs were recombined into pAD/PL DEST vector (Invitrogen) for adenovirus production and subsequent delivery into human myeloid cells. The IRF5-HA fragment was subsequently transferred into the modified pBent vector containing 1-strep-tag. The 5'wt/3'wt and 5'wt/

Submitted January 6, 2010; accepted March 1, 2010. Prepublished online as *Blood* First Edition paper, March 17, 2010; DOI 10.1182/blood-2010-01-263020.

*T.K. and D.S. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2010 by The American Society of Hematology

3' mut TNF luciferase-reporter constructs provided by Mr T. Smallie (Kennedy Institute) were used to generate 5' mut/3' wt and 5' mut/3' mut constructs with mutated sites κ B2/2 ξ /2a.⁵ IRF5 DBD (amino acid 1-131) were polymerase chain reaction (PCR) amplified and cloned into bacterial expression vector pET21d (Novagen). All constructs were verified by sequencing. The sequences and restriction maps are available upon request.

Cell culture

All reagents used for cell culture were tested for endotoxin and only used if the endotoxin levels were less than 20 pg/mL (Lonza). All cell cultures were maintained at 37°C in 5% CO₂ and 95% humidity in the appropriate media supplemented with 10% fetal calf serum (Gibco) and 1% penicillin/streptomycin (PAA). HEK-293–TLR4-CD14/Md2 cells (Invivogen) were cultured in Dulbecco modified Eagle medium (DMEM; PAA) supplemented with 10 mg/mL blasticidin and 50 mg/mL HygroGold (Invivogen) per the manufacturer's instruction. Enriched populations of human monocytes were obtained from the blood of healthy donors by elutriation as described previously.⁴ MDMs and MDDCs were obtained after 5 to 7 days of culturing human monocytes in RPMI 1640 (PAA) supplemented with 100 ng/mL macrophage colony-stimulating factor (M-CSF) or 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL IL-4 (Peprotech). MDMs, MDDCs, and cell lines were stimulated with 100 ng/mL LPS (Alexis Biochemicals) unless indicated otherwise.

ELISA

Cytokine secretion was quantified with specific enzyme-linked immunosorbent assays (ELISAs) for human TNF (BD Bioscience), human IFN- γ (BD Bioscience), and human IFN- λ 1/IL-29 (R&D Systems) according to manufacturer instructions. Absorbance was read at 450 nm by a spectrophotometric ELISA plate reader (LabSystems Multiscan Biochromic) and analyzed using Ascent LabSystems software. All samples were analyzed in triplicate in a volume of 50 μ L.

Mixed lymphocyte reaction

Human MDDCs were plated in 96-well, flat-bottom tissue plates at 2×10^4 cells per well. T lymphocytes were isolated from the blood of healthy donors by elutriation, analyzed by FACS, and used if purity was more than 90%. T lymphocytes were added to MDDCs at 5×10^5 so that the final MDDC/T cell ratio was 1:25. Control cultures contained medium or T lymphocytes, or MDDCs alone. A total of 10 μ g/mL anti-TNFR1 antibody (MAB 625; R&D Systems) or IgG control antibody (MAB 002; R&D Systems) was added to the cocultures after 6 hours or 24 hours. Cultures were established in duplicate and incubated at 37°C in 5% CO₂ for a total of 72 hours. After culture, supernatants were collected and stored at –20°C for detection of cytokines.

RNA interference

siRNA-mediated knockdown was performed using On-target plus SMART pool reagents (Dharmacon) designed to target human IRF5 and NF- κ B RelA. Lipofectamine RNAiMAX (Invitrogen) and DharmaFECT I (Dharmacon) were used as the siRNA transfection reagents for HEK-293–TLR4-Md2/CD14 cells and MDDCs, respectively, according to the manufacturers' instructions. Multiple siRNAs were used to validate the knockdown specificity and exclude off-target effects.

Adenoviral infection

Infections of MDMs were performed in 96-well plates in triplicate at a multiplicity of infection (MOI) of 50:1. Cells were seeded in serum-free, antibiotics-free RPMI containing the desired number of viral particles in a final volume of 50 μ L. The plates were incubated overnight at 37°C followed by aspiration of the supernatants and replacement with 100 μ L of standard media per well. Cells were allowed to recover for a further 24 hours before experimental assay.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells using a QiaAmp RNA Blood mini kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen) and 18-mer oligo dTs (Eurofins MWG Operon). The gene expression was analyzed by 2-standard curve or $\Delta\Delta$ Ct methods where appropriate based on the quantitative real-time PCR with TaqMan primer sets for human TNF and PO (Applied Biosystems) in a Corbett Rotor-gene 6000 machine (Corbett Research Ltd).

Luciferase gene reporter assay

HEK-293–TLR4-CD14/Md2 cells were seeded into polylysine-coated 96-well plates at a density of 30 000 cells per well. Next day, cells were transfected with 10 ng of the indicated expression vector, 50 ng of TNF luciferase reporter, and 50 ng of pEAK8-Renilla using the Lipofectamine 2000 protocol (Invitrogen). Total amount of DNA was kept at 120 ng per well. At 48 hours after transfection, the activity of the reporters were measured using the Dual-Glo Luciferase system (Promega) optimized for 96-well plate format according to the manufacturer's protocol. Each experiment was performed in triplicate.

Nuclear and total protein extracts and Western

Cells were grown on 10-cm² dishes and exposed to vehicle and agents; reactions were terminated by washing cells twice with ice-cold phosphate-buffered saline (PBS). Cells were then removed by scraping and transferred to Eppendorf tubes. Nuclear or total protein extracts were prepared as previously described.⁵ Equal amounts of proteins were resolved by Novex Tris-glycine gel (Invitrogen), transferred onto Hybond-N membranes (Amersham Biosciences), and subjected to incubation with antibodies against IRF5 (ab2932; Abcam), followed by detection with horseradish peroxidase (HRP)-conjugated secondary antibodies and the chemiluminescent substrate solution ECL (GE Healthcare).

EMSA

Oligonucleotide probes were radiolabeled with [α -³²P]dCTP (Perkin Elmer): κ B4 (Forward [F]: agctGGGCATGGGAATTTCCAACCTCT; Reverse [R]: agctGAGTTGGAAAATCCCATGCC); κ B4a (F: agctAACTCTGGGAATTC-CAATCCTT; R: agctAAGGATTGGAATTTCCAGAGT T); κ B4b (F: agctCT-TGCTGGGAAAATCCTGCAG; R: agctGCTGCAGGATTTTCCAGCAAG); ISRE1 (F: agctGAAGCCAAGACTGAAACCAGCATT; R: agctTAATGCT-GGTTT CAGCTTTGGCTTC); ISRE2 (F: agctCCGGTCAGAATGAAA-GAAGAAGG; R: agctC CTTCTTCTTTTCAATTCTGACC CGGT); ISRE5 (F: agctGGAGAAGAAACCGAGACAGAAGG TG; R: agctCACCTTCTGTC TCGTTTCTTCTCC). 'ISRE'16 (F: agctTTTGCTTAGAAAAGAAACATG-GTCTC; R: agctGAG ACCATGTTTCTTTTCTAAGCAAA); 'ISRE'17 (F: agctACATAAAACAAAGCCCAACAGAATATTC; R: agctGGAATATTCTGT-TGGGCTTTGTTTATGT); and PRDI-III (IFN- β) (F: agctGGGAACTG-AAAGGGAAAGTGAAAGTGG; R: agctCCACTTTCACCTTTCCCTT-TCAGTTTCCC). Binding reactions with 50 ng of bacterially expressed and purified IRF5 DBD and electrophoretic mobility shift assay (EMSA) gel separation were performed as previously described.⁵

