

THE ROLE OF NF-κB IN ANTIGEN PRESENTATION AND INFLAMMATION

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«Εάν μή έλπηται ανέλπιστον, ούκ εξευρήσει, ανεξερεύνητον εόν καί άπορον»

Ηράκλειτος

This thesis is dedicated to my parents Theodoros and Despina, and my brother Dimitris, whose love and support is the most important thing in my life, my grandmother Panayiota whose words are still inspiring me, and my best friend Seraphim Karakostas who lost his life in a tragic accident during my Ph.D. study

ABSTRACT

In this thesis, I employed a highly efficient genetic modification method consisting of replication-deficient adenoviruses, recently developed at the KIR Divison, to dissect intracellular signalling pathways involved in the regulation of the immune response in physiologically relevant primary cells. My studies concentrated on the pathways that lead to NF-κB activation in dendritic cells (DC) and macrophages, as NF-κB has been previously shown to be required for antigen presentation and cytokine production in these cell types. First, I found that IκB kinase 2 (IKK2), an upstream activator of NF-κB, plays a major role in NF-κB activation in DC as it is essential for efficient antigen-presenting function and CD40 ligand-induced cytokine production. IKK2 is also important in CD40 ligand-induced cytokine production in macrophages. However, its role in LPS-induced NF-κB activation and cytokine production is receptor- and lineage-restricted as IKK2 is not involved in LPS-induced activation in either DC or macrophages but is in human umbilical vein endothelial cells (HUVEC). Next, I examined NF-κB-inducing kinase (NIK), another upstream activator of NF-κB, and found that NIK is redundant with respect to DC NF-κB activation and antigen presentation as expression of an inhibitory form of it was unable to block these functions. Nevertheless, overexpression of its wild-type form was a potent activator of DC antigen-presenting function by activating IKK2 and coordinating the production of cytokines, chemokines, adhesion and costimulatory molecules. This strong adjuvant effect of NIK was not only limited to *in vitro* studies but could be also reproduced in mice *in vivo*. Thus, in a model of genetic immunization, incorporation of NIK into the adenoviral vector could increase the immunogenicity of a vector-encoded antigen and skew it towards a T_{H1} profile. Finally, I investigated the role of MyD88, an adaptor molecule involved in IL-1R/Toll-like receptor signalling. Although inhibition of MyD88 activity could block IL-1 or LPS signalling in HUVEC or human skin fibroblasts, in contrast in immature DC or macrophages this resulted in NF-κB activation and cytokine production in the absence of additional stimulation. This paradoxical effect suggested that MyD88 is involved in maintaining an inhibitory signal in DC and macrophages that prevents ligand-independent TLR signalling.

In summary, these studies highlight the importance of validating intracellular signalling pathways in the relevant cell types, and demonstrate that information obtained *in vitro* can be used successfully to manipulate the immune response *in vivo*. In addition, they suggest that IKK2 is a therapeutic target for the treatment of allograft rejection and chronic inflammation, and define a novel family of immunostimulatory molecules that consists of wild-type NIK, inhibitory forms of MyD88 and possibly other NF-κB activating intracellular signalling molecules that could be useful for enhancing immunogenicity of genetic vaccines.

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Declaration

This dissertation is the result of my own work with the exception of a few figures. In chapter 5, figure 5.14 was the result of Dr. T.Stonehouse's work, whereas figure 5.10 was the result of my collaboration with Dr. T.Stonehouse. In chapter 6, the immunization of mice, the bleeding and the excision of lymph nodes were performed in collaboration with Dr. R.Williams who was my teacher for the *in vivo* experiments.

Abbreviations

Adβ-gal	β-galactosidase
AdIκBα	adenoviral vector encoding porcine IκBα
AdIKK2wt	adenoviral vector encoding wild-type IκB kinase 2
AdIKK2dn	adenoviral vector encoding dominant negative IκB kinase 2
AdMyD88wt	adenoviral vector encoding wild-type MyD88
AdMyD88dn	adenoviral vector encoding dominant negative MyD88
Ad0	adenoviral vector containing no insert
APC	antigen-presenting cell
ARE	AU rich element
ATP	Adenosine Triphosphate
β-gal	β-galactosidase
β-ME	β-Mercaptoethanol
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CIA	Collagen-Induced Arthritis
CD	Cluster Determinant
CD25	IL-2R α-chain
CD40L	CD40 Ligand
CD45	Leukocyte common antigen
CD80	B7-1
CD86	B7-2
CFA	Complete Freud's Adjuvant
CMV	Cytomegalovirus
CNTF	Ciliary Neurotrophic Factor
CREB	CAMP Response Element Binding Protein
CTP	Cytosine triphosphate
DAG	Diacylglycerol
DC	Dendritic Cell
DD	Death Domain
dH_2O	Deionized Water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
DTH	Delayed-Type Hypersensitivity
DTT	Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis

ECL	Enhanced Chemoluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunoabsorbant Assay
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular-signal Regulated kinase
FACS	Fluorescent Activated Cell Sorting
FCS	Foetal Calf Serum
FDG	2 mM fluorescein Di-(β-galactopyranoside)
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
HBSS	Hank's Buffered Salt Solution
HCl	Hydrochloric Acid
HEPES	4-2(hydroxyethyl)-1-piperazineehenesulphonic acid
HLA	Human Leucocyte Antigen
H_2O_2	Hydrogen peroxide
HRP	HorseRadish Peroxidase
HSF	Human Skin Fibroblast
Hsp	Heat Shock Protein
HUVEC	Human Umbilical Vein Endothelial Cell
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IκB	Inhibitor of κB
IKAP	IKK Associated Protein
IKK	IκB kinase
IKKdn	IKK dominant negative
IKKwt	IKK wild-type
IL-	Interleukin
IL-1ra	Interleukin 1 Receptor Antagonist
IP	Immunoprecipitate
IRAK	IL-1 Receptor Associated protein Kinase
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal Kinase
LBP	LPS-binding Protein
LIF	Leukaemia Inhibitory Factor
LN	Lymph Node
LPS	Lipopolysaccharide

LT	Lymphotoxin
MAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein kinase
MAPKAP	MAPK-Activated Protein Kinase
MBP	Myelin Basic Protein
MCM	Monocyte-Conditionned Medium
M-CSF	Macrophage Colony-Stimulating Factor
MEKK	MAPK/ERK kinase kinase or MKKK
MEKKwt	MEKK wild-type
MHC	Major Histocompatibility Complex
MKK	MAPK Kinase
MKKK	MKK Kinase
MKKKK	MKKK Kinase
MLR	Mixed Lymphocyte Reaction
mRNA	Messenger RNA
MTT	(3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide
MVA	Modified Vaccinia Virus
MyD88	Myeloid Differentiation primary response protein 88
MyD88dn	MyD88 dominant negative
MyD88wt	MyD88 wild-type
NaCl	Sodium Chloride
NaF	Sodium Fluoride
$NaVO_4$	Sodium Orthovanadate
NF-κB	Nuclear Factor of κB
NIK	NF-κB-inducing kinase
NIKdn	NIK dominant negative
NIKwt	NIK wild-type
NK	Natural Killer cell
NO	Nitric Oxide
PAGE	Polyacrylamide Gel Electrophoresis
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PDGF	Platelet-Derived Growth Factor
PE	Phycoerythrin
PGE	Prostaglandin
PI	Phosphatidylinositol
PI3K	PI 3-Kinase
PKA	Protein Kinase A

PKB	Protein Kinase B
PKC	Protein Kinase C
PMA	Phorbol Myristoyl Acetate
PMSF	Phenyl Methyl Sulphonyl Fluoride
PVDF	Polyvinyl Difluoride
R	Receptor
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
rpm	Revolution Per Minute
RPMI	Roswell Part Memorial Institute medium number 1640
SAPK	Stress-Activated Protein Kinase
s.c.	Subcutaneous
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error Mean
Ser	Serine
SH	Src Homology
SODD	Silencer of Death Domains
STAT	Signal Transducer and Activator of Transcription
TAK	TGF-Activating Kinase
TBE	Tris-Borate EDTA
TCR	T Cell Receptor
TE	Tris-EDTA
TEMED	N'N'N'N'-Tetraethyl methylene diamine
TF	Transcription Factor
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TMB	Tetramethylbenzide Dichloride
TNF	Tumour Necrosis Factor
TNF-R	Tumour Necrosis Factor Receptor
T_H	T helper cell
Thr	Threonine
Tyr	Tyrosine
UV	Ultraviolet
VCAM	Vascular Cell Adhesion Molecule

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CHAPTER 1

INTRODUCTION

1.1. Immunity and the immune response

1.1.1 The immune system

To fight infectious agents and protect us from disease, vertebrates employ a remarkably adaptive defence system, the immune system, that has evolved to protect them from invading pathogenic microorganisms and probably cancer. This uses an enormous variety of cells and proteins, and is capable of specifically recognizing, generating an immune response and then eliminating an apparently limitless variety of foreign pathogens, without in most cases damaging its host.

Immune responses can be described as either "innate" or "adaptive". Innate immune responses are present before the arrival of the pathogen and comprise of anatomic and physiologic defence barriers (skin and mucous membranes) as well as non-specific phagocytic and inflammatory processes to attack non-specifically invading microorganisms. Adaptive immune responses specifically recognise and selectively eliminate foreign microorganisms, viruses and molecules. Unlike innate immune responses, adaptive immune responses display specificity, diversity and memory, and in many cases they confer life-long protective immunity to re-infection with the same pathogen.

Adaptive immune responses can be "humoral" or "cell-mediated". Humoral responses involve the activation of B cells and the production of specific antibodies against the pathogen that result in its opsonization, phagocytosis or complement-mediated killing. Cellular immune responses involve the recognition of specific pathogens by T lymphocytes that become activated and initiate appropriate effector responses. Important cell types involved in adaptive immunity are monocytes/macrophages and dendritic cells; they present pathogen to T cells and result in their activation and proliferation. Other major cell types that play a role in immune responses are polymorphonuclear granulocytes such as neutrophils, basophils and eosinophils, mast cells, natural killer (NK) cells, platelets and endothelial cells, although their role seems to be more important in innate immunity and effector mechanisms of adaptive immunity. The properties of cells involved in the immune system are summarized in table 1.1.

	CELL TYPE	FUNCTIONS
Lymphoid	Helper T lymphocytes	Stimulate B lymphocyte growth and differentiation, and they activate macrophages.
	Cytotoxic T lymphocytes	Kill virally infected or tumour cells and they activate macrophages
	B lymphocytes	Present antigen to T lymphocytes and produce antibodies
	Large granular lymphocytes or natural killer (NK) cells	Involved in the early responses to virally infected or tumour cells and they mediate antibody-dependent cellular cytotoxicity.
Myeloid	Mononuclear phagocytes (monocytes/macrophages)	Phagocytose foreign particles and dead cells, they present antigen to T lymphocytes, they induce fibroblast and vascular endothelial cell proliferation important in tissue repair and they recruit other inflammatory cells.
	Neutrophils	Phagocytose foreign particles and dead cells.
	Eosinophils	Phagocytose IgE-coated particles and mediate immediate-type hypersensitivity reactions.
	Basophils/Mast cells	Involved in IgE-mediated hypersensitivity.
	Platelets	Mediate blood clotting.
	Dendritic cells	Present antigen to T lymphocytes
Endothelial cells		Regulate the recruitment of effector cells and the blood clotting process.

Table 1.1: The major functions of the cells of the immune system.

With the exception of endothelial cells, all other cell types of the immune system are derived from the pluripotent stem cells in the bone marrow (table 1.1) (Zon L.I. 1995; Eichmann A. et al. 1997; Morrison S.J. et al. 1997). Pluripotent progenitor cells give rise consecutively to progenitor cells of more limited potential and central to that process are the myeloid (granulocyte-monocyte progenitor) and the common lymphoid progenitor cells. Lymphocytes are derived from the common lymphoid progenitor. T cells develop from their precursors in the thymus, whereas mammalian B cells differentiate in the fetal liver and the adult bone marrow.

Monocytes/macrophages, dendritic cells and neutrophils are mainly derived from the granulocyte-macrophage (myeloid) progenitor in the bone marrow whereas eosinophil, basophil and mast cell committed precursors are derived from the pro-myeloid-lymphoid precursor (figure 1.1).

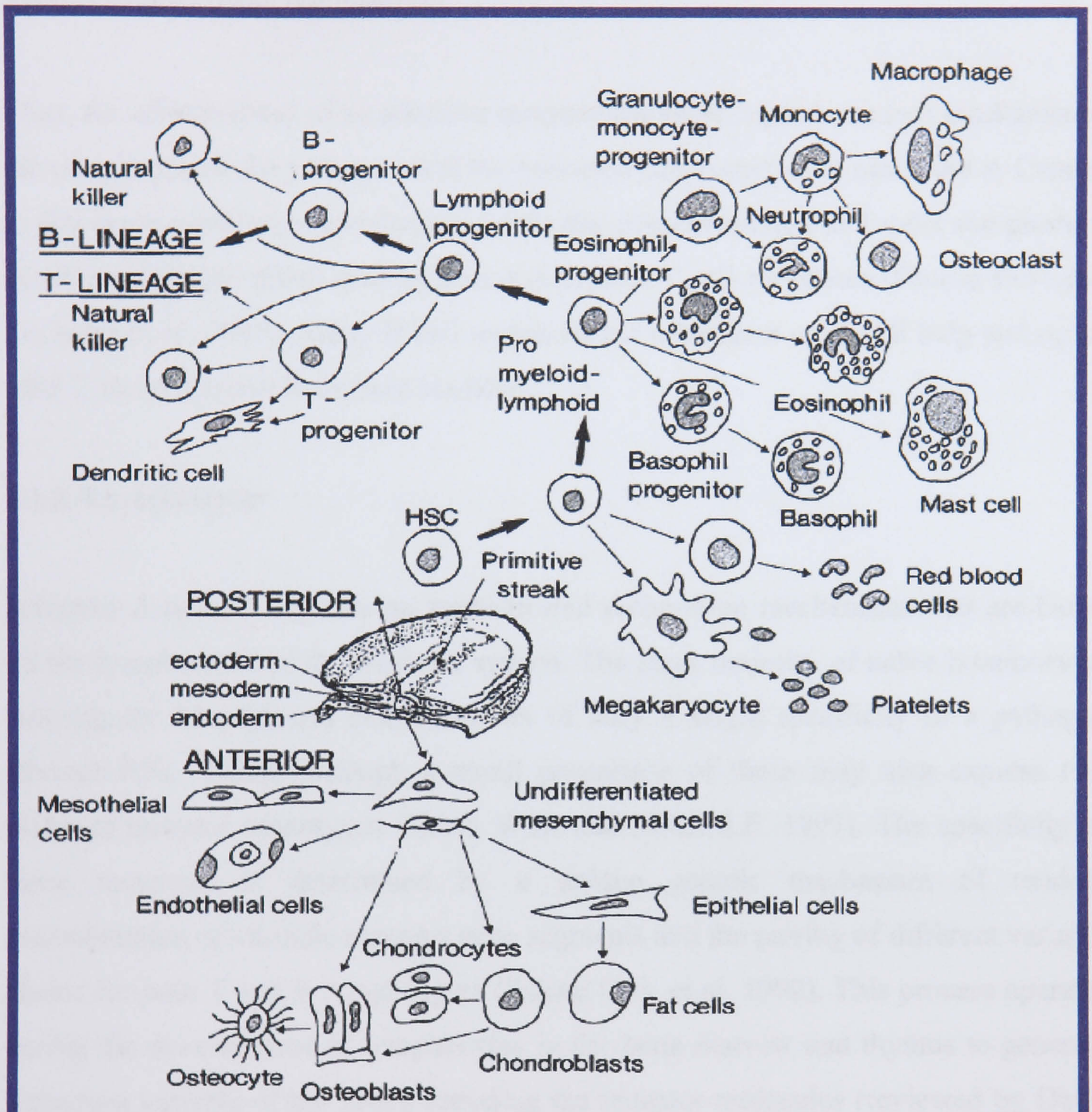


Figure 1.1: The development of the cells of the immune system. Intraembryonic mesoderm gives rise to pluripotent haematopoietic stem cells (HSC) and undifferentiated mesenchymal cells. B cells, T cells, monocytes/macrophages, granulocytes and dendritic cells are derived from the haematopoietic stem cells, whereas endothelial cells originate from mesenchymal stem cell precursors.(Borrowed from Melchers F. and Rolink A. 1999)

Lymphocytes interact with non-lymphoid cells in organized tissues known as central or peripheral lymphoid organs. The central lymphoid organs are the bone marrow and the thymus, and are involved in the maturation of T and B lymphocytes. After maturation, both cell types enter the circulation and migrate through the blood to the peripheral lymphoid organs that consist of the spleen, lymph nodes and mucosal

lymphoid tissues. Lymphocytes are continually recirculating through them, as is antigen trapped and carried there from all sites by antigen-presenting cells. In the presence of an infection, lymphocytes that recognize the infectious agent are arrested in the lymphoid tissue, where they proliferate and differentiate into effector cells capable of combating the infection.

Thus, the effectiveness of an adaptive immune response depends on two mechanisms: the recognition of the pathogen and the initiation of a response to eliminate it. Central to that are macrophages and dendritic cells that present antigen to T cells and produce cytokines that can direct and regulate subsequent T cell responses (Fearon D.T. and Locksley R.M. 1996). Many B cell responses are dependent on T cell help and occur after T cell responses have been initiated.

1.1.2. Lymphocytes

Adaptive immunity depends on sophisticated recognition mechanisms that are based on the lymphocytes of the immune system. The large majority of naïve lymphocytes entering the bloodstream bear receptors of only a single specificity of a pathogen (Burnet F.M. 1959), although a small proportion of them may also express two different receptor clonotypes (Heath W.R. and Miller J.F. 1993). The specificity of these receptors is determined by a unique genetic mechanism of random recombination of variable receptor gene segments and the pairing of different variable chains for both T and B lymphocytes (Schatz D.G. et al. 1992). This process operates during the development of lymphocytes in the bone marrow and thymus to generate numerous variants of the genes encoding the receptor molecules (reviewed by Davis M.M. and Bjorkman P.J. 1988). Therefore, although individual lymphocytes carry receptors of only one specificity, the specificity of each lymphocyte is different, and thus the millions of lymphocytes present in the body generate millions of different specificities. During the lifetime of an individual only those lymphocytes that encounter an antigen to which their receptor binds will be activated to proliferate and differentiate into effector cells. This is known as the clonal selection theory (Burnet F.M. 1959).

Activation of B lymphocytes

B cell activation induces the proliferation of mature B cells and their differentiation into antibody-secreting cells or into memory cells (DeFranco A.L. 1987). Under these circumstances, B cells encounter antigen for the first time and the production of antibody is termed a primary antibody response. If memory cells encounter antigen again, a secondary antibody response of greater magnitude takes place. This occurs more promptly, is composed of antibodies with higher affinity for the antigen, and is dominated by immunoglobulins expressing gamma, alpha, or epsilon C regions (IgG, IgA or IgE) rather than by IgM, which is the dominant immunoglobulin of the primary response. This process where a single H-chain V region becomes expressed with genes encoding C regions other than μ and δ is referred to as immunoglobulin isotype switching (Lorenz M. et al. 1995; Stavnezer J. 1996).

The differentiation of activated B cells into memory cells takes place in the specialized microenvironment of the germinal centers in the spleen and lymph nodes (Kelsoe G. 1996; MacLennan I.C.M. 1994). The increase in B cell antibody affinity also takes place in the germinal centers and depends on somatic hypermutation with antigen-mediated selection of cells that bear receptors with heightened avidity for the antigen (Han S.H. 1995; Wagner S.D. and Neuberger M.S. 1996). This process is known as affinity maturation.

B cells can be activated when they encounter an antigen that can be recognized by its cell surface immunoglobulin (B cell receptor). The activation process may be a direct one if the antigen can induce cross-linkage of membrane immunoglobulin antigens. This requires that the antigen expresses more than one copy of an epitope complementary to the binding site of the receptor. Such a requirement is fulfilled by polysaccharides of microorganisms, envelope proteins of viruses and some other antigens with repetitive epitopes (Feldmann M. et al. 1972; DeFranco A.L. 1987; Mond J.J. et al. 1995). However, most antigens do not have repetitive epitopes and are not capable of cross-linking surface immunoglobulins. The initiation of B cell responses in this case requires additional signals delivered by a specialized subset of T

cells, T helper cells, that recognize antigen on the surface of B cells and are thereby stimulated to deliver activating signals to them (DeFranco A.L. 1987).

Activation of T lymphocytes

T cells differ from B cells in their mechanism of antigen recognition. T cells recognize antigen displayed as peptides in the binding groove of MHC molecules present on the surface of other cells known as antigen-presenting cells and can not recognize antigenic determinants of intact, unprocessed molecules. T lymphocytes are activated and induced to proliferate in the lymphoid tissues (reviewed by Picker L.J. and Butcher E.C. 1996) where they can efficiently interact with phagocytic cells such as macrophages and B cells that serve the role of antigen-presenting cells (Liu Y. and Janeway C.A. Jr 1992; Rudd C.E. 1996; Steinman R.M. et al. 1993). The most important antigen-presenting cells, however, are the dendritic cells. Immature precursors of dendritic cells trap antigen in the periphery and migrate to the lymphoid tissues where they acquire a distinctive branched morphology to efficiently present antigen to T cells.

Normally, naïve recirculating lymphocytes migrate through the lymphoid tissue before returning to the bloodstream via the lymph. On recognizing its specific antigen, a lymphocyte stops migrating, becomes activated and within hours enlarges in size. Then, it starts to divide, normally duplicating two to four times every 24 hours for 3-5 days, so that each naïve lymphocyte gives rise to a clone of around 1000 progeny cells of identical specificity. These cells then differentiate into effector cells. T cells acquire the ability to kill infected cells and also to activate other cells of the immune system such as B cells to secrete antibody. When T lymphocytes become activated, changes in the expression of adhesion molecules are induced (van Seventer G.A. et al. 1991; MacKay C.R. 1992). T cells then stop circulating through the blood and lymph and instead migrate through the endothelial cells at the sites of infection, signalled by cytokines and chemokines released by inflammatory cells at these sites.

Effector cells have only a limited lifespan, and after eliminating the pathogen and removing the antigen, most of the antigen-specific T cells undergo apoptosis. A few, however, persist and continue to circulate in the blood. This ensures that a more rapid

and effective response will be generated on a second infection with the same pathogen, and therefore provides lasting immunity (Beverley P. 1996; Sprent J. and Tough D.F. 1994; Zinkernagel R.M. et al. 1996). This phenomenon, known as immunological memory, is the most important biological consequence of the development of adaptive immunity and forms the logical basis for vaccination (Beverley P.C. 1997).

1.1.3. Recognition and effector mechanisms of adaptive immunity

The mechanisms the immune system uses for the recognition and the destruction of the pathogen vary and depend on the particular characteristics of the pathogen. The surface immunoglobulins of B cells serve to recognize antigen outside cells whereas the T cell receptor of T cells is used to detect antigen which is inside cells.

Elimination of extracellular pathogens and toxins by antibodies

Antibodies are the first line of defense of the adaptive immune response and are found in the blood plasma and in extracellular fluids. Antibodies can protect from pathogens in various ways. First, they can bind and neutralize bacteria and their toxic products, preventing them from infecting cells and/or damaging them (Robbins F.C. and Robins J.B. 1986; Mandel B. 1976). Second, they can enable phagocytic cells to ingest and destroy the invading pathogen, especially in cases where the phagocytes cannot recognize it on their own (Gounni A.S. et al. 1994). Third, they activate a system of plasma proteins known as complement that can directly destroy bacteria and also enable phagocytes to engulf and destroy bacteria that they would not recognize otherwise (Tomlinson S. 1993). Different isotypes of antibodies engage a distinct set of effector mechanisms for eliminating the pathogen once it is recognized, as summarized in table 1.2.

Functional activity	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization	+	-	++	++	++	++	++	-
Opsonization	-	-	+++	-	++	+	+	-
Sensitization for NK cell killing	-	-	++	-	++	-	-	-
Sensitization of mast cells	-	-	-	-	-	-	-	++++
Activation of complement	++++	-	++	+	++	-	+	-

Table 1.2: Specialized functions of each human immunoglobulin (Ig) isotype. These are graded from dominant effector functions (++++) to major (+++), less major (++), minor (+) and non-existent (-) functions (adapted from Janeway C.A. Jr. and Travers P. 1997)

Elimination of intracellular pathogens and activation of B cell responses

Antibodies can detect pathogens in the blood and the extracellular fluid spaces. However, some bacterial pathogens and parasites and all viruses have evolved to replicate inside cells where they cannot be detected by antibodies. To eliminate these invaders, cell-mediated immune responses involving T cells are employed.

Central to the cell-mediated immune responses are $CD4^+$ T helper (T_H) cells that determine both the specificity and the mechanism of cell-mediated immunity. T_H cells develop into T_{H1} or T_{H2} subsets depending on the antigen presentation mechanisms (Mosmann T.R. and Coffman R.L. 1989; Constant S. et al. 1995; Pfeiffer C. et al. 1995; Wang L.F. et al. 1996; Rissoan M.C. et al. 1999) and the local patterns of cytokine and hormone expression involved in their activation. For example, if a pathogen triggers the release of IL-12 and IFNγ from macrophages and NK cells (Hsieh C.S. et al. 1993; Bendelac A. et al. 1995), the T_H subset that develops will be biased towards T_{H1}, whereas release of IL-4 and IL-10 will bias it towards T_{H2} (Paul W.E. and Seder R.A. 1994; Scott P. 1993).

These subsets of T_H cells represent different patterns of differentiation from the same precursors. In the mouse, it has been suggested that naive (T_{HP}) cells that have not been previously stimulated release only IL-2 but after short term stimulation they develop into T_{H0} cells and start releasing IFNγ, IL-2, IL-4, IL-5 and IL-10. After chronic stimulation, the specialized T_{H1} and T_{H2} subsets arise (Romagnani S. 1991).

T_{H1} cells release IL-2 and IFNγ, while T_{H2} cells release IL-4, IL-5, IL-6 and IL-10 (Kamogawa Y. et al. 1993; Mosmann T.R. and Sad S. 1996). Some cytokines such as IL-3, GM-CSF and TNFα are released by both cell types. More recently, a third class of helper T lymphocytes, T_{H3}, has been defined that has the profile of T_{H2} cytokine secretion but is distinguished by its ability to secrete TGF-β, a cytokine involved in the suppression of T_{H1} cells (Roncarolo M.G. and Levings M.K.2000; O'Garra A. et al. 1997).

These distinct patterns of cytokine secretion direct the response towards particular effector mechanisms. T_{H1} cells tend to activate macrophages to combat infections of intracellular bacteria. They stimulate antibacterial mechanisms of the phagocyte, including the fusion of macrophage lysosomes with vesicles containing intracellular bacteria, and at the same time they release cytokines that attract macrophages to the site of infection. T_{H2} cells, on the other hand, play a major part in the destruction of extracellular pathogens by activating B cells and increasing production of eosinophils and mast cells. T_{H3} cells are thought to play a suppressor role in immune responses and may be important in oral tolerance (reviewed by MacDonald T.T. 1998). Once established, each of these cytokine patterns is able to suppress the other: IFNγ from T_{H1} cells inhibits proliferation of T_{H2} cells, while IL-4 and IL-10 from T_{H2} cells inhibit cytokine secretion of T_{H1} cells (reviewed by Mitchison N.A. et al. 1999).

When the pathogen is a virus, the immune response depends on $CD8^+$ cytotoxic T cells (T_C) to clear the infection. T_C cells recognize body cells infected with viruses.and kill them before viral replication gets completed (Griffiths G.M. 1995). $CD8^+$ cytotoxic T cells are more abundant when T_{H1} activity is dominant, and are less prevalent when T_{H2} is dominant (Hamann D. et al. 1999). Nevertheless, there have been reports of T_c subtypes, with T_{C1} cells secreting IFNγ but not IL-4 or IL-5, and T_{C2} cells secreting IL-4 and IL-5 but not IFNγ (Croft M. et al. 1994; Carter L.L. and Dutton R.W. 1996). No clear differences, however, in the effector functions of these two subtypes have been defined.

All the effects of T lymphocytes, from their activation and proliferation to effector function, depend upon interactions with cells containing and presenting foreign

proteins. The molecules that display peptide antigen to T cells are membrane glycoproteins encoded in a cluster of genes known as the major histocompatibility complex (MHC). There are two types of MHC molecules, MHC class I and II that have some very important differences but share most of their major structural features (Bjorkman P.J. et al. 1987; Madden D.R. et al. 1992; Fremont D.H. et al. 1996; Madden D.R. 1995). The most important structural feature that they share is the outer extracellular domains that form a long cleft in which peptide fragments are trapped during the synthesis and assembly of the MHC molecule inside the cell (figure 1.2). The MHC molecule is then transported to the cell surface where it displays the bound peptide to T cells.

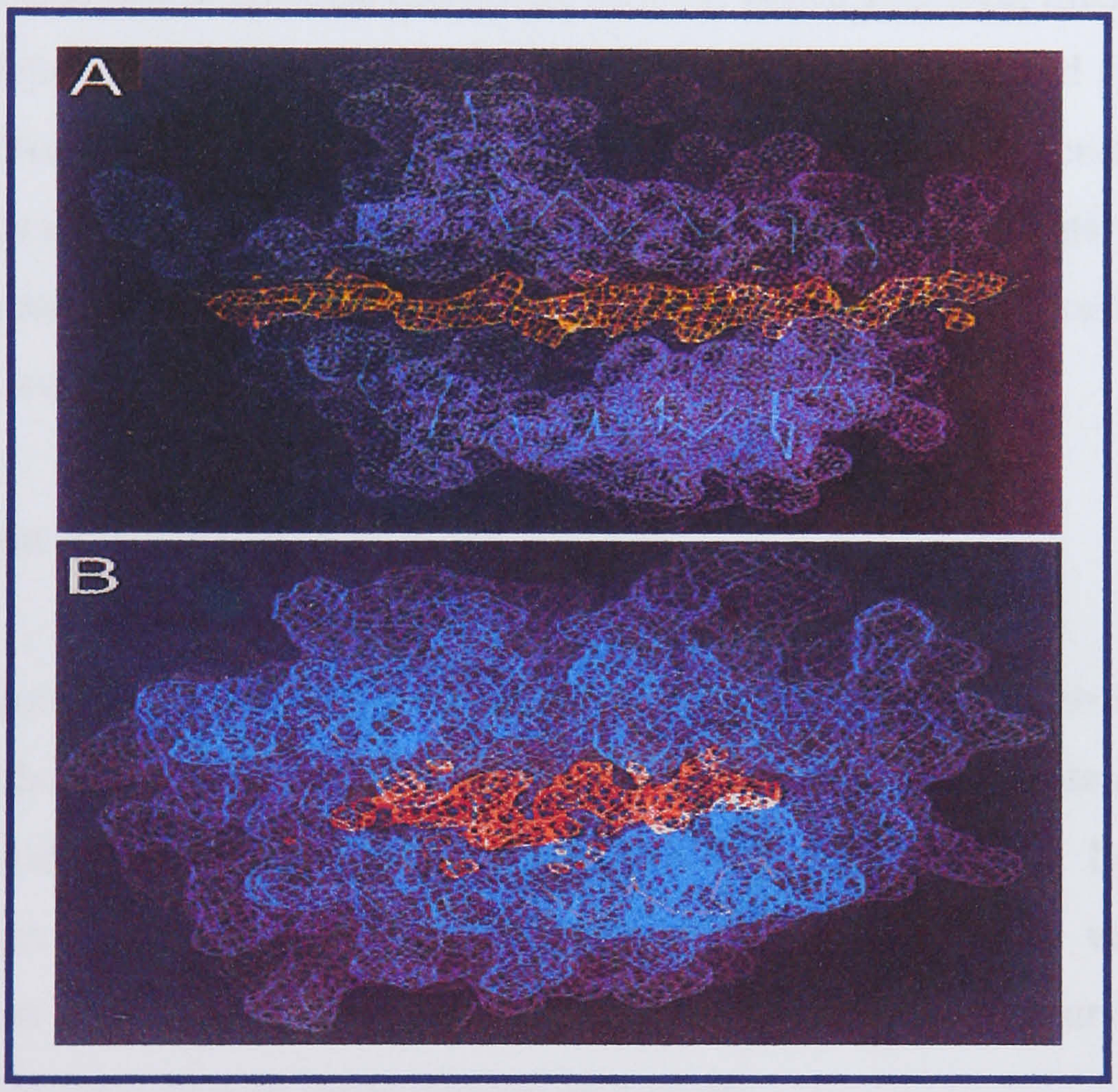


Figure 1.2: Electron density pictures of (A) the MHC class II and (B) the MHC class I binding clefts. With blue is the surface of the MHC molecule and with red is the bound peptide (Borrowed from Bjorkman P.J. et al. 1987 and Brown J.H. et al. 1993)

The most important difference between the two classes of MHC molecules is the source of the peptides that they trap and carry to the cell surface. MHC class I molecules collect peptides derived from proteins synthesized in the cytosol, and are thus able to display fragments of viral proteins on the cell surface (Morrison L.A. et al. 1986; Song R. and Harding C.V. 1996; York I.A. and Rock K.L. 1996). MHC class II molecules bind peptides derived from proteins in intracellular membrane-

bound vesicles, and can in that way display peptides derived from pathogens living in macrophage vesicles or internalized by phagocytic cells or B cells (Cresswell P. 1994; Konig R. et al. 1996). MHC class I molecules bearing viral peptides are recognized by cytotoxic T cells that kill the infected cell. MHC class II molecules bearing peptides from intracellular or phagocytosed bacteria are recognized by T_{H1} or T_{H2} cells.

1.1.4. Monocytes and macrophages

Macrophages are a family of mononuclear phagocytes that are widely distributed throughout the body, both inside and outside lymphohaematopoietic organs. They vary in lifespan and phenotype depending on their origin and local microenvironment, and their functions are mainly homeostatic, regulating the local and systemic milieu through diverse plasma membrane receptors and varied secretory products. Macrophages generate signals that influence the growth, differentiation and death of other cells and they recognize and engulf senescent and abnormal cells (reviewed in Todd R.F. and Schlossman S. 1984).

Growth and differentiation of macrophages

Macrophages originate from precursors in the yolk sac that migrate to fetal liver, then spleen and bone marrow. In the fetus, mature macrophages proliferate actively during tissue remodelling in developing organs. In the normal adult, however, tissue macrophages do not self-renew extensively, unless they are in a very specialized environment such as the lung or pituitary. Sometimes after injury, considerable replication at local sites of inflammation can occur.

Macrophages are ultimately derived from the pluripotent haematopoietic progenitor cell that through the myeloid progenitor cell gives rise to monocytes, dendritic cells and granulocytes. Monocytes are circulating cells that can differentiate, in turn, into macrophages, dendritic cells or osteoclasts. The whole growth and differentiation process is tightly regulated by specific growth factors such as IL-3, M-CSF, GM-CSF, IL-4 and IL-13 and growth inhibitors such as IFNα/β, TGFβ and LIF, and further modulated by interactions with adjacent stromal and other cells (fibroblasts,

endothelial cells and T cells) through c-kit-ligand (SCF), Ftl-3-ligand and other interactions. The growth ability of a macrophage precursor decreases progressively and markedly during differentiation from the stem cell to the committed precursors and then monocytes and macrophages. The main stages involved in macrophage differentiation are summarized in figure 1.3. Elicited macrophages respond more vigorously than resident macrophages to growth stimuli in vivo and in vitro but the molecular basis for that is not known.