Chromatin immunoprecipitation

A total of 7×10^6 MDDCs or HEK-293–TLR4-CD14/Md2 cells were fixed by adding 1% formaldehyde (final concentration) for 5 minutes at room temperature. Nuclear extracts were subjected to 6×12 -second pulses of sonication using Vibra-Cell VCX130 (Sonics) at 20% amplitude. For immunoprecipitation reaction, nuclear extracts were precleared with protein G–Sepharose bead slurry (GE Healthcare) for 2 hours, and then incubated with 2 μ g of antibodies against IRF5 (ab2932; Abcam), RelA, or Pol II (sc-372 and sc-899; Santa Cruz Biotechnology) overnight at 4°C with rotation. Immunocomplexes were then collected with protein G–Sepharose beads for 30 minutes, rigorously washed, and eluted. For Re–chromatin immunoprecipitation (ChIP), RelA ChIP eluates were subsequently incubated with either IRF5 antibody or no antibody control and processed as

described. Cross-linked protein-DNA complexes were reversed by incubating them at 65°C overnight, and DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN). The immunoprecipitated DNA fragments were then interrogated by real-time PCR using SYBR Premix Ex Taq II master mix (Takara Bio) and the following primers for TNF locus: control region, (TGTGTGTCTGGGAGTGAGAACT and TCTTCTCAGCT-TCTCCTTTGCT); region A, (CCACAGCAATGGGTAGGAGAATGT and GAGGTCTCTGGAGGCTCTTTCACT); region B, (GGAAGCCAAGACT-GAAACCAGCA and CCGGGAATTCACAGACCCCACT); region C, (TCCCTCAACCCCGTTTCT and TAGGACCCTGGAGGCTGAAC); region D, (AACTTTCCAATCCCCGCC and GGTGTGCCAACAAC-TGCCTT); region E, (CAGCAAGGACAGCAGAGGAC and TCCCGGAT-CATGCTTTTCACT); region F, (GGCAGTCAGTAAGTGCTCTCCAA and TACCTACAACATGGGCTACAGG); region G, (ACAGCTTTGATCCCT-GACATCT and CTCCGTGTCTCAAGGAAGTCTG); region H, (ATATTC-CCCATCCCCAGGAAACA and CTGCAACAGCCGGAATCTCACC); region I, (GAGGACCTCACTCAGCCCTT and CGGCAGTTCGGTTCCT-TGTT); region J, (ACTGGTCTTTGTGGTGAAGGAG and GAACTAGT-GGGCTCAAGTGGT); region K, (GCTATGATCATGCCACTGTACCC and TACCACATGGTTTCTCCTGCC); and region L, (GCTGAAAGT-CAGCCATGAAGTA and CACTTAGGGTGTCCCATTTAGG). Data were analyzed using Rotogene 6000 software (Corbett Research Ltd). All primer sets were tested for specificity and equal efficiency before use.

Immunoprecipitation

HEK-293-TLR4-CD14/Md2 cells were transfected with ONE-strep-IRF5-HA construct or corresponding empty vector. At 24 hours after transfection cells were fixed with 1% formaldehyde for 10 minutes at room temperature before high salt lysis and affinity purification on Strep-Tactin MacroPrep sepharose (IBA). The eluates were de-cross-linked by incubating at 65°C overnight before separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Exogenous IRF5 and endogenous RelA were detected by immunoblotting with anti-HA-HRP (12013819001; Roche) and anti-RelA (sc-372; Santa Cruz Biotechnology). Alternatively, cells were transfected with RelA-FLAG or BAP-FLAG control protein. At 24 hours after transfection, cells were lysed and affinity-purified with anti-FLAG M2 Sepharose beads (Sigma). Exogenous RelA and endogenous IRF5 were detected by immunoblotting with anti-FLAG-HRP (A8592; Sigma) and anti-IRF5 (ab2932; Abcam). Interaction of endogenous RelA and IRF5 was detected by overnight incubation of the cell lysates with goat anti-IRF5 antibody (ab2932; Abcam) or no antibody control before precipitation with protein G beads. IRF5 was detected by immunoblotting with mouse anti-IRF5 antibody (sc-56714; Santa Cruz Biotechnology), while RelA was detected by immunoblotting with anti-RelA. Triton X-100-extracted nuclei and DNase I digestion of chromatin was performed as described previously.¹⁷

Bioinformatics and statistical analyses

The nucleotide sequence were inspected with transcription factor binding site searching software JASPAR¹⁸ and Genomatrix¹⁹ for the presence of putative IFN-sensitive response element (ISRE) sites (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Statistical analysis was performed using 1-way analysis of variance (ANOVA) with Dunnett multiple comparison posttest or Student *t* test where appropriate (**P* < .05; ***P* < .01; ****P* < .001).

Results

Sustained TNF secretion by MDDCs is important for efficient T-cell activation

Myeloid cells (eg, macrophages and DCs) are the major producers of the key immune modulator TNF in response to TLR4 stimulation.²⁰ TNF protein is below the limit of detection in the supernatants of resting cells (Figure 1A). After 4 hours of LPS stimulation,

TNF is secreted at similar levels in MDMs and MDDCs (early phase). However, a marked difference in TNF production was observed in MDMs and MDDCs stimulated with LPS for 24 hours (late phase). Although the level of TNF significantly decreased in MDMs, there was an increase in TNF levels in MDDCs (Figure 1A) in each individual blood donor (supplemental Figure 1A).

Human TNF acting through TNF receptor is involved in DC maturation from bone marrow precursors^{21,22} and activation of type I helper (Th1), measured by the release of IFN- γ .²³ Thus, we examined whether the late-phase secretion of TNF by MDDCs is needed for IFN- γ production by T cells. MDDCs were stimulated with LPS for 2 hours and exposed to human T cells extracted and purified from the peripheral blood of major histocompatibility complex (MHC)-unmatched donors in a mixed lymphocyte reaction (MLR). Antibodies against TNF receptor 1 (TNFR1) or isotype IgG control were added to the reaction. T cells incubated with MDDCs treated with anti-IgG antibodies produced high levels of IFN- γ (Figure 1B), whereas the control reactions (MDDCs or T cells cultured on their own) secreted no detectable IFN- γ . Blocking TNF at 6 hours after setting the MLR reaction resulted in strong reduction of IFN- γ , but no effect was observed when anti-TNFR1 antibodies were added to the reaction after 24 hours, suggesting that most T cells are in an activated state after the prolonged exposure to TNF (Figure 1B).

Thus, the observed sustained expression of TNF by MDDCs might be of benefit to both their maturation and antigen-presenting function and is essential for establishing a robust Th1 phenotype.

IRF5 protein is highly expressed in MDDC and controls late-phase TNF secretion

The observed differential LPS-induced secretion of TNF by human DCs and macrophages (Figure 1A) prompted us to examine the molecular mechanisms of this phenomenon. Myeloid cells from IRF5^{-/-} mice show impaired induction of proinflammatory cytokines, including TNF, upon stimulation by different TLR ligands.⁸ We hypothesized that the difference in TNF secretion profile in MDDCs and MDMs might be due to the difference in IRF5 expression in these cells. We examined levels of IRF5 protein after human monocyte differentiation into MDMs and MDDCs. No increase in the levels of IRF5 protein was observed in MDMs, even after 5 days of differentiation (Figure 1C). However, expression of IRF5 protein was detected after 1 day of monocyte differentiation into MDDCs and remained at an elevated level until day 7 (Figure 1C). Significantly, whereas at least 3 different IRF5 isoforms were observed in human monocytes, only some of them accounted for high levels of IRF5 in MDDCs: one is likely to be IRF5v3/v4.¹¹

Next, we looked at the effect of ectopic IRF5 expression in MDMs that have low levels of endogenous IRF5 protein (Figure 1C) on T-cell activation. MDMs were infected with adenoviral expression vector encoding HA-tagged IRF5 or the corresponding empty vector pBent. At 48 hours after infection, no significant effect on the resting cells (measured by endogenous IFN- λ 1 response) was observed. Exposure of T cells to MDMs with elevated levels of IRF5 protein resulted in increase of IFN- γ secretion to the levels comparable with that of T cells exposed to MDDCs (Figure 1D). Thus, we argued that IRF5 might be responsible for sustained secretion of TNF. To test this hypothesis, MDMs were infected with adenoviral expression vector encoding HA-tagged IRF5 or IRF3 (as a control) or pBent. IRF5-HA and IRF3-HA vectors expressed similar levels of proteins (supplemental Figure 1B), but only IRF5 resulted in a significant increase in TNF secretion (Figure 1D; supplemental Figure 1C), whereas only

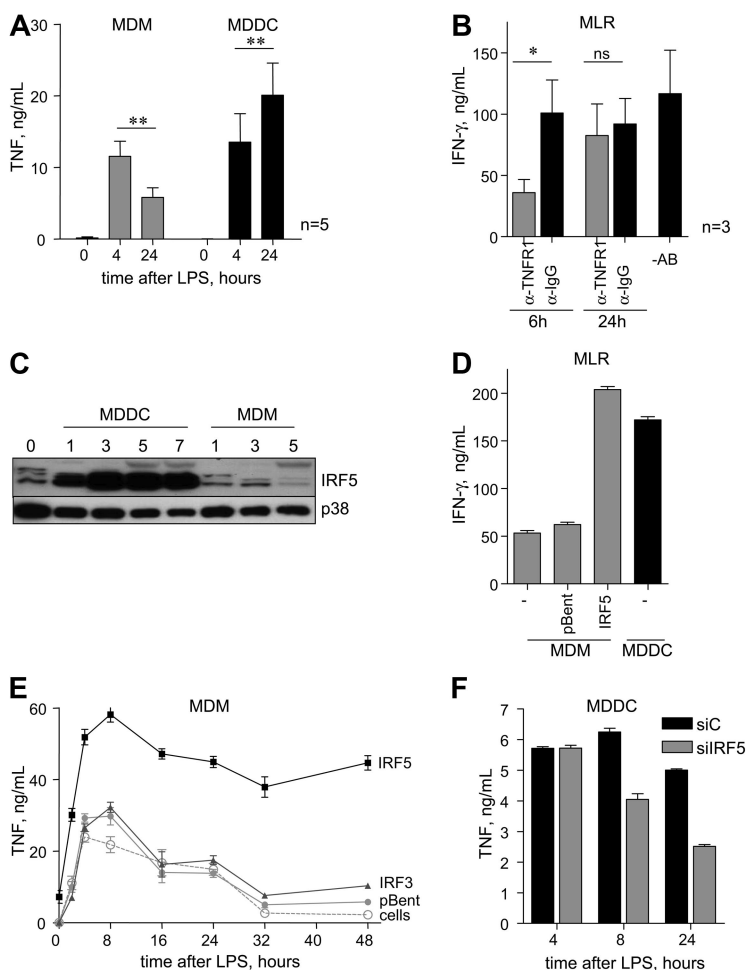


Figure 1. IRF5 protein is highly expressed in MDDCs and control late-phase TNF secretion. (A) Monocyte-derived dendritic cells (MDDCs) and monocyte-derived macrophages (MDMs) were stimulated with lipopolysaccharide (LPS; 10 ng/mL) for 4 hours and 24 hours, and secreted tumor necrosis factor (TNF) was measured by ELISA. Data show means $\pm$ standard error of the mean (SEM) of 5 independent experiments each using monocytes derived from a different donor. $**P < .01$ (Student *t* test). (B) MDDCs were stimulated with LPS for 2 hours and then were cocultured with T lymphocytes. Anti-TNFR1 or anti-IgG control antibodies were added 6 hours or 24 hours after coculture start. IFN- γ secretion was determined by ELISA after 72 hours of coculture. Data show means $\pm$ SEM of 3 independent experiments. $*P < .05$. (C) Cells were collected at day 0 (monocytes); days 1, 3, 5, and 7 (MDDCs) after differentiation with granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) and IL-4 (10 ng/mL); and days 1, 3, and 5 after differentiation with M-CSF (50 ng/mL; MDMs); total protein extracts were then subjected to Western blot analysis. p38 MAPK was used as loading control. Representative blots of 5 independent experiments each using monocytes derived from a different donor. (D) MDMs were left untreated (cells) or infected with adenoviral vectors encoding IRF5 or empty vector (pBent), stimulated with LPS for 2 hours, and cocultured with T lymphocytes. IFN- γ secretion was determined by ELISA after 72 hours of coculture. Data show means $\pm$ SD and are representative of 3 independent experiments each using MDMs derived from a different donor. (E) MDMs were left untreated (cells) or infected with adenoviral vectors encoding IRF5, IRF3, or empty vector (pBent) and stimulated with LPS for the indicated time. The amount of secreted TNF protein was determined by ELISA. Data show means $\pm$ SD and are representative of 3 independent experiments each using MDMs derived from a different donor. (F) MDDCs were transfected with siRNAs targeting IRF5 (siIRF5) and stimulated with LPS (10 ng/mL) for the indicated time. TNF secretion was compared with control cells transfected with nontargeting siRNA (siC). Data shown are the means $\pm$ SD and are representative of 2 independent experiments each using MDDCs derived from a different donor.

IRF3 induced IFN- λ 1 (supplemental Figure 1D), consistent with the previously published data.¹⁶ Strikingly, TNF secretion in MDMs with overexpression of IRF5 remained at a steady sustained level up to 48 hours after LPS stimulation (Figure 1E), similar to that of MDDCs with high levels of endogenous IRF5 (Figure 1C). siRNA-mediated inhibition of IRF5 in MDDCs (supplemental Figure 1E) resulted in reduction of TNF secretion at 8 and 24 hours after LPS stimulation (Figure 1F), supporting the notion that IRF5 may be required for the late-phase TNF expression.

Taken together, these results suggest that sustained TNF secretion by MDDCs leading to robust T-cell activation is likely to be a consequence of a high level of IRF5 protein in these cells.

IRF5 is involved in transcriptional regulation of *TNF*

We next sought to investigate whether IRF5 is involved in transcriptional regulation of the *TNF* gene. In human MDDCs, stimulation with LPS resulted in a rapid up-regulation of *TNF* mRNA expression, which reached the peak between 1 and 2 hours but remained at a steady level until 8 hours after stimulation (Figure 2A). Consistent with the observed differences in protein secretion, *TNF* mRNA expression in MDMs was characterized by more transient kinetics (supplemental Figure 2A), whereas siRNA-mediated inhibition of IRF5 reduced *TNF* mRNA expression (Figure 2A). The observed inhibition was statistically significant when analyzed in multiple blood donors (supplemental Figure 2B). In the same cells, siRNA-mediated inhibition of NF- κ B RelA, a transcription factor previously shown to be important for an

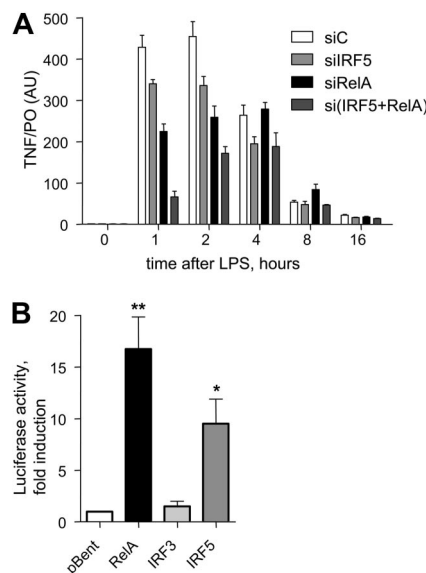
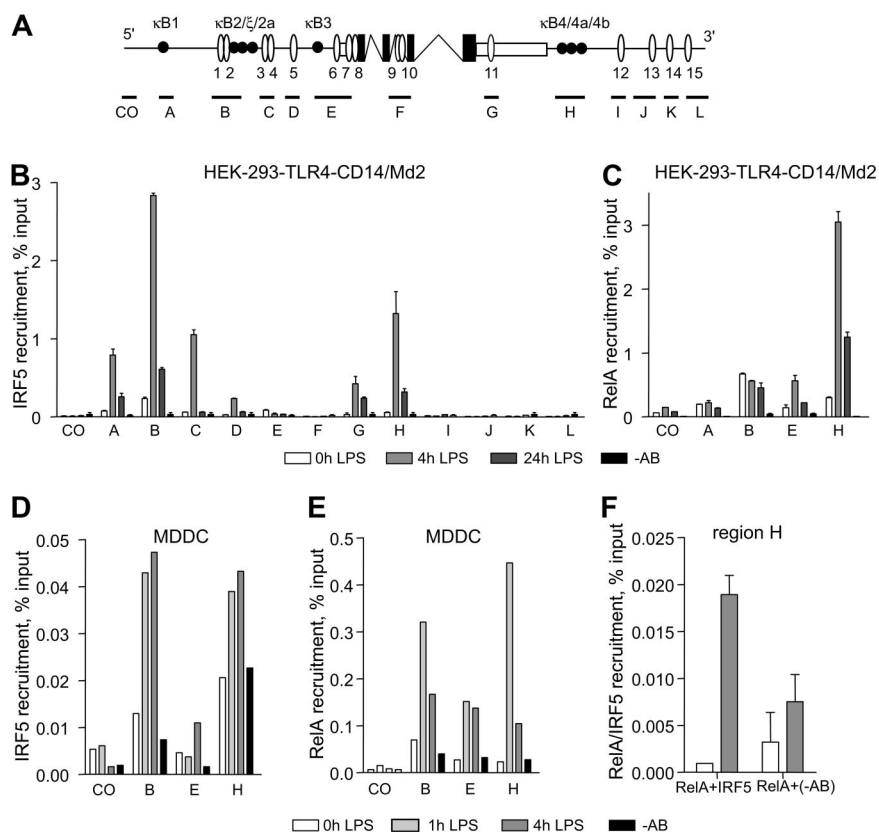