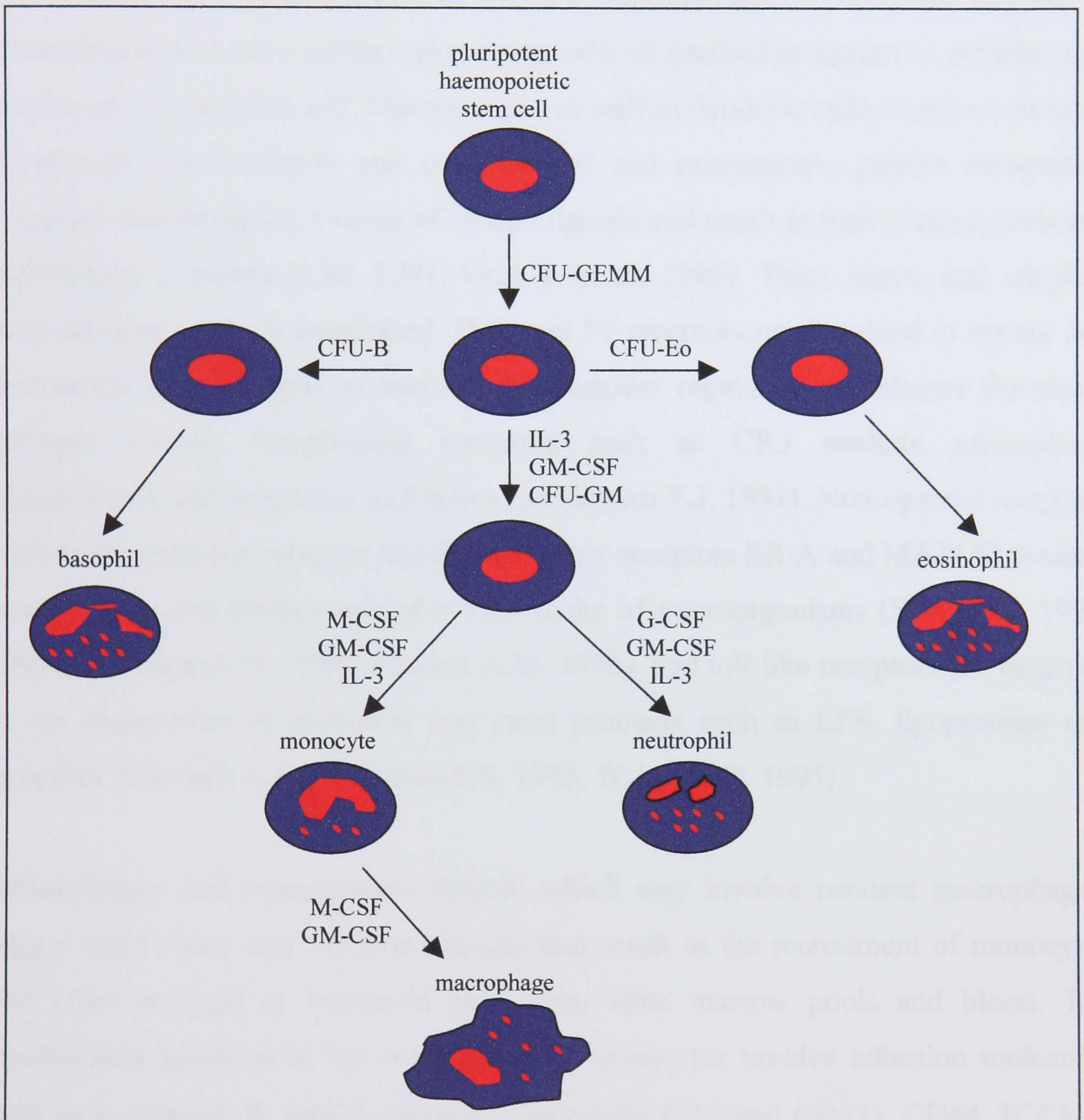


Figure 1.3: The growth and differentiation process of myeloid cells that gives rise to monocytes and macrophages. It requires M-CSF, GM-CSF, IL-3 and colony forming units (CFU).

Little is known about the factors that influence macrophage longevity and turnover. Growth factors such as M-CSF (CSF-1) enhance macrophage survival and prevent induction of apoptosis. Members of the TNF-TNF receptor family may also play a

role in determining macrophage survival, especially in induced macrophage populations. Tissue macrophages can vary greatly in life span, from days to months. Apart from inflammatory stimuli, local and systemic environmental factors such as hormones (estrogen) are known to influence macrophage turnover.

Macrophage activation and effector function

The initiation and localization of an immune response depend on the recognition by macrophages and other antigen presenting cells of particulate agents or proteins that are foreign or modified self. Macrophages as well as dendritic cells express a number of opsonic (i.e. antibody and complement) and non-opsonic pattern recognition receptors that recognize a range of related ligands and result in their phagocytosis and endocytosis (Yamada K.M. 1991; Ofek I. et al. 1995). Thus, innate and adaptive immune responses are interlinked. Different Fc receptors are involved in uptake and destruction of pathogens as well as the negative regulation of effector functions, whereas various complement receptors such as CR3 mediate macrophage phagocytosis, cell migration and activation (Brown E.J. 1991). Non-opsonic receptors such as the mannose receptor and the scavenger receptors SR-A and MARCO mediate phagocytosis and endocytosis of a wide range of microorganisms (Stahl P.D. 1992; Ofek I and Sharon N. 1988; Pearson A.M. 1996), and toll-like receptors are involved in the recognition of microbial and yeast products such as LPS, lipoproteins and zymosan (Ulevitch R.J. and Tobias P.S. 1995; Wright S.D. 1995).

Inflammatory and immunologic stimuli, which may involve resident macrophages, trigger local tissue and vascular changes that result in the recruitment of monocytes and other myeloid or lymphoid cells from bone marrow pools and blood. The mechanisms involved in the recruitment of monocytes involve adhesion molecules such as L-selectin, β_1 and β_2-integrins (especially CR3 and CD31), CD44, ICAM-1 and VCAM (Patarroyo M. 1994), cytokines such as TNFα, IFNγ, IL-12 and their receptors (Mantovani A. 1997), and chemokines such as MCP-1, MCP-3 and MIP-1α/β (Greaves D.R. and Schall T.J. 2000).

Some of the major effector functions of activated macrophages include antimicrobial antiviral and antineoplastic activity (Adams D.O. and Hamilton T.A. 1984).

Antimicrobial activity of macrophages uses reactive nitrogen and oxygen intermediates as well as oxygen-independent mechanisms (Knight J.A. 2000). High levels of nitric oxide inactivate Fe-S-containing enzymes and lead to metabolic failure and cell death of microbes (Weinberg E.D. 1984; Nathan C.F. and Hibbs J.B. Jr. 1991; MacMicking J. et al. 1997), whereas reactive oxygen intermediates mediate cell injury by disrupting the structure of proteins, lipids, and nucleic acids and by activating thiol groups in enzymes (Miller R.A. and Britigan B.E. 1997). Oxygen-independent antimicrobial mechanisms include growth inhibition by acidification of the phagosome, iron removal by iron-binding proteins, and digestion by lysozyme and lysosomal hydrolases (Payne S.M. 1993; Cornelissen C.N. and Sparling P.F. 1994; Adams D.O. and Hamilton T.A. 1984). Finally, macrophages are non-permissive for viral replication in them. This is very important for antiviral defenses as for a number of viruses, host susceptibility depends on their ability to replicate in macrophages and mononuclear phagocytes.

Macrophages have also been described to have antineoplastic effects. Large numbers of macrophages are commonly found within tumours and inflammatory macrophages but not resident macrophages, acquire competence to lyse neoplastic cells in a contact-dependent, non-phagocytic manner. It has been shown that IFNγ renders macrophages capable of selectively binding tumour cells and that an activation stimulus such as LPS induces full cytolytic competence. Factors secreted by macrophages in conjunction with factors secreted by other cells can prime macrophages for cytolytic competence. For example, IFNγ and TNFα are synergistic in the induction of tumouricidal activity whereas prostaglandin E stimulates tumouricidal activity in activated macrophages but depresses that of resident macrophages. Somatotropin is as effective as IFNγ in priming macrophages. The macrophage products hydrogen peroxide, nitric oxide and the complement components are also important in the cytolytic mechanisms of macrophages. To prevent macrophage anti-neoplastic action some tumours secrete substances such as TGF-β or IL-10 to suppress macrophage activation and growth.

Macrophages in immunoregulation

Macrophages are very important in immunoregulation as they secrete products such as cytokines and chemokines that influence and modulate the immune response. First, they produce IL-12 and IL-18 that direct naïve T cells towards the T_{H1} subtype and induce cell-mediated immunity (Scott P. 1993b; Fearon D.T. and Locksley R.M. 1996). IL-12 and IL-18, in turn, induce IFNγ production from T cells that activates macrophages further. This process can be blocked by the T_{H2} products IL-4, IL-10 and IL-13 that inhibit the generation of IL-12 by human monocytes and macrophages.

Second, macrophages secrete GM-CSF, TNFα and IL-1 that enhance dendritic cell antigen-presenting function by up-regulating the expression of MHC, CD80, CD86, CD40 and other costimulatory molecules. IL-10 produced by macrophages and T_{H2} cells down-regulates dendritic cell antigen-presentation and can block this enhancement of antigen presentation. IL-1 has multiple effects on T cells and especially T_{H0} and T_{H2}. It enhances IL-2 and IFNγ production, induces IL-2 receptors on T cells and augments cytotoxic T lymphocyte activity. TNFα also affects T cells. It augments T cell responsiveness to antigen directly or via stimulation of other cytokines such as IL-6, and its up-regulates the expression of the IL-2R. In combination with IL-2 and IL-6, TNFα can also induce T cell activation in the absence of T cell receptor engagement (Unutmaz D. et al. 1994; Sebbag M. et al. 1997). Chronic exposure, however, of T cells to TNFα, however, has been shown to down-regulate T cell responsiveness and proliferation, suggesting that a autoregulatory mechanism is taking place (Cope A. et al. 1994). TNFα affects macrophages too, by stimulating them to produce the pro-inflammatory cytokines IL-1 and IL-6, and the more anti-inflammatory prostaglandin E_2. IL-10 is a potent inhibitor of T cell activation and IL-2 production by both direct effects on T cells and indirect effects on antigen-presenting cells. Other macrophage products that suppress T cell activation include hydrogen peroxide, arginase, ROIs, nitric oxide and IL-1ra.

The importance of macrophages in orchestrating the immune response has been realized after studies on the mechanisms of chronic inflammation. It has been found that the macrophage product TNFα directs pro-inflammatory cytokine production

such as IL-1, IL-6 (Fong Y. et al. 1989; Brennan F.M. et al. 1989) and by neutralizing TNFα, remission of inflammatory and destructive processes could be observed (Elliott M.J. et al. 1994a; Elliott M.J. et al. 1994b). This mechanism was initially discovered in the rheumatoid synovium but has been shown since to be more universal and take place in Crohn's disease too (Van Dullemen H.M. et al. 1995). The investigation of the physiological triggers of macrophage factors such as TNFα, as well as the signalling pathways that lead to TNFα production have recently attracted great attention due to their therapeutic potential.

1.1.5. Dendritic cells

T and B lymphocytes are the mediators of adaptive immunity, but their function is under the direct or indirect control of DC, unique antigen-presenting cells in their ability to induce primary immune responses and immunological memory (Banchereau J. and Steinman R.M. 1998; Hart D.N. 1997). DC originate from the bone marrow and give rise to circulating precursors that home to tissues in an immature form characterized by high phagocytic but limited antigen-presenting capacity. In the presence of inflammatory stimuli, immature DC start maturing towards a state of decreased phagocytic ability. Their antigen-presenting ability increases and they migrate to lymphoid organs where they stimulate the rare antigen-specific T cells and, thus, initiate an immune response. DC present antigen to $CD4^+$ T helper cells, which in turn regulate $CD8^+$ cytotoxic T cells and B cells, as well as macrophages, eosinophils and natural killer cells (figure 1.4).

Dendritic cell development in mice

In mice, two distinct pathways of DC development have been clearly identified, the myeloid and the lymphoid. Support for this has come from mutant and knockout mice; RelB knockout mice are deficient in myeloid but not lymphoid DC, whereas mice carrying a mutant Ikaros gene that is important in lymphoid development are deficient in lymphoid DC (Burkly L. et al. 1995; Wu L. et al.1997; Weih F.E. et al. 1995). Myeloid DC can arise from myeloid-committed precursors cultured with GM-CSF (Inaba K. et al. 1992; Scheicher et al. 1992) or from monocytes cultured with

GM-CSF and IL-4 (Sallusto F. and Lanzavecchia A.J. 1994). Lymphoid DC originate from lymphoid-committed precursors and express CD8α (Saunders D et al. 1996; Steinman RM et al. 1997; Vremec D. et al. 1992; Wu L. et al. 1996; Shortmann K et al. 1998). Although only $CD8\alpha^+$ DC have been shown to be of lymphoid origin, thymic DC are very similar in phenotype to $CD8\alpha^+$ splenic and lymph node DC suggesting that they may be of lymphoid origin too (Vremec D. and Shortman K. 1997).

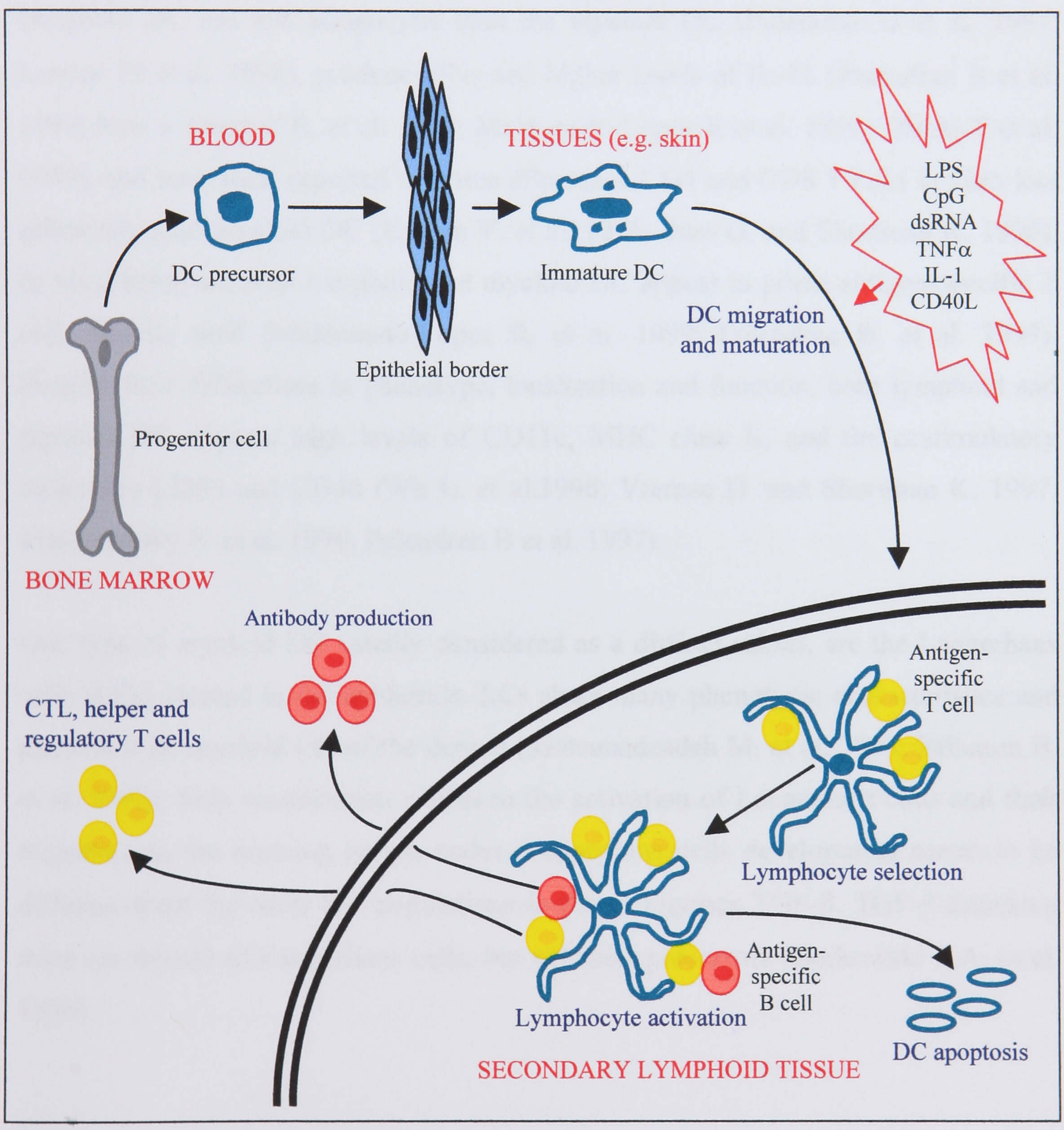


Figure 1.4: The life cycle of dendritic cells. Bone marrow-derived DC precursors enter tissues as immature DC. In tissues, they continuously capture and process antigen till they encounter an inflammatory stimulus that triggers their activation, migration to the regional lymph nodes and maturation. Mature DC present antigen with increased efficiency to antigen-specific T cells which proliferate and activate B cells. Thus, effector immune responses involving helper, cytotoxic (CTL) and regulatory T cells, as well as antibody-secreting B cells are initiated.

Lymphoid DC are localized in the T cell-rich areas of the periarteriolar lymphatic sheaths (PALS) in the spleen and lymph nodes. Myeloid DC, on the other hand, are localized in the marginal zone bridging channels of the spleen (Steinman R.M. et al. 1997; Pulendran B et al. 1997; De Smedt T et al.1996; Leenen P.J. et al. 1998) but can also migrate to the PALS under the influence of pro-inflammatory signals such as LPS (De Smedt T. et al.1996) or parasite extracts (Reis e Souza C.R. et al. 1997). The different distribution of the two DC subsets suggests different functions, as well. The lymphoid DC are less phagocytic than the myeloid DC (Pulendran B et al. 1997; Leenen PJ et al. 1998), produce IFNγ and higher levels of IL-12 (Pulendran B et al. 1997; Reis e Souza C.R. et al. 1997; Maldonado-Lopez R et al. 1999; Ohteki T et al. 1999), and have been reported to prime allogeneic CD4 and CD8 T cells in vitro less efficiently than myeloid DC (Kronin V. et al. 1996; Suss G. and Shortman K. 1996). In vivo, however, both lymphoid and myeloid DC appear to prime antigen-specific T cells equally well (Maldonado-Lopez R. et al. 1999; Pulendran B. et al. 1999). Despite their differences in phenotype, localization and function, both lymphoid and myeloid DC express high levels of CD11c, MHC class II, and the costimulatory molecules CD86 and CD40 (Wu L. et al.1996; Vremec D. and Shortman K. 1997; Maraskovsky E. et al. 1996; Pulendran B et al. 1997).

One type of myeloid DC usually considered as a distinct subset, are the Langerhans cells (LCs) located in the epidermis. LCs share many phenotypic characteristics and markers with myeloid DC of the dermis (Mohamadzadeh M. et al. 1993; Salomon B. et al. 1998). Skin sensitization results in the activation of Langerhans cells and their migration in the draining lymph nodes. Langerhans cells development seems to be different from the other DC populations in that it requires TGF-β. TGF-β knockout mice are devoid of Langerhans cells, but not their precursors (Borkowski T.A. et al. 1996).

Dendritic cell development in humans

Human DC are very heterogenic in many aspects including their lineage origins, localization, maturation stages and functional differences. They are localized in the skin epidermis and dermis (Langerhans and dermal/interstitial DC), the marginal zones of the spleen (splenic marginal DC), the T cell areas of the lymph nodes (T-zone interdigitating DC), the germinal centers of the lymph nodes (germinal-center DC), the thymus (thymic DC), the liver (liver DC) and the blood (blood DC).

In humans, similarly to mice, DC originate from myeloid and lymphoid precursors (Galy A. et al. 1995; Res P. et al. 1996) (figure 1.5). Immature myeloid DC can be derived from $CD11c^+$ $CD14^+$ monocytes (Romani N et al. 1994; Sallusto F. and Lanzavecchia A. 1994; Zhou L.J. and Tedder T.F. 1996), as well as lineage-negative (LIN^{neg}) $CD11c^+$ blood DC (Ito T. et al. 1999) under the influence of GM-CSF and IL-4 or TNFα. In the presence of TGF-β (Ito T. et al. 1999), $CD11c^+$ blood DC differentiate into Langerhans cells. Myeloid DC resembling Langerhans and dermal DC can also be derived from $CD34^+$ haematopoietic progenitors under the influence of GM-CSF, IL-4, TNFα and TGF-β as before (Caux C. et al. 1996; Caux C. et al. 1992; Caux C. et al. 1997).

Distinct factors regulate the survival and differentiation of $CD11^-$ DC precursors, originally described as plasmacytoid monocytes (Grouard G. et al. 1997; Olweus J. et al. 1997; Cella M. et al. 1999a; Palucka K. and J.Banchereau. 1999; Siegal F.P. et al. 1999). These cells require IL-3 but not GM-CSF for survival, otherwise they die rapidly after isolation. They obtain a mature DC phenotype after CD40L activation. $CD11^-$ DC precursors have been found in adult blood, express the pre-T cell receptor and contain precursors of mature $CD4^+$ $TCR\alpha/\beta^+$ T cells (Bruno L et al. 1997).

Monocytes and $CD11c^-$ $IL\text{-}3R\alpha^+$ plasmacytoid DC precursors display many phenotypic differences. Monocytes express significant levels of CD11b, CD13, CD14, CD33 and CD45RO, whereas plasmacytoid precursors express low GM-CSFRα and high IL-3α consistent with their in vitro cytokine responsiveness. In addition,

immature monocyte-derived DC, but not immature CD11c$^-$, DC have high endocytic/phagocytic capacity.

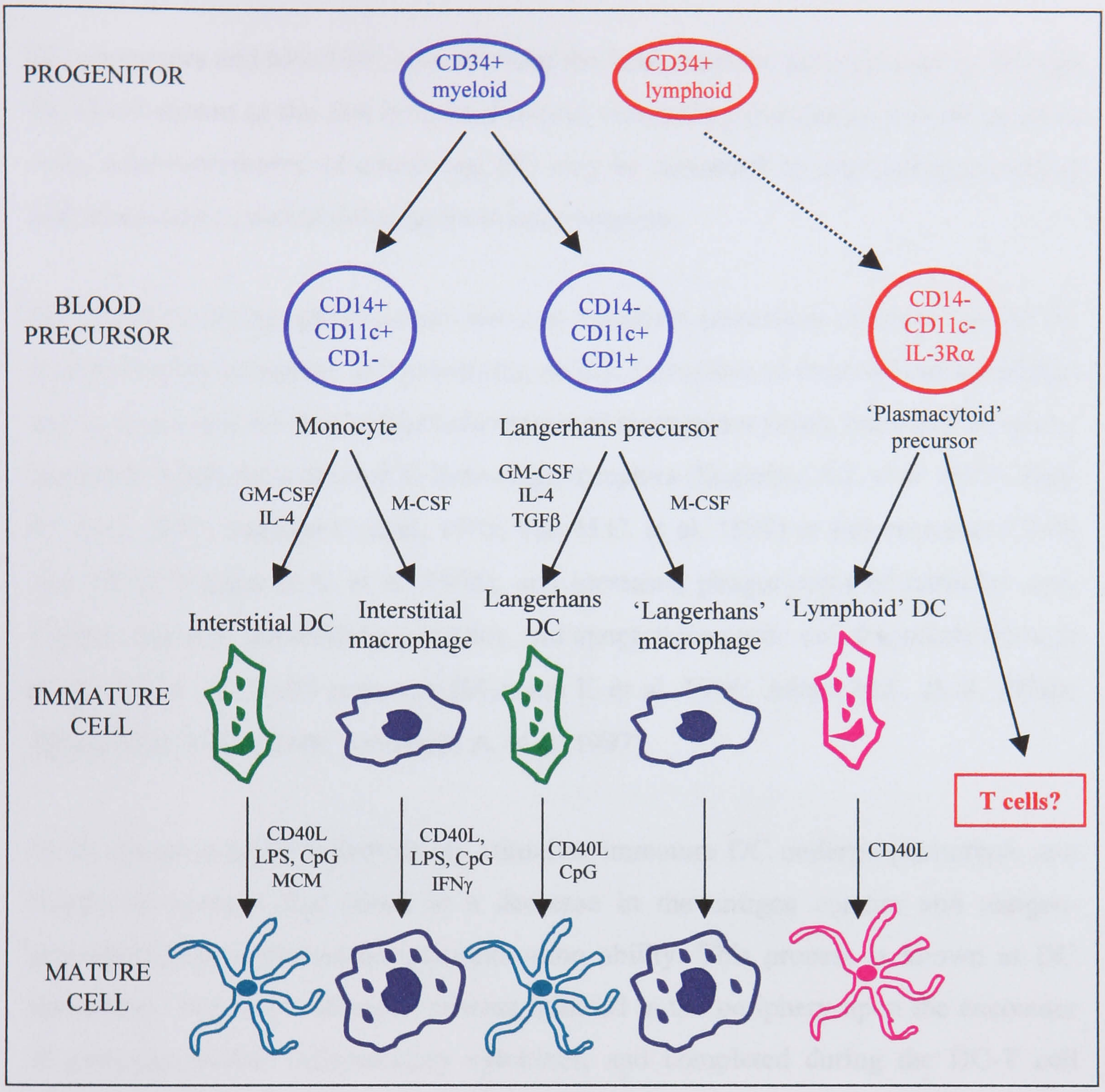


Figure 1.5: Subsets of human dendritic cells and related macrophages. GM-CSF and IL-4 result in the devepoment of dendritic cells from myeloid progecitors, whereas M-CSF induces macrophage differentiation. IL-3, on the other hand, is required for the generation of dendritic cells from lymphoid progenitors (Adapted from Banchereau J. et al. 2000).

There have been suggestions that DC and macrophages may interconvert in vivo as culture of monocytes and CD11c$^+$ DC with GM-CSF or M-CSF in vitro gives rise to macrophages (Caux C. et al.1996; Palucka K.A. et al. 1998). Moreover, IL-10 has been shown to convert human DC infected with M.tuberculosis to a macrophage-like phenotype with down-regulated antigen-presenting molecules and increased antimicrobial activity (Fortsch D. et al. 2000).

Antigen-presentation and T cell activation

DC precursors and blood DC migrate from the bone marrow, and presumably through the blood stream to the non-lymphoid tissues where they eventually become resident cells. This recruitment of circulating DC may be increased in response to cytokines and chemokines produced during local inflammation.

DC found in non-lymphoid tissues have an immature phenotype characterized by an increased antigen-capture and processing ability. A number of mechanisms have been shown to account for that and include increased macropinocytosis, increased receptor-mediated endocytosis through C-lectin-type receptors (Engering A.J. et al. 1997; Jiang W. et al. 1995; Sallusto F. et al. 1995; Tan M.C. et al. 1997) or Fcγ receptors CD64 and CD32 (Fanger N.A. et al. 1996), and increased phagocytosis of particles such viruses, bacteria, intracellular parasites, and apoptotic/necrotic cell fragments through CD36, αvβ3 and αvβ5 receptors (Matsuno K et al. 1996; Albert M.L. et al. 1998a; Albert M.L. et al. 1998b; Rubartelli A. et al. 1997).

In the presence of an inflammatory stimulus, immature DC undergo phenotypic and functional changes that result in a decrease in the antigen capture and antigen-processing, but increased antigen-presenting ability. This process is known as DC maturation, and is a continuous process initiated in the periphery upon the encounter of pathogen and/or inflammatory cytokines, and completed during the DC-T cell interaction in the lymph nodes. Several molecules such as LPS (Rescigno M. et al. 1999), bacterial DNA (Akbari O. et al. 1999; Hacker H et al. 1998; Hartmann G. et al. 1999), double-stranded RNA (Cella M et al. 1999), the pro-inflammatory cytokines TNFα and IL-1 (Sallusto F. et al. 1995), and the engagement of CD40 (Cella M. et al. 1996; Koch F. et al. 1996) and TRANCE receptor (Josien R. et al. 1999) receptors have been shown to trigger DC maturation. This is regulated by pro-inflammatory and anti-inflammatory signals in the local microenvironment such as IL-10, TGF-β and prostaglandins.

Maturation of DC is accompanied by the loss of endocytic and phagocytic receptors, the up-regulation of costimulatory molecules such as CD40, CD58, CD80, CD86 and MHC class I and II, the down-regulation of CD68 and up-regulation of DC-lysosome-associated membrane protein (DC-LAMP) and various morphological changes (reviewed by Cella M. et al. 1997). Morphological changes include the loss of adhesive structures, the cytoskeleton reorganization, and the acquisition of high cellular motility (Winzler C. et al. 1997). An actin-binding protein, p55 fascin, is expressed in high levels in blood DC and interdigitating DC located in the T cell areas of the lymph nodes, and is required for dendritic projections in maturing Langerhans cells (Ross R. et al. 1998).

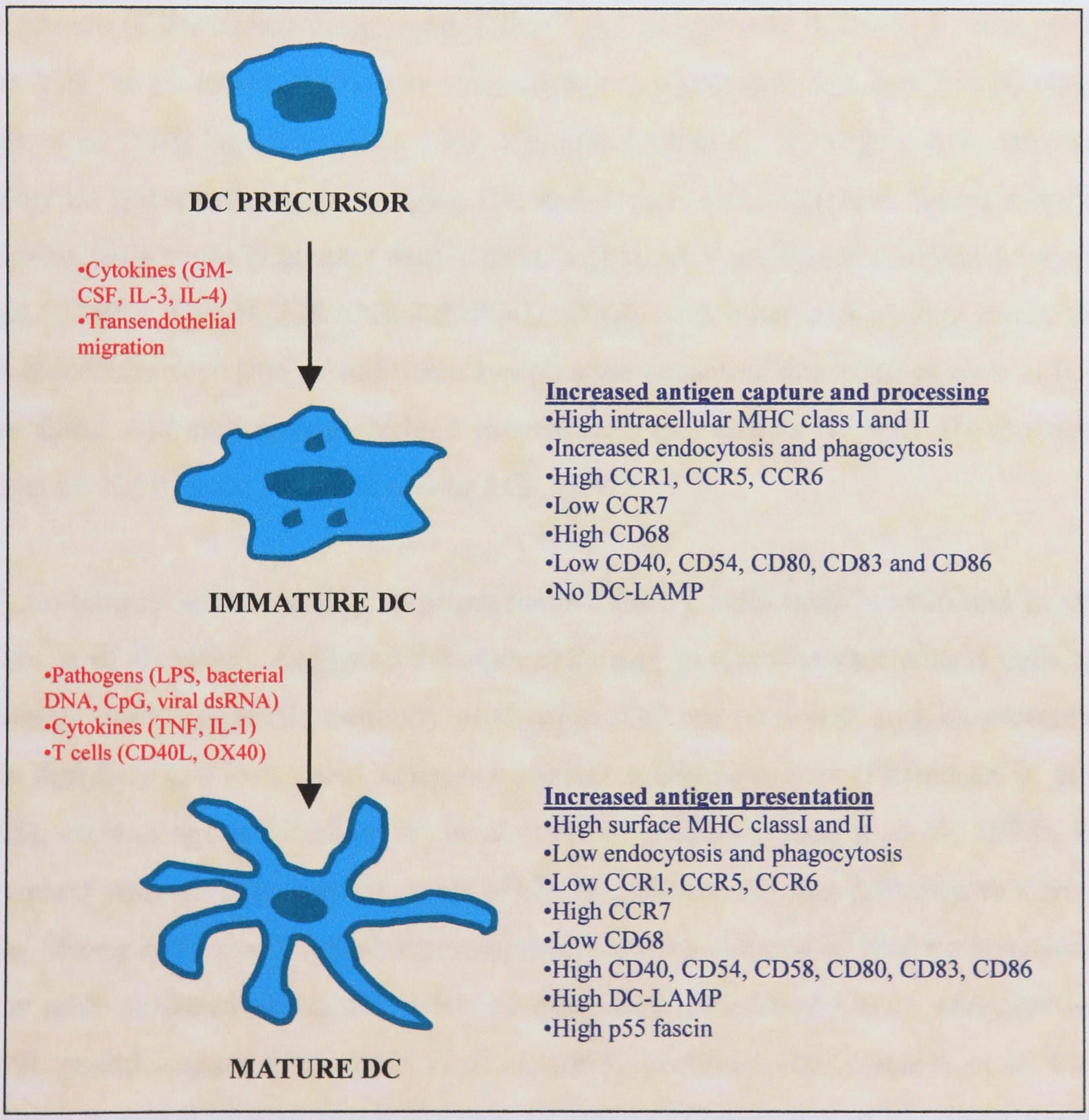


Figure 1.6: Phenotypic changes triggered by dendritic cell maturation (Adapted from Banchereau J. et al. 2000).

At the same time, maturation triggers the down-regulation or desensitization of MIP-3α receptors, and the up-regulation of CCR7 and 6Ckine (Dieu M.C. et al. 1998; Sallusto F. et al. 1998; Sozzani S. et al. 1999; Yoshida R. et al. 1997; Chan V.W. et al. 1999). This prevents MIP-3α from positioning immature DC to non-lymphoid tissues (Dieu M.C. et al. 1998; Power C.A. et al. 1997), and allows MIP-3β and SLC to attract mature DC towards the lymph stream (Gunn M.D. et al. 1998; Saeki H. et al. 1999). When mature DC enter the draining lymph nodes, MIP-3β and 6Ckine produced by the T cell zone, result in their localization into the paracortical areas (Dieu M.C. et al. 1998; Ngo V.N. et al. 1998). The newly arriving DC may then become themselves a source of MIP-3β and 6Ckine, resulting in the amplification and persistence of the chemotactic signal (Dieu M.C. et al. 1998; Sallusto F. et al. 1998; Ngo V.N. et al. 1998). These two chemokines can also attract naïve T cells (Gunn M.D. et al. 1998; Ngo V.N. et al. 1998; Campbell J.J. et al. 1998) and, thus, are likely to play an important role in bringing DC and T cells close together. When maturing dendritic cells come in contact with T cells, they receive additional maturation signals from CD40L, TRANCE/RANK-L/OPG-L, OX40 and other cell-surface molecules, that induce the secretion of additional lymphocyte-attracting chemokines such as IL-8, fractalkine, and macrophage-derived chemokiness (Kanazawa N. et al. 1999; Adema G.J. et al. 1997; Tang H.L. and Cyster J.G.. 1999).

DC are unique in their ability to prime naïve $CD4^+$ T cells both in vitro and in vivo (Inaba K et al. 1990). Activated T helper cells can, in turn, interact with B cells and stimulate antigen-specific antibody production. DC are so potent antigen-presenting cells that they can even break tolerance against soluble antigens (Pulendran B. et al. 1998), viral antigens (Shimizu Y. et al. 1998), tumours (Gong J. et al. 1998), and transplant antigens (Steptoe R.J. et al. 1997). In addition, DC can prime naïve $CD8^+$ T cells. Strong cytotoxic T lymphocyte responses can be induced in vivo by injection of mice with antigen-bearing DC such as allogeneic (McKinney E.C. and Streilein. 1989), peptide-pulsed (Takahashi H. et al. 1993), protein-loaded (Nair S. et al. 1993; Brossart P. and Bevan M.J. 1997), DNA-transfected (Rouse R.J. et al. 1994), virally-transfected (Specht J.M. et al. 1997) or RNA-pulsed DC (Ashley D.M. et al. 1997). Although DC can activate $CD8^+$ T cells directly (Young J.W. and Steinman R.M. 1990; Bhardwaj N. et al. 1994; Bohm W. et al. 1995), $CD4^+$ T cell help is often

needed. DC have also been shown to be required for naïve CD4 T cells survival (Brocker T. 1997) and immune T cell memory (Ludewig B. et al. 1999a).

Regulation of T cell-mediated immunity

In mice, as in humans, DC functions are distinct and dependent on their location, cytokine microenvironment and mouse strain. Splenic $CD8\alpha^+$ lymphoid DC prime naïve CD4 T cells to produce T_{H1} cytokines, whereas splenic $CD8\alpha^-$ myeloid DC prime naïve CD4 T cells to produce T_{H2} cytokines (27, 31). This may depend on their ability to produce IL-12 as IL-12-deficient mice fail to induce T_{H1} responses (Magram J. et al. 1996). Lymphoid DC can be induced to make large amounts of IL-12 (Pulendran B. et al. 1997; Reis e Sousa C.R. et al. 1997; Maldonado-Lopez R. et al. 1999; Ohteki T. et al. 1999) and IFNγ (Ohteki T. et al. 1999), whereas myeloid dendritic cells do not make IL-12 and may induce T_{H2} cytokine profiles through through the release of IL-13 (McKenzie G.J. et al. 1998) and IL-6 (Rincon M. et al. 1997), and the expression of OX40L (Flynn S et al. 1998). CD80 and CD86 may also be involved in T cell polarization as there is evidence that CD80 promotes T_{H1} responses whereas CD86 promotes T_{H2} responses (Freeman G.J. et al. 1995; Kuchroo V.K. et al. 1995).

Mature Langerhans and dermal DC, on the other hand, are equally potent in stimulating the proliferation of naïve T cells, but only dermal DC can induce the IL-2-driven differentiation of naïve B cells in vitro (Caux C et al. 1997). Moreover, and although both subsets express IL-12 after CD40 ligation, only dermal DC express IL-10 (de Saint-Vis B. et al. 1998). Dermal DC have a 10-fold higher efficiency in antigen capture than Langerhans cells. Langerhans cells are poor stimulators of antigen-specific $CD4^+$ T cell clones (Mommaas A.M. et al. 1999) but more potent $CD8^+$ T cell stimulators than monocyte-derived DC (Mortarini R. et al. 1997).

In humans, monocyte-derived $CD11c^+$ DC have been reported to polarize naïve T cells towards a T_{H1} phenotype whereas $CD11c^-$ plasmacytoid DC polarize towards a T_{H2} phenotype (Rissoan M.C. et al. 1999), but this may be dependent on the differentiation/maturation stage of DC. Thus, immature $CD11c^-$ DC precursors may

induce T_{H0} subsets and may need to mature to induce T_{H2} responses (Rissoan M.C. et al. 1999). Soluble factors that affect DC differentiation such as IL-10, TGF-β, steroids (Piemonti L. et al. 1999) or prostaglandin E2 (Buelens C. et al. 1995; Caux C. et al. 1994; De Smedt T. et al. 1997; Kalinski P. et al.1998; Liu L et al. 1998a) may also convert DC that normally induce T_{H1} cells to induce T_{H2} cells. The induction of a T_{H1} or T_{H2} profile depends principally on the production of IL-12 (Macatonia S.E. et al. 1995). $CD11c^+$ but not $CD11c^-$ DC can secrete IL-12 upon activation. The influence of distinct DC subsets to T cell cytokine profiles does not only apply to CD4 T cells but also to CD8 T cells and NK T cells (Liu Y.J. et al. 2000).

T cell tolerance

DC are also very important in tolerance. Thymic DC are capable of mediating T cell negative selection in fetal organ cultures in vitro (Matzinger P. and Guerder S. 1989) and against superantigens in vivo (Inaba M. et al. 1991; Mazda O. et al. 1991). Thymic DC have been shown to induce tolerance to myelin basic protein and prevent the development of experimental autoimmune encephalomyelitis (Khoury S.J. et al. 1995). But although the role of DC in negative selection is well demonstrated (Brocker T. et al. 1997a), their role in peripheral tolerance still needs confirmation. The strongest evidence comes from studies showing that DC can abrogate tolerance against soluble antigens (Pulendran B. et al. 1998), viral antigens (Shimizu Y. et al. 1998), tumours (Gong J. et al. 1998), transplant antigens (Steptoe R.J. et al. 1997) and neonates (Ridge J.P. et al. 1996).

Regulation of B cells and humoral immunity

B cells proliferate, undergo somatic hypermutation, isotype switching, affinity selection, and differentiation into memory B cells or plasmablasts in the germinal centers of the spleen and the lymph nodes. Germinal centres (GC) also contain T cells, follicular DC (FDC) and germinal center DC (GCDC). GCDC are clearly distinct from FDC can activate B cells directly by inducing a strong expansion of GC B cells and skewing their differentiation into plasmablasts in the presence of CD40 activation and cytokines such as IL-2 or IL-15 (Dubois B. et al. 1999). *In vitro*, blood

DC, $CD4^+CD11c^+$ DC or monocyte-derived DC also shared the capacity to trigger GC B cell proliferation, whereas Langerhans cells or the *in vitro*-related CD1a-intermediate derived DC displayed little effect (Dubois B. et al. 1999). These results together with other recent studies (Dubois B. et al. 1997; Fayette J. et al. 1997; Dubois B. et al. 1998) suggest that DC may directly regulate B cell activation during the course of the humoral response.

1.1.6. Cytokines in the immune response

Cytokines

Cytokines are small soluble proteins or glycoproteins that act as local messengers in all important biological processes including cell growth, development, repair, fibrosis, inflammation and immunity. They are most frequently produced by leukocytes although they can be produced by cells of any type, and they signal by binding to their respective cell surface cytokine receptors (Oppenheim J.J. and Feldmann M. 2000).

Cytokines can be divided into families on the basis of their similarity in amino acid sequence, and most importantly in three-dimensional structure. Thus, the haematopoietin family comprises cytokines that form four α helices while members of the TNF family form a jelly roll motif. Other important families are the IL-1 family, the IFNα/β, the PDGF and the TGFβ families. The largest family is probably the chemokine family which is itself subdivided into two major groups, the C-X-C and C-C chemokine families (table 1.3).

Most cytokines are single polypeptide chain proteins, but some homodimers (IFNγ, IL-10, TGFβ) or homotrimers (TNFα and other members of the TNF family) also exist (reviewed in Feldmann M. et al. 1996). Only IL-12 is a heterodimer. Although cytokines were originally considered to be secreted, membrane-bound forms have now been documented for TNFα, IL-1, IFNγ, FGF, TGF-α, TGF-β, M-CSF and SCF. The cell surface forms of these proteins are capable of signalling via their appropriate receptor.

Family	Cytokines
Haematopoietins	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-11, IL-12, IL-13, IL-15, EPO, LIF, GM-CSF, G-CSF, Onco M, CNTF, GH and tpo
TNF family	TNFα, LTα, LTβ, CD40L, CD30L, CD27L, 4-1BBL, OX40, OPG and FasL
IL-1	IL-1α, IL-1β, IL-1ra, IL-18, bFGF, aFGF and ECGF
PDGF	PDGF A, PDGF B, M-CSF
TGFβ	TGFβ, BMPs (1,2,4 etc)
C-X-C chemokines	IL-8, gro α/β/γ, NAP-2, ENA 78, GCP-2, PF4, CTAP-3, Mig, γIP-10
C-C chemokines	MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β, RANTES

Table 1.3: Cytokine families grouped by structural similarity (adapted from Feldmann M. et al. 1996)

Cytokine receptors

Cytokines interact with cells through specific cell surface receptors of high affinity, the density of which is usually between 10 to 10000 sites/cell. On the basis of their structural similarity, cytokine receptors can also be subdivided into families. Thus, the largest is the haematopoietin receptor family that is characterized by an extracellular region composed of one or more domains containing four conserved cysteines and a tryptophan-serine-X-tryptophan-serine amino acid motif in the membrane proximal domain. Others include the immunoglobulin receptor family that has immunoglobulin-like motifs, the TNF receptor family that contains cysteine-rich domains, the IFN receptor family, the TGFβ receptor family, and the chemokine receptor that is characterized by the seven transmembrane segments structure common to the rhodopsin receptor family.

However, it is now becoming apparent that in most instances more than one polypeptide is needed to constitute a functional receptor. The IL-2 receptor, for example consists of three different polypeptide chains whereas the IL-6 receptor of two. In many cases, there are chains common to several receptors such as the IL-2Rγ chain that also associates with the IL-4, IL7, IL-9 and IL-15 receptors, the gp130

chain that associates with IL-6, CNTF, oncostatin M, LIF, IL-11 and cardiotropin-1 receptors, and the β chain of the IL-3 receptor that associates with the GM-CSF and IL-5 receptors too. The TNF receptor, on the other hand, consists of trimers of the same chain. In addition to cell surface receptors, soluble receptors corresponding to the ligand-binding domains of cytokine receptors have been described. Some soluble receptors may act as inhibitors of cytokines (Heaney M.L. 1998).

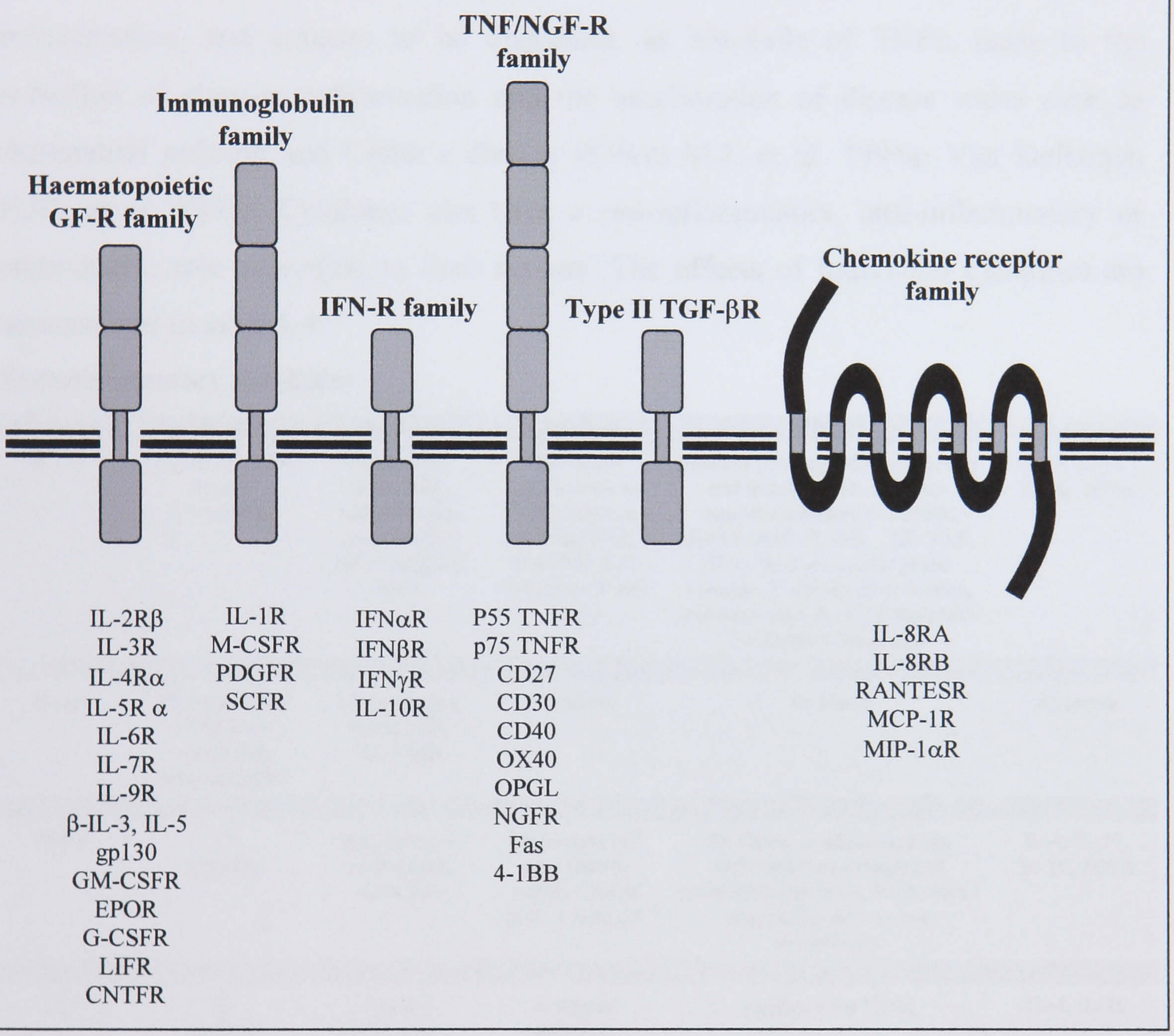


Figure 1.7: Cytokine receptors can be grouped broadly into six families based on their molecular structure (adapted from Feldmann M. et al. 1996)

Properties of cytokines

Cytokines form a network by either inducing or suppressing the production of either cytokines (Dinarello C.A. et al. 1986; Balkwill F.R. and Burke F. 1989). Cells of one type appear to communicate with cells of another type through the production of

cytokines. For instance, the cytokine products IL-1 and IL-6 of the antigen-presenting cell can signal to a T cell to help produce IL-2, which may then act on both itself or neighbouring T cells to proliferate and produce other cytokines such as IFNγ and GM-CSF. These latter proteins may then feedback to upregulate the activities of the antigen-presenting cell, thus illustrating how cytokines can act in networks. Similarly, TNFα can induce IL-1, both of which can in turn induce IL-6 and GM-CSF (Dinarello C.A. et al. 1986; Brennan F.M. et al. 1989). This network is seen in inflammation, and appears to be important, as blockade of TNFα leads to the reduction of chronic inflammation and the amelioration of disease states such as rheumatoid arthritis and Crohn's disease (Elliott M.J. et al. 1994a; Van Dullemen H.M. et al. 1995). Cytokines can have a pro-inflammatory, anti-inflammatory or chemotactic role according to their actions. The effects of individual cytokines are summarized in table 1.4.

Proinflammatory cytokines

Cytokine	MW (KDa)	Sources	Inducers	Effects	Inhibited
IL-1α	33 (membrane-bound) 17 (soluble)	Most cells, especially macrophages, keratonocytes and endothelial cells	Microbial agents such as LPS, injurous agents, TNF, GM-CSF, IL-1, substance P and IFNγ	Induces prostaglandin production and metalloproteinases, up-regulates adhesion molecules, cytokines (IL-2, IFN, CSF, TNF, IL-6, IL-8 etc), acute phase proteins, T and B cell activation, and stimulates the Hypothalamic-Pituitary-Adrenal axis	IL-4, IL-13, IL-10, TGFβ
IL-1β	33 processed to 17 by IL-1-converting enzyme (ICE)	Macrophages, neutrophils, astrocytes	As above	As above	As above
TNFα	17 trimeric	Macrophages, neutrophils, astrocytes	Depend on cell type but are mostly similar to IL-1 inducers	As above. In addition, it up-regulates macrophage and neutrophil activation, HLA class I expression and tumour cytotoxicity.	IL-4, IL-13, IL-10, TGFβ
LT	25 trimeric	T and B lymphocytes, NK cells	Antigens, mitogens	As above for TNFα	IL-4, IL-10
IL-6	20	Macrophages, fibroblasts, B and T cells	Like IL-1 and TNFα	B cell growth, Ig production, acute phase proteins, stem cell and NK cell activation, platelet production, mesanglial cell proliferation, osteolysis, IFN production	As above
IFNγ	20-25 dimer	CD4 and CD8 T cells, CD4 T_{H1} cells, NK cells	T cell activation, IL-12, IL-18, IL-2	Induces class I and II. Activates monocytes, primes for cytokine production and up-regulates cell surface receptors. Involved in CTL differentiation, blocks T_{H2} activity and promotes the DTH. It inhibits B cell proliferation and IgE production. Activates neutrophils and NK cells.	IL-10, TGFβ

Anti-inflammatory cytokines

Cytokine	MW (kDa)	Sources	Inducers	Effects
IL-10	18 dimer	Macrophages, T and B cells, keratinocytes	LPS for macrophages, others for T cell activation	Inhibits macrophage cytokine production (IL-1, IL-6, TNFα, GM-CSF, IL-12), antigen presentation, down-regulates cytokine receptor expression and up-regulates cytokine inhibitors (sTNF-R, IL-1ra), induces strong B cell activation, Ig production and MMP expression.
TGFβ	25	Most cells	Depends on cell type	Activates connective tissue growth and synthesis of extracellular matrix inhibitor of haemopoiesis, immune cell activity and inflammation
IL-4	20	T cells and mast cells	T cell activation and IgE production	T cell growth factor, inducer of T_{H2} cells, , coactivator of B cell growth and Ig secretion, and activator of most cells and basophils. Inhibits macrophage proinflammatory cytokine production (IL-1, TNF, IL-6 etc)
IL-13		T cells, mast cells	T cell activation and IgE production	Coactivator of B cell growth and Ig secretion, and activator of most cells and basophils. Inhibits macrophage proinflammatory cytokine production (IL-1, TNF, IL-6 etc)

Chemotactic cytokines (Chemokines)

Chemokine	MW (kDa)	Sources	Inducers	Effects
IL-8 (C-X-C)		Amost all cells, fibroblastss, macrophages and neutrophils	Depends on cell type, LPS and pro-inflammatory cytokines such as TNFα, IL-1	Attracts neutrophils, basophils, eosinophils, T cells, induces keratinocyte proliferation, and neutrophil activation (degranulotion, enzyme release, respiratory burst)
GROα/β/γ (C-X-C)		Almost all cells	IL-1, TNFα, LPS etc.	Neutrophil chemotaxis and activation, melanoma cell proliferation, fibroblast proliferation, basophil chemoattraction
ENA78 (C-X-C)		Epithelial cells	IL-1, TNFα etc.	Attracts and activates neutrophils
IP-10 (C-X-C)		Most cells	IL-1, TNFα, LPS etc.	Chemoattractant of monocytes and T cells
MCP-1 (C-C)		Monocytes, fibroblasts	IL-1, TNFα, LPS etc.	Monocyte and basophil chemotaxis and activation
MCP-2 (C-C)		Keratinocytes, endothelium		Monocyte and basophil chemotaxis and degranulation
MIP-1α (C-C)		Monocytes, lymphocytes	IL-1, TNFα, LPS, T cell activators	Monocyte chemotaxis, T cells (particularly $CD8^+$), B cells
MIP-1β (C-C)		Monocytes, lymphocytes	IL-1, TNFα, LPS, T cell activators	Monocyte chemotaxis, T cells (particularly $CD8^+$), B cells
RANTES (C-C)		Lymphocytes	T cell activators	Monocyte chemotaxis

Table 1.4: Properties of cytokines and chemokines (Adapted from Feldmann M. et al. 1996)

1.2. Signalling pathways in host defense and inflammation

The generation of an immune response depends, first, on the recognition of pathogens by cells of the immune system, and second, on the initiation of appropriate innate and adaptive effector mechanisms. The recognition of pathogens depends partly on the recently discovered toll-like receptor (TLR) family, whereas the effector mechanisms require the production of the cytokines such as TNFα and IL-1 that orchestrate an inflammatory cascade of cytokine, chemokine and acute phase protein production (reviewed by Aggarwal B.B. and Puri R.K. 1997). The intracellular signalling pathways activated by the TNF, the IL-1 and the toll-like receptors are of major interest due to their potential therapeutic applications and are reviewed here.

1.2.1. TNF receptor signalling

Two types of TNF receptor are known, the p55 and the p75 TNF-R (CD120a and CD120b), which belong to the TNF receptor superfamily (Loetscher H. et al. 1990a; Loetscher H. et al. 1990b; Smith C.A. et al. 1990; Gray P.W. et al. 1990). Their expression differs, with the p55 TNF-R being almost ubiquitous, and the p75 TNF-R being chiefly expressed on haematopoietic cells (reviewed in Aggarwal B.B. and Natarajan K. 1996).

As with many other receptors, signal transduction through the TNF receptors depends on receptor aggregation following ligand binding. Although TNFα is trimeric, it is not clear whether trimerization of the receptor is needed, as dimerization using monoclonal antibodies has also be shown to be sufficient for triggering intracellular signalling events (Engelmann H. et al. 1990). Dimerization-induced signalling, however, requires both the conformational change and translocation of the receptor (Kapas L. et al. 1992) and may not be physiologiaclly relevant. Other evidence has suggested that dimerization of the extracellular domains of the TNF receptors is actually inhibitory and prevents the intracellular domains from spontaneous signalling (Naismith J.H. et al. 1995). The recent discovery of the "suppressors of death domains" (SODD) has further complicated the picture. These proteins bind to the

death domain of the p55 TNF-R and the DR3 TNF-R family member, and prevent their aggregation in the absence of stimulus (Jiang Y. et al. 1999). Upon TNFα binding, SODD dissociates, allowing the death domains to interact and signal.

Both of the TNF receptors are thought to signal by using a number of adaptor proteins to recruit kinases and downstream signalling molecules (Darnay B.G. et al., 1994a; Darnay B.G. et al. 1994b). No intrinsic enzymatic activity has been associated with the TNF receptors themselves.

Adaptor proteins

The two TNF receptors use different adaptor proteins that reflect their different intracellular domains, and mediate distinct functions. The p55 TNF-R has a death domain (DD) in its intracellular portion known to be required for TNFα-induced apoptosis. The death domain uses a structure of six amphipathic α-helices in an antiparallel arrangement to mediate homotypic interactions with other death domain-containing proteins such as the TNF-R associated death domain (TRADD), the Fas associated death domain (FADD/MORT1), the RAIDD/CRADD and the serine/threonine kinase RIP (Hsu H. et al. 1995; Chinnaiyan A.M. et al. 1996; Duan H. and Dixit V.M. 1997; Ahmad M. et al. 1997; Hsu H. et al. 1996b; Stanger B.Z. et al. 1995; Ting A.T. et al. 1996).

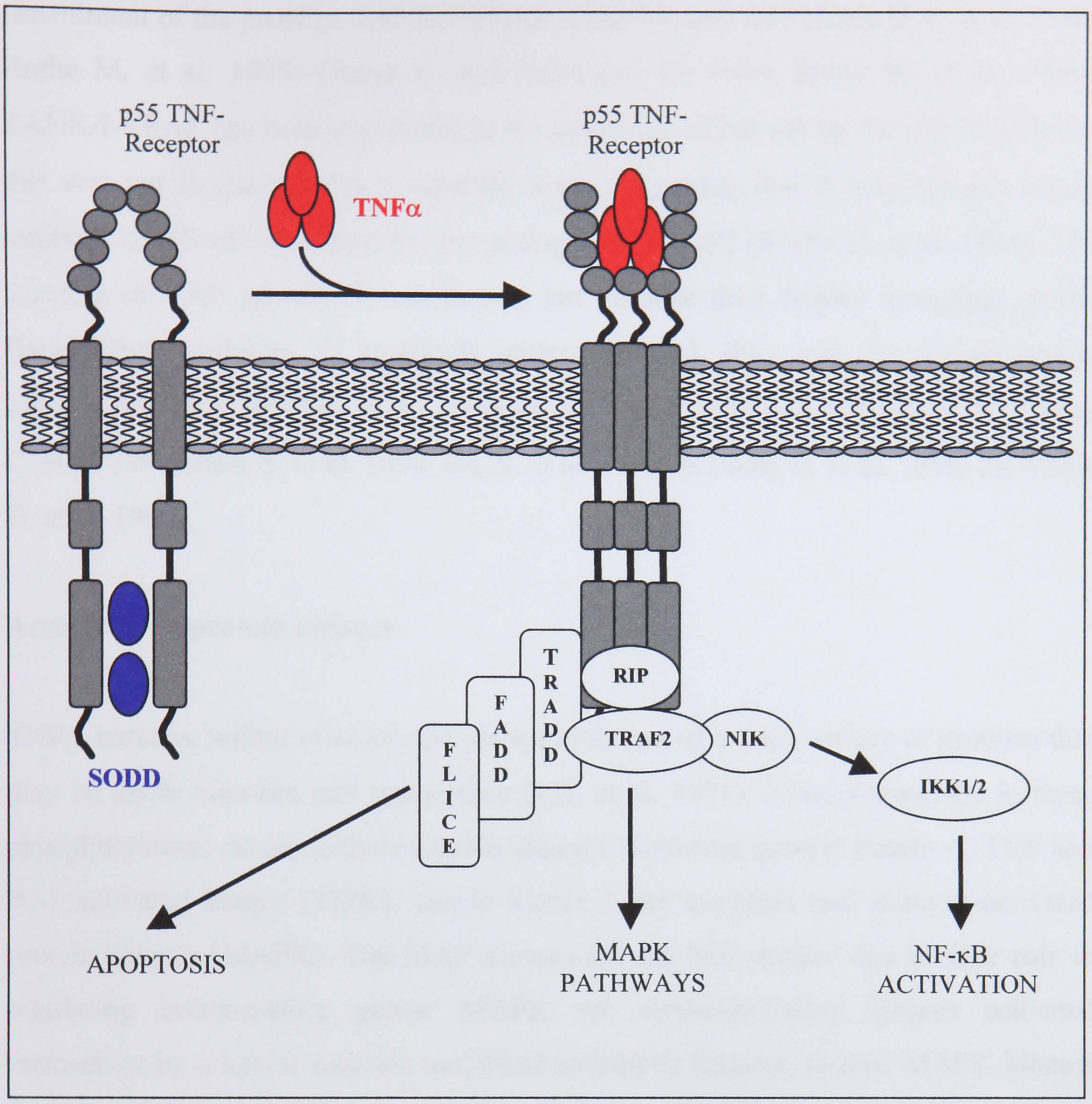


Figure 1.8: The p55 TNF receptor. In the absence of stimulation, the TNF-R exists in dimers. SODD proteins prevent the p55 TNF-R from signalling. Upon ligand binding, SODD proteins are released, the receptor trimerizes, and via receptor-associated proteins, it induces signalling cascades that lead to MAPK and NF-κB activation, and apoptosis.

TNFα signalling through the p55 TNF-R can induce apoptosis as well as NF-κB activation. These signals have been shown to bifurcate at the level of TRADD (Hsu H. et al. 1996b), where TRADD interacts with FADD to transduce the apoptotic signal and TRAF2 to induce NF-κB activation. In addition to TRAF2, MORT1 and RIP can also induce NF-κB activation when overexpressed (Hsu H. et al. 1996b), but the physiological significance of this pathway is uncler.

The p75 TNF-R does not possess a death domain but seems to form a heterodimeric complex with TRAF1 and TRAF2 (Rothe M. et al. 1994). This allows the

recruitment of the proteins TANK/I-TRAF, CIAP1/2 and A20 (Song H.Y. et al. 1996; Rothe M. et al. 1995; Cheng G. and Baltimore D. 1996; Rothe M. et al. 1996). TANK/I-TRAF has been implicated in the activation of NF-κB by the p75 TNF-R but this was put in question by a separate study suggesting that it may actually be an inhibitor of NF-κB activation by interacting with TRAF2 (Rothe M. et al. 1996). The function of cIAP proteins is not known but because they display homology to the Baculovirus inhibitors of apoptosis proteins (IAPs), they may be anti-apoptotic. Finally, A20 may function as an inhibitor of NF-κB activation and apoptosis (Ferran C. et al. 1998; Hess S. et al. 1998; Hu X. et al. 1998; Wissing D. et al. 1998; De Valck D. et al. 1999).

Activation of protein kinases

TNFα induces, within minutes, the phosphorylation of a wide variety of proteins that may be differ between cell types (Guy G.R. et al. 1991). Kinases involved in these phosphorylation events include protein kinase C isoforms, protein kinase A, TNF and IL-1 activated kinase (TIPK), casein kinase I-like enzymes and mitogen-activated protein kinases (MAPK). The MAP kinases are the best studied due to their role in regulating inflammatory genes. MAPK are serine/threonine kinases activated themselves by a kinase cascade too. Dual specificity kinases, termed MAPK kinases (MKK), activate MAPK by serine and tyrosine phosphorylation. MAPKK are activated, in turn, by another group of serine/threonine kinases collectively known as MAPKK kinases (MKKK or MEKK). The mode of activation of MAPKKKs is unclear but may involve small G proteins and upstream MAPKKK kinases.

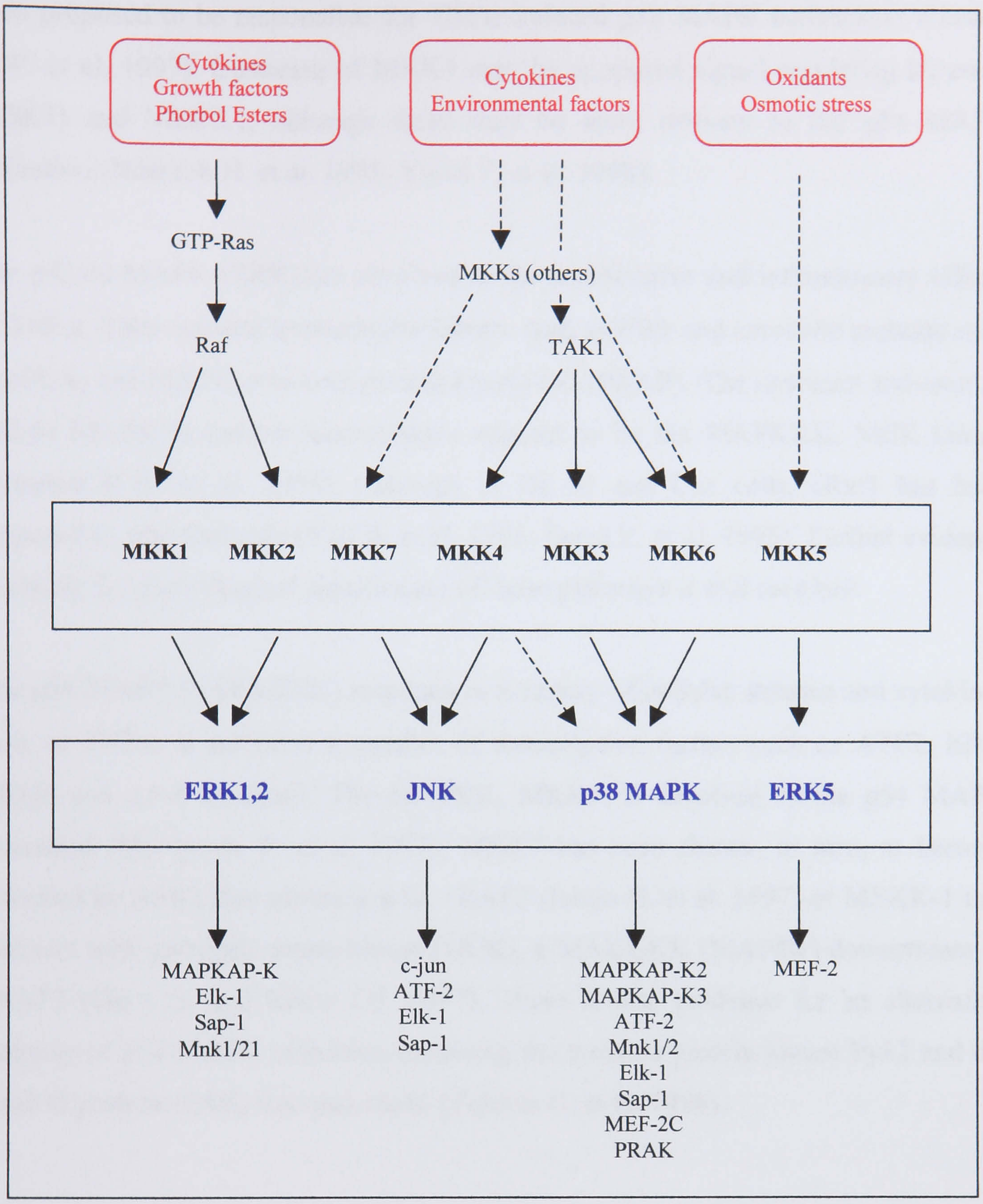


Figure 1.9: The MAPK cascade. The MAPK cascade is activated by various stimuli and results in the phosphorylation of numerous downstream targets involved in inflammation and cellular stress responses.

In response to TNFα, all three types of MAPK, p38, p42/44 and p54 MAPK, get activated. The p38 MAPK cascade has been implicated in the regulation of various inflammatory genes in response to TNFα stimulation, including IL-6, IL-8 and TNFα itself, through its effects upon transcription, mRNA stability and translation. The p38 MAPK pathway modulates the transcription factor ATF2 as well as the cytosolic proteins cytosolic phospholipase A_2 ($cPLA_2$) and Hsp27. The MAPKK3 (MKK3) has

been proposed to be responsible for TNFα-induced p38 MAPK activation (Winston B.W. et al. 1997). Upstream of MKK3 may be apoptosis signal-regulating kinase 1 (ASK1) and MEKK1, although these may be more relevant to the p54 MAPK activation (Nishitoh H. et al. 1998; Yujiri T. et al. 1998).

The p42/44 MAPK (ERK) are involved in the proliferative and inflammatory effects of TNFα. They activate transcription factors such as Elk1 and cytosolic proteins such as $cPLA_2$ and MAPK activated protein kinase (MAPKAP). The upstream activator of p42/44 MAPK in murine macrophages appears to be the MAPKKK, MEK kinase (Winston B.W. et al. 1995), although in HL-60 and Cos cells, cRaf1 has been proposed to play that role (Yao B. et al. 1995; Berra E. et al. 1995). Further evidence to clarify the physiological significance of these pathways is still required.

The p54 MAPK (SAPK/JNK) responds to a variety of cellular stresses and cytokines such as TNFα. It activates a number of transcription factors such as ATF2, Elk1, CREB and AP-1 (fos/jun). The MAPKK, MKK7, is involved in the p54 MAPK activation (Moriguchi T. et al. 1997). MKK7 has been shown, in turn, to become activated by ASK1 that interacts with TRAF2 (Ichijo H. et al. 1997) or MEKK-1 that interacts with germinal centre kinase (GCK), a MAKKKK (MAP4K) downstream of TRAF2 (Shi C.S. and Kehrl J.H. 1997). There is also evidence for an alternative pathway of p54 MAPK activation involving the tyrosine protein kinase Pyk2 and the small G proteins PAK, Rac and cdc42 (Tokiwa G. et al. 1996).