Figure 2. IRF5 is involved in transcriptional regulation of *TNF*. (A) MDDCs were transfected with siRNAs targeting IRF5 (siIRF5), RelA (siRelA), or both [si(IRF5+RelA)] and stimulated with LPS (10 ng/mL) for the indicated time. *TNF* mRNA expression was compared with control cells transfected with nontargeting siRNA (siC). Data shown are the means $\pm$ SD and are representative of 4 independent experiments each using MDDCs derived from a different donor. (B) HEK-293 cells were cotransfected with the *TNF* 5'-wt/3'-wt reporter plasmid and equal amounts of expression plasmids encoding for human IRF5, RelA, IRF3, or empty vector (pBent). At 48 hours after transfection, cells were harvested and luciferase activity was measured as described. Data are presented as a fold over pBent $\pm$ SEM from 4 independent experiments. $*P < .05$; $**P < .01$ (1-way ANOVA).

Figure 3. IRF5 is recruited to the 5' upstream and 3' downstream region of *TNF*. (A) Schematic of the *TNF* locus. Protein coding and noncoding exons are shown in black and white. Putative ISREs are allocated as white ovals; κ B sites are shown as black circles. The approximate amplicon size of primer sets spanning the *TNF* locus (A to L) are indicated by black lines. CO indicates a control primer set containing neither an ISRE nor a κ B site. (B-C) HEK-293-TLR4-Md2/CD14 cells were left unstimulated or stimulated with LPS (1 μ g/mL) for 4 hours and 24 hours and analyzed by ChIP with antibodies specific to IRF5 (B) or RelA (C). (D-E) MDDCs were left unstimulated or stimulated with LPS for 1 hour and 4 hours, followed by ChIP with antibodies specific to IRF5 (D) or RelA (E). (F) Corecruitment of RelA and IRF5 to region H was assessed by re-ChIP analysis with antibodies against RelA followed by IRF5-specific antibodies. (B-F) Data show mean percentage input relative to genomic DNA (gDNA) plus or minus SD of a representative experiment. -AB indicates a no-antibody control.



efficient TNF production by human MDDCs,²⁴ resulted in reduction of TNF mRNA expression at the initial phase of gene induction (1-2 hours after LPS stimulation; Figure 2A). Within this time window, depletion of both IRF5 and RelA had the strongest effect on mRNA expression (Figure 2A), indicating that RelA and IRF5 may cooperate in controlling transcription of the *TNF* gene.

To investigate whether IRF5 can directly modulate transcription of the *TNF* gene, we used a gene-reporter plasmid in which the luciferase gene was flanked by 1171 nt 5' upstream and 1252 nt 3' downstream of the *TNF* gene. This construct encompassed all evolutionary conserved sequences in the region and contained known κ B sites.^{25,26} It was coexpressed with HA-tagged IRF5, IRF3, and NF- κ B subunits in HEK-293 cells, and luciferase activities were compared with empty vector pBent. RelA- and IRF5-transfected cells showed a significant increase in luciferase activity (Figure 2B). Other NF- κ B subunits or IRF3 had little or no effect (Figure 2B; supplemental Figure 2C). Of interest, a deletion of the IRF5 DNA-binding domain (IRF5 Δ DBD) or a point alanine to proline mutation in it (IRF5 A68P) previously shown to act as dominant-negative mutants of IRF5,^{27,28} resulted in a major drop in luciferase activity (supplemental Figure 2D).

We concluded that IRF5 along with RelA is likely to be directly involved in the transcriptional regulation of the human *TNF* gene. Although the initial phase of *TNF* induction depends on both factors, only IRF5 appears to be crucial for maintaining prolonged *TNF* transcription in MDDCs. Moreover, the DBD of IRF5 is required for the optimal level of *TNF* gene up-regulation.

IRF5 is recruited to the 5' upstream and 3' downstream regions of the *TNF* gene in response to LPS stimulation

To further address the involvement of IRF5 in the *TNF* gene regulation, we systematically analyzed the recruitment of IRF5 to

the *TNF* locus. A well-conserved N-terminal DBD of IRF factors recognizes a class of DNA sequences known as ISRE, 15 of which were computationally mapped to this locus together with known κ B sites: κ B1, κ B2/2a, κ B3, and κ B4/4a/4b (Figure 3A; supplemental Table 1). A series of primers spanning the locus and encompassing ISRE sites was designed and used in the quantitative ChIP assay (Figure 3A).

HEK-293-TLR4-CD14/Md2 cells responsive to LPS were used to investigate the effect of LPS stimulation on recruitment of IRF5 to the *TNF* locus. Increased occupancy of IRF5 was observed at regions A, B, C, G, and H 4 hours after LPS stimulation, followed by a decrease after 24 hours (Figure 3B). Taking into consideration the average ChIP fragment size of around 500 bp and the proximity of the sequences amplified, some degree of overlap in regions A through C was inevitable and might have accounted for the observed symmetrical distribution of enrichment at regions A, B, and C. Whereas the enrichment of IRF5 signal at region B was expected due to the presence of putative ISRE 1/2 that can interact with IRF5 in vitro (supplemental Figure 3A), it was surprising to observe the recruitment of IRF5 at region H because this region contains no putative ISREs (Figure 3A). Moreover, we observed no IRF5-DNA binding at κ B4/4a/4b binding sites in region H or at additional nonconsensus 'ISRE'16 and 'ISRE'17 sites in the vicinity of region H (supplemental Table 1; supplemental Figure 3A). We also investigated the recruitment of NF- κ B RelA to the *TNF* locus and observed LPS-induced binding of RelA to regions B, E, and H (Figure 3C), which correlated with the distribution of multiple NF- κ B-binding regions.