Phospholipase activation cascades

TNF receptor signalling induces the activation of phosphatidylcholine-phospholipase C (PC-PLC) and the generation of diacylglycerol (DAG) (Schutze S. et al. 1990). This activates the atypical PKCζ and the acid sphingomyelinase that have been implicated in NF-κB activation (Schutze S. et al. 1994; Heller R.A. et al. 1994; Muller G. et al., 1995). Activation of acid sphingomyelinase has been shown to map to the death domain of the p55 TNF-R (Wiegmann K. et al. 1994). However, individuals defective in acid sphingomyelinase, show no defects in TNFα-induced NF-κB (Kuno K. et al.

1994). TNFα also induces neutral sphingomyelinase activity by using FAN, a p55 TNF-R-associated protein (Adam-Klages S. et al. 1996). By inducing ceramide production, TNFα activates ceramide activated kinase (CAPK) and phosphatase (CAPP) (Mathias S. et al. 1991; Dobrowsky R.T. and Hannun Y.A. 1993). CAPK may be important in the upstream activation of Raf1 and consequently p42/44 MAPK (Yao B. et al., 1995), and the insulin signalling adapter molecule IRS-1 (Peraldi P. et al. 1996). CAPK phosphorylates IRS-1 at a serine residue and in that way impairs its activation by tyrosine phosphorylation (Peraldi P. et al. 1996). This could provide a reason for the development of TNFα-induced insulin resistance (Peraldi P. and Spiegelman B. 1998).

Apoptosis and caspase-induced cascades

Caspases are cysteine proteases that play a crucial role in the induction of apoptosis. They normally exist as inactive precursors but upon induction of apoptosis they become activated by proteolytic cleavage at internal caspase substrate sites, resulting in a cascade of caspase activation (Earnshaw W.C. et al. 1999). Three caspases are able to interact with the p55 TNF-R. Caspace 8 (MACH/FLICE/Mch5) and caspase 10 have in their N-terminal death domains (DD) that can interact with the N-terminal DD of FADD/MORT1 (Boldin M.P. et al. 1996; Muzio M. et al., 1996; Fernandes-Alnemri T. et al. 1996; Vincenz C. and Dixit V.M. 1997). Caspase 2, on the other hand, has an N-terminal recruitment domain (CARD) that binds through homophilic interactions to a CARD domain in RAIDD (Duan H. and Dixit V.M. 1997; Ahmad M. et al. 1997). Of these caspases, only caspase 8 has been shown to be involved in the induction of apoptosis without the use of overexpression studies. Caspase 8 activates sequentially caspase 9, 3, 7 and then 6 (Pan G. et al. 1998; Hirata H. et al. 1998). The downstream substrates of caspases involved in the apoptotic pathway are not well defined, but include DFF and caspase activated DNase (CAD), two enzymes with a role in DNA fragmentation (Enari M. et al. 1998; Sakahira H. et al. 1998).

1.2.2. IL-1 signalling

IL-1 receptors

There are two forms of the IL-1 receptor, both of which are members of the immunoglobulin superfamily. The type I IL-1 receptor (IL-1RI) is a widely expressed 80 KDa transmembrane glycoprotein, comprising a 319 amino acid extracellular region with three Ig-like domains, a 20 amino acid transmembrane and a 213 amino acid cytosolic region (Sims J.E. et al. 1989).

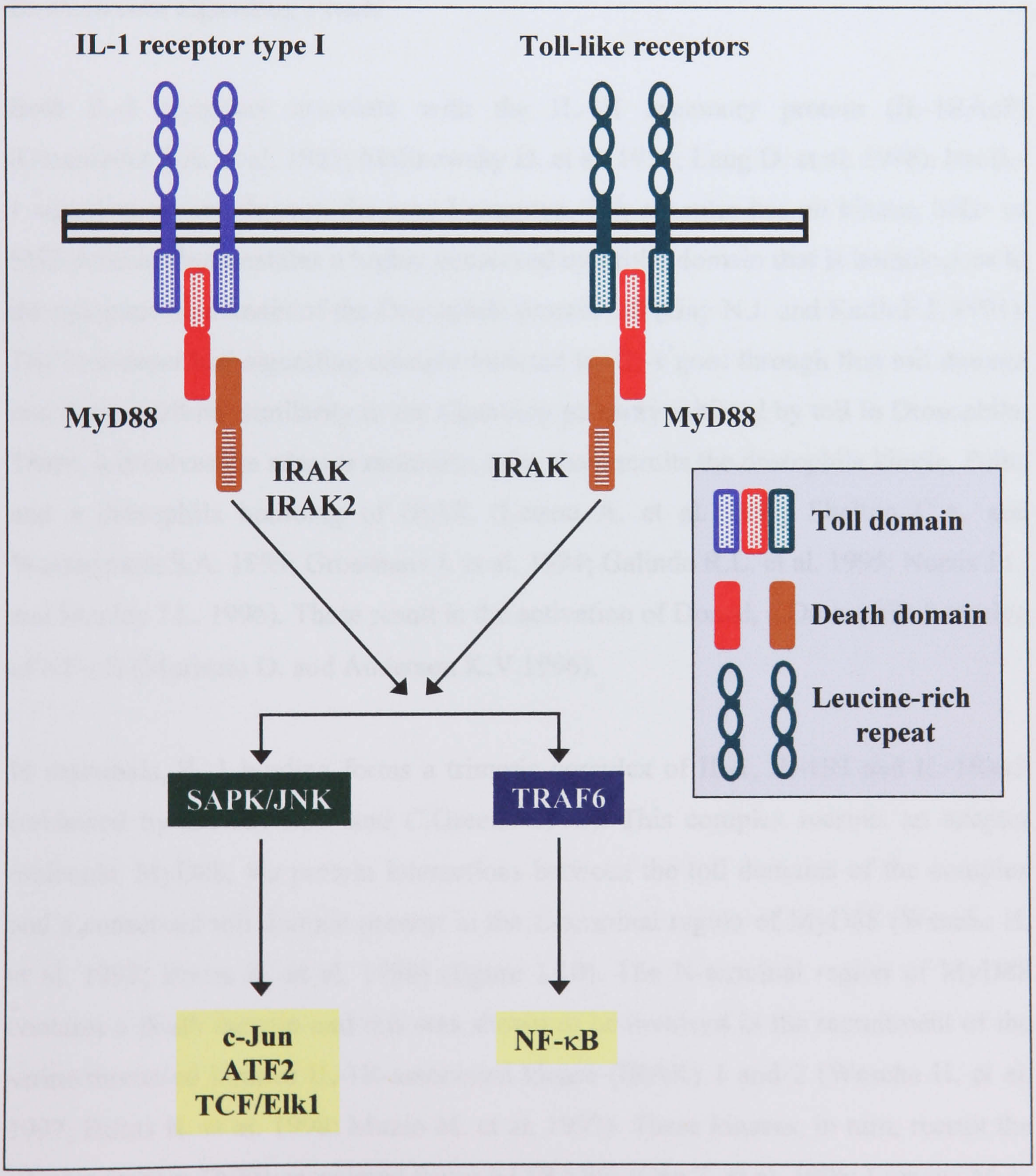


Figure 1.10: Toll-like receptors and the IL-1 receptor trigger a similar intracellular signalling cascade. This involves MyD88, IRAK, TRAF6, JNK and NF-κB activation.

The type II IL-1 receptor (IL-1RII) resembles the type I receptor in that it has an extracellular part of 330 amino acids that comprises three Ig domains, and a transmembrane part of 26 amino acids. It differs, however, in that its intracellular portion of 29 amino acids is very short and incapable of signaling. Thus, the IL-1RII acts as a decoy receptor by binding to IL-1 and reducing its free concentration (Colotta F. et al. 1993). In addition, there is a soluble form of the IL-1RII (sIL-1RII) that functions to sequester circulating IL-1, rendering it inactive (Colotta F. et al. 1993).

Downstream signalling events

Both IL-1 receptors associate with the IL-1R accessory protein (IL-1RAcP) (Greenfeder S.A. et al. 1995; Malinowsky D. et al. 1998; Lang D. et al. 1998), but IL-1 signaling occurs through the type I receptor. This receptor has no kinase, SH2- or SH3-domains but contains a highly conserved cytosolic domain that is homologous to the cytoplasmic domain of the Drosophila protein toll (Gay N.J. and Keith F.J. 1991). The best-described signalling cascade initiated by IL-1 goes through that toll domain and shows striking similarity to the signalling pathway initiated by toll in Drosophila. There, it involves the adaptor molecule, tube, that recruits the drosophila kinase, Pelle, and a drosophila homolog of IRAK (Letsou A. et al. 1993; Shelton C.A. and Wassermann S.A. 1993; Grosshans J. et al. 1994; Galindo R.L. et al. 1995; Norris J.L. and Manley J.L. 1996). These result in the activation of Dorsal, a Drosophila homolog of NF-κB (Morisato D. and Anderson K.V.1996).

In mammals, IL-1 binding forms a trimeric complex of IL-1, IL-1RI and IL-1RacP (reviewed by O'Neill L.A. and C.Greene 1998). This complex recruits an adaptor molecule, MyD88, via protein interactions between the toll domains of the complex and a conserved toll domain present in the C-terminal region of MyD88 (Wesche H. et al. 1997; Burns K. et al. 1998) (figure 1.10). The N-terminal region of MyD88 contains a death domain and this was shown to be involved in the recruitment of the serine/threonine kinases IL-1R-associated kinase (IRAK) 1 and 2 (Wesche H. et al. 1997; Burns K. et al. 1998: Muzio M. et al. 1997). These kinases, in turn, recruit the adaptor protein TNFR-associated factor 6 (TRAF6) (Cao Z. et al. 1996; Lomaga M.A.

et al. 1999), and the signalling cascade progresses further through the IKK complex to the activation of NF-κB (figure 1.13). It is not clear how IκB kinases get activated as recent evidence from knockout mice indicates that the requirement for NF-κB-inducing kinase (NIK), long-believed to be essential for the activation of NF-κB, may be cell-type specific (Garceau N. et al. 2000). Other kinases such as the MAPKKK TGF-β-activated kinase 1 (TAK1) and the ECSIT and MEKK-1 have been proposed to play that role (Ninomiya-Tsuji J. et al. 1999; Kopp E. et al. 1999). Apart from NF-κB, IL-1 signalling induces the activation of the transcription factors NF-IL-6 and IL-1 nuclear factor (Stricker K. et al., 1993) and the activation of MAPKs (Freshney N.W. et al., 1994; Kracht M. et al., 1994) and β-casein kinase (Guesdon F. et al., 1993).

Upon IL-1 binding to its receptor, several other kinases are activated too, but the mechanism by which these components interact with the above cascade has not been determined (reviewed by O'Neill L.A. and Greene C. 1998). A small G protein in IL-1 signalling may also be involved (Singh R. et al. 1999; Jefferies C.A. and O'Neill L.A. 2000). Phosphatidylinositol 3-kinase appears to associate with the cytoplasmic region of IL-1RacP and to initiate a parallel signalling pathway that leads to the transactivation of NF-κB subunits by phosphorylation (Sizemore N et al. 1999).

1.2.3. Toll-like receptors signalling

Central to the recognition mechanisms of the innate immune system are a number of germline-encoded receptors known as toll-like receptors (TLRs) due to their homology with the Drosophila Toll receptor. In the fruit fly, the toll pathway was first identified by its role in body development (reviewed by Belvin M.P. and Anderson K.V. 1996). Then, it was shown that individual toll receptors and toll-like receptors activate specialized anti-fungal or antibacterial genes through the activation of the NF-κB transcription factors (Engstrom Y. 1993). Currently, seven Drosophila and ten human genes with a toll domain have been identified in the genome databases and more are certain to appear in the near future (reviewed by O'Neill L.A. and Dinarello C.A. 2000).

All members of the toll family are membrane proteins that cross the membrane once and share similar extracellular domains, that include 18-31 leucine-rich repeats (LRRs), and similar cytoplasmic domains of approximately 200 amino acids, that are also similar to the cytoplasmic domain of the IL-1 receptor (the Toll/IL-1-receptor homologous region or TIR). The extracellular domains of the TLR family are quite divergent, making it likely that different ligands activate different receptors. The extracellular domains of TLR2 and TLR4 are only 24% identical, and the extracellular domains of human and mouse TLR4 are only 53% identical, whereas the cytoplasmic domains of human and mouse TLR4 are 83% identical.

Activators of Toll-like receptors

At present, only the activators for two of the mammalian TLR, TLR2 and TLR4, have been identified. These are the cell wall components LPS of gram-negative bacteria, peptidoglycan and lipoteichoic acid (LTA) of gram positive bacteria, and mannan of fungi (Ulevitch R.J. and Tobias P.S. 1995; Wright S.D. 1995). The first experiments implicating TLR in the responses to pathogens came from transfection of the cloned receptors into cell lines that could not respond to LPS. These provided evidence that TLR2, but not TLR4, was sufficient to confer responsiveness of the embryonic kidney 293 cell line to LPS (Yang R.B. 1998; Kirschning C.J. et al. 1998). LPS induced the rapid activation of NF-κB and the release of IL-8. This response, required both the LPS-binding protein (LBP) and CD14 that are necessary for LPS signalling (Yang R.B. 1998). However, direct binding of LPS to TLR2 was of low affinity with a dissociation constant (K_d) of 500-700 nM, and put in question the physiological significance of these experiments. In addition to LPS, transfection experiments showed that TLR2 may also involved in the recognition of peptidoglycan and lipoteichoic acid, the cell wall components of gram positive bacteria (Schwandner R. et al. 1999; Yoshimura A. et al. 1999). Clarification of the physiological role of TLR2 came from the study of TLR2-deficient mice; TLR2 is required for the immune response against several gram positive bacterial cell wall components, as well as Staphylococcus aureus, peptodoglycans, and zymosan (Underhill D.M. et al. 1999; Takeuchi O. et al. 1999). A subsequent study suggested that the responsiveness of TLR2 could have been due to protein contaminants in the LPS preparation (Hirschfeld M. et al. 2000).

In parallel with the above studies, positional cloning of the classical mouse *lps* mutant demonstrated that TLR4 but not TLR2, is specifically required for the response to LPS. Mutations in the *lps* gene prevent most of the normal responses to LPS, including B cell proliferation, macrophage cytokine secretion, opsonization, NO production, and LPS-induced septic shock. Cloning of the *lps* locus by two independent groups demonstrated that the mutations alter the mouse *tlr4* gene (Poltorak A. et al. 1998; Qureshi S.T. et al. 1999), and the *lps* phenotype was also confirmed by a targeted mutation in the Tlr4 gene (Hoshino K. et al. 1999). Subsequent experiments showed that TLR4 can mediate activation of NF-κB in response to LPS if the appropriate cell line is used. The fact that initial experiments involving biochemical techniques failed to suggest a TLR4 involvement in LPS signalling, points out the dangers of using non-relevant cell lines and of overinterpreting transfection data and overexpression studies, in which the amount of protein generated is in excess of the physiological levels.

What is not clear is whether microbial components act as direct ligands to TLRs or whether they promote the production of classical ligands. The binding affinities for LPS-CD14-LBP complexes are lower than what would be expected if LPS is a direct ligand. Recently, a novel secreted protein, MD-2, was shown to increase the response of TLR4 to LPS, suggesting that accessory proteins may increase the affinity of TLRs for ligands such as LPS (Shimazu R. et al. 1999). Evidence for an alternative model for TLR activation comes from studies in the fruitfly, where active Spätzle, the apparent toll ligand, is produced by localized proteolytic processing of an inactive secreted precursor protein, triggered by a locally modified peptidoglycan (Levashina E.A. et al. 1999). This suggests that LPS might similarly trigger protease cascades that activate the classical protein ligand pathway. The fact that TLRs may not necessarily function as pattern-recognition receptors for infectious determinants but may instead recognize proteolytic fragments of self-proteins such as Spätzle is a very important observation. It implies that, in order to proceed, immune responses may not need the presence of foreign or infectious agents, but may use endogenous signals of distress or damage (Gallucci S. et al. 1999). This is a departure from the models of tolerance

based strictly on self/nonself discrimination (Matzinger P. 1999; Medzhitov R. and Janeway C.A. Jr 2000).

Signalling through toll-like receptors

The homology of the intracellular portion of toll-like receptors to the intracellular portion of the IL-1RI suggested that they may also use an analogous framework for signalling (figure 1.10). Thus, TLR4 interacts with the adaptor protein MyD88 (Muzio M. et al. 1998a; Medzhitov R. et al. 1998; Kawai T. et al. 1999) to recruit IRAK and TRAF6 (Muzio M. et al. 1998b; Lomaga M.A. et al. 1999). TRAF6 mediates NF-κB activation, but the p54 MAPK activation bifurcates earlier in the pathway as overexpression of dominant-negative versions of TRAF6 do not block its activation (Muzio M. et al. 1998). Of the other mammalian TLRs, only TLR2 has been studied and is also thought to activate a similar intracellular signalling pathway (Yang R.B. et al. 1998; Kirsching C. et al. 1998; Yang R.B. et al. 1999)

The importance of MyD88 and IRAK in toll-like receptor signalling, however, has been complicated by the analysis of knockout mice. MyD88-deficient mice display defects in NF-κB activation in response to the structurally similar cytokines IL-1 and IL-18 (Adachi O. et al. 1998) and are highly resistant to LPS-induced shock and cytokine production. However, LPS-induced MAPK and NF-κB activation remain almost normal, displaying only delayed kinetics (Kawai T. et al. 1999). A similar phenotype of only delayed NF-κB activation was also displayed by macrophages of IRAK-deficient mice (Kanakaraj P. et al. 1998; Swantek J.L. et al. 2000). TRAF-6-deficient mice, on the other hand, show a loss of NF-κB activation in response to both IL-1 and LPS stimulation (Lomaga M.A. et al. 1999). These findings suggest that LPS may use in addition to MyD88/IRAK an additional yet unidentified mediator to activate NF-κB through TRAF6 and MAPKs.

1.2.4. NF-κB activation

NF-κB transcription factors exist in virtually all cells. They were first described as B-lymphocyte specific nuclear proteins, essential for the transcription of

immunoglobulin kappa (κ) light chains (Sen R. and Baltimore D. 1986). They were later shown to have pleiotropic functions in immunity but also development and cell survival.

Mammalian cells contain five NF-κB subunits-relA (p65), relB, c-rel, p50 and p52-which form various homo- and hetero-dimers (Ghosh S. et al. 1990; Kieran M. et al. 1990; Meyer R. et al. 1991; Nolan G.P. et al. 1991; Ruben S.M. et al. 1991; Ryseck R.P. et al. 1992). The p50 and p52 subunits, which lack transactivation domains, are produced by processing of 105 kDa and 100 kDa precursor molecules, respectively. Members of the NF-κB family are characterized by a conserved N-terminal 300 amino acid sequence known as the 'rel homology' domain that is responsible for dimerization, DNA-binding, nuclear localisation and interaction with the I-κB family of proteins (described below). The p65/relA, relB and c-rel members also have a transactivation domain in their C-terminal that can modify their activation potential (Bull et al. P. 1990; Schmitz M.L. and Baeuerle P.A. 1991; Ruben S.M. et al. 1992; Rysek R.P. et al., 1992).

In the vast majority of cells, NF-κB is sequestered in the cytoplasm in an inactive form complexed with inhibitor of NF-κB (IκB) proteins. The IκB family consists of IκBα, IκBβ, IκBε and Bcl-3 (reviewed by Baldwin A.S. 1996), and is characterized by the presence of several ankyrin-like motifs important for the interaction with NF-κB, and a C-terminal PEST motif important for the regulation of IκB stability and protease sensitivity (Sun S.C. et al. 1994).

NF-κB is rapidly activated by a large spectrum of chemically diverse agents and cellular stress conditions including bacterial lipopolysaccharides, microbial and viral pathogens, cytokines such as TNFα and IL-1, and growth factors (reviewed by Baeuerle P.A. and Baltimore D. 1996). Activation involves the phosphorylation, polyubiquitination and subsequent degradation of IκB by the 26S proteasome, a major pathway for the degradation of intracellular proteins in eukaryotic cells (Chen Z. et al. 1995; Scherer D.G. et al. 1995; Traenckner E.B. and Baeuerle P.A. 1995). The amino acid residues Ser32 and Ser36 of IκBα were identified as essential for the phosphorylation process (Brown K. et al. 1995; Traenckner E.B. et al. 1995) whereas

Lys21 and Lys22 for the ubiquitination process (Scherer D.G. et al. 1995). IκB degradation leads to the exposure of a nuclear translocation sequence of the NF-κB dimer, allowing its nuclear translocation and DNA binding (Beg A.A. et al. 1993; Brown K. et al. 1993; Henkel T. et al. 1993; Mellits K.H. et al. 1993). NF-κB interacts with the consensus sequence 5'-GGGPuNNPyPyCC-3' which is present in numerous immunologically relevant genes.

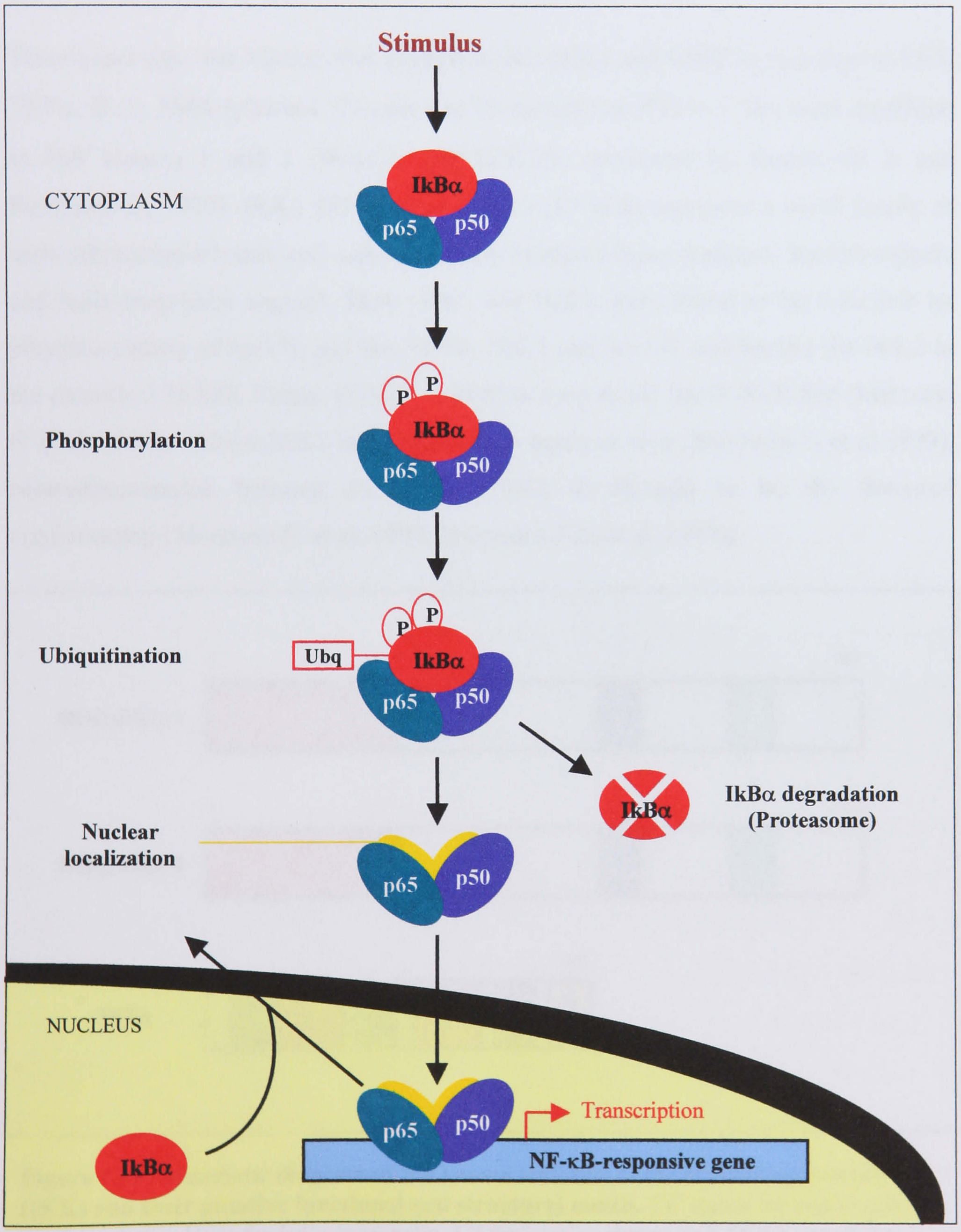


Figure 1.11: The current mechanism of IκBα degradation and NF-κB activation.

The IκB kinases

Although different inducers of NF-κB activity utilise distinct signalling pathways, they must converge at some point to target IκB for proteolytic degradation. Recent evidence has suggested that the point of convergence is the IκB kinase complex.

Three years ago, the kinases that phosphorylate IκBα and IκBβ in response to LPS, TNFα, IL-1, PMA (phorbol 12-muristate 13-acetate) or HTLV-1 Tax were identified as IκB kinases 1 and 2 (IKK1/α and IKK2/β) (reviewed by Stancovski I. and Baltimore D. 1997). IKK1 (85 kDa) and IKK2 (87 kDa) represent a novel family of serine/threonine kinases and contain closely related kinase domains, leucine-zippers and helix-loop-helix regions. Both IKK1 and IKK2 were found to be inducible by phosphorylation of Ser176 and Ser180 for IKK1 and Ser177 and Ser181 for IKK2 in the canonical MAPK kinase (MKK) activation loop motif Ser-X-X-X-Ser (Mercurio F. et al. 1997). Whilst IKK2 homodimers can occur in vivo (Mercurio F. et al 1999), heterodimerisation between IKK1 and IKK2 is thought to be the favoured conformation (Mercurio F. et al. 1997; Woronicz J.D. et al. 1997).

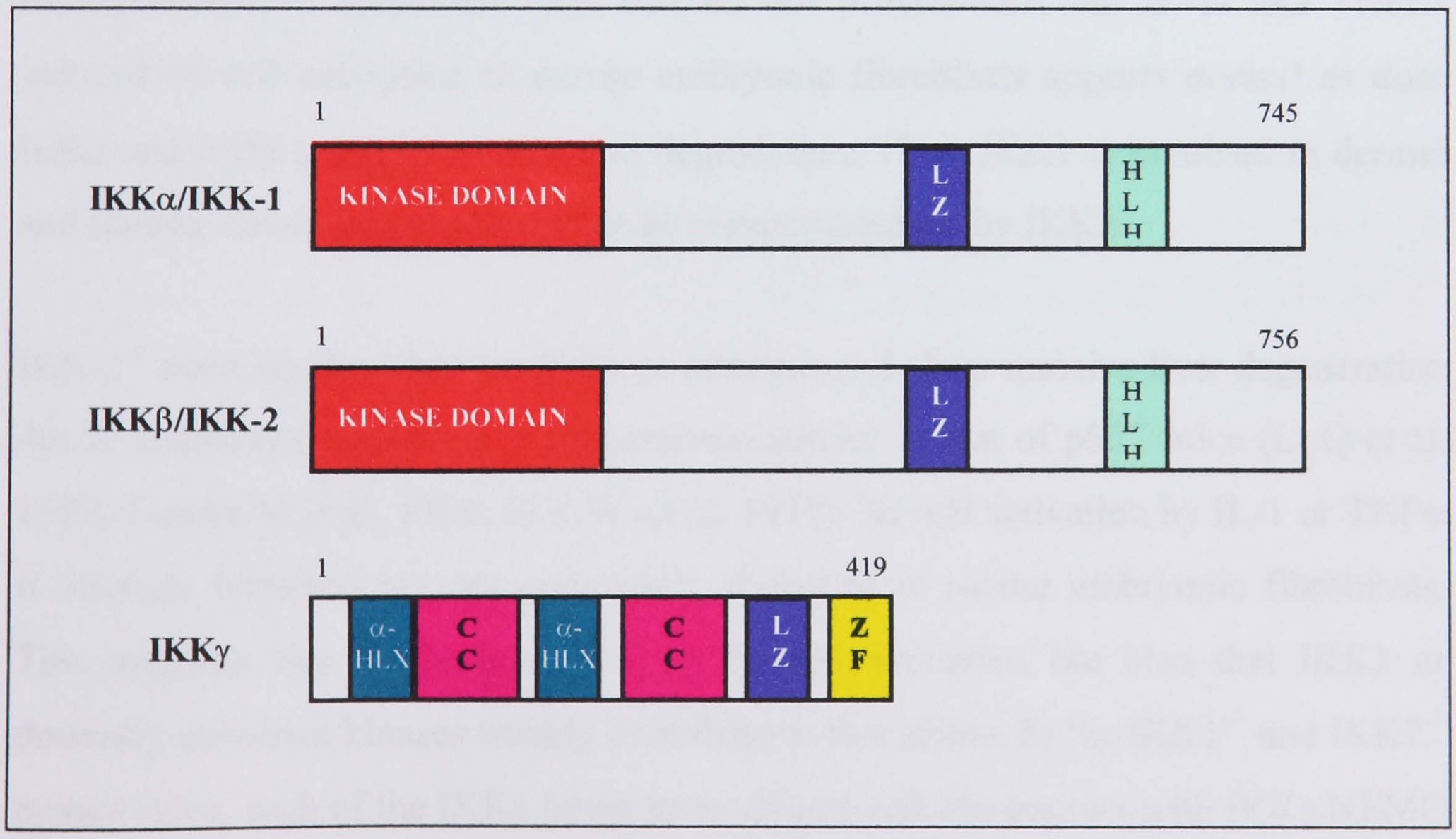


Figure 1.12: Schematic diagram of the known subunits of the IκB-kinase complex (IKK) and their putative functional and structural motifs. CC stands for coiled coil, α-hlx for α-helix, HLH for helix-loop-helix, LZ for leucine zipper, and ZF for zink finger. (Adapted from Karin M. et al. 2000)

IKK1 and IKK2 are part of a high molecular weight (700 kDa) kinase complex (DiDonato J.A. et al 1997; Mercurio F. et al 1997; Regnier C.H. et al. 1997) that also contains regulatory subunits such as NEMO/IKKγ and IKAP. IKKγ is composed of coiled-coil motifs, including a leucine zipper, and is essential for NF-κB activation by LPS, TNFα, IL-1, Tax or PMA (Yamaoka S. et al. 1998; Rothwarf D.M. et al. 1998; Mercurio F. et al. 1999). IKAP (IKK-associated protein) was suggested to be a scaffold protein able to bind IKKs and NF-κB-inducing kinase (NIK) and to assemble them into an active kinase complex (Cohen L. et al. 1998). Its precise role, however, is not clear.

Biochemical and transfection experiments have suggested non-equivalent functions of IKK1 and IKK2. IKK2 was shown to have a higher kinase activity for IκBα and to be the predominant kinase responsible for the phosphorylation of IκBα in response to TNFα, IL-1, LPS and dsRNA (O'Connell M.A. et al. 1998; Algarte M. et al. 1999; Delhase M. et al 1999; Li X.H. et al. 1999). More dramatic differences, however, became obvious by the generation of IKK1- and IKK2-deficient mice. IKK1$^{-/-}$ mice have shorter limbs and skull, a fused tail, and die perinatally. They have hyperproliferative epidermal cells that do not differentiate, but IL-1- and TNFα-induced NF-κB activation in mouse embryonic fibroblasts appears normal as does IκBα and IκBβ phosphorylation and degradation. Thus, IKK1 is involved in dermal and skeletal development and cannot be compensated for by IKK2.

IKK-2$^{-/-}$ mice, on the other hand, die as embryos and show massive liver degeneration due to hepatocyte apoptosis, a phenomenon similar to that of p65$^{-/-}$ mice (Li Q et al. 1999; Tanaka M et al. 1999; Li Z.W. et al. 1999). NF-κB activation by IL-1 or TNFα is strongly impaired but not completely abolished in mouse embryonic fibroblasts. This suggests that IKK2 is crucial for NF-κB activation but also that IKK1 or presently unknown kinases weakly contribute to this action. In the IKK1$^{-/-}$ and IKK2$^{-/-}$ mutant mice, each of the IKKs forms homodimers and can interact with IKKγ/NEMO in the absence of the other (Li X.H. et al. 1999; Li Q. et al. 1999); however, it is not known if homodimers play a normal physiological role. As the distinct phenotypes observed in IKK1- and IKK-2-deficient mice suggest that each kinase responds to

different stimuli, specific adaptor proteins or upstream kinases should exist allowing each of the IKKs to perform different tasks.

Recently, a new serine/threonine kinase IKK-i (Shimada T. et al. 1999) which shares 30% overall identity with IKK1 or IKK2 was shown to phosphorylate IκBα preferentially at Ser36 and to stimulate an NF-κB reporter gene. However, although IKK-i mRNA was strongly induced by LPS, its kinase activity was not regulated by any known NF-κB stimuli, suggesting that it may not be a physiological activator of NF-κB.

Moreover, other kinases such as $p90^{rsk}$, PKR (dsRNA-dependent kinase), casein kinase II and the catalytic subunit of DNA-dependent kinase have also been shown to phosphorylate IκB (Schouten G.J. et al. 1997; Kumar A. et al. 1994; Li S. and Sedivy J.M. 1993; Liu L. et al. 1998b). The kinase activities of these enzymes, though, have not been shown yet to specifically phosphorylate ser32 and ser36 of IκBα.

Direct phosphorylation of Tyr42 in the N-terminal domain of IκBα have also been suggested to be important in the activation of NF-κB by an alternative mechanism (Imbert V. et al. 1996; Abu-Amer Y. et al. 1998). The tyrosine phosphatase inhibitor pervanadate and hypoxia, but also TNFα, have been shown to induce tyr42 phosphorylation of IκBα, leading to the activation of NF-κB in the absence of IκB degradation. The $p56^{lck}$ kinase and the c-Src and PI-3 kinases, respectively, have been implicated in that process (Imbert V. et al. 1996; Abu-Amer Y. et al. 1998; Beraud C. et al. 1999). This alternative mechanism of NF-κB activation appears to be cell type-specific, as Tyr42 phosphorylation of IκBα reduces the activation of NF-κB in response to TNFα in HeLa but not U937 cells (Singh S. et al. 1996).

Finally, another atypical activation pathway is observed in cells exposed to short-wavelength UV radiation (254 nm). UV radiation induces the degradation of IκBα via the 26S proteasome, but this process is not mediated by phosphorylation of either Ser-32 and Ser-36 or Tyr-42 (Bender K. et al. 1998; Li N. and Karin M. 1998). The mechanism utilized by UV light is unknown. Nevertheless, NF-κB activation by UV



light, pervanadate or hypoxia is considerably slower and weaker than it is in response to activation by TNFα, IL-1 or LPS.

Upstream regulation of the IKK complex

Several mitogen-activated protein kinase kinase kinases (MAP3Ks) have been suggested to be inducible IKK kinases. Overexpression of either NIK, MEKK1 (MAPK/ERK kinase kinase 1), MEKK2 or MEKK3 (Lee F.S. et al. 1998; Nemoto S. et al. 1998; Zhao Q. and Lee F.S. 1999) leads to activation of IKKs and NF-κB reporter genes. Kinase inactive versions of IKK1 or IKK2 inhibit NF-κB activation by MAP3Ks, implying that these kinases act upstream of the IKK complex. NIK and MEKK-1 can phosphorylate both IKK1 and IKK2 in vitro although NIK displays greater specificity for IKK1 and MEKK1 for IKK2 (Regnier C.H. et al. 1997; Woronicz J.D. et al. 1997; Lee F.S. et al. 1998; Ling L. et al. 1998; Nakano H. et al. 1998; Nemoto S. et al 1998).

Other MAP3K-like kinases were shown to connect NIK and MEKK1 to receptor complexes (figure 1.13). First, transforming growth factor β activated kinase 1 (TAK1) together with its activator TAK1-binding protein 1 (TAB1) may link TRAF-6 to the NIK/IKK cascade to mediate NF-κB activation by IL-1 (Ninomiya-Tsuji J. et al. 1999). Second, TANK-binding kinase 1 (TBK1), a novel kinase related to IKK1 or IKK-2 and 48% identical to IKK-I, has been shown to mediate TRAF-2, -5,-6 and TANK-induced NF-κB activation (Pomerantz J.L. and Baltimore D. 1999). TBK1 forms a complex with TANK andTRAF-2 that functions upstream of NIK and the IKK complex, but TBK1 does not appear to be required for the activation of NF-κB by TNFα, IL-1 or CD40.

On the other hand, the adapter protein ECSIT (evolutionarily conserved signalling intermediate in toll/IL-1 pathways) may link TRAF-6 to MEKK1 as it promotes the processing of MEKK-1 to an 80 kDa form that correlates with NF-κB activation (Kopp E. et al 1999). In addition, tumour progession locus 2 (TPL-2 also known as Cot) was proposed to mediate signals from TCR-CD3 and CD28 to NIK (Lin X et al. 1999) although further evidence is needed.

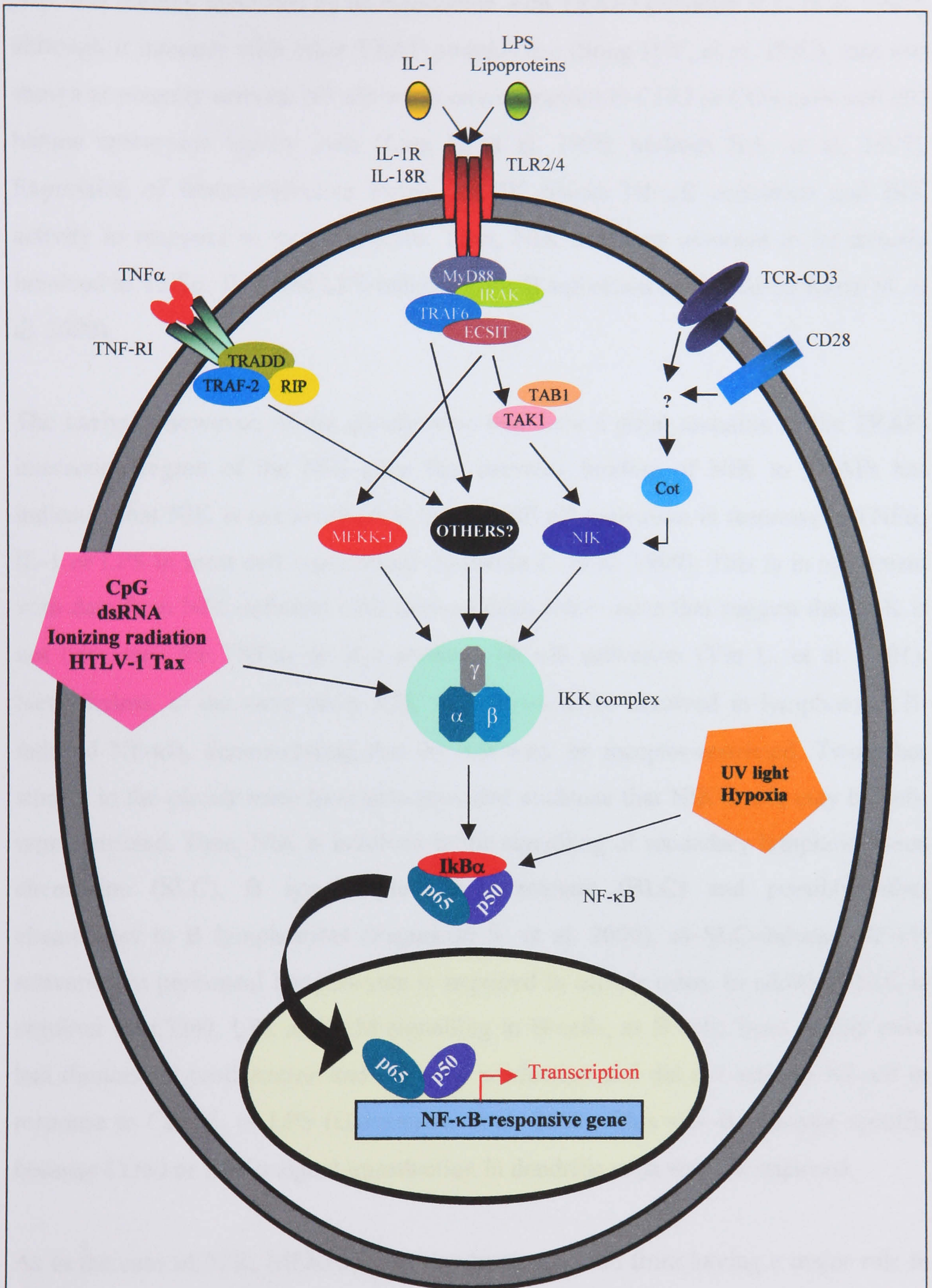


Figure 1.13: A schematic model of NF-κB activation. Various pathways initiated by stimuli such as TNFα, IL-1, LPS, dsRNA, or ionizing radiation, result in the the phosphorylation of the IKK complex, and the activation and nuclear translocation of NF-κB. Not all the pathways, however, converge at the level of the IKK complex as UV light- and hypoxia-induced NF-κB activation seem to operate through a different mechanism (Modified from Karin M. et al. 2000).

NIK was initially identified by its association with TRAF2 (Malinin N.L. et al. 1997), although it interacts with other TRAF proteins too (Song H.Y. et al. 1997), and was shown to potently activate NF-κB when overexpressed in CHO or COS cells and 293 human embryonic kidney cells (Ling L. et al. 1998; Malinin N.L. et al. 1997). Expression of kinase-defective forms of NIK blocks NF-κB activation and IKK activity in response to most inducers. Thus, NIK has been assumed to be directly involved in TNFα, IL-1 and LPS-induced NF-κB activation (reviewed by Karin M. et al. 2000).

The analysis, however, of the *aly/aly* mice that have a point mutation in the TRAF-interacting region of the NIK gene that prevents binding of NIK to TRAFs has indicated that NIK is not involved in IKK or NF-κB activation in response to TNFα, IL-1 or LPS in most cell types tested (Shinkura R. et al. 1999). This is in agreement with data from NIK-deficient cells derived from *Nik*-/- mice that suggest that NIK is not necessary for TNFα- or IL-1-induced NF-κB activation (Yin L. et al. 2001). Nevertheless, in the same study NIK was shown to be involved in lymphotoxin β-induced NF-κB, demonstrating that its role may be receptor-restricted. Two other studies in the *aly/aly* mice have also provided evidence that NIK's role may be cell-type restricted. Thus, NIK is involved in the signalling of secondary lymphoid tissue chemokine (SLC), B lymphocyte chemoattractant (BLC) and possibly other chemokines to B lymphocytes (Fagarasan S. et al. 2000), as SLC-induced NF-κB activation in peritoneal lymphocytes is impaired in *aly/aly* mice. In addition, NIK is required for CD40, LPS and IgM signalling in B cells, as B cells from *aly/aly* mice had diminished proliferation and antibody production and did not activate NF-κB in response to CD40L or LPS (Garceau N. et al. 2000). This was B cell-type specific because CD40 or TNFα signal transduction in dendritic cells was not impaired.

As in the case of NIK, MEKK-1 has also been excluded from having a major role in IKK and NF-κB activation. MEKK1-deficient mice show no abnormality in TNFα or IL-1 regulation of NF-κB activation in macrophages or fibroblasts (Yujiri T. et al. 2000), and MEKK1 seems to be only required for fibroblast and epithelial cell migration. Therefore, it appears that the IKK complex is the most likely point of convergence for many NF-κB-inducing signalling pathways.

Transactivation of NF-κB

Increasing evidence suggests that NF-κB subunits can also interact directly with kinases that phosphorylate them and regulate their activity. The major domain sensitive to phosphorylation is located in the C-terminal region of NF-κB family members and is known as the transactivation domain. Both stimulatory (Naumann M. and Scheidereit C. 1994; Hayashi T. et al. 1993) and inhibitory (Neumann T. et al. 1995) phosphorylations of p65 have been reported, and several upstream kinases have been implicated in the transactivating phosphorylation event, including PI-3K, Akt (PKB), p38 MAPK and p42/44 MAPK (Reddy S.A. et al. 1997; Sizemore N. et al. 1999; Ozes N.O. et al. 1999; Kane L.P. et al. 1999).

1.3. Host defense against infection

The immune response against infectious agents depends on the nature of the pathogen, with different mechanisms operating in response to viruses, bacteria, fungi, or parasites.

1.3.1. Immunity to viruses

Viruses are extremely diverse in their ability to infect, persist and initiate disease in a host. Entry often takes place through mucosal surfaces or through broken skin. Replication usually occurs at epithelial surfaces, followed in some cases by the spread of the virus to the bloodstream to infect other tissues. Recovery from the infection involves the elimination of the virus from the host. Some viruses, however, such as the herpes virus persist in a latent non-infectious form that can be reactivated, and other viruses such as the hepatitis B virus and the lymphocytic choriomeningitis virus can maintain an infectious form despite the presence of an immune response.

The initial defense barrier against virus infection is the integrity of the body surfaces. Once viruses manage to bypass that, early "non-specific" innate immune responses get activated. These involve activation of natural killer cells and the production of interferon α/β that directly inhibits viral replication (Muller U. et al. 1994; Van den

Broek MF et al. 1995) and enhances natural killer cell/macrophage activation (Biron C.A. 1997; Gidlund M. et al. 1978; Santoli D. et al. 1978) as well as antigen presentation (figure 1.14) (Lindahl P. et al. 1976).

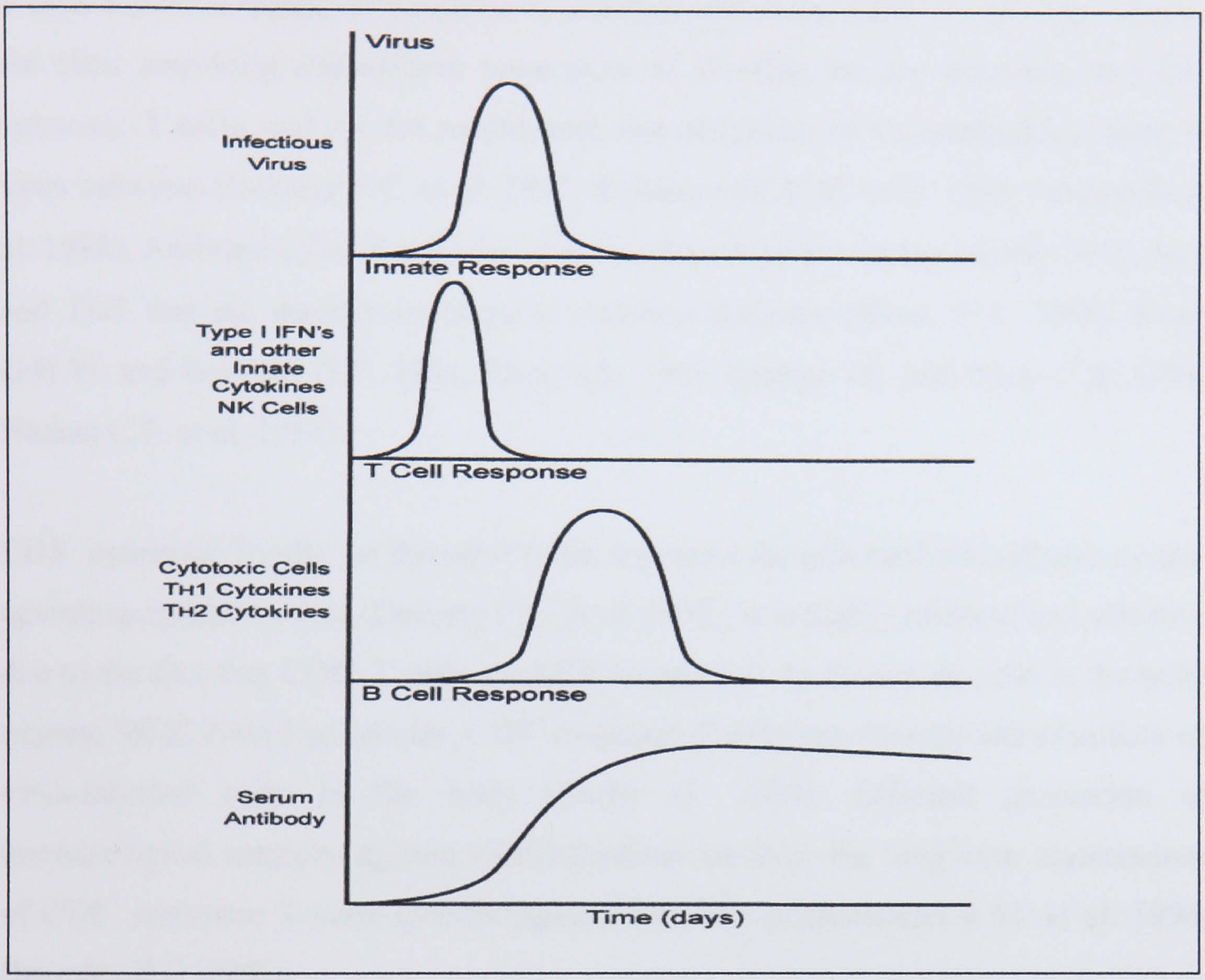


Figure 1.14: Kinetics of host responses during a primary acute viral infection. Innate immune responses characterized by the production of IFNs and the activation of NK cells take place first. Adaptive immune responses then follow, and involve T and B cell activation (borrowed from Ahmed R. and Biron C.A. 1999).

If the infection persists, adaptive immune responses start to take place with the appearance of helper and cytotoxic T cells (Tough D.F. et al. 1996) and antiviral antibodies, mainly of the IgA and IgG isotype. Antibodies are very important in preventing the spread of the virus to the bloodstream or to other cells and tissues. Antibodies can be generated against any viral protein but of particular importance are antibodies directed against glycoproteins that are expressed on the viral envelope. These are very useful in controlling infection by neutralizing the viral infection of other cells (Bachmann M.F. et al. 1997; Topham D.J. and Doherty P.C. 1998). Complement can also damage the virus envelope, but this is not considered to be a

major mechanism in the defense against viruses as individuals with complement deficiencies are not predisposed to severe viral infection but get bacterial infections instead (Kazatchkine M.D. et al. 1987)

T cells exhibit a variety of functions in antiviral immunity. $CD4^+$ T cells are required for class switching and affinity maturation of B cells, for the induction of $CD8^+$ cytotoxic T cells, and for the recruitment and activation of macrophages at sites of virus infection (Doherty P.C. et al. 1997; Koszinowski U.H. et al. 1991; Ahmed R. et al. 1988). Antiviral $CD4^+$ T cells are of a T_{H1} phenotype producing mainly IFNγ, IL-2 and TNF that are directly involved in antiviral defenses (Biron C.A. 1994; Wong G.H.W. and Goeddel D.V. 1986; Chan S.H. 1991; Orange J.S. and Biron C.A. 1996; Nathan C.F. et al. 1983).

$CD8^+$ cytotoxic T cells, on the other hand, represent the principal surveillance system operating against viruses (Doherty P.C. et al. 1997). It is highly efficient and selective due to the fact that $CD8^+$ T cells are MHC-restricted. As almost all cells in the body express MHC class I molecules, $CD8^+$ cytotoxic T cells can identify and eliminate all virus-infected cells in the body (Berke G. 1994). Efficient generation of immunological memory against viral infections involves the long-term maintenance of $CD8^+$ cytotoxic T cells specific against the virus (Zinkernagel R.M. et al. 1996; Beverley P.C. 1996)

1.3.2. Immunity to bacteria

As with viral infections, the first line of body's defense against pathogenic bacteria consists of simple barriers to the entry or establishment of the infection. Thus, the skin and exposed epithelial surfaces have innate protective mechanisms. Intact skin cannot be penetrated by most bacteria, and proteins such as defensins, or fatty acids produced by the skin are toxic to many microorganisms (reviewed by Neutra M.R. et al. 1996). Epithelial surfaces are cleansed by ciliary action in the trachea or by flushing; in the urinary tract. Moreover, the stomach and the vagina provide an acidic environment in which most bacterial strains cannot survive. In practice, only a small minority of the potentially pathogenic microorganisms around us manage to gain access to the tissues.

If the microorganisms do manage to enter the tissues, they are combated by further elements of the innate immune system (Bancroft G.J. et al. 1991). The types of recognition are broad-spectrum mechanisms that evolved before antigen-specific T cells and immunoglobulins, allowing protective responses to be triggered by common bacterial components. Central to this are toll-like receptors, a family of receptors that have been conserved from Drosophila to humans and have been shown to be involved in the recognition of the bacterial components lipopolysaccharide, lipoarabonomannan and lipoproteins as well as the fungal carbohydrate zymosan (Ulevitch R.J. and Tobias P.S. 1995). Signalling through toll-like receptors results in the activation of phagocytes that release cytokines such as TNFα and IL-1 and various chemokines. Overall, this leads to the increased permeability of the vascular endothelium, the systemic activation of phagocytic cells, the increased recruitment of leukocytes, and the increased adhesion and diapedesis of phagocytes to the infected tissue. In addition, TNF and IL-12 activate natural killer cells that release IFNγ which in turn feeds back to augment macrophage activation, phagocytic ability and antigen presentation (Tracey K.J. and Cerami A. 1989; Biron C.A. and Gazzinelli R.T. 1995; Chan S.H. et al. 1991).

Activation of complement via the alternative pathway may also result in the killing of some bacteria such as gram-negative bacteria that have an outer lipid bilayer susceptible to the lytic complex (Brown E.J. 1991; Frank M.M. and Fries L.F. 1991). The complement, at the same time, releases C3a and C5a that are chemotactic to neutrophils and also cause smooth-muscle contraction and mast cell degranulation (Hopken U.E. et al. 1996). Histamine and leukotrienes (LTB_4) released contribute to further increases of vascular permeablity, and attachment to bacteria of cleaved derivatives of C3 are important in subsequent opsonization by phagocytes. Ultimately, most bacteria are killed by macrophages that employ reactive oxygen and nitrogen intermediates (Miller R.A. and Britigan B.E. 1997; MacMicking J. et al. 1997), cationic proteins such as defensins (Ouellette A.J. and Selsted M.E. 1996), or other antomicrobial mechanisms such as transient pH changes in the phagolysosome to kill microorganisms (Adams D.O. and Hamilton T.A. 1984).

Apart from activating complement and killing gram-negative bacteria, antibodies are particularly important in protecting against damage from bacterial toxins. Antibodies can neutralize bacterial toxins or even extracellular matrix-degrading enzymes preventing the spreading of the microorganism or the tissue damage induced by it. Secretory IgA responses are very useful in preventing bacteria from binding epithelial cells in external and mucosal surfaces. IgG1, IgG3 and IgM responses in man are the most efficient complement-fixing antibodies and are also the subclasses with the highest affinity for Fc receptors (reviewed by Burton D.R. and Woof J.M. 1992; Jefferis R. et al. 1994).

Protection from bacteria growing extracellularly requires mainly antibody responses that can inhibit the spread of the microorganism and the damage that can occur due to toxins and other substances released. However, some bacterial strains have developed mechanisms that permit them to live inside damaged or metabolically deranged host phagocytes, or escape killing by moving out of the phagolysosomes into the cytoplasm (reviewed by Finlay B.B. and Cossart P. 1997; Small P.L. et al. 1994). Others, such as Mycobacterium leprae, can invade non-phagocytic cells that have little antimicrobial potential. To clear infections of such pathogens, the organism develops $CD8^+$ cytotoxic T lymphocyte responses to bacterial antigens presented by antigen-presenting cells (Kaufmann S.H. 1988; Kaufmann S.H. 1993; Liu C.C. et al. 1996; Kagi D. et al. 1996). These result in the killing of cells harboring pathogenic microorganisms and exposure of them to fresh activated phagocytic cells, circulating antibodies or other antimicrobial mechanisms. If $CD8^+$ cytotoxic T cell responses are compromised as, for example, in the β_2 microglobulin knock-out mice, susceptibility to M.tuberculosis is substantially increased (Ladel C.H. et al. 1995). Thus, intracellular bacterial infections require T cell differentiation to a T_{H1} phenotype and cell-mediated immune responses, whereas extracellular bacterial infections require antibody responses favoured by a T_{H2} phenotype.

1.3.3. Immunity to parasites

Parasitic infections typically stimulate both antibody- and cell-mediated defense mechanisms, but the effectiveness depends upon the particular parasite and the stage

of infection. In general, antibody is most effective against extracellular parasites in blood and tissue fluids, whereas cell-mediated responses are required to eliminate intracellular parasites. Many parasites (e.g. malarial parasites) can exist in both extracellular and intracellular forms according to the stage of infection; consequently, both antibody with complement and cell-mediated immune responses are needed.

Before host immunity has been initiated or achieved, the complement provides an early line of defense as several parasites carry molecules in their surface coats that activate the alternative pathway. Tissue macrophages, monocytes and neutrophils also have an anti-parasitic activity that is greatly enhanced specifically in response to parasitic antigens when adaptive immune mechanisms take place. Macrophages, for example, act as effector cells to inhibit the multiplication of parasites or even to kill them (Green S.J. et al. 1990; Stenger S. et al. 1996). They secrete cytokines such as TNF, IL-1, IL-12 and colony-stimulating factors (Finkelman F.D. et al. 1997) that enhance immunity by activating natural killer cells, neutrophils, eosinophils and platelets. In addition, macrophages can kill many parasites by phagocytosis and ingestion, or by acting as killer cells through antibody-dependent cell-mediated cytotoxicity (Langermans J.A. et al. 1994). Activation of macrophages is a general feature of the early stages of infection with macrophage effector functions being enhanced through IFNγ secreted by natural killer cells and later by T cells (Scharton T.M. and Scott P. 1993).

The effector properties displayed by macrophages are also seen in neutrophils that are phagocytic and can also kill parasites by oxygen-dependent and independent mechanisms. Neutrophils are activated by cytokines such as IFNγ, TNFα and GM-CSF. Extracellular destruction by neutrophils is mediated by H_2O_2, whereas granular components are involved in the intracellular destruction of ingested organisms. Neutrophils migrate to parasite-infected inflammatory lesions and probably act to clear parasites from bursting cells. Similarly to macrophages, they can participate in antibody-dependent cytotoxic reactions.

Eosinophils are less phagocytic than neutrophils. They degranulate in response to perturbation of their surface membrane, and their activities are enhanced by cytokines

such as TNF and GM-CSF. Most of their activities, however, are controlled by antigen-specific mechanisms. Eosinophils act together with mast cells to degranulate in response to local IgE (Weller P.F. 1994; Gounni A.S. et al. 1994). Platelets can also exert an effector function; They bear Fcε receptors on their surface membrane, by which they mediate antibody-dependent cytotoxicity associated with IgE.

T cells are central to the development of adaptive immune responses to parasites (Sher A. and Coffman R.L. 1992). The type of T cell responsible for controlling an infection, varies with the parasite and the stage of infection. For example, $CD4^+$ T cells mediate immunity against the blood stage of Plasmodium, whereas the $CD8^+$ cells protect against its liver stage (Nardin E.H. and. Nussenzweig R.S. 1993). In the latter case, $CD8^+$ T cells secrete IFNγ that inhibits the multiplication of parasites within the hepatocytes, and destroy infected hepatocytes. $CD8^+$ T cells do not affect the blood stage parasites because erythrocytes do not express MHC class I. The immune response against T.cruzi or T.gondii, on the other hand, depends not only on $CD4^+$ and $CD8^+$ T cells but also on NK cells and antibody production (Tarleton R.L. et al. 1996).

Similarly, the T helper phenotype varies. In the early stages of an infection there may be a mixture of both T_{H1} and T_{H2} subsets. As T_H subsets are mutually antagonistic, the type of T cell that finally predominates will decide the outcome of the infection. In malaria, T_{H1} cells act against the liver stage of malaria, whereas elimination of the blood stage of malaria occurs in the spleen via activated effector cells and antibody-dependent cytotoxicity (Heinzel F.P. et al. 1989; Scharton T.M. and Scott P. 1993; Soong L. et al. 1997).

T_{H1} immune responses are very important for protection against intracellular protozoa. IFNγ released activates macrophages to kill the protozoa (e.g. L.major, T.cruzi and T. gondii) that live within them, and enhances effector responses against other parasites (Heinzel F.P. et al. 1989). In contrast, T_{H2} cells are necessary for the elimination of intestinal worms, and this involves mucosal mast cells and eosinophils activated by antigen-stimulated T cells (Madden K.B. et al. 1991; Finkelman F.D. et al. 1997; Roberts C.W. et al. 1996; Weller P.F. 1994; Gounni A.S. et al. 1994). Mast cells

produce a number of cytokines such as IL-3, IL-4, GM-CSF and TNFα that cause changes in the permeability of the gut and shedding of the epithelium which may also help eject some protozoan parasites. Alteration of the mucosal permeability allows complement and serum antibodies to leak into the gut lumen. In helminth infections, however, both T_{H1} and T_{H2} responses are important and the relative contribution of each is still unclear (Mahmoud A.A. 1989; Else K.J. 1994).

Many parasitic infections induce non-specific antibody production, known as hypergammaglobulinaemia (Pritchard D.I. 1983). This is probably due to parasitic substances acting as B cell mitogens. Levels of total immunoglobulins are raised with high IgM levels in trypanosomiasis and malaria, and high IgG levels in malaria and visceral leishmaniasis. Specific antibody production, though, is very important in controlling parasitic infections. Antibodies can act directly on protozoa damaging them by activating complement, neutralize parasites by blocking their attachement to the host, enhance phagocytosis by macrophages or induce antibody-dependent cell mediated cytotoxicity (Mangold B.L. and Dean D.A. 1986; White W.I. et al. 1991). Different antibody isotypes may have different effects on the outcome of the infection, as development of immunity to parasites in children seems to depend upon a switch from IgG4 to IgE that occurs with age.

1..3.4. Vaccination

Vaccination constitutes the most successful manipulation of the immune sustem to date because it takes advantage of its specificity and inducibility. It grew from the successes of Jenner and Pasteur against smallpox and cholera, and has resulted in the eradication or virtual elimination of various human diseases (Jenner E. 1778; Pasteur L. 1880). Vaccination is history's most cost effective public health tool.

There are several requirements for successful vaccination and they vary according to the nature of the pathogen. For extracellular organisms, for example, vaccines need to induce strong antibody responses as a mechanism to fight infection, whereas for intracellular organisms antibody responses need to be combined with effective cytotoxic T lymphocyte responses. In either case, both T and B cell responses must be

primed. Ideally, host defense should be stimulated at the sites of infection of the pathogen, such as mucosal surfaces. In addition, successful vaccines must be safe, protective in the majority of the population, and provide long-lived immunity. Currently available vaccines in general use in man are summarized in table 1.5. They comprise live attenuated, whole killed and conjugate vaccines.

DISEASE	VACCINE TYPE	ADMINISTRATION
Tetanus	Toxoid	Three doses between 2-6 months of age and boosting every 10 years
Diphtheria	Toxoid	
Pertussis	whole killed	
Polio	killed (Salk) or attenuated (Sabin)	
Measles	Attenuated	Given together (MMR vaccine) at 12-18 months
Mumps	Attenuated	
Rubella	Attenuated	
Tuberculosis	BCG	Given in the UK between 10-14 years
Hepatitis B	Surface antigen (conjugate vaccine)	Given to people at risk
Rabies	Killed	Given to people at risk
Meningitis	Polysaccharide	Given to travellers
Yellow fever	Attenuated	
Cholera	Mutant	
Typhoid	Killed	
Hepatitis A	killed/attenuated	
Influenza	Killed	Given to people at risk

Table 1.5: Currently available vaccines (Adapted from Playfair J. 1998).

Although the most effective vaccines are by far live attenuated microorganisms and viruses, or sometimes closely related non-pathogenic microorganisms, they carry some risk to revert back to the wild-type and also to be potentially lethal to immunosuppressed or immunodeficient individuals. For that reason, acellular vaccines are preferred and the commonest type of them is whole killed pathogens and conjugate vaccines. Conjugate vaccines consist of individual components of the microorganism or virus and are safer than whole-killed pathogens that still contain toxins and constituents that can cause tissue damage. Single isolated constituents of a

microorganism, however, are poorly immunogenic and very rarely induce the appropriate immune response, with the exceptions of toxoid vaccines against tetanus and diphtheria toxins. To enhance the immunogenicity of pathogen-derived constituents in order to generate immunity, adjuvants are used. A very wide range of materials, with numerous and distinct mechanisms of action, have been used as adjuvants (reviewed by Singh M. and O'Hagan D. 1999) but despite that, only aluminium-based mineral salts have obtained approval for their use in humans by the US Food & Drug Administration (Rockville, MD).

Most of the currently available vaccines induce antibody responses capable of mediating long-term protection. In contrast, when cell-mediated immunity is required for protection, the success has been very limited; there are no vaccines for most viral or parasitic diseases. Over the past 5-10 years, the understanding of the mechanisms involved in antigen presentation and cell-mediated immunity, and the advent of DNA technology has lead to the development of DNA vaccination, a novel approach to induce cell-mediated protection.

A standard DNA vaccine consists of genes of the pathogen cloned into a DNA vector (e.g. a bacterial plasmid) and optimized for high expression in mammalian cells. When injected intramuscularly or subcutaneously, DNA vaccines elicit T_{H1} and cytotoxic T lymphocyte responses by mechanisms that are believed to involve direct transfection of dendritic cells, transfection of somatic cells and cross-priming of dendritic cells by somatic cells (Corr M. et al. 1996; Doe B. et al. 1996; Condon C. et al. 1996; Raz E. et al. 1994; Albert M.L. et al. 1998). DNA vaccines have been shown to induce protective immunity against L.major, malaria, M.tuberculosis and others in a number of animal models (Gurunathan S. et al. 2000b). Currently, an extensive amount of work is under way to optimize DNA vaccines by incorporating into the vaccine cytokine and chemokine genes that can enhance immunity (Gurunathan S. et al. 2000a) or by using non-pathogenic/attenuated microorganisms or viruses as vehicles to enhance the efficiency of DNA delivery into antigen-presenting cells.

During the last decade, vaccines have attracted attention in cancer therapy as well. It has been found that tumours express modified or mutant proteins that are antigenic and can be recognized by T cells. The ability of the immune sytem to detect tumour

cells and destroy them is known as immune surveillance (Marincola F.M. et al. 2000). Tumours, however, escape this immune surveillance by down-regulating adhesion or costimulatory molecules necessary to elicit a primary T cell response, by mutating their antigens to generate escape mutants from the immune response, by losing expression of particular MHC class I molecules or by producing immunosuppressive cytokines (Bodmer W.F. et al. 1993; Moller P. and Hammerling G.J. 1992; Storkus W.J. et al. 1987; Tada T. et al. 1991; Torre-Amione G. et al. 1990). In order to break the tolerance of the immune system to tumour antigens and to induce an immune response against them vaccines are being investigated. Various forms of vaccines have been used (Raychaudhuri S. and Rock K.L. 1998) with great success in animal models and encouraging results in the first human clinical trials.

1.4. Aims of this thesis

During the last decade, great progress has been made towards understanding the signalling pathways that take place in immune and inflammatory processes. However, there has been a large degree of controversy whether observations made in cell lines that are selected for long term *in vitro* growth represent physiologically relevant observations.

An approach to circumvent that problem has been the generation of mice deficient in signalling components to directly examine their role *in vivo*. Although this offers great advantages over traditional approaches, it allows compensation mechanisms to operate during development and it often results in non-viable mice. This limits the amount of information that can be obtained.

In this thesis, I have used another approach for studying the role of signalling components that is based on the use of replication-deficient recombinant adenoviruses as an efficient gene transfer technique into human primary cells. This approach overcomes one of the major obstacles of studying the biochemical signalling pathways in primary cells, which is the difficulty of transfecting DNA (Stacey K.J. et al. 1993; Zhong L. et al. Eur J Immunol 1999). In addition, it allows the study of endogenous genes instead of using artificial reporter constructs.

The aims of this study were:

- To investigate the role of **IKK2**, **NIK** and **MyD88** in NF-κB activation, cytokine production and **antigen presentation** in human primary **dendritic cells**.

- To examine whether the role of **IKK2**, **NIK** and **MyD88** in NF-κB activation is **lineage-restricted** by comparing dendritic cells, macrophages, skin fibroblasts and umbilical vein endothelial cells.

- To **validate *in vitro* observations on antigen presentation *in vivo*** by setting up a model of genetic immunization with adenoviral vectors.

The data presented in the following chapters will challenge some of the current concepts of NF-κB activation, and will propose a novel way of enhancing antigen presentation *in vivo*.

CHAPTER 2

MATERIALS AND METHODS

2.1. Reagents

2.1.1. Cytokines and antibodies

Recombinant human cytokines used in these studies are summarized in table 2.1.

Table 2.1: Sources of human recombinant cytokines

Cytokine	Species	Source
Il-1α	human	Hoffman La Roche, USA
IL-2	human	Hoffman La Roche, USA
IL-4	human	Peprotech, London, UK
IL-6	human	Sandoz, Basel. Switzerland
Il-8	human	Biosource, Nivelles, Belgium
IL-10	human	Schering-Plough Research Institute, NJ, USA
IL-12	human	Genetics Institute Inc. USA
IL-15	human	Iimmunex, Seattle, WA, USA
IL-18	human	R&D Systems, Abindon, UK
sCD40L	human	Peprotech, London, UK
TNFα	human	Centre of Molecular and Macromolecular Studies, Lodz, Poland
IFNγ	human	Bender Wien, Vienna, Austria
M-CSF	human	Genetics Institute Inc., MA, USA
GM-CSF	human	Genetics Institute Inc., MA, USA
MIP1α	human	R&D Systems, Abindon, UK
RANTES	human	R&D Systems, Abindon, UK
MCP-1	human	R&D Systems, Abindon, UK
MCP-3	human	R&D Systems, Abindon, UK
IP-10	human	R&D Systems, Abindon, UK
IL-4	mouse	R&D Systems, Abindon, UK
IFNγ	mouse	R&D Systems, Abindon, UK

Murine monoclonal and rabbit polyclonal anti-human antibodies used for FACS staining (FC), cell culture (CC), western blotting (WB) and supershifts (SS) are summarized in tables 2.2 and 2.3.