Next, we validated the pattern of IRF5 and RelA binding to the *TNF* locus in MDDCs stimulated with LPS for 0, 1, and 4 hours. Strong enrichment in both IRF5 and RelA recruitment was observed at regions B and H (Figure 3D-E), reproducible in

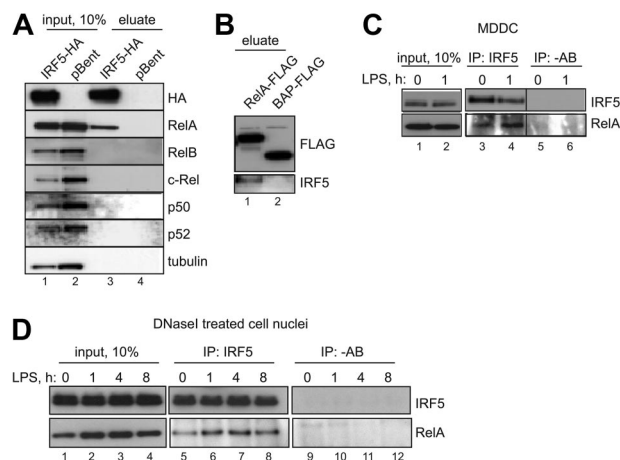


Figure 4. IRF5 specifically interacts with RelA. (A) HEK-293-TLR4-Md2/CD14 cells were transfected with human IRF5 tagged with ONE-strep-tag (N-terminus) and HA-tag (C-terminus; lanes 1 and 3) or an empty vector pBent (lanes 2 and 4) and fixed with formaldehyde. Cross-links were reversed by heating and immunoblotted for bait IRF5 (anti-HA antibodies), or NF- κ B subunits and tubulin. (B) HEK-293-TLR4-Md2/CD14 cells were transfected with RelA-FLAG (lane 1) or BAP-FLAG (lane 2). Cell lysates were immunoprecipitated with M2 anti-FLAG Sepharose and immunoblotted for bait RelA (anti-FLAG antibodies) or IRF5. (C) MDDCs were stimulated with LPS for 1 hour or left untreated. The endogenous interaction between RelA and IRF5 was examined by immunoprecipitation (IP) with anti-IRF5 antibody and immunoblotting with anti-RelA antibody. -AB indicates a mock IP. (D) Nuclear pellet from Triton-extracted HEK-293-TLR4-Md2/CD14 cells was solubilized with DNaseI, and endogenous interaction between RelA and IRF5 was examined after IP as in panel C.

5 independent blood donors (supplemental Figure 3B). Importantly, LPS stimulation resulted in rapid transcription of the full-length nascent *TNF* transcript (estimated by recruitment of Pol II to the *TNF* 3' downstream region H), which was robustly maintained at least up to 4 hours after stimulation (supplemental Figure 3D).

In summary, in response to LPS stimulation IRF5 along with RelA is efficiently recruited to the 5' upstream and 3' downstream regions of the human *TNF* gene. Significantly, the lack of putative ISRE binding sites in the 3' downstream region of the gene strongly suggested that recruitment of IRF5 to this region might be mediated via its interactions with other TFs or accessory proteins.

IRF5 forms specific physical interactions with RelA

To tease out whether IRF5 recruitment to region H may be mediated via its interactions with RelA, we performed sequential ChIP analysis of the region and found that IRF5 recruitment was codependent on RelA after LPS stimulation (Figure 3F). This finding prompted us to investigate whether IRF5 and RelA interact physically.

IRF5 with an N-terminal ONE-strep-tag and a C-terminal HA-tag was expressed in HEK-293 cells. Figure 4 shows that in conditions similar to ChIP analysis (ie, in vivo cross-linking with formaldehyde), ectopically expressed IRF5, purified over a Strep-Tactin column, efficiently pulls down endogenous RelA (Figure 4A, compare lanes 3 and 4). To determine whether this interaction was specific, we immunoblotted for other NF- κ B family members: Rel-B, c-Rel, p50 and p52, or a control protein tubulin. None of these resulted in a positive interaction (Figure 4A). Furthermore, we conducted a complementary experiment in which human RelA containing C-terminal FLAG tag was expressed in HEK-293 cells and immunoprecipitated in the absence of cross-linking agents on anti-FLAG Sepharose. Specific interactions between ectopically expressed RelA and endogenous IRF5 were observed. No interac-

tion was detected between a control FLAG-tagged bacterial alkaline phosphatase (BAP) and IRF5 (Figure 4B, compare lanes 1 and 2).

Next, we examined whether an interaction between the endogenous RelA and IRF5 could be detected in MDDCs, and if this interaction may be induced by LPS stimulation. IRF5 was immunoprecipitated from the cells stimulated with LPS for 0 or 1 hour using anti-IRF5 antibodies. The Western blot for RelA revealed a specific interaction with IRF5 (Figure 4C). A densitometry analysis of quantities of the bait and target proteins indicated that the quantity of RelA bound to IRF5 was somewhat higher in LPS-stimulated cells (Figure 4C lane 4).

Finally, we asked the question whether the observed RelA-IRF5 interactions are dependent on the simultaneous binding of both TFs to DNA (ie, RelA and IRF5 interact only when bound to corresponding κ B and ISRE binding sites in proximity to each other). To address this, we extracted nuclei from HEK-293-TLR4-CD14/Md2 cells stimulated with LPS for 0, 1, 4, and 8 hours and subjected the chromatin to DNaseI digestion. Subsequent precipitation of endogenous immune complexes with anti-IRF5 antibodies revealed that RelA interacted with IRF5 even in the absence of DNA bridging (Figure 4D lanes 5-8). Once again, the number of RelA-IRF5 complexes increased with LPS stimulation, corresponding to the rise in nuclear RelA (Figure 4D lanes 1-4).

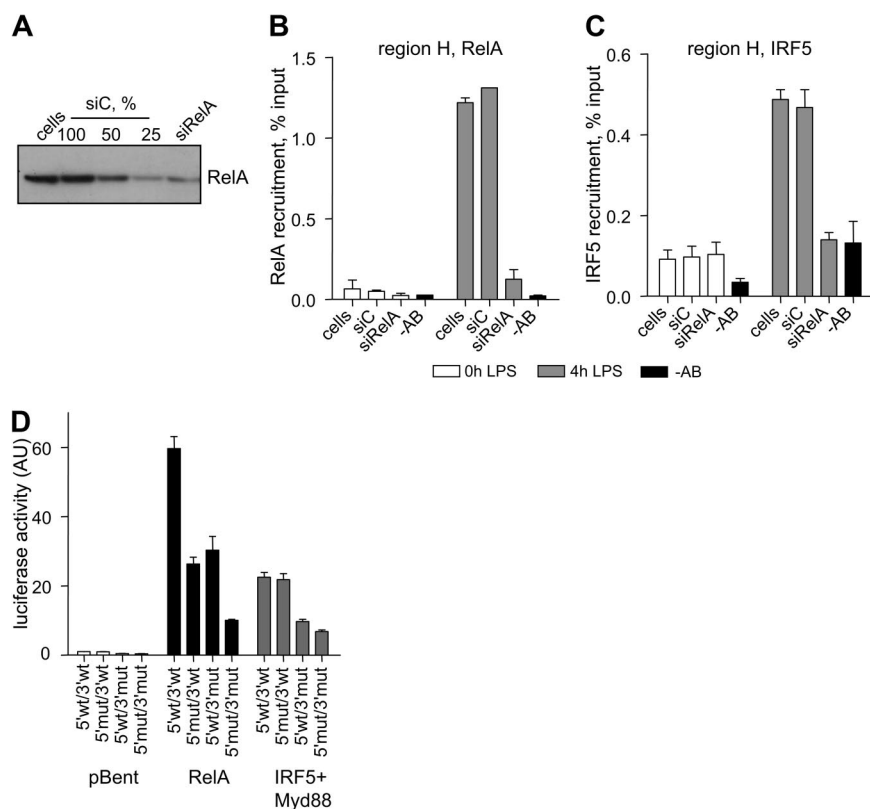
In summary, IRF5 can specifically interact with RelA but not other 4 NF- κ B subunits. This interaction is not dependent on IRF5 binding to DNA, and the quantity of RelA-IRF5 complexes is increased in response to LPS stimulation. Thus, we hypothesized that IRF5 recruitment to the 3' downstream region of the *TNF* gene lacking putative ISRE sites is a consequence of direct physical interactions between DNA-bound RelA and IRF5.

RelA is required for IRF5-dependent *trans*-activation of the *TNF* gene

To test this hypothesis, we first analyzed IRF5 recruitment to the *TNF* locus in the cells in which the levels of RelA were significantly reduced. In HEK-293-TLR4-CD14/Md2 cells, siRNA-mediated knockdown of RelA resulted in approximately 75% reduction in RelA protein (Figure 5A) and about a 10-fold decline in its recruitment to region H after 4 hours of LPS stimulation (Figure 5B). As predicted, the IRF5 recruitment to the same region was prevented (Figure 5C). Of interest, when we analyzed RelA and IRF5 recruitment to region B in RelA-depleted cells, we observed only partial reduction in IRF5 recruitment (supplemental Figure 4), consistent with the view that IRF5 can bind directly to DNA at this region (supplemental Figure 3A).