Table 2.2: Antibodies used for ELISA and flow cytometry

Specificity	Antibody	Species	Source
ELISA Antibodies			
Anti-human			
TNFα	61E71 (coat)	Mouse	Dr W. Buurman, Netherlands
TNFα	(detection)	Rabbit	Dr W. Buurman, Netherlands
IL-1β	ILB1-H6 (coat)	Mouse	Dr D. Webb, Syntex, CA, USA
IL-1β	IL1B1-H67 (detect)	Mouse	Dr D. Webb, Syntex, CA, USA
IL-6	LNI 314-14 (coat)	Mouse	Dr F. Di Padova, Basel, Switzerland
IL-6	LNI 314-110 (detect)	Mouse	Dr F. Di Padova, Basel, Switzerland

IL-8	4.1.13 (coat)	Mouse	Dr Wong, Genetech, CA, USA
IL-8	A5.12.14 (detect)	Mouse	Dr Wong, Genetech, CA, USA
IL-12	C11.5.14 (coat)	Mouse	Genetics Institute Inc., MA
IL-12	12H4/1.2.8 (detect)	Mouse	Genetics Institute Inc., MA
IL-15	34505.11 (coat)	Mouse	R&D Systems, Abindon, UK
IL-15	34593.11 (detect)	Mouse	R&D Systems, Abindon, UK
IL-18	52713.11 (coat)	Mouse	R&D Systems, Abindon, UK
IL-18	AYQ01 (detect)	Goat	R&D Systems, Abindon, UK
MIP1α	14215.41 (coat)	Mouse	R&D Systems, Abindon, UK
MIP1α	UU027111 (detect)	Goat	R&D Systems, Abindon, UK
RANTES	21414.211	Mouse	R&D Systems, Abindon, UK
RANTES	VC017011 (detect)	Goat	R&D Systems, Abindon, UK
MCP-1	23007.11 (coat)	Mouse	R&D Systems, Abindon, UK
MCP-1	AKY018031 (detect)	Goat	R&D Systems, Abindon, UK
MCP-3	36320.11 (coat)	Mouse	R&D Systems, Abindon, UK
MCP-3	AUP018021 (detect)	Goat	R&D Systems, Abindon, UK
IP-10	33036.211 (coat)	Mouse	R&D Systems, Abindon, UK
IP-10	AAP017011 (detect)	Goat	R&D Systems, Abindon, UK
Anti-mouse			
IL-4	CX1010031	Goat	R&D Systems, Abindon, UK
IL-4	XR050031	Goat	R&D Systems, Abindon, UK
IFNγ	UA019061	Goat	R&D Systems, Abindon, UK
IFNγ	WB029061	Goat	R&D Systems, Abindon, UK
Flow cytometry antibodies			
CD3/CD19	Leucogate	Mouse	Becton Dickinson, CA, USA
CD14/CD45	Leucogate	Mouse	Becton Dickinson, CA, USA
IgG2a control	M049218	Mouse	Pharmingen,UK
CD80	M046450	Mouse	Pharmingen, UK
CD86	M030253	Mouse	Pharmingen, UK
HLA-DR	M042986	Mouse	Pharmingen, UK
HLA-A,B,C	M029593	Mouse	Pharmingen, UK
CD25	M044582	Mouse	Pharmingen, UK

Table 2.3: Antibodies used for western blotting (WB), immunoprecipitation (IP) and supershifts (SS)

Specificity	Antibody	Species	Use	Source
Flag-epitope	M2	Mouse	WB, IP	Sigma, Dorset, UK
IκBα	C-15	Rabbit	WB	Santa Cruz Biotech, CA, USA
IκBβ	N-20	Rabbit	WB	Santa Cruz Biotech, CA, USA
IKK2	H-470	Rabbit	WB	Santa Cruz Biotech, CA, USA
NIK	SAK14	Sheep	WB	Prof. J. Satlakvala, London, UK
MyD88	F-19	Goat	WB	Santa Cruz Biotech, CA, USA
IRAK	19703	Rabbit	WB	Upstate Biotech, NY, USA
p42 MAPK	(K-23)-G	Rabbit	WB	Santa Cruz Biotech, CA, USA
β-galactosidase	D19-2F3-2	mouse	WB	Roche Mol. Biochem., USA
Phospho-IκBα	Lot 2	Rabbit	WB	New England Biolabs
Phospho-p38 MAPK	Lot 6	Rabbit	WB	New England Biolabs

Phospho-p42/44 MAPK	Lot 10	Rabbit	WB	New England Biolabs
Phospho-p54 MAPK	Lot 8	Rabbit	WB	New England Biolabs
Ig control	H-249	Rabbit	SS	Santa Cruz Biotech, CA, USA
p65 (rel-A)	H-286	Rabbit	SS	Santa Cruz Biotech, CA, USA
RelB	E-259	Rabbit	SS	Santa Cruz Biotech, CA, USA
C-rel	H-085	Rabbit	SS	Santa Cruz Biotech, CA, USA
p50	H-119	Rabbit	SS	Santa Cruz Biotech, CA, USA
p52	F-259	Rabbit	SS	Santa Cruz Biotech, CA, USA
Donkey anti-rabbit-IgG-HRP $F(ab')_2$	184:181	Donkey	WB 2nd layer	Amersham Life Sciences, UK
Rabbit anti-mouse-IgG-HRP	097(101)/1203	Rabbit	WB 2nd layer	DAKO, Cambridge, UK
Rabbit anti-sheep-IgG-HRP	097(101)/1103	Rabbit	WB 2nd layer	DAKO, Cambridge, UK

For western blotting, second layer antibodies were donkey anti-rabbit IgG HRP $F(ab')_2$ fragments (Amersham Pharmacia, UK), rabbit anti-mouse IgG HRP or rabbit anti-sheep-IgG HRP (DAKO, Cambridge, UK).

2.1.2. Enzyme inhibitors

Table 2.4: Synthetic enzyme inhibitors

Inhibitor	Target	Source
Aprotinin	Serine proteases	Sigma, Dorset, UK
Leupeptin	Serine/cysteine proteases	Sigma, Dorset, UK
Pepstatin	Aspartic proteases	Sigma, Dorset, UK
Phenyl-methyl-sulphonyl-fluoride (PMSF)	Serine proteases	Sigma, Dorset, UK
Sodium fluoride	Protein phosphatases	Sigma, Dorset, UK
Sodium orthovanadate	Protein phosphatases	Sigma, Dorset, UK
β-glycerophosphate	Protein Phosphatases	Sigma, Dorset, UK

2.2. Buffers

2.2.1. General Buffers

(i) Phosphate buffered saline (PBS)
Made from 10X concentrated solution

4.3 mM Na_2HPO_4
1.4 mM KH_2CO_3
1.4 mM KCl
137 mM NaCl
pH 7.0

(ii) Tris EDTA buffer (TE)

Tris-HCl, pH8.0
1 mM EDTA

(iii)	Immunoprecipitation buffer	1% (v/v) Triton X-100 5 mM HEPES pH7.4 150 mM sodium chloride 0.1% (w/v) SDS 0.5% (w/v) sodium deoxycholate 1 mM EDTA 25 mM β-glycerophosphate 1 mM sodium orthovanadate 10 mM sodium fluoride

2.2.2. Tissue culture media

(i)	Monocyte and dendritic cell culture medium	RPMI 1640 containing 5% (v/v) heat inactivated foetal calf serum
(ii)	Human umbilical vein endothelial cells medium	RPMI 1640 containing 10% (v/v) heat inactivated foetal calf serum, 10% (v/v) heat-inactivated newborn bovine serum, 15 μg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 500 U/ml heparin (CD Pharmaceuticals Ltd., Wrexham, UK)
(iii)	T lymphocyte culture medium	RPMI 1640 containing 10% (v/v) heat inactivated human AB+ serum
(iv)	K5 and L47 mouse fibroblasts	RPMI 1640 containing 1% heat inactivated foetal calf serum, 1 % penicillin/streptomycin and 2 mg/ml gentamycin (G418)
(v)	Human skin fibroblasts medium	DMEM containing 5% (v/v) foetal calf serum, 1% penicillin/streptomycin
(vi)	Human embryonic kidney 293 cells medium	DMEM containing 5% (v/v) foetal calf serum, 1% penicillin/streptomycin
(vii)	Mouse peritoneal macrophages medium	DMEM containing 5% (v/v) foetal calf serum, 1% penicillin/streptomycin and 20 μM of β-mercaptoethanol
(viii)	Mouse lymph node cells medium	DMEM containing 5% (v/v) foetal calf serum, 1% penicillin/streptomycin and 20 μM of β-mercaptoethanol

2.2.3. FACS buffers

(i)	FACS wash buffer	PBS containing 1% FCS, 0.02% azide
(ii)	FACS fixing buffer	PBS containing 1% paraformaldehyde,

		0.02% azide

2.2.4. ELISA buffers

(i)	ELISA coating buffer	PBS
(ii)	ELISA wash buffer	PBS containing 0.05% Tween 20
(iii)	Buurman wash buffer	Distilled H^2O containing 0.01% Tween 20
(iv)	ELISA blocking buffer	PBS containing 2% bovine serum albumin (BSA)
(v)	glycine substrate buffer, pH 10.4	0.1M glycine 0.001M $MgCl_2$ 0.001M $ZnCl_2$

2.2.5. SDS-PAGE solutions

(i)	Solution A	30% acrylamide 0.8% bis-acrylamide
(ii)	Solution B	1.5 M Tris-HCl, pH 6.8
(iii)	Solution C	0.5 M Tris-HCl, pH 8.8
(iv)	Running buffer	19 mM Tris-base 192 mM glycine 0.1% SDS
(v)	Transfer buffer	25 mM Tris-base 150 mM glycine 20% methanol
(vi)	Stripping buffer	62.5 mM Tris-Hcl, pH 6.7 2% SDS 100 mM β-mercaptoethanol
(vii)	Sample buffer	125 mM Tris-HCl, pH 6.8 20% glycerol 0.01% bromophenol blue 5% β-mercaptoethanol

2.2.6. Lysis buffers

(i)	Hypotonic lysis buffer	10 mM HEPES 1.5 mM $MgCl_2$ 10 mM KCl pH 7.9

(ii)	Hypertonic extraction buffer	5 mM HEPES 1 mM $MgCl_2$ 0.2 mM EDTA 0.5 M NaCl 25% glycerol pH 7.0
(iii)	Bradford reagent for protein assay	0.1% (w/v) Coomassie brilliant blue G 5% (v/v) methanol Orthophosphoric acid

Added fresh: 0.5mM DTT, 0.5mM PMSF, 30 μg/ml leupeptin, 5 μg/ml aprotinin and 5 μg/ml pepstatin.

2.2.7. Electrophoretic mobility shift assay (EMSA)

(i)	10X T4 Polynucleotide kinase buffer	700 mM Tris.HCl pH 7.6 100 mM magnesium chloride 50 mM Dithiothreitol (DTT)
(ii)	5X DNA binding buffer	50 mM Tris.HCl pH 7.5 20% (v/v) glycerol 250 mM sodium chloride 5 mM magnesium chloride 2.5 mM EDTA 2.5 mM DTT 0.25 mg/ml poly(dI-dC)
(iii)	Tris EDTA buffer (TE)	10 mM Tris.HCl pH 8.0 1 mM EDTA
(iv)	10X Tris-Borate-EDTA buffer (TBE)	0.89 M Tris.HCl pH 8.0 0.89 M boric acid 2 mM EDTA

2.2.8. Adenoviral purification buffers

(i)	Saturated CsCl solution	~35 ml of 0.01 M Tris 1 mM EDTA CsCl added until saturation
(ii)	PBS^{2+} (1 litre)	880 ml H_2O 100 ml 10X PBS 10 ml 1% w/v $CaCl_2$ 10 ml 1% w/v $MgCl_2$
(iii)	1.4 g/ml density CsCl (0.534 g/ml concentration in 1litre)	534 g $CsCl_2$ in 1 litre H_2O

(iv)	1.34 g/ml density CsCl (0.453 g/ml concentration in 250 ml)	212.5 ml of 1.4 g/ml CsCl 37.5 ml PBS^{2+}

2.2.9. β-galactosidase detection

For the detection of β-galactosidase, the following solutions were used:

(i)	FDG staining solution	PBS 10 mM HEPES pH 8.0 4% (v/v) foetal calf serum
(ii)	FDF stain	2 mM fluorescein Di-(β-galactopyranoside)

2.3. Tissue culture

2.3.1. Media

The following tissue culture media and solutions were obtained as 1 X sterile solutions from BioWhittaker, Berkshire, UK.

1) RPMI 1640, containing 25mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) and 2 mM L-glutamine

2) Hank's balanced salt solution (HBSS), containing 0.35 g/L sodium bicarbonate (without calcium or magnesium)

3) Dulbecco's modified Eagle's medium (DMEM)

4) Modified Eagle's medium (MEM)

5) Antibiotics solution, containing 100 U/ml penicillin and 100 μg/ml streptomycin

2.3.2. Sera

Foetal calf serum (FCS), newborn bovine serum (NBS) and normal human AB+ serum (HS) were purchased from Sigma (UK) and BioWhittaker (UK), respectively. They were thawed in a waterbath at 37° C, prior to heat-inactivation at 56° C for 30 minutes. Sera were kept at -20° C as 50 ml aliquots.

2.3.3. Endotoxin testing

All media, sera, antibodies, cytokines, proteins and pharmacological agents were tested for the presence of endotoxin prior to their use by the BioWhittaker Limulus amebocyte assay (BioWhittaker, Berkshire, UK). A flat-bottomed microplate (falcon, Becton Dickinson, Oxford, UK) was equilibrated to 37° C. A solution of endotoxin (0.1Eu/ml equivalent to 100 pg/ml) was prepared and serially diluted in LPS-free water (BioWhittaker, Berkshire, UK). 50 µl/well were added in the 96-well plate along with 50 µl test sample or blank (LPS-free water). Then, another 50 µl of limulus amebocyte lysate were added to each well and incubated for 10 min at 37° C. 100 µl of the substrate solution (at 37° C) were added to each well and incubated for a further 6 minutes prior to termination of the reaction by the addition of 100 µl stop reagent. Absorbance was read at 410 nm. The sensitivity of the assay was <10 pg/ml. All assays were kindly performed by Patricia Green.

2.3.4. LPS and lipid A

Salmonella typhimirium LPS and E. coli lipid A were purchased from Sigma Chemical Company (UK).

2.3.5. Cell lines

The cell lines used for the purpose of these studies are listed in table 2.5.

Table 2.5: Cell lines used in this study

HeLa	Human epithelial cells	ATCC, MD, USA
HEK 293	Human embryonic kidney	ATCC, MD, USA
K5 transfectants	Mouse control fibroblasts	Dr D.Gray (UK) and Prof. Schimpl (Germany)
L47 transfectants	Mouse fibroblasts expressing mouse CD40L	Dr D.Gray (UK) and Prof. Schimpl (Germany)

HeLa and HEK 293 cells were maintained in 5% FCS 1% penicillin/streptomycin DMEM, whereas transfected mouse fibroblasts were maintained in 1% FCS 1% penicillin/streptomycin.

2.3.6. Human skin fibroblasts and human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (Jaffe E.A. et al. 1973) and maintained in RPMI, in the presence of 10% FCS, 10% NCS, 1% penicillin/streptomycin, 15 μg/ml endothelial growth supplement (Sigma, Poole, UK) and 50 IU/ml heparin. Primary human skin fibroblasts (HSF) were obtained from circumcision specimens cultured in 5% FCS 1% penicillin/streptomycin DMEM in 75 cm^2 tissue culture flasks.

2.3.7. Preparation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained by density centrifugation of leukopheresis residues from healthy volunteers (North London Blood Transfusion Service, Colindale, UK). Heparinised residues were diluted 2X with HBSS and 25 ml were carefully layered over equal volumes of Ficoll-Hypaque lymphoprep (Nycomed, Oslo, Norway) in 50 ml sterile tubes prior to centrifugation for 30 minutes at 2000 rpm at room temperature. After centrifugation, the interface layer was collected and washed twice with HBSS (centrifuged for 10 minutes at 2000 rpm). PBMC were then collected and resuspended in 30 ml of RPMI containing 5% FCS.

2.3.8. Isolation of human peripheral blood T cells and monocytes

Peripheral blood T cells and monocytes were obtained from PBMC after cell separation in a Beckman JE6 elutriator. Elutriation was performed in RPMI containing 1% FCS (elutriation medium). Briefly, the cell suspension was pumped into a funnel-shaped elutriation chamber at a constant predetermined flow rate. As elutriation medium travels through the chamber, the velocity decreases as the chamber gets wider, creating a velocity gradient from the narrow end of the chamber to the widest part (figure 2.1).

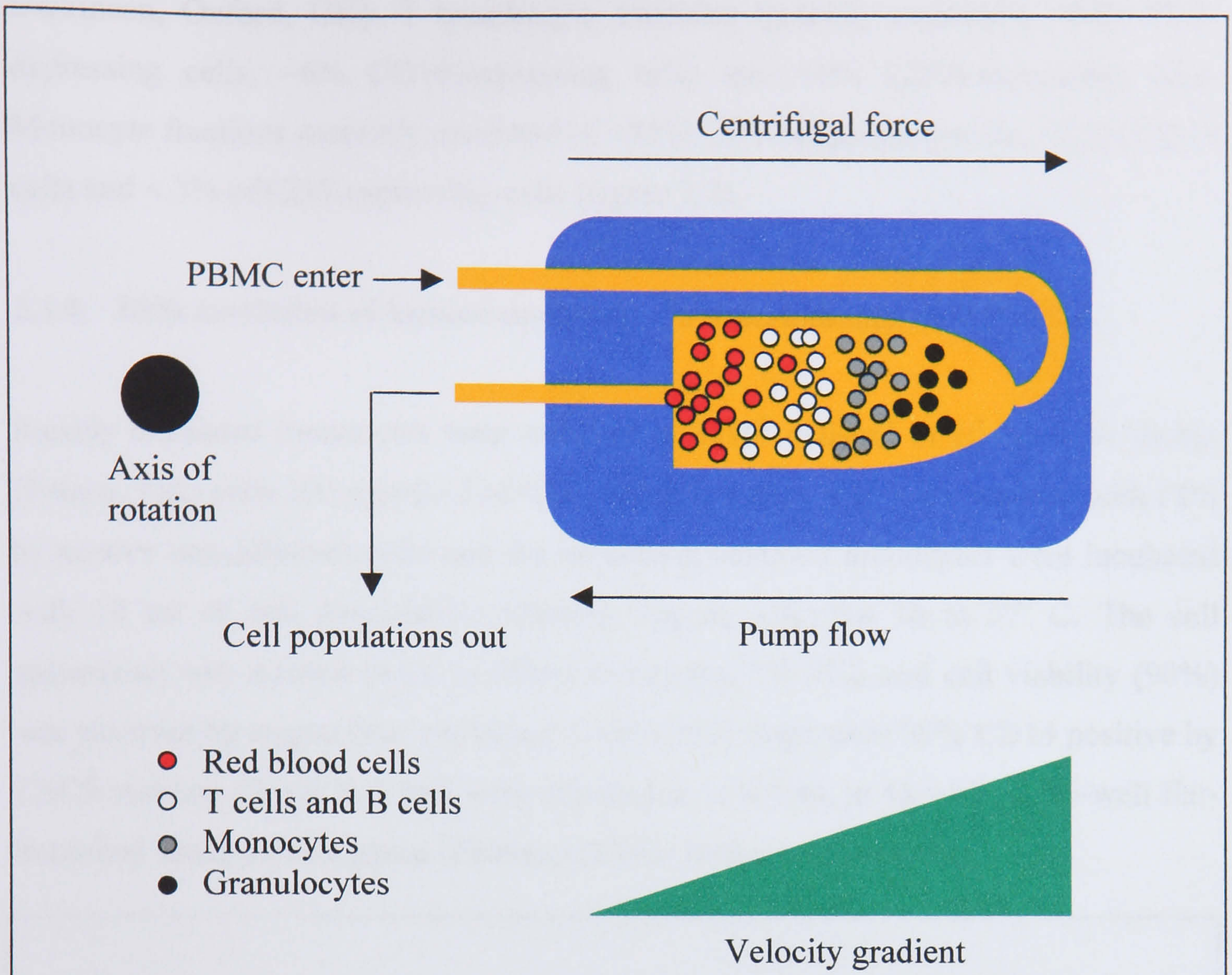


Figure 2.1: Centrifugal elutriation used for the purification of peripheral blood T cells and monocytes. Illustration of a cross-section of an elutriation chamber. The chamber rotates clockwise around the axis at 2500 rpm creating a centrifugal force. The peristaltic pump creates an opposing flow. The funnel-shaped chamber causes the velocity of the fluid to slow, forming a defined gradient. This results in cells of different sizes and densities to migrate to the point where the two opposing forces balance. Thus, bigger cells such as granulocytes and macrophages are placed at the back of the elutriation chamber as they receive a stronger centrifugal force due to their bigger mass. Smaller cells such as T cells and B cells migrate to the front. By gradually increasing the flow rate, cells of increasing size can be eluted. Good purities of T cells and monocytes can be obtained by using this method.

Cells migrate through the chamber under the influence of two opposite forces, the centrifugal force pushing them in the backward direction and the fluid velocity, pushing them in the forward direction. Smaller cells receive a lower centrifugal force and thus localize near the outlet of the chamber whereas larger cells remain near the inlet of the chamber as they are under the influence of a higher centrifugal force due to their size (figure 2.1). By gradually increasing the flow rate, successive fractions of increasingly large cells were washed out of the rotor and collected. Lymphocyte and monocyte purity was assessed by flow cytometry using fluorochrome-conjugated anti-human monoclonal antibodies against CD45, CD3, CD14 and CD19 (Becton

Dickinson, Oxford, UK). T lymphocyte fractions typically contained ~80% CD3-expressing cells, ~6% CD19-expressing cells and <1% CD14-expressing cells. Monocyte fractions routinely consisted of >85% CD14-expressing cells, <0.5% CD19 cells and < 3% of CD3-expressing cells (figure 2.2).

2.3.9. Differentiation of human monocytes to macrophages with M-CSF

Freshly elutriated monocytes were cultured at $1x10^6$ cells/ml in 10 cm petri dishes (Falcon, UK) with 100 ng/ml of M-CSF. After 2-3 days they were washed with PBS to remove non-adherent cells and the remaining adherent monocytes were incubated with 10 ml of cell dissociation solution (Sigma, UK) for 1h at 37° C. The cell suspension was washed twice in RPMI containing 5% FCS and cell viability (90%) was assessed by trypan blue exclusion. Cells at this stage were 99% CD14 positive by FACS staining (figure 2.2) and were cultured at $1x10^6$/ml, in 48-well or 96-well flat-bottomed tissue culture plates (Falcon, UK) for further experiments.

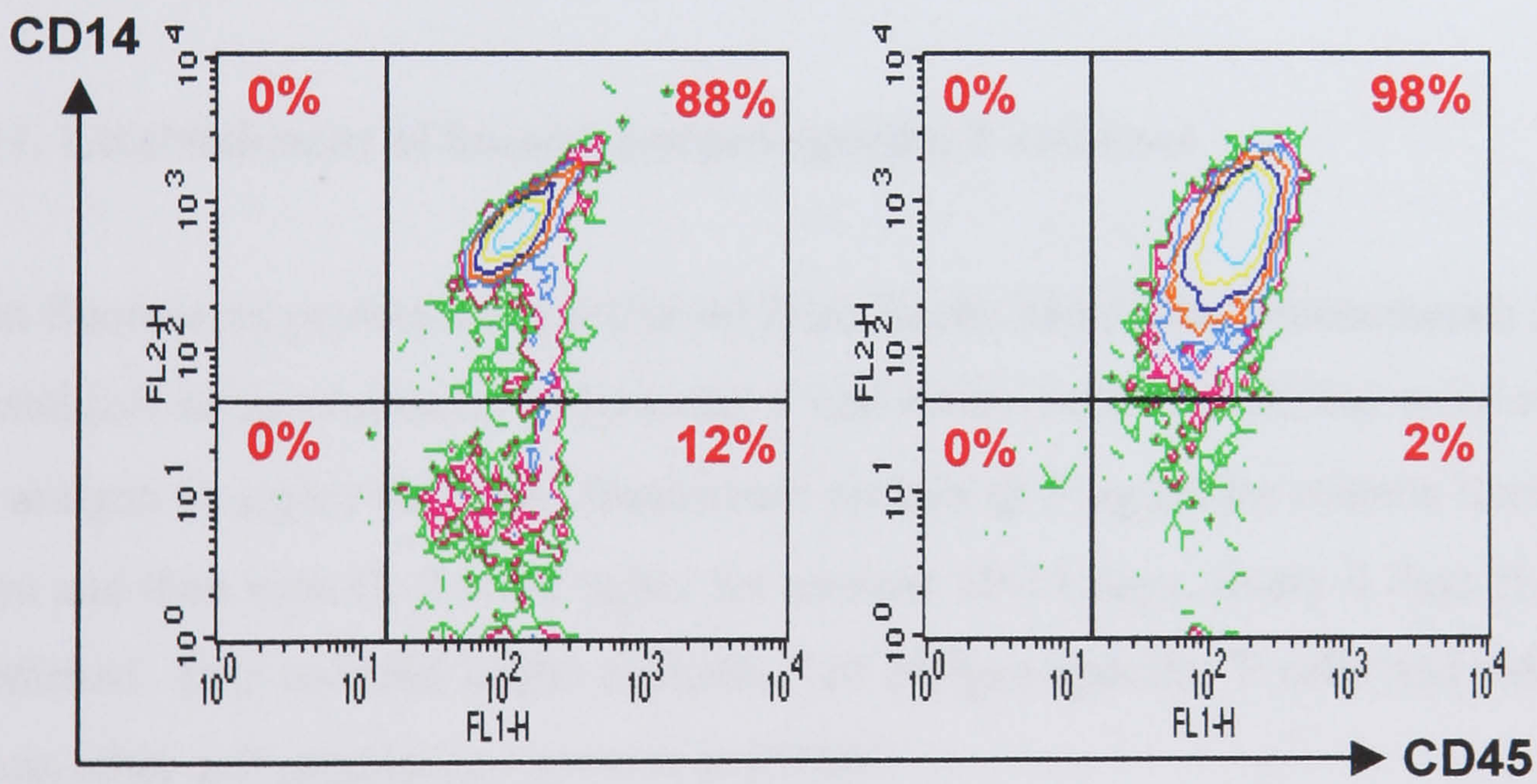


Figure 2.2: Monocyte purity after centrifugal elutriation and after treatment for 2 days with M-CSF and differentiation into a macrophage-like phenotype. After centrifugal elutriation, human peripheral blood monocytes were collected and stained by FACS for the presence of CD14/CD45. Similarly, peripheral blodd monocytes differentiated with M-CSF for 2 days were stained for CD14/CD45. Cells were examined by flow cytometry and the percentage of $CD14^+$ cells are shown.

To stimulate macrophages 10 ng/ml LPS, 1μg/ml lipid A, 30 μg/ml of soluble CD40L or $1x10^6$ cells/ml of transfected mouse fibroblasts were used. For LPS/lipid A blocking experiments, 10 μg/ml of an anti-TLR4 monoclonal antibody (HTA1216, mouse IgG1; Shimazu R. et al. 1999) were added to the cultures 30 min before stimulation.

2.3.10. Differentiation of human monocytes to dendritic cells

Freshly elutriated monocytes were cultured at $1x10^6$ cells/ml in 10 cm petri dishes (Falcon, UK) in 5% FCS RPMI supplemented with 50 ng/ml GM-CSF and 10 ng/ml IL-4 for 5-6 days, as previously described (Sallusto F. and Lanzavecchia A. 1994). At day 3, cytokines were replenished. This method presents several advantages as compared to differentiation of dendritic cells directly from blood or bone marrow precursors. Besides being easy and giving high numbers of cells, it generates a homogenous population of cells with a stable "immature DC" phenotype. This phenotype can be pushed to maturation by addition of TNFα or LPS (100 ng/ml) for a further 2-3 days to the DC culture (Sallusto F. and Lanzavecchia A. 1994).

2.3.11. Establishment of human antigen-specific T cell lines

Green fluorescent protein was purchased from Roche Molecular Biochemicals (USA). To establish antigen-specific polyclonal T cell lines, $1x10^6$ PBMC/ml were cultured with antigen (1 μg/ml for green fluorescent protein or 5 μg/ml for tetanus toxoid) for 7 days and then with IL-2 at 20 ng/ml for another 10-14 days. Every 4 days IL-2 was replenished. This resulted in the expansion of antigen-specific T cells and cell death of most other cell populations present in PBMC.

After 17-21 days of culture, a restimulation step was included by culturing the T cells with autologous irradiated PBMC at a 1:1 ratio and fresh antigen in the absence of IL-2. After 4 days IL-2 was added for another 10-14 days. This restimulation cycle was repeated at least 4 times before use of antigen-specific T cells in further experiments (Lanzavecchia A. 1984).

2.3.12. Proliferation assays of human antigen-specific T cells

To assess the specificity of antigen-specific T cell lines, $1x10^5$ of antigen-specific T cells were cultured with $1x10^5$ autologous irradiated PBMC and various concentrations of antigen in 96-well flat-bottomed microtiter plates as previously described (Lanzavecchia A. 1984). After 3 days, cells were pulsed with [^{3}H]Thymidine overnight and harvested the following day.

To measure dendritic cell-induced antigen-specific T cell proliferation, $1x10^5$ antigen-specific T cells were cultured with graded doses of irradiated or mitomycin-treated dendritic cells that were unpulsed, pulsed with antigen, uninfected or adenovirus-infected. [^{3}H]-Thymidine incorporation was measured after 2 days. All antigen-specific proliferation assays were done in triplicates.

2.3.13. Mixed lymphocyte reaction (MLR)

To assay the immunostimulatory capacity of non-irradiated or irradiated (3000 rad from a ^{137}Cs source) DC, uninfected, adenovirus-infected, LPS-treated or monocyte-conditioned medium-treated DC were cultured in graded doses with $1x10^5$ of allogeneic elutriated T cells in quadruplicate in a 96-well flat-bottom microtiter plate (Falcon). Proliferation was measured on day 5 by thymidine incorporation after a 16h pulse with [^{3}H] thymidine (0.5 μCi/well; Amersham Life Science, UK).

2.3.14. Elicitation and isolation of mouse peritoneal exudate macrophages

Eight to ten week old DBA/1 mice were injected intraperitoneally with 2 ml of a freshly prepared 1% soluble potato starch solution. Five days later, mice were sacrificed by asphyxiation and 7-8 ml of HBSS was injected into their peritoneal cavity. The bloated peritoneal cavity was then gently massaged with a kneading action, and the mouse's abdomen was carefully opened up and the peritoneal lavage gently collected into a polypropylene tube. Thereafter, peritoneal cells were washed

three times and cultured in DMEM containing 5% FCS, 1% penicillin/streptomycin 20 μM of β-mercaptoethanol.

2.3.15. Cryopreservation of cells

Cells were resuspended in 5% FCS RPMI at a maximum concentration of 10 millions per ml, aliquoted in 1 ml cryovials and chilled on ice. Then, dimethylsulfoxide (DMSO, Sigma, USA) was added at a final concentration of 10%. The cells were slowly frozen in sealed polystyrene foam containers at -70°C, and transferred to a liquid nitrogen tank for long term storage. To thaw the cells, cryovials were immersed quickly in a water bath at 37°C and DMSO was removed by washing the cells twice in 20 ml of complete medium. Cell viability was estimated by the trypan blue exclusion test.

2.4. Adenovirus production and handling

2.4.1. Adenoviruses

The construction of adenoviral vectors involves two major stages. First, the gene of interest is cloned into a small plasmid, the 'shuttle' or 'transfer' vector, that contains elements of the adenoviral genome. To produce competent virus particles, the shuttle vector is then recombined by homologous recombination with the actual adenoviral genome (Danthinne X. and Imperiale M.J. 2000)

Adenoviruses used in these studies are listed in table 2.6 and have been constructed through the homologous recombination of the Ad5 genome with the shuttle vector in HEK 293 cells. They have the E1 and E3 regions of the adenoviral genome deleted; this not only prevents adenoviral replication, but also removes the adenoviral genes known to subvert intracellular signalling (Hayder H. and Mullbacher A. 1996). Without the E1 region, the expression of the remaining adenoviral genes is also extremely inefficient.

Table 2.6: Adenoviruses used in this study

Adenovirus	Insert cDNA	Serotype	Shuttle Vector	Source
Ad0(0) or Ad0	No insert	Ad5	pJM17	Drs A.Byrnes M.Wood Oxford, UK
Adβ-gal(0)	β-galactosidase	Ad5	pJM17	Drs A.Byrnes M.Wood Oxford, UK
Ad0(GFP)	Green fluorescent protein	Ad5	pAd.BM5	Quantum Biotech, Canada
AdIκBα(0)	Porcine IκBα	Ad5	pJM17	Dr R. de Martin, Austria
AdIKK2dn(0)	Mutated human IKK2 (Single point mutation of K44→A) Dr R. de Martin, Austria	Ad5	pJM17	Dr R. de Martin, Austria
AdIKK2wt(GFP)	Human IKK2	Ad5	AdKS17 (modified AdTrack)	Dr C. Smith, KIR Division, ICSM, UK
AdNIKdn(GFP)	Mutated human NIK (Point mutations of KK429/430AA) D. Wallach	Ad5	AdKS17 (modified AdTrack)	Dr C. Smith, KIR Division, ICSM, UK
AdNIKwt(GFP)	Human NIK	Ad5	AdKS17 (modified AdTrack)	Dr C. Smith, KIR Division, ICSM, UK
AdMyD88dn(GFP)	Mutated mouse MyD88 (DD truncation and point mutation) Dr K. Burns Lausanne, Switzerland	Ad5	AdKS17 (modified AdTrack)	Mrs C. Andrews, KIR Division, ICSM, UK
AdMyD88wt(GFP)	Mouse MyD88 by Dr Xu, Southwestern, USA	Ad5	AdKS17 (modified AdTrack)	Mrs C. Andrews, KIR Division, ICSM, UK

2.4.2. Adenovirus plaque assay

Plaque assays were used for determining the concentration, or titre, of viruses from large scale grow ups or from donated stocks, or for making plaque-purified archives for future grow ups. Titre is measured in viral plaque forming units per ml (pfu/ml) and is essential for integrity and reproducibility between experiments. Thus, 10 µl of purified virus were subjected to 10-fold serial dilutions in serum-free DMEM to achieve dilutions of $1x10^{-6}$ to $1x10^{-10}$, each within a volume of 1 ml. This was used to infect one well of a six-well tissue culture plate, containing HEK 293 cells one day post-confluence. A negative control that consisted of serum-free medium alone was used. Following infection for 1 h at 37°C, media were removed and cells were carefully overlayed with 0.75% (w/v) agar/MEM kept at 42° C. MEM medium consisted of 4% (v/v) FCS and 2% (v/v) penicillin/streptomycin, and the agar/MEM solution was allowed to solidify at room temperature for 5 mins before being returned to a 37°C tissue culture incubator .(He T.C. et al. 1998). After 10 days, plaques were counted for each dilution, and the titre of the virus was calculated accordingly. Typically, viral grow ups yielded titres between $5x10^{9}$ to $2x10^{10}$ pfu/ml. The term "pfu" describes the number of infectious viral particles present.

2.4.3. Adenovirus plaque purification

Plaque purification was used for the maintenance of good viral stocks with archives being derived from colonies of single adenoviruses. Following the plaque assay, plaques were selected and approximately 1-5 µl of medium from within the plaque was removed by puncturing the agar overlay with a 10 µl Gilson pipette. The virally infected media were then diluted into 0.5 ml of serum-free DMEM and incubated overnight at 37° C. This allows the dissociation of virus particles from the cellular debris or the agar constituents that can subsequently be grown to larger titres by successive infection and lysis of increasing numbers of 293 human embryonic kidney cells.. Thus, the 0.5 ml were used to infect one well of a 12-well tissue culture plate. After incubation of the cells for 1-2 h at 37°C, another 0.5 ml of complete medium was added. Cells were left in the incubator until a full cytopathic effect (cpe) was observed (3-5 days post-infection). Cytopathic effects are characterized by the

degeneration of the cell monolayer due to cell detachment and lysis caused by adenoviral replication.

2.4.4. Small scale adenoviral amplification

To achieve viral amplification, adenovirus produced after full cpe of a well of a 12-well plate was successively transferred to 293 cell monolayers of increasing surface area. Thus, one fifth of the adenoviral titre obtained from a 6-well plate was used to infect a well of a 6-well plate in a volume of 1 ml, under serum-free conditions, for 1 hr. Then, 1 ml of growth media containing 4% (v/v) FCS and 2% (v/v) penicillin/streptomycin DMEM was added, and cells were left in culture until a full cpe could be observed (normally 2-3 days).

At this stage, adenoviruses were screened for expression of the recombinant transgene. This was done by infecting a well of a 6-well plate with crude adenoviral extracts from the 12-well plate stage, and by lysing the cells the following day in order to assess the expression of the protein of interest by western blotting. Each plaque picked after the plaque assay of either gifted or recently grown up adenoviruses was screened in that way. The presence of high levels of expression of the recombinant transgene in all plaques tested was indicative of a high quality recombinant adenovirus stock. After this screening process, aliquots of the adenoviral extracts were kept as plaque-purified archives and stored at $-70^{o}C$ for future use. Western blots from the screening of plaques from large scale grow ups of recombinant adenoviruses used in this study are shown in figure 2.3.

2.4.5. Large scale adenoviral amplification

Half of the crude lysate of the 6-well stage containing the recombinant adenovirus was used to infect a confluent 75 cm^2 flask of 293 cells in a total volume of 5 ml. Again serum-free conditions were used. After 1 hr, 5 ml of 4% (v/v) FCS 2% (v/v) penicillin/streptomycin DMEM were added into the 75 cm^2 flask, and infection was allowed to proceed for 2-3 days until a full cytopathic effect was observed. Cells and media were then harvested and subjected to three freeze thaw cycles to release the viral particles from infected cells that had not yet lysed.

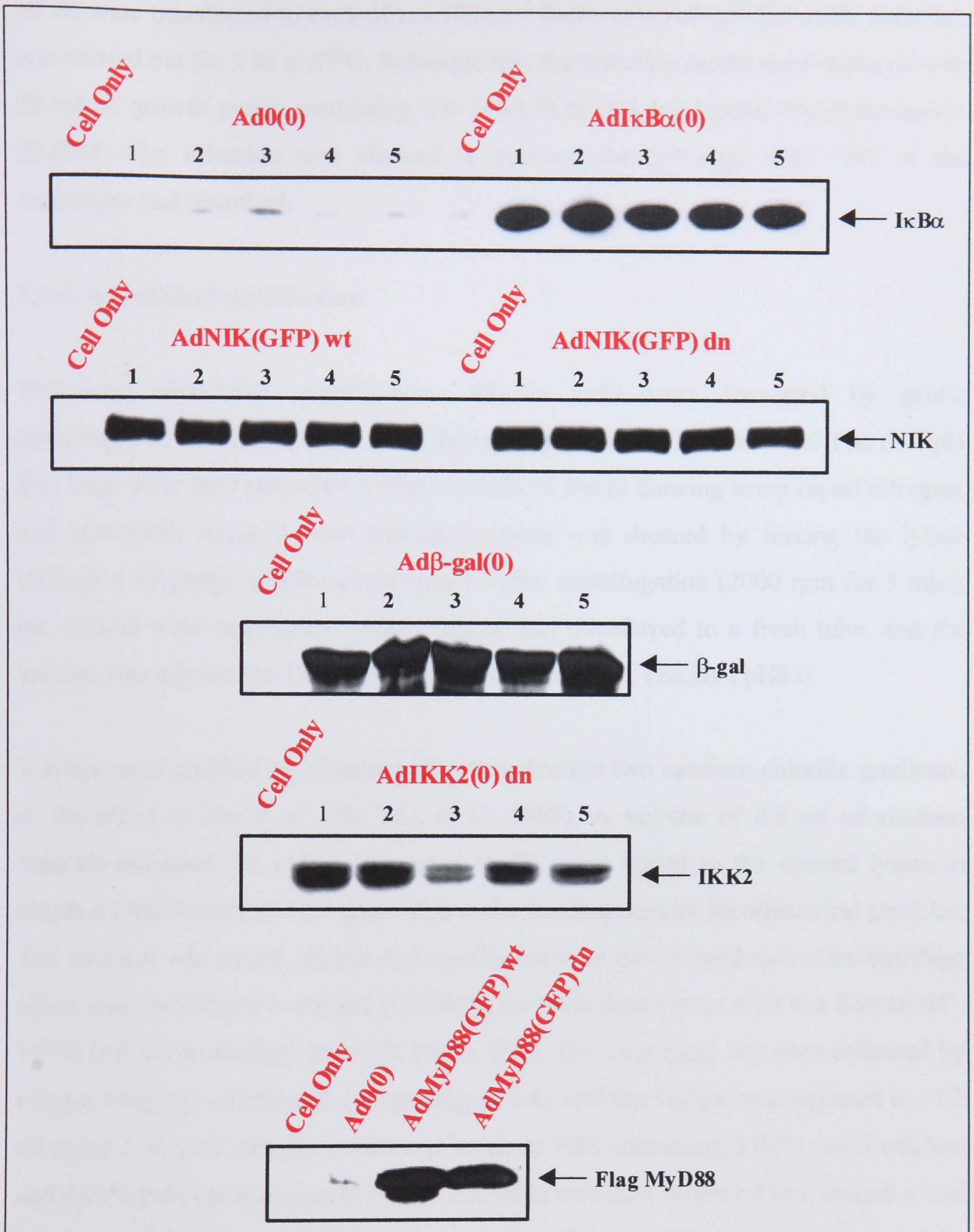


Figure 2.3: Screening of individual colonies of recombinant adenoviruses for transgene expression. Plaques picked from the plaque assay of recombinant adenoviruses were used to infect 12-well and subsequently 6-well plates of 293 cells. Some 293 cells were then collected, lysed and extracts collected for the examination of transgene expression. The presence of the transgene in all colonies tested was a confirmation of the quality of recombinant adenoviruses used in this study.

The volume of the crude lysate was adjusted to 100 ml with serum-free DMEM and 10 ml were transferred to each of ten 175 cm^2 flasks of confluent 293 cells. Infection was carried out for 1 hr at 37°C. Subsequently, the infection media were replaced with 20 ml of growth media containing 2% (v/v) FCS, 1% (v/v) penicillin/streptomycin DMEM. The infection was allowed to proceed for 2-3 days until 70% of the monolayer had detached.

2.4.6. Adenoviral purification

Following adenoviral amplification, all the cells were harvested by gentle centrifugation (1000 rpm for 5 min), and resuspended in 10 ml of 0.1M Tris.HCl pH 8.0. They were then subjected to three rounds of freeze thawing using liquid nitrogen, and chromatin released from disrupted nuclei was sheared by forcing the lysate through a 19-gauge needle several times. After centrifugation (2000 rpm for 5 min), the cleared viral supernatant was collected and transferred to a fresh tube, and the volume was adjusted to 11.4 ml by the addition of 0.1M Tris.HCl pH8.0.

Viruses were purified by ultracentrifugation through two caesium chloride gradients, as described in He *et al.* (He T.C. et al. 1998). A volume of 6.8 ml of caesium chloride-saturated TE pH8.0 (saturated at 4°C) was added to the cleared lysate to obtain a final density of 1.34 g/ml. This is the banding density for adenoviral particles. The solution was mixed, distributed equally between two polyallomer ultracentrifuge tubes, and centrifuged overnight (180,000g for more than 8 hr at 4°C) in a Sorvall RC-M150 GX ultracentrifuge (Sorvall, Herts, UK). The viral band was then collected by using a 19-gauge needle and syringe (figure 2.4), and the volume was adjusted to 17.2 ml using 1.34 g/ml density caesium chloride in PBS containing 0.01% (w/v) calcium and 0.01% (w/v) magnesium (PBS^{2+}). The virus was then subjected to a second round of ultracentrifugation (180,000g for more than 8 hr at 4°C) and was recovered by using a 19-gauge needle and syringe in a volume not exceeding 2.5 ml.

Recombinant adenovirus was subsequently subjected to further purification to remove casium chloride salts. This was achieved by column chromatography using a PD10 column (Amersham Pharmacia, Amersham, UK). The column was equilibrated in 25 ml of PBS^{2+} solution, and the purified virus solution (adjusted to 2.5 ml by addition of

PBS^{2+} solution) was introduced into the top of the column. Elution was carried out according to the manufacturers' instructions by addition of 3.5 ml of PBS^{2+} solution to the column. This 3.5 ml flow through contains caesium chloride-free virus in PBS^{2+} solution and was collected. Glycerol was added to a final concentration of 10% (v/v), and the virus was sterilised by passage through a 0.2 μm filter, aliquoted and stored at –70°C. Aliquots of the virus were always tested for the presence of LPS or for replication-competence. The test for replication competence consisted of infecting human skin fibroblasts with newly purified adenovirus, and examining the ability of the adenovirus to lyse the cells. If human skin fibroblats remained healthy 2-3 weeks after adenoviral infection, this was indicative of a replication-deficient adenoviral stock.

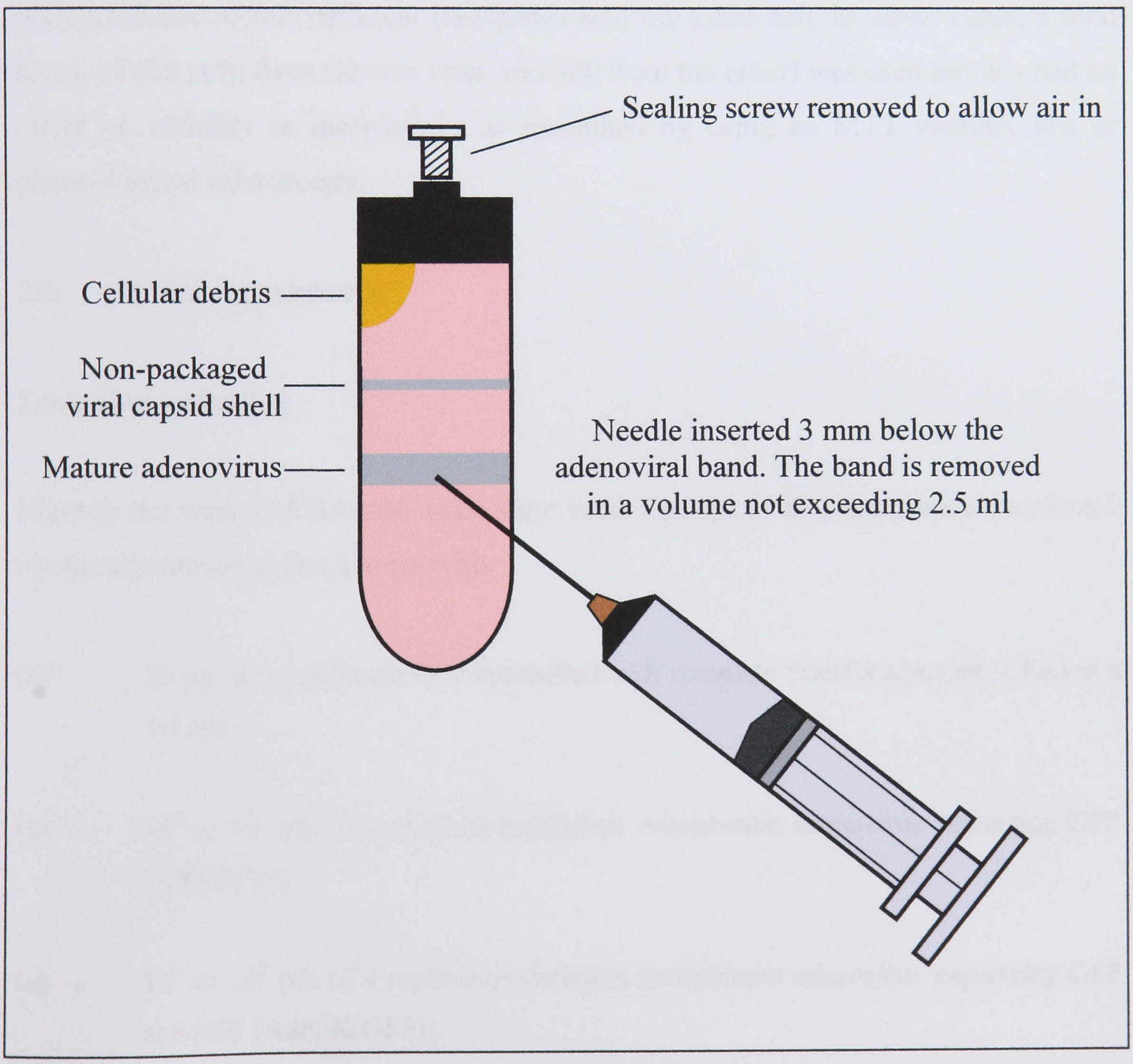


Figure 2.4: Isolation of the mature adenoviral band after caesium chloride density-gradient centrifugation. The density of 1.34 g/ml of caesium chloride used for ultracentrifugation results in the separation of packaged mature adenoviral particles from non-packaged capsid particles and cellular debris. After removing the sealing screw to allow air to enter, the viral band was isolated by using a 5 ml syringe attached to a 19-gauge needle to puncture the soft walled polyallomer ultracentrifuge tube.

2.4.7. Adenoviral infection of cells

For adenoviral infection, HSF and HUVEC were plated at 10^4 cells/ml, whereas monocyte-derived DC were plated at $1x10^6$ cells/ml. Cells were infected in serum-free medium with replication-deficient adenoviruses at a multipicity of infection (m.o.i.) of 100 unless otherwise stated. The term "m.o.i." is used to describe the ratio of infectious viruses per cell used (100:1 in this case). After 2 hours, the medium containing free adenovirus was removed, and complete medium was added. Cells were left for 1-2 days in culture in order to overexpress the transgene of interest, and were further used in experiments. For double adenoviral infection, two different viruses were added together to the cultures during the infection process. This allows the expression of two different transgenes into the same cell. In some cases, a total m.o.i. of 600 (100 from the one virus and 500 from the other) was used but this had no effect on viability or morphology as examined by using an MTT viability test or phase-contrast microscopy.

2.5. *In vivo* experiments

2.5.1. Immunization

Eight to ten week old BALB/c mice were used. Groups of five mice were vaccinated by the subcutaneous (s.c.) route with:

(a) 20 μg of recombinant GFP emulsified with complete Freud's adjuvant (CFA) at a 1:1 ratio

(b) 10^6 or 10^7 pfu of a replication-deficient recombinant adenovirus expressing GFP [Ad0(GFP)]

(c) 10^6 or 10^7 pfu of a replication-deficient recombinant adenovirus expressing GFP and NIK [AdNIK(GFP)].

(d) 10^6 or 10^7 pfu of a replication-deficient adenovirus without insert [Ad0(0)].

A total volume of 100 µl/mouse was injected at the base of the tail. As a control, a group of five naïve animals was used.

In some cases, adenovirus-immunized mice received a booster injection of 10^6 pfu of recombinant adenovirus 56 days after primary immunization.

2.5.2. Bleeding

Mice were tail-bled 14, 28 and 56 days after the primary immunization, or 14 days after the boosting vaccination (figure 2.5). Sera were then collected by centrifuging whole blood at 12000 rpm for 10 minutes and keeping the top layer. Samples were maintained at -20°C prior to the examination of antibody levels present (section 2.7.4).

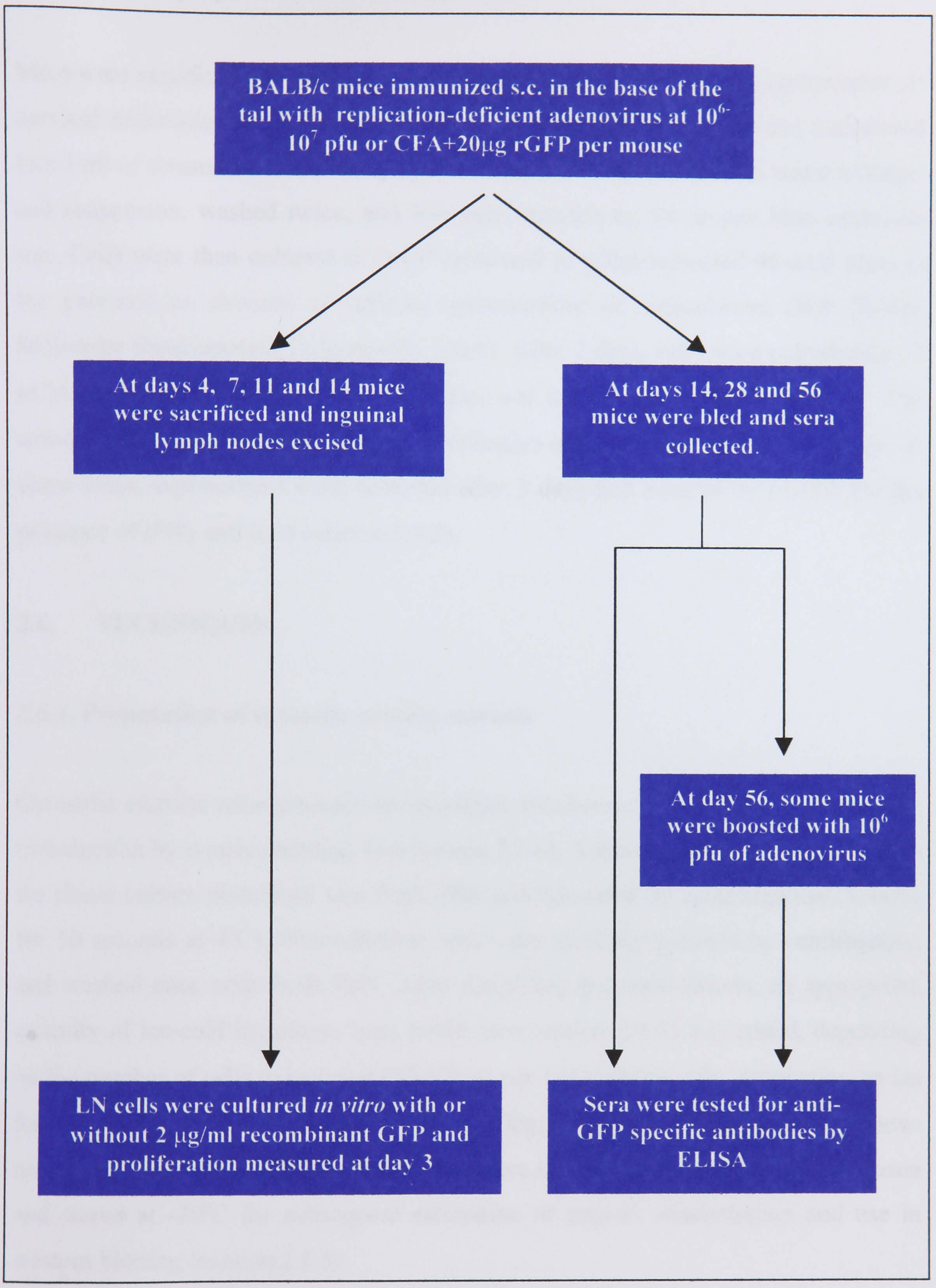


Figure 2.5: Summary of the protocol of the *in vivo* experiments

2.5.3. *Ex vivo* lymph node cell cultures

Mice were sacrificed 4, 7, 11 and 14 days after immunization by CO_2 asphyxiation or cervical dislocation. Inguinal lymph nodes were excised aseptically and transferred into 3 ml of serum-free DMEM. Lymph node cells were teased apart to make a single-cell suspension, washed twice, and live cells counted by the trypan blue exclusion test. Cells were then cultured at $5x10^5$ cells/well in a flat-bottomed 96-well plate in the presence or absence of various concentrations of recombinant GFP (Roche Molecular Biochemicals, Indianapolis, USA). After 2 days, cells were pulsed with 10 μCi/well of tritiated thymidine overnight, and harvested the following day. The amount of radioactivity incorporated is indicative of lymph node cell proliferation. In some cases, supernatants were collected after 3 days and assayed by ELISA for the presence of IFNγ and IL-4 (section 2.7.3).

2.6. TECHNIQUES

2.6.1. Preparation of cytosolic protein extracts

Cytosolic extracts were prepared to investigate biochemical events involved in signal transduction by western blotting (see session 2.2.6). Adherent cells were scraped from the tissue culture plate/flask into fresh PBS and harvested by centrifugation (13000g for 10 seconds at 4°C). Non-adherent cells were similarly pelleted by centifugation and washed once with fresh PBS. After discarding the supernatants, an appropriate quantity of ice-cold hypotonic lysis buffer (see session 2.2.6) was added, depending on the number of cells to be lysed (50-100 μl per $1x10^6$ cells). After incubation on ice for 10 minutes, lysates were centrifuged (13000g, 5 minutes, 4°C) in order to remove nuclei and cell debris. The cleared lysates were then removed to fresh tubes, frozen and stored at -20°C for subsequent estimation of protein concentration and use in western blotting (session 2.6.5).

2.6.2. Preparation of nuclear protein extracts

Nuclear protein extracts were prepared to study the NF-κB activation and translocation from the cytosol to the nucleus and its DNA-binding ability. After lysis of cells in hypotonic lysis buffer (see section 2.2.6) , nuclei were pelleted by centrifugation (13000g for 5 minutes at 4°C), washed once in hypotonic lysis buffer to remove contaminating cytosolic proteins, and then resuspended in hypertonic extraction buffer for 1-2 hours at 4°C under agitation. Hypotonic lysis buffer prevents leaching of proteins out of the nucleus during lysis, whereas hypertonic extraction buffer makes the nuclear membrane porous, allowing nuclear proteins to escape into solution. After centrifugation (13000g for 10 minutes at 4°C) supernatants containing the nuclear protein were removed to fresh tubes and stored at -70°C. This method of nuclear extracts preparation is based on that of Whiteside et al., 1992. Protein concentration of nuclear extracts was determined as described in section 2.6.3.

2.6.3. Protein concentration assay

Before using cytosolic or nuclear extracts in any further experimental procedure (e.g. western blotting, electrophoretic mobility shift assays), it was necessary to determine their protein concentration in order to ensure that equivalent amounts of protein were present in each sample. Protein concentrations were assessed by the Bradford assay. Briefly, 20 µl of appropriately diluted extracts were added in triplicates in a 96-well tissue culture plate along with 20 µl of known concentrations of BSA (Sigma, UK) ranging from 10-1000 µg/ml. 200 µl of Bradford reagent (section 2.2.6) were then added to each well, and absorbance was measured at 595 nm in a spectrophotometer (Multiscan Bichromatic, Labsystems). From the linear standard curve formed by the range of BSA concentrations, protein concentrations in the cytosolic and nuclear extracts were determined.

2.6.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The separation of cellular proteins for subsequent analysis was achieved by gel electrophoresis according to the SDS-discontinuous system described by Laemmli (Laemmli U.K. et al. 1970). This system is based on the Ornstein-Davis buffering system with the addition of SDS to denature proteins and confer equal charge densities on all polypeptide species present, resulting in electrophoretic separation on the basis of molecular size alone. Before being loaded onto the gel, samples were boiled at 95°C for 5 minutes in the presence of 4x sample buffer. Molecular weight markers were run alongside samples to allow sizing of proteins (SeeBlue markers, Invitrogen, CA, USA).

Table 2.7: SDS-PAGE gel recipes

	Resolving gels		Stacking gel
	10%	**12.5%**	
Distilled H_2O	10 ml	7.5 ml	-
Solution A	10ml	12.5 ml	1 ml
Solution B	-	-	9 ml
Solution C (2x)	10 ml	12.5 ml	-
TEMED	20 µl	20 µl	10 µl
10% APS	150 µl	150 µ	80 µ

The BioRad gel apparatus was used according to the manufacturers instructions. Solutions used are listed in table 2.2.5. N,N,N',N'-tetramethyl-1, 2-diaminoethane (TEMED) and ammonium persulphate (APS) were purchased from Sigma (UK).

2.6.5. Western Blotting

Protein transfer.

Proteins separated by SDS-PAGE can subsequently be transferred from the gel matrix onto a hydrophobic membrane, nitrocellulose (Schleicher&Schuel, Dassel, Germany) or polyvinyl difluoride (PVDF) (Millipore, Bedford, MA), allowing specific proteins

to be identified by probing the membrane with specific antibodies. Transfer of proteins is achieved by placing the gel in an electric field perpendicular to the direction of electrophoresis. This results in negatively charged, SDS-coated proteins migrating towards the anode and, if a membrane is placed between the gel and the anode plate, proteins will be immobolised on the membrane. Membranes were equilibrated in transfer buffer (PVDF membrane was first soaked in methanol) before being placed onto 3 layers of filter paper also pre-wetted with transfer buffer. The gel was then placed on top of the membrane ensuring that there are no bubbles between them, and a further 3 layers of wet filter paper were layered on top of the gel. The gel sandwich was then transfered to a Biorad transfer chamber filled with transfer buffer. Transfer buffer temperature was maintained at 4°C by using a water-circulating cooling pump and a constant voltage of 100V was applied to the chamber. The transfer process was allowed to take place for 1-2 hours. Membranes were stained with Ponceau-S (Sigma) to visualize transferred proteins and uniformity of the lanes. Membranes were destained by washing in PBS/Tween-20 (0.1%) three times for 10 minutes.

Immunoblotting.

After transfer, membranes were blocked by gentle agitation in 5% Marvel milk powder (Cadbury, Birmingham, UK) in PBS/Tween-20 (0.1%) for at least 1 hour at room temperature to prevent non-specific binding of antibodies. The membrane was then incubated with the primary layer antibody raised against the protein of interest for either 1-2 hours at room temperature or overnight at 4°C. After removal of the primary layer, membranes were washed three times for 10 minutes in PBS/Tween-20 (0.1%). Primary layer antibodies were typically polyclonal rabbit, murine or sheep antibodies and a secondary, HRP-conjugated anti-rabbit, anti-mouse or anti-sheep antibody was used to visualize the proteins. Secondary antibody was diluted in blocking buffer and the membrane was incubated in this solution for 1-2 hours at room temperature. The membrane was then washed for a further three times in PBS/Tween before being soaked in the chemiluminescent substrate solution ECL (Amersham Life Sciences Ltd.) for 1-2 minutes. Excess ECL was removed by blotting the membrane in Saran wrap (Dow Chemical Company, UK) and antibody-bound proteins were visualised using Hyperfilm MP (Amersham Life Sciences Ltd). Exposed films were developed using an automatic film processor (AGFA, UK).

Reprobing membranes.

When needed, a membrane was stripped of the bound antibodies by using a β-mercaptoethanol-containing stripping buffer and gentle agitation at 58°C for 2-3 hours. The membrane was then washed thoroughly in PBS/Tween-20 (0.1%) to remove all traces of the stripping buffer before reblocking and incubating with the new primary antibody. This procedure was not repeated more than two times for any membrane.

2.6.6. Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays (EMSA) provide a simple method for the detection and quantification of DNA binding complexes such as transcription factors. The technique is based on the principle that DNA fragments or oligonucleotides bound to protein complexes migrate through non-denaturing polyacrylamide gels slower than free oligonucleotides. EMSA reactions are performed by incubating nuclear protein extracts with ^{32}P-end labelled oligonucleotides that contain putative protein-binding sites for the transcription factor of interest. Protein:DNA complexes are then formed, and when separated by non-denaturing gel electrophoresis, these retarded complexes can be visualized by autoradiography or by phosphoimaging (FLA-2000, Fujifilm, UK). Oligonucleotide end labelling and DNA binding reactions were carried out using the Gel Shift Assay System of Promega (Southampton, UK).

Radiolabelling oligonucleotide DNA probes

For labelling of 3.5 pmoles of the double-stranded oligonucleotide probe, the following reaction mixture was assembled and incubated at 37°C for 1 hour:

Consensus probe (1.75 pmol/ml)	2 µl
10X T4 Polynucleotide kinase buffer	1 µl
[γ-^{32}P]-ATP (3000Ci/mmol at 10 µCi/µl)	1 µl
Nuclease-free water	5 µl
T4 Polynucleotide kinase (10U/µ)	1 µl
Total volume =	10 µl

The reaction was terminated by the addition of 1 µl of 0.5M EDTA pH8.0 prior to the addition of 89 µTE pH 8.0. The labelled oligonucleotide probe was then purified from unincorporated label by chromatography using a 1 ml Sepharose G-25 column equilibrated in TE pH 8.0 (Microspin™, Pharmacia Life Sciences, NJ, USA). The activity of the probe was determined by adding 1 µl of the labelled probe into 1 ml of scintillation fluid, before reading on a β-scintillation counter (Wallac LC14-10). Typically, the activity of end-labelled probes was about 100,000 cpm per µl.

DNA binding reaction

Reaction volumes of 9 µl were set up containing equal protein amounts from the nuclear extracts adjusted with nuclease-free water and including 2 µl of 5X DNA binding-buffer (section 2.2.6). 1 µl of labelled oligonucleotide probe was then added to each reaction mixture, and samples were left for 30 min at room temperature on a gentle shaker. This step allows protein:DNA complexes to form.

Electrophoresis and autoradiography

Samples were immediately loaded onto non-denaturing 5% (w/v) polyacrylamide gels (1.5ml TBE, 23.5 ml distilled water, 5 ml 30% acrylamide [37.5:1 bis], 200 µl APS and 20 µl TEMED) after the addition of 1 µl of 10X DNA-loading buffer (section 2.7). Before the addition of the samples, gels had been pre-electrophoresed for 2-3 hours at 200 V. Samples were electrophoresed with 0.5X TBE buffer, until the dye reached 3-4 cm from the bottom of the gel. Electrophoresis was stopped at that point, gels disassembled and dried onto filter paper (BioRad model583 gel drier). Gels wer then monitored by autoradiography using Hyperfilm MP (Amersham Life Sciences) at –70°C, or by phosphoimaging using the FLA-2000 phosphoimager (Fujifilm, UK).

2.7. IMMUNOLOGICAL METHODS

2.7.1. Immunofluorescence staining and flow cytometry

This technique is based upon the tagging of individual cells with fluorochromes, whose intensity correlates directly with the level of antigen expression. Cells are put through the flow cytometer where each cell passes through a laser beam. If a cell is labelled with a fluorochrome, it emits light at a pre-determined wavelength that can be detected. The light is scattered according to the relative intensity of fluorochromess bound to each cell, which in turn reflects the density of antigen expression. Antibodies used for immunostaining are listed in table 2.2.

For FACS staining, cells were first harvested. For adherent cells where surface receptors need to be intact, a warm 2% EDTA in PBS solution was used for 20 min at 37°C. After cells were in solution, they were washed once and then resuspended in ice-cold FACS washing buffer. All subsequent incubations were performed at 4°C. For each analysis, $5x10^5$ cells were incubated with the relevant conjugated or unconjugated antigen-specific antibody or isotype control for 30 min and then washed twice with FACS washing buffer. For directly conjugated antibodies, cells were directly examined by flow cytometry, whereas for unconjugated antibodies, cells were incubated for a further 30 min with a 1:100 dilution of goat anti-nouse IgG conjugated to either phycoerythrin (PE; Southern Biotechnology Associates, USA) or fluorescein isothiocyanaate (FITC; Southern Biotechnology Associates, USA). After two further washing steps in FACS washing buffer, cells were ready for analysis on a FACScan flow cytometer (Becton and Dickinson). The CellQuest (Becton Dickinson) software was used.

2.7.2. β-galactosidase staining

Control adenoviruses encoding the bacterial β-galactosidase (LacZ) gene were routinely used to infect cells in order to optimize viral infectivity conditions. After infection, cells were left for 24-48 hrs to express the LacZ protein. Subsequently, they were detached by using trypsin, and washed in FDG-staining solution (section). The

cells were resuspended in 100 µl of FDG staining solution and incubated for 10 min in a 37°C water bath. Fluorescein-Di-β-galactopyranoside (FDG) is a fluorimetric substrate of β-galactosidase (Sigma, Dorset, UK) yielding fluorescein as a consequence of enzyme activity. Thus, 40 µl of FDG stain (section 2.9) were added to the cells and incubated for exactly 1 min. The reaction was immediately quenched by the addition of 10 volumes of ice-cold staining solution. In addition to stopping the reaction, the drop in temperature and the increase in volume prevent the leaching of free fluorescein between cells. Samples were incubated for 30 min on ice, and were then examined for β-galactosidase activity by flow cytometry.

2.7.3. Enzyme linked immunoabsorbent assays (ELISA)

Cytokine levels in cell culture supernatants were measured by standard 2 or 3 layer sandwich ELISA techniques using specific monoclonal or polyclonal antibodies. The appropriate coating antibody was diluted to the working concentration (see table 2.8) in PBS (unless otherwise stated) and 50 µl of this was dispensed into polystyrene, 96-well flat-bottomed microtitre plates (Nunc-Immuno plate II, BRL, Uxbridge, Middlesex, UK). Plastic sealing film was used to cover the coated plates which were stored at 4°C overnight. Coating antibody was then removed and the wells washed twice with PBS/0.01% Tween-20 (H_2O/0.01% Tween-2-for the hTNF ELISA). Uncoated sites were blocked by incubation with 200 µl of blocking buffer (2% BSA/PBS) for 1 hour at room temperature. The wells were washed again prior to the addition of 50 µl volumes of cytokine standards or assay samples (in triplicate), neat or diluted appropriately. Cytokine standards were serially diluted in the same tissue culture medium of the samples. After incubation for 2 hours at room temperature or overnight at 4°C, the standards and samples were aspirated from the plate and the wells washed twice with wash buffer as before, prior to the addition of 50 µl of the detection antibody, diluted to the relevant concentration of 0.1% BSA/PBS. The detection antibody was left on the plate at room temperature for 2 hours before its removal and further washing of the plate.

Two layer ELISA: Where the detection antibody itself was biotinylated, the plate was incubated for 1 hour with 50 µl Streptavidin-HRP (Amersham Life Sciences Ltd) per

well (diluted to the appropriate concentration in PBS/0.5% BSA). Alternatively, in the case of the human TNFα ELISA, where the detection antibody is not biotinylated, an anti-rabbit HRP conjugate (goat anti-rabbit Ig-HRP, Immunoresearch Laboratories Inc., USA) was used.