Next, we examined the effect of site-specific mutations in the κ B sites on the ability of IRF5 to activate the *TNF* gene. A panel of 4 gene-reporter constructs was used in this analysis: (1) 5'wt/3'wt (as in Figure 2B); (2) 5'wt/3'wt (mutated κ B2/2 ϵ /2a sites in the *TNF* 5' upstream); (3) 5'wt/3'wt (mutated κ B4/4a sites in the 3' *TNF* downstream); and (4) 5'wt/3'wt (mutated all κ B sites just described). The reporter constructs were coexpressed with HA-tagged IRF5 and RelA in HEK-293 cells, and luciferase activities were compared with empty vector pBent. As expected, removal of either 5' upstream or 3' downstream κ B sites diminished the ability of RelA to drive the gene-reporter activity (Figure 5D). However, the *trans*-activation of the reporter constructs by IRF5 (supported by ectopically expressed Myd88) appeared to be largely unaffected by mutations in the 5' upstream κ B sites, suggesting that IRF5 does not use κ B2/2 ϵ /2a sites for its binding to the *TNF* 5' upstream and is likely to involve the identified ISRE 1

Figure 5. RelA is required for IRF5-mediated activation of *TNF*. (A-C) HEK-293-TLR4-Md2/CD14 cells were transfected with siRNA against RelA (siRelA) or with nontargeting siRNA (siC) and used in ChIP analysis of RelA and IRF5 recruitment. Data indicate mean percentage input relative to gDNA $\pm$ SD of a representative experiment. -AB indicates a no-antibody control. (A) A total of 75% of RelA protein was degraded estimated by serial dilutions of the siC control sample analyzed by Western blotting. (B) Reduction in LPS-induced RelA recruitment to region H in siRelA-treated cells. (C) Reduction in LPS-induced IRF5 recruitment to region H in siRelA-treated cells. (D) HEK-293-TLR4-Md2/CD14 cells were transfected with the RelA, IRF5, and MyD88 expression constructs together with the *TNF* 5' upstream/luciferase/*TNF* 3' downstream reporter plasmids: 5'wt/3'wt indicates wild-type construct; 5'wt/3'mut indicates mutated κ B2 (GTGAATTCCTCC $\rightarrow$ tTGAATTCCTCC), κ B β (GTGATTTTCC $\rightarrow$ aTccTTTCC), and κ B2a (GGGCTGTCTCC $\rightarrow$ taGCTGTGCTCC) sites in the *TNF* 5' upstream; 5'wt/3'mut indicates mutated κ B4 (GGGAATTTCC $\rightarrow$ cGcAATgTgC) and κ B4a (GGGAATTTCCA $\rightarrow$ cGcAAGTgCA) sites in the *TNF* 3' downstream; and 5'mut/3'mut indicates all κ B sites mutated. Data show means $\pm$ SD and are a representative of 3 independent experiments, each performed in triplicate.



and 2 sites. However, the *trans*-activation of the reporter construct with mutations in κ B4/4a sites by IRF5 was significantly reduced, indicating that IRF5 activity depends on NF- κ B binding to this region (Figure 5B). Low amounts of endogenous RelA detected in the nuclei of resting HEK-293-TLR4-CD14/Md2 cells (data not shown) appeared to provide a necessary DNA anchor for IRF5.

Thus, IRF5 recruitment to the *TNF* 3' downstream region is mediated by way of a complex assembly with RelA and does not involve a direct contact to DNA. Importantly, another mode of function of IRF5 in *TNF* regulation is a direct recruitment to the *TNF* gene 5' upstream. The 2 functional modes also imply the possibility of a higher order enhancer structure at the *TNF* locus, possibly involving IRF5-RelA-mediated intrachromosomal looping.

Discussion

Production of the key immune modulator *TNF* is both cell and stimulus specific. Myeloid cells are the major producers of *TNF* in response to TLR4 stimulation.²⁰ Consequently, a tight control of the amount and duration of *TNF* expression by these cells is critical for a self-limited immune response. Here, we aimed to understand the molecular bases of differential *TNF* expression in human DCs and macrophages. We demonstrate that IRF5 appears to be a defining factor in maintaining the *TNF* gene transcription in MDDCs. Remarkably, we unravel a complex molecular mechanism used by IRF5 to control the human *TNF* gene expression: 2 spatially separated regulatory regions and 2 independent modes of actions are involved.

IRF5 is highly expressed in MDDCs but not other myeloid cells (Figure 1). During differentiation MDDCs acquire a particular phenotype, characterized among other markers by higher levels of RelB and c-Rel.²⁹ Important for understanding the mechanisms of

sustained *TNF* expression, RelB was previously shown to replace RelA at the promoters of macrophage-derived chemokine and Epstein-Barr virus (EBV)-induced molecule 1 ligand chemokine genes and to prolong their transcription in MDDCs.³⁰ We also observed an increase in the RelB and c-Rel levels during monocyte differentiation into MDDCs, but not into MDMs (supplemental Figure 5A). However, neither RelB nor c-Rel was able to drive transcription of *TNF* (supplemental Figure 2), whereas RelA, whose level was similar in all human myeloid cell types (supplemental Figure 5A), had a strong *trans*-activating effect (Figure 2). This led us to conclude that RelA was likely to participate in the initial phase of *TNF* activation, which is indistinguishable between MDDCs and MDMs, but other MDDC-specific factors, such as IRF5, may contribute to the observed extended expression of *TNF* in MDDCs. Indeed, forced expression of IRF5 in MDMs led to prolonged *TNF* secretion (Figure 1), whereas depletion of IRF5 in MDDCs resulted in reduction of *TNF* expression, particularly at a later time (4 hours) after LPS stimulation (Figure 2; supplemental Figure 2). Although we cannot formally rule out other factors that might feed into the *TNF* expression system at a later time, the ability of IRF5 to activate the *TNF* gene-reporter construct (Figure 2) and its efficient recruitment to the *TNF* locus (Figure 3) strongly suggest a direct role for IRF5 in *TNF* gene induction in response to LPS.

TNF is an early primary response gene whose mRNA expression in MDDCs is induced approximately 100-fold within 30 minutes after LPS treatment (supplemental Figure 2). The genomic locus encompassing the *TNF* gene is open to regulatory proteins and in murine bone marrow-derived macrophages (BMDMs) does not require nucleosome remodeling complexes for its activation.³¹ Consistent with this notion, we find a significant accumulation of Pol II molecules at the transcription start site (TSS) of the gene even in resting MDDCs (supplemental Figure 6), akin to the results

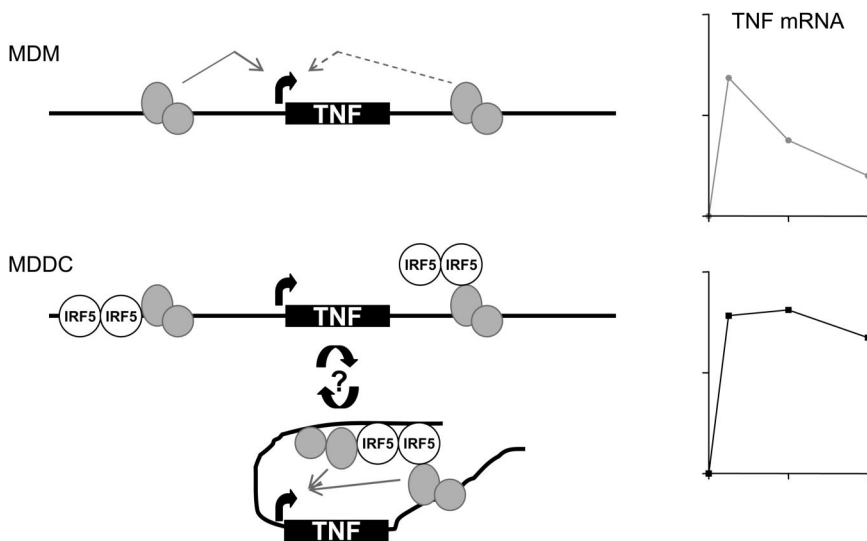


Figure 6. A model for IRF5-RelA-mediated induction of *TNF* in myeloid cells. LPS-induced recruitment of NF-κB RelA-containing complexes (gray ovals) to the 5' upstream and 3' downstream regions leads to transient TNF expression in MDMs. The mechanisms of transmitting the activating signal from 3' downstream to Pol II requires further investigation. IRF5 binding to DNA at the 5' upstream and to RelA at the 3' downstream establishes the possibility for region circularization and recycling of Pol II molecules, leading to sustained TNF expression in MDDCs.

obtained in mouse BMDMs.^{32,33} LPS stimulation, however, results in a robust recruitment of RelA and IRF5 to both 5' upstream region B and 3' downstream region H (Figure 3) and in a significant induction of Pol II recruitment to the 3' downstream region of the gene (supplemental Figure 6). This suggests an increase in production of full-length nascent *TNF* transcripts upon LPS stimulation of MDDCs, in addition to induction of splicing of already generated nascent transcripts reported by Hargreaves et al.³²

The recruitment of IRF5 to the 5' upstream region is likely to involve direct binding to DNA via the identified ISRE sites (supplemental Figure 3), whereas the recruitment of IRF5 to the 3' downstream region is mediated via protein-protein interactions with RelA (Figure 4). These interactions are induced after stimulation of MDDCs with LPS, while no other NF-κB subunits appear to complex with IRF5 (Figure 4). Previous studies demonstrated that IRF3, another member of the IRF family, forms in vitro interactions with RelA via its Rel homology domain (RHD).³⁴ Considering that the RHD is a highly conserved domain present in all NF-κB proteins, the exclusiveness of IRF5 interactions with RelA is somewhat surprising. Further work is needed to map the interface of RelA-IRF5 interactions.

Regions B and H are characterized by high level of sequence conservation,^{25,35} and contain cell type-specific DNaseI hypersensitivity sites.^{35,36} Moreover, the *TNF* 5' upstream and 3' downstream regions have been shown to physically interact by forming an intrachromosomal loop, the topology that could promote the reinitiation of transcription.³⁵ This model may be of a particular relevance to TNF expression by MDDCs, in which a cooperative action of RelA and IRF5 at both the 5' upstream and downstream regions appears to be essential for maintaining *TNF* gene transcription over a prolonged period of time. Here, the locus circularization may be directed via newly unraveled protein-protein interactions between RelA and IRF5 (Figure 4). The observed DNA-binding-independent corecruitment of IRF5 to the 3' downstream region (Figure 5) further supports the possibility of high-order enhancer structure at the locus (Figure 6).

Why is TNF secretion maintained for longer in MDDCs than in MDMs (Figure 1)? DCs are professional antigen-presenting cells (APCs) that are crucial for both innate and adaptive responses to infection. They sense invading pathogens and respond by secreting various cytokines as well as by up-regulating the expression of MHC II and costimulatory molecules, essential for efficient antigen

presentation to T cells.³⁷ The mature DCs migrate to the draining lymph nodes, where they initiate Th1 differentiation. TNF acting through the TNF receptor is involved in DC maturation from bone marrow precursors.^{21,22} A recent study demonstrated that TNF blockade impaired DC survival and function in RA.³⁸ Our data showing that TNF produced by DCs is a key factor in human Th1 activation support this study. Moreover, it is the late-phase TNF secretion that is needed to achieve the full activation potential (Figure 1). Macrophages, on the other hand, do not migrate to the draining lymph nodes but accumulate in large numbers at a site of inflammation, secrete inflammatory cytokines, and attract other immune cells via chemotaxis.³⁹ Thus, a mechanism which would restrain the degree and duration of TNF secretion by macrophages would be important for ensuring the resolution of acute inflammatory response, thereby limiting tissue damage.

Another question is how IRF5 is activated in MDDCs by TLR4 signaling. Takaoka et al demonstrated that ectopically expressed IRF5 translocates to the cell nuclei in response to LPS, and that this translocation is dependent on the presence of Myd88.⁸ We observed endogenous nuclear IRF5 even in resting MDDCs or HEK-293-TLR4-CD14/Md2 cells, and its level was not increased after LPS stimulation (supplemental Figure 5B), although we could not exclude the possibility of active nuclear export-import of IRF5 induced by phosphorylation at the previously described serine residues⁴⁰ due to the lack of phosphospecific antibodies. In the same cells, endogenous IRF3 showed a clear pattern of induced nuclear translocation (supplemental Figure 5B), which corresponded to its phosphorylated form (data not shown). It is worth noting, however, that an ectopically expressed mutant of IRF5 in which the described critical serines at positions 427 and 430^{9,40} were substituted with alanines was still transcriptionally active in the TNF reporter assay (G.R., unpublished data, 2009), suggesting that IRF5 may not need to be phosphorylated at these residues to activate transcription. We also did not observe any loss in IRF5 *trans*-activating potential when lysines 401 and 402, implicated in another Myd88-induced posttranslational modification of IRF5, K63-linked polyubiquitination,⁴¹ were substituted with arginines (G.R., unpublished data, 2009). Because the overexpression data generated in the HEK-293 cell line may be misleading, we plan to test these and other mutants of IRF5 in complementation experiments in the cells from IRF5-deficient

mice to elucidate the impact of these mutations on IRF5 activation and function in vivo.

Regulation of IRF5 activity is an important issue because the excessive activation of this protein may lead to pathology. Interestingly, another member of the IRF family, IRF4, was shown to act as a negative regulator of TLR signaling by inhibiting the production of selected IRF5-dependent genes, including TNF, via direct competition with IRF5 for interactions with Myd88.⁴² In mice, IRF4 was observed to be differentially expressed in DCs and regulate the development of a specific DC subset, conventional DCs.⁴³ In humans, IRF4 was also found to be expressed in MDDCs but not MDMs,⁴⁴ suggesting that a self-controlled IRF5-IRF4 regulatory system might have developed to finely modulate TLR signaling pathways and production of IRF5-dependent inflammatory cytokines.

In summary, sustained TNF secretion in human MDDCs is mediated by cooperative action of IRF5 and RelA at the 5' upstream and 3' downstream regions of the *TNF* gene. TLR4 stimulation induces protein-protein interactions between RelA and IRF5 and allows for DNA-independent recruitment of IRF5 to the *TNF* 3' downstream region. IRF5 may assist in formation of a high-order enhancer structure linking together the regulatory regions in the *TNF* 5' upstream and 3' downstream and allowing for maintaining of transcription over a longer time (Figure 6). Based on the resistance of IRF5^{-/-} mice to lethal endotoxic shock, impaired production of proinflammatory cytokines, and deficiency in Th1 immune response, IRF5 was proposed as a target for therapeutic interventions.⁸ Here, we define RelA-IRF5 interactions as a putative target for cell-specific modulation of *TNF* expression and possible other selected inflammatory mediators.

References

- Jongeneel CV. Regulation of the TNF alpha gene. *Prog Clin Biol Res*. 1994;388:367-381.
- Collart MA, Baeuerle P, Vassalli P. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol Cell Biol*. 1990;10(4):1498-1506.
- Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV. Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J Exp Med*. 1990;171(1):35-47.
- Foxwell B, Browne K, Bondeson J, et al. Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. *Proc Natl Acad Sci U S A*. 1998;95(14):8211-8215.
- Udalova IA, Knight JC, Vidal V, Nedospasov SA, Kwiatkowski D. Complex NF-kappaB interactions at the distal tumor necrosis factor promoter region in human monocytes. *J Biol Chem*. 1998;273(33):21178-21186.
- Honda K, Taniguchi T. Toll-like receptor signaling and IRF transcription factors. *IUBMB Life*. 2006;58(5-6):290-295.
- Sakaguchi S, Negishi H, Asagiri M, et al. Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. *Biochem Biophys Res Commun*. 2003;306(4):860-866.
- Takaoka A, Yanai H, Kondo S, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature*. 2005;434(7030):243-249.
- Barnes BJ, Kellum MJ, Field AE, Pitha PM. Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes. *Mol Cell Biol*. 2002;22(16):5721-5740.
- Chen W, Lam SS, Srinath H, et al. Insights into interferon regulatory factor activation from the crystal structure of dimeric IRF5. *Nat Struct Mol Biol*. 2008;15(11):1213-1220.
- Manci ME, Hu G, Sangster-Guilty N, et al. Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms: multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. *J Biol Chem*. 2005;280(22):21078-21090.
- Graham RR, Kozyrev SV, Baechler EC, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat Genet*. 2006;38(5):550-555.
- Dieguez-Gonzalez R, Calaza M, Perez-Pampin E, et al. Association of interferon regulatory factor 5 haplotypes, similar to that found in systemic lupus erythematosus, in a large subgroup of patients with rheumatoid arthritis. *Arthritis Rheum*. 2008;58(5):1264-1274.
- Han SW, Lee WK, Kwon KT, Lee BK, Nam EJ, Kim GW. Association of polymorphisms in interferon regulatory factor 5 gene with rheumatoid arthritis: a metaanalysis. *J Rheumatol*. 2009;36(4):693-697.
- Miceli-Richard C, Comets E, Loiseau P, Puechal X, Hachulla E, Mariette X. Association of an IRF5 gene functional polymorphism with Sjogren's syndrome. *Arthritis Rheum*. 2007;56(12):3989-3994.
- Thomson SJ, Goh FG, Banks H, et al. The role of transposable elements in the regulation of IFN-lambda1 gene expression. *Proc Natl Acad Sci U S A*. 2009;106(28):11564-11569.
- Fujita M, Kiyono T, Hayashi Y, Ishibashi M. In vivo interaction of human MCM heterohexameric complexes with chromatin. Possible involvement of ATP. *J Biol Chem*. 1997;272(16):10928-10935.
- Vlieghe D, Sandelin A, De Bleser PJ, et al. A new generation of JASPAR, the open-access repository for transcription factor binding site profiles. *Nucleic Acids Res*. 2006;34(Database issue):D95-D97.
- Genomatix software. Available at <http://www.genomatix.de/>. Accessed February 2008.
- Grivnenkov SI, Tumanov AV, Liepinsh DJ, et al. Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects. *Immunity*. 2005;22(1):93-104.
- Abe K, Yarovsky FO, Murakami T, et al. Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo. *Blood*. 2003;101(4):1477-1483.
- Ritter U, Meissner A, Ott J, Korner H. Analysis of the maturation process of dendritic cells deficient for TNF and lymphotoxin-alpha reveals an essential role for TNF. *J Leukoc Biol*. 2003;74(2):216-222.
- Aspalter RM, Eibl MM, Wolf HM. Regulation of TCR-mediated T cell activation by TNF-RII. *J Leukoc Biol*. 2003;74(4):572-582.
- Yoshimura S, Bondeson J, Foxwell BM, Brennan FM, Feldmann M. Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines. *Int Immunol*. 2001;13(5):675-683.
- Kuprash DV, Udalova IA, Turetskaya RL, Kwiatkowski D, Rice NR, Nedospasov SA. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol*. 1999;162(7):4045-4052.

Acknowledgments

We are grateful to Dr Daniel Wong (Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom) for help with IRF5 DBD expression and purification, Mr Tim Smallie (Kennedy Institute of Rheumatology [KIR], London, United Kingdom) for providing 5'wt/3'wt and 5'wt/3'mut *TNF* gene-reporter constructs, and Dr Fui G. Goh and Mr Scott Thomson (KIR) for advice and useful suggestions on RNAi and ChIP. We also thank Drs Jonathan Dean, Matt Pierce, and Lynn Williams (KIR) for critical reading of the manuscript and helpful comments.

The research leading to these results was supported by MRC collaborative grant 82189 to I.A.U. and has received funding from the European Community's Seventh Framework Program FP7/2007-2013 under grant agreement no. 222008.

Authorship

Contribution: T.K., D.S., G.R., K.B., and A.L. performed research; T.K., D.S., and I.A.U. designed research and analyzed data; and T.K., D.S., and I.A.U. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Irina A. Udalova, Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, 65 Aspenlea Rd, London, W6 8LH, United Kingdom; e-mail: i.udalova@imperial.ac.uk.

26. Kuprash DV, Udalova IA, Turetskaya RL, Rice NR, Nedospasov SA. Conserved kappa B element located downstream of the tumor necrosis factor alpha gene: distinct NF-kappa B binding pattern and enhancer activity in LPS activated murine macrophages. *Oncogene*. 1995;11(1):97-106.
27. Schoenemeyer A, Barnes BJ, Mancini ME, et al. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *J Biol Chem*. 2005;280(17):17005-17012.
28. Yang L, Zhao T, Shi X, et al. Functional analysis of a dominant negative mutation of interferon regulatory factor 5. *PLoS ONE*. 2009;4(5):e5500.
29. Neumann M, Fries H, Scheicher C, et al. Differential expression of Rel/NF-kappaB and octamer factors is a hallmark of the generation and maturation of dendritic cells. *Blood*. 2000;95(1):277-285.
30. Sacconi S, Pantano S, Natoli G. Modulation of NF-kappaB activity by exchange of dimers. *Mol Cell*. 2003;11(6):1563-1574.
31. Ramirez-Carrozzi VR, Nazarian AA, Li CC, et al. Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. *Genes Dev*. 2006;20(3):282-296.
32. Hargreaves DC, Hornig T, Medzhitov R. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell*. 2009;138(1):129-145.
33. Ramirez-Carrozzi VR, Braas D, Bhatt DM, et al. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell*. 2009;138(1):114-128.
34. Ogawa S, Lozach J, Benner C, et al. Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell*. 2005;122(5):707-721.
35. Tsytsykova AV, Rajsbaum R, Falvo JV, Ligeiro F, Neely SR, Goldfeld AE. Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers. *Proc Natl Acad Sci U S A*. 2007;104(43):16850-16855.
36. Taylor JM, Wicks K, Vandiedonck C, Knight JC. Chromatin profiling across the human tumour necrosis factor gene locus reveals a complex, cell type-specific landscape with novel regulatory elements. *Nucleic Acids Res*. 2008;36(15):4845-4862.
37. Ueno H, Klechevsky E, Morita R, et al. Dendritic cell subsets in health and disease. *Immunol Rev*. 2007;219:118-142.
38. Baldwin HM, Ito-Ihara T, Isaacs JD, Hilken CM. TNF(alpha) blockade impairs dendritic cell survival and function in rheumatoid arthritis [published online ahead of print April 7, 2010]. *Ann Rheum Dis*. doi:10.1136/ard.2009.110502.
39. Glaros T, Larsen M, Li L. Macrophages and fibroblasts during inflammation, tissue damage and organ injury. *Front Biosci*. 2009;14:3988-3993.
40. Cheng TF, Brzostek S, Ando O, Van Scoy S, Kumar KP, Reich NC. Differential activation of IFN regulatory factor (IRF)-3 and IRF-5 transcription factors during viral infection. *J Immunol*. 2006;176(12):7462-7470.
41. Balkhi MY, Fitzgerald KA, Pitha PM. Functional regulation of MyD88-activated interferon regulatory factor 5 by K63-linked polyubiquitination. *Mol Cell Biol*. 2008;28(24):7296-7308.
42. Negishi H, Ohba Y, Yanai H, et al. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci U S A*. 2005;102(44):15989-15994.
43. Tamura T, Tailor P, Yamaoka K, et al. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol*. 2005;174(5):2573-2581.
44. Lehtonen A, Veckman V, Nikula T, et al. Differential expression of IFN regulatory factor 4 gene in human monocyte-derived dendritic cells and macrophages. *J Immunol*. 2005;175(10):6570-6579.