Three layer ELISA: For the IL-6 ELISA, incubation with the detection antibody is followed by a further hour incubation with a goat anti-rabbit biotinylated antibody prior to incubation with streptavidin-HRP, as above.

After removal of the HRP conjugate, plates were washed 5 times with the washing buffer. Then, a 100 µl of a 1:1 mixture of peroxidase solution (H_2O_2) and TMB peroxidase substrate (Kinkegaard and Pery Laboratories, Gaithesburg, USA) was added. The colour was allowed to develop before the addition of 100 µl of 2M H_2SO_4 to stop the reaction, and absorbance was read at 450 nm on a spectrophotometric ELISA plate reader (Multiscan Bichromatic, Labsystems, UK)) using the DeltaSoft II.4 software programme (Biometallics Inc., USA). A summary of antibodies/conjugates used for ELISA are presented in table

Table 2.8: Summary of antibodies and standard curve concentrations used for ELISA

Human TNFα	Coat Detect Goat anti-rabbit HRP Standard curve range	1:1000 1:1000 1:12000 10,000-14 pg/ml
Human IL-1β	Coat Detect Streptavidin HRP Standard curve range	15 µg/ml 2 µg/ml 1:2000 10,000-14 pg/ml
Human IL-6	Coat Detect Goat anti-mouse biotin Streptavidin HRP Standard curve range	3 µg/ml 1 µg/ml 1:10000 1:2000 10,000-14 pg/ml
Human IL-8	Coat Detect Streptavidin HRP Standard curve range	2 µg/ml 1 µg/ml 1:2000 10,000-14 pg/ml
Human IL-12	Coat Detect Streptavidin HRP Standard curve range	1:1000 1:1000 1:2000 10,000-14 pg/ml

Human IL-15	Coat Detect Streptavidin HRP Standard curve range	2 µg/ml 200 ng/ml 1:2000 10,000-14 pg/ml
Human IL-18	Coat Detect Streptavidin HRP Standard curve range	10 µg/ml 1 µg/ml 1:2000 5,000-6.8 pg/ml
Human MIP1α	Coat Detect Streptavidin HRP Standard curve range	4 µg/ml 40 ng/ml 1:2000 5,000-6.8 pg/ml
Human RANTES	Coat Detect Streptavidin HRP Standard curve range	4 µg/ml 5 µg/ml 1:2000 5,000-6.8 pg/ml
Human MCP-1	Coat Detect Streptavidin HRP Standard curve range	2 µg/ml 50 ng/ml 1:2000 5,000-6.8 pg/ml
Human MCP-3	Coat Detect Streptavidin HRP Standard curve range	2 µg/ml 100 ng/ml 1:2000 5,000-6.8 pg/ml
Human IP-10	Coat Detect Streptavidin HRP Standard curve range	4 µg/ml 100 ng/ml 1:2000 5,000-6.8 pg/ml
Mouse IL-4	Coat Detect Streptavidin HRP Standard curve range	2 µg/ml 1 µg/ml 1:2000 5,000-7 pg/ml
Mouse IFNγ	Coat Detect Streptavidin HRP Standard curve range	2 µg/ml 1 µg/ml 1:2000 5,000-7 pg/ml

2.7.4. Measurement of mouse antibodies to green fluorescent protein

A method for the measurement of anti-GFP-specific antibodies was developed by modification of a solid phase ELISA technique for antibody detection previously described for the detection of anti-collagen antibodies (Williams et al. 1992). The method consisted of the following steps:

(a) Polystyrene microtitre plates (Falcon) were coated with 1 μg/ml of rGFP in PBS, overnight at 4°C.

(b) The plates were washed in PBS containing 0.05% (w/v) Tween 20 (Sigma) twice, and then blocked using 2% BSA in PBS for 1 hr at room temperature.

(c) Plates were again washed twice in PBS/Tween, and samples were diluted in PBS containing 0.01% BSA and incubated for 2 hr at room temperature.

(d) After two washing steps in PBS/Tween, an alkaline phosphatase or HRP-conjugated anti-mouse antibody diluted in PBS/0.01% BSA was added for 1 hr at room temperature.

Table 2.9: List of the anti-mouse antibodies used in this study for the detection of GFP-specific antibodies. These are directly conjugated to either alkaline phosphatase (AP) or Horseradish Peroxidase (HRP)

Anti-mouse antibody	Conjugate	Dilution	Source
Ig	Sheep-HRP	1:3000	Amersham Life Sciences, UK
IgG	Sheep-AP	1:2500	The Binding Site, Birmingham, UK
IgG1	Sheep-AP	1:2500	The Binding Site, Birmingham, UK
IgG2a	Sheep-AP	1:4000	The Binding Site, Birmingham, UK
IgM	Goat-AP	1:30000	The Binding Site, Birmingham, UK

(e) Plates were then washed four times with PBS/Tween, and 50 μl of the substrate solution were added per well. For alkaline phosphatase-conjugated antibodies, the substrate p-nitrophenyl phosphate (Sigma) in glycine substrate buffer, pH 10.4, was

added and incubated at 37^0C until a colour reaction developed (normally 1-2 hours). For HRP-conjugated antibodies, a 1:1 mixture of peroxidase solution (H_2O_2) and TMB peroxidase substrate (Kinkegaard and Pery Laboratories, Gaithesburg, USA) was used. The colour was allowed to develop (normally within 5 minutes) before the addition of 100 µl of 2M H_2SO_4 to stop the reaction. Absorbance was read at 414 or 450 nm for alkaline phosphatase or HRP antibodies, respectively, on a spectrophotometric ELISA plate reader (Multiscan Bichromatic, Labsystems, UK)) using the DeltaSoft II.4 software programme (Biometallics Inc., USA). Arbitrary antibody units were calculated as described in Williams R.O. et al. (1992). The 50% O.D. point of the antibody levels induced by rGFP and CFA immunization was used to define "100 arbitrary units".

2.8. Statistical Analysis

All statistical analysis was done by using the GraphPad Prism statistics package (GraphPad Software Inc., USA). To calculate p values for the statistical sgnificance of certain observations, a *student's two-tailed t test* or a *one-way ANOVA Dunnet's multiple comparisons test* was used.

CHAPTER 3

HETEROGENEITY OF IKK2 REQUIREMENT IN NF-κB ACTIVATION REVEALED BY ADENOVIRAL GENE TRANSFER INTO DENDRITIC CELLS AND MACROPHAGES

3.1. Introduction

Major cell types involved in adaptive immunity are monocytes/macrophages and dendritic cells. Macrophages phagocytose foreign particles and dead cells, present antigen to T lymphocytes, induce fibroblast and vascular endothelial cell proliferation important in tissue repair and the recruitment of effector cells, and attract other inflammatory cells themselves.

DC (DC), in turn, share many of the functions exerted by macrophages, but also have a unique ability to activate naïve T cells and regulate adaptive immunity. DC direct both the quality and the extent of the immune response, but their ability to stimulate T cell proliferation depends on their activation status. In the presence of an inflammatory stimulus such as bacterial LPS, TNFα or contact with activated T cells, immature DC undergo a process of functional activation or 'maturation' by increasing their ability to present antigen. During that process, DC up-regulate MHC, adhesion and costimulatory molecule expression, and leave the tissues to reach the secondary lymphoid organs where they select and activate antigen-specific T cells (reviewed by Cella M. et al. 1997).

Although DC activation is a critical event for the induction of immune responses, little is known about the signalling pathways involved in this process. Recently, NF-κB has been implicated to play a major role in efficient antigen presentation both in mouse and human DC. In mouse DC, the serine protease inhibitor N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) that blocks NF-κB nuclear translocation has been shown to inhibit TNFα production and up-regulation of MHC class II and CD86 (Rescigno M. et al. 1998) in response to LPS. In humans, my colleagues (Yoshimura S. et al. 2001a) and others (Oyama T. et al. 1998) have shown that overexpression of IκBα, the natural inhibitor of NF-κB, into DC blocks antigen presentation. IκBα abrogates the capacity of DC to induce the allogeneic mixed lymphocyte reaction via a mechanism involving down-regulation of class II and costimulatory molecules as well as cytokine production (Yoshimura S. et al. 2001a). Moreover, it has been shown that the NF-κB family member, relB, is necessary for dendritic cell development and efficient antigen presentation. Undifferentiated skin

DC numbers in *relB*-deficient mice were normal, but the cutaneous delayed-type hypersensitivity response was impaired, suggesting that DC differentiation and not development had been affected by the disruption of relB (Burkly L. et al.; Weih F. et al. 1995). In humans, active nuclear relB correlates with enhanced antigen-presenting function of DC (Pettit A.R. et al. 1997).

Studies on signalling pathways involved in DC maturation have been hampered by the low number of DC that can be obtained and the lack of efficient genetic modification techniques (Zhong L. et al. 1999). The first problem has recently been circumvented; DC can now be prepared in high numbers by culturing peripheral blood monocytes with GM-CSF and IL-4 (Sallusto F. and Lanzavecchia A. 1994; Romani N. et al. 1996). To bypass the second problem, I developed an efficient adenoviral gene transfer technique to transfect immature DC, based on a similar method used for macrophages (Foxwell B. et al. 1998). Using replication-deficient adenoviruses, I have examined the involvement of IκB-kinase 2 (IKK2) in DC NF-κB-activation, cytokine production and antigen-presenting function. I have also extended these studies to other primary cell types involved in inflammatory responses such as macrophages, human umbilical vein endothelial cells (HUVEC) and human skin fibroblasts (HSF). The findings presented in this chapter will challenge the current belief that IKK2 is the upsteam kinase responsible for NF-κB activation in response to TNFα, IL-1 and LPS, and suggest that at least in the case of LPS, this is cell-type specific.

3.2. Efficient adenoviral gene transfer into primary human cells

One of the major obstacles of studying the biochemical signalling pathways in primary cells is the difficulty of transfecting DNA. DC and macrophages, in particular, are extremely inefficient in taking up DNA probably because they are specialized to phagocytose and degrade foreign bodies such as DNA (Stacey K.J. et al. 1993; Zhong L. et al. 1999). Low transfection efficiencies do not permit the study of endogenous genes which requires instead the use of reporter constructs.

To overcome these problems we have developed a very efficient technique of gene transfer based on replication-deficient adenoviruses. Using $E1^-E3^-$ recombinant adenoviruses, we have been able to transfect with efficiencies greater than 95% a wide range of primary cells including immature DC, macrophages, HUVEC and HSF.

Immature dendritic cells

Recently, my colleagues demonstrated that human DC cells can be efficiently infected by recombinant adenoviruses (Yoshimura S. et al. 2001a) without the need to be combined with cationic lipids (Dietz A.B. and Vuk-pavlovic S. 1998). This study, however, was done in mature DC, a cell type with a very limited lifespan induced during an immune response. For the purposes of this study, I wanted to investigate signalling pathways in immature DC, the cells that normally reside in tissues where they take up antigen, and that are more important for therapeutic intervention. Thus, I optimized the conditions for adenoviral gene transfer into immature DC to achieve maximum infection efficiencies with the minimum amount of cellular stress caused.

Immature DC were generated from human peripheral blood monocytes after 5 days culture with GM-CSF and IL-4 (Sallusto F.and Lanzavecchia A. 1994; Romani N. et. 1996; Bender A. et al. 1996). Examination of these cells for the expression of adenoviral receptors by FACS, showed that they express high levels of $\alpha V\beta 5$ but negligible $\alpha V\beta 3$ (figure 3.1). In addition, they express the coxsackie virus and adenovirus receptor (CAR) and MHC class I that have been proposed to be the receptors that mediate the entry of the adenovirus into the cells (Bergelson J.M. et al. 1997; Tomko R.P. et al. 1997; Hong S.S. et al. 1997).

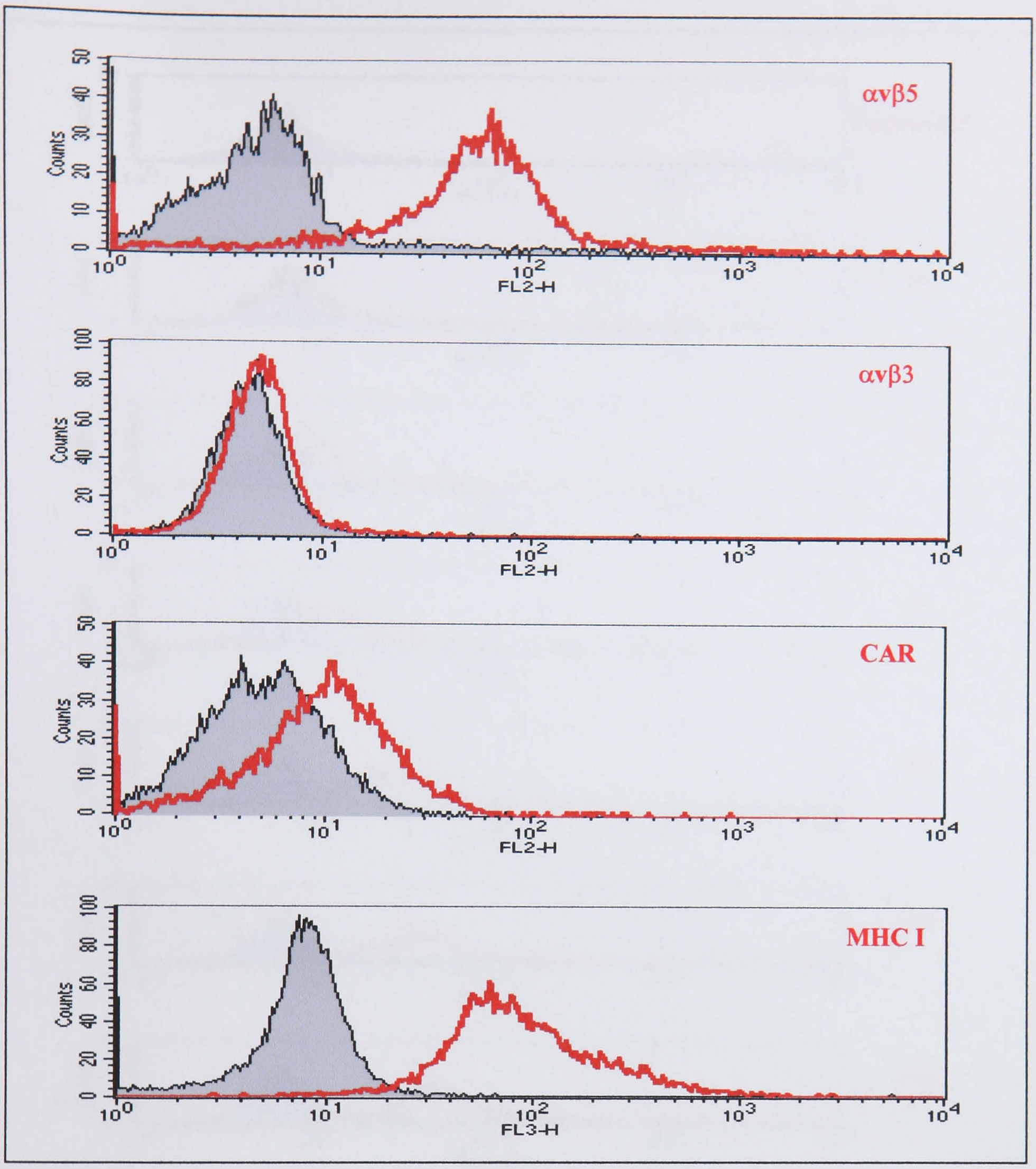


Figure 3.1: Immature DC express CAR, MHC class I, and the αvβ5 but not αvβ3 integrin. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in 5% FCS 1% p/s RPMI. DC were then collected and examined for the expression of CAR, MHC I, αVβ3 and αVβ5 integrin expression by FACS staining. A representative of four independent experiments is shown.

The expression of adenoviral receptors and co-receptors on immature DC, prompted me to investigate their infectivity by using a replication-deficient adenoviral vector encoding the bacterial protein β-galactosidase under the control of the cytomegalovirus promoter [Adβ-gal(0)]. Addition of FDG, a colorigenic substrate of β-galactosidase, allows the infection efficiency of cells to be examined by FACS and calculated by using the crossover point between stained and unstained cells in the FACS histogram plot. Interestingly, I found that at a multiplicity of infection as low as 100, greater than 95% of DC were infected (figure 3.2).

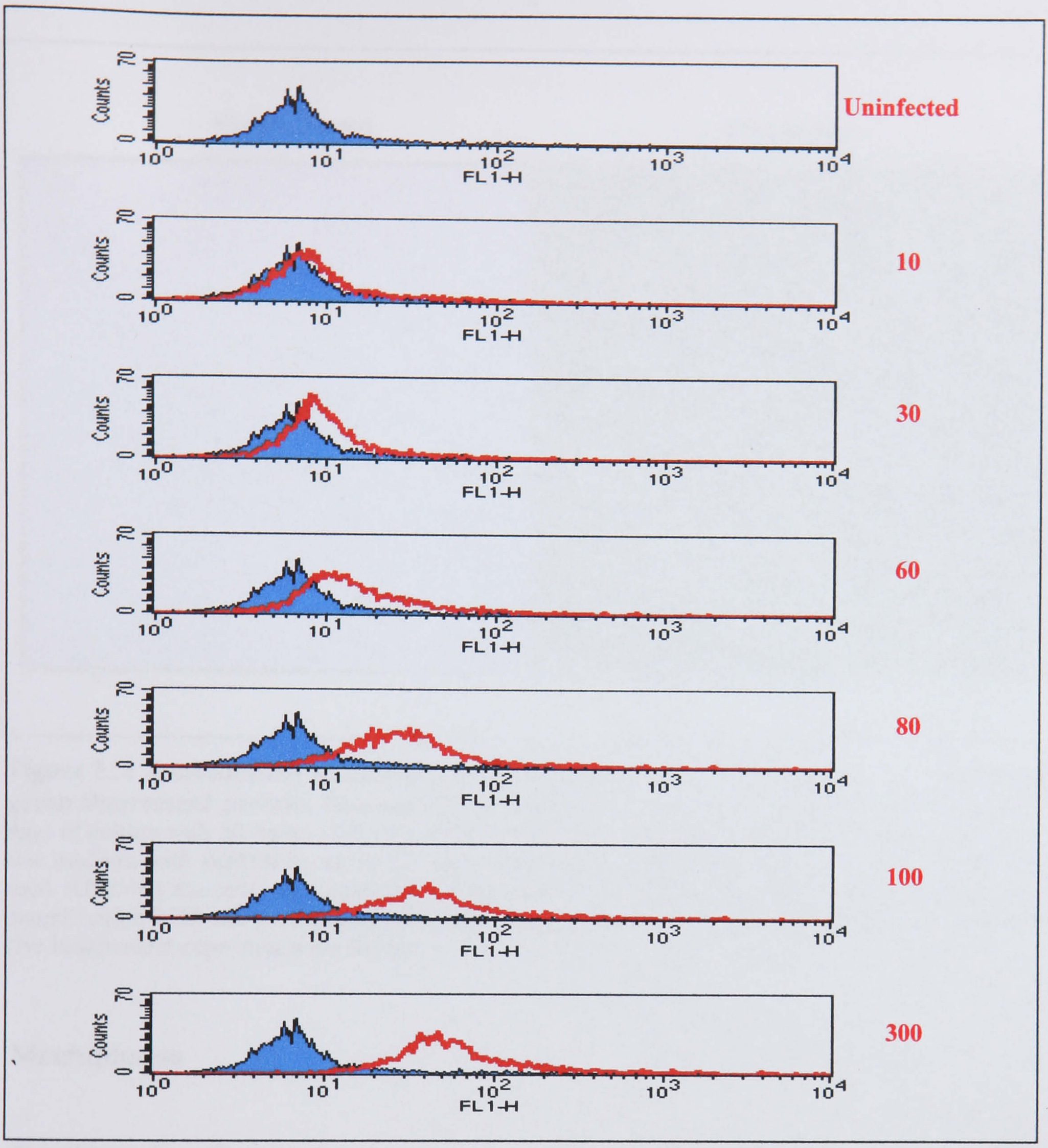


Figure 3.2: Efficient adenoviral gene transfer into immature DC is achieved at a multiplicity of infection of 100. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected for 2h in serum-free medium with Ad0(β-gal), an $E1^-E3^-$ adenovirus carrying the gene of β-galactosidase (β-gal). A wide range of m.o.i. was used. After 48h, the cells were examined for the presence of β-galactosidase by the FDG assay. Adβ-gal-infected cells are shown in red, whereas control uninfected cells are shown in blue. A representative of three independent experiments is shown.

To confirm the previous results, I also used a replication-deficient adenoviral vector encoding green fluorescent protein (GFP), a jellyfish protein that fluoresces under U.V. light and can be visualized by fluorescence microscopy. Indeed, a m.o.i. of 100 resulted in >95% of immature DC expressing GFP (figure 3.3). This m.o.i. was used in all subsequent studies involving DC.

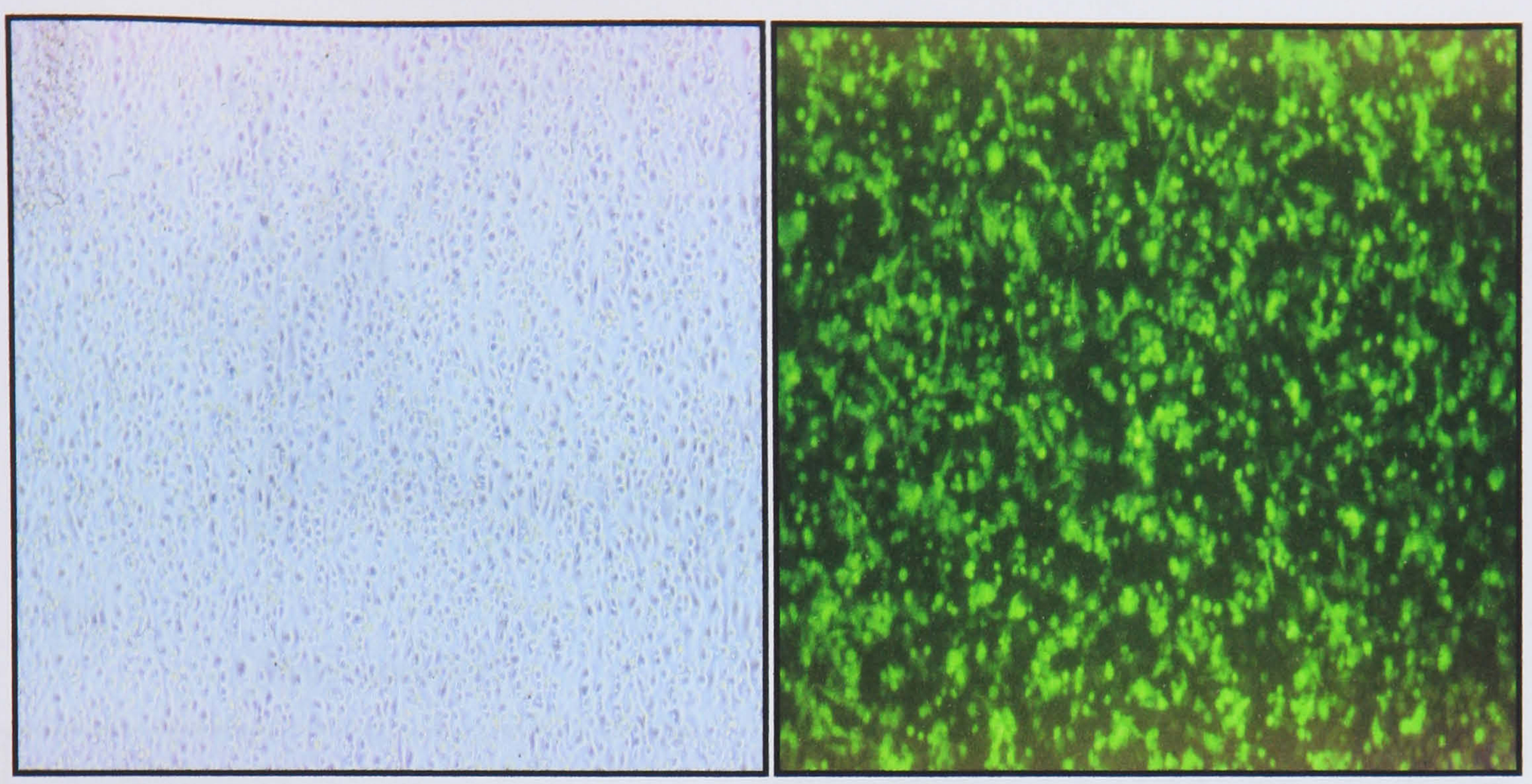


Figure 3.3: A multiplicity of infection of 100 results in >95% of immature DC expressing green fluorescent protein. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were infected for 2h in serum-free medium with Ad0(GFP), an $E1^-E3^-$ adenovirus carrying the gene of GFP. A m.o.i. of 100 was used. After 48h, the cells were examined for the presence of GFP by fluorescence microscopy (original magnification 20X) and photographed. Representative phase-contrast (a) and fluorescent (b) pictures of five independent experiments are shown.

Macrophages

Conditions for efficient adenoviral gene transfer into macrophages have recently been optimized by my colleagues (Foxwell B. et al. 1998). Resting monocytes express very low levels of αV integrins (Wickham T. et al. 1995). Differentiation, however, of monocytes to macrophages with M-CSF for 2-3 days results in the up-regulation of αVβ5 but not αVβ3 (Foxwell B. et al. 1998). This permits efficient adenoviral infection of 90% at a m.o.i. of 50 and almost 100% at a m.o.i. of 100 (Foxwell B. et al. 1998).

To confirm these observations, I used Ad0(β-gal) and Ad0(GFP) to infect M-CSF-differentiated macrophages, and examined infection efficiency by FDG staining and

fluorescence microscopy, respectively. Consistently with previous findings, I found that a m.o.i. of 100 achieved infectivity levels of greater than 95% (figure 3.4).

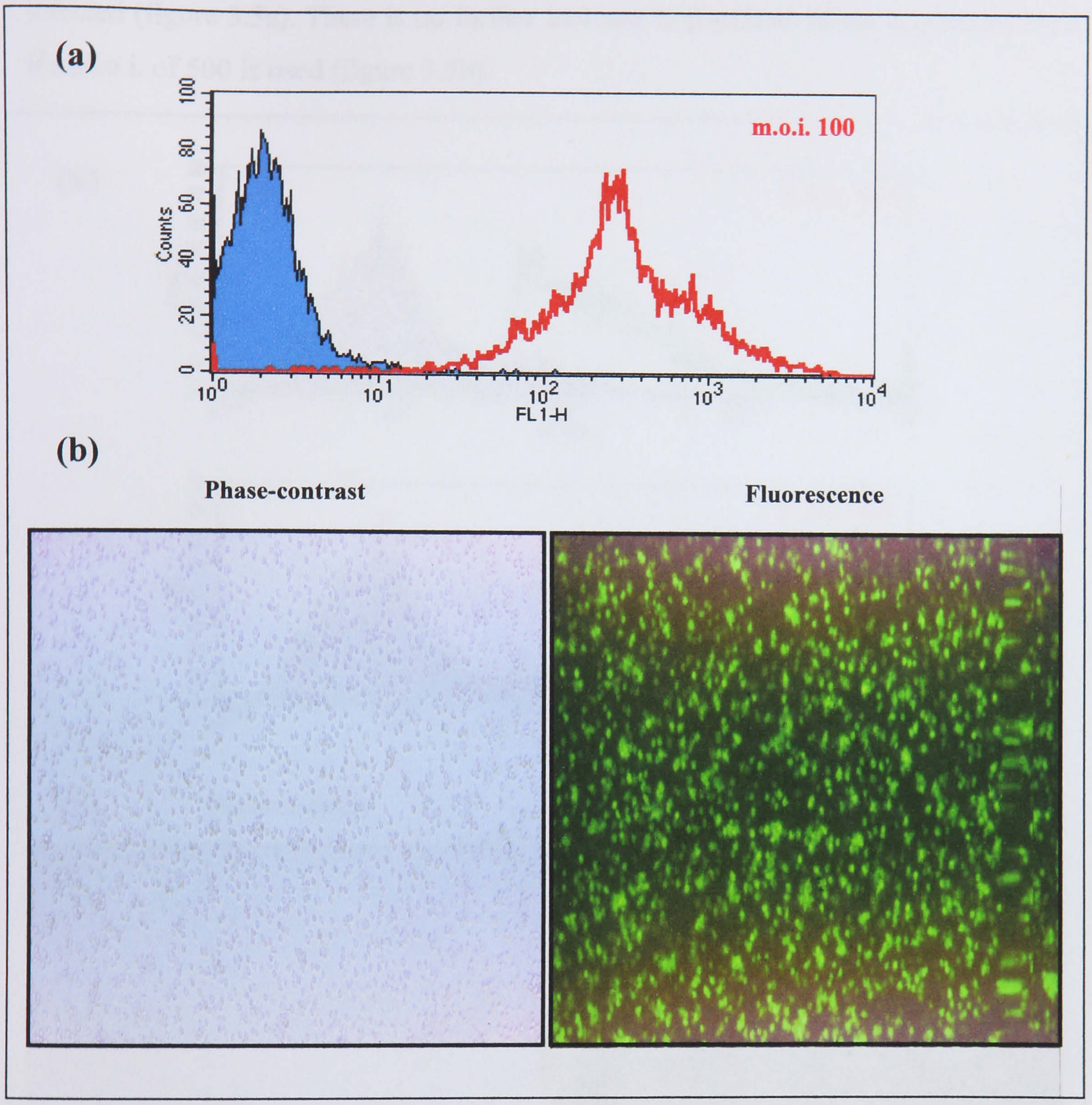


Figure 3.4: Efficient adenoviral gene transfer into human M-CSF-differentiated macrophages is achieved at a m.o.i. of 100. Human peripheral blood monocytes were cultured with 100 ng/ml in 5% FCS 1% p/s RPMI. Then, they were left uninfected or infected for 2h in serum-free medium with Ad0(β-gal) or Ad0(GFP), $E1^-E3^-$ adenoviruses carrying the gene of β-galactosidase or GFP, respectively. A m.o.i. of 100 was used. After 48h, the cells were examined for the presence of β-galactosidase by FDG staining (a), and GFP by fluorescence microscopy (magnification 20X) (b). A representative of three independent experiments is shown.

Human umbilical vein endothelial cells

Primary cultures of HUVEC have been shown to express the cocksackievirus and adenovirus receptor (CAR) and to be efficienctly infected by adenovirus at a multiplicity of infection of 50-100 (Wrighton C.J. et al. 1996; Carson S.D. et al.

1999). Using adenoviruses expressing β-galactosidase or GFP, I confirmed this finding; at a m.o.i. of 100, more than 95% of subconfluent HUVEC cells can be infected (figure 3.5a). There is no further increase in β-galactosidase expression even if a m.o.i. of 500 is used (figure 3.5b).

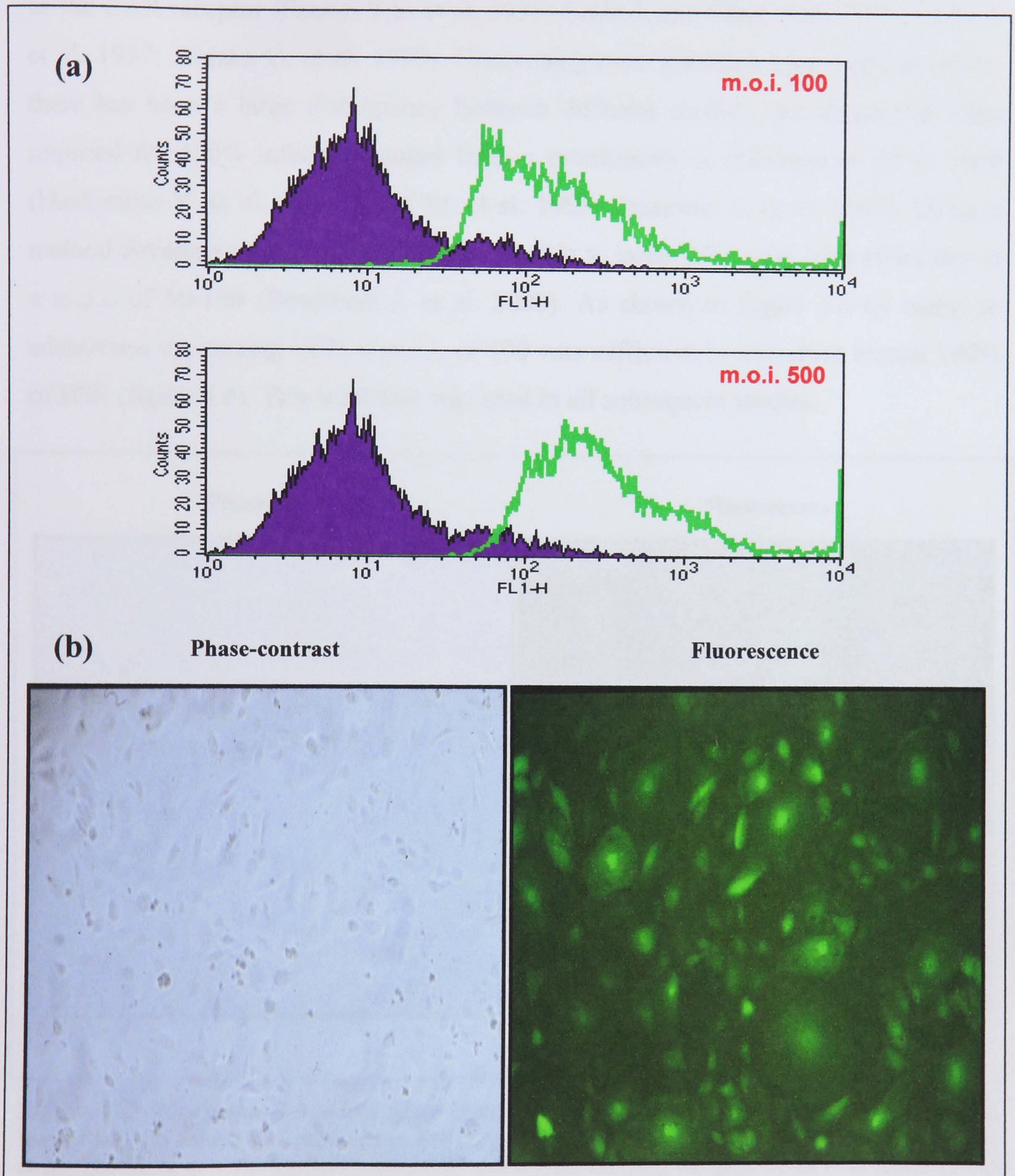


Figure 3.5: Efficient adenoviral gene transfer into human umbilican vein endothelial cells is achieved at a multiplicity of infection of 100. Human umbilical vein endothelial cells were cultured in 10% FCS 10% NBS 1% p/s RPMI. Then, they were left uninfected or infected for 2h in serum-free medium with Ad0(β-gal) or Ad0(GFP), $E1^-E3^-$ adenoviruses carrying the gene of β-galactosidase or GFP, respectively. For Ad0(β-gal), two m.o.i. of 100 and 500 were used, whereas for Ad0(GFP) only a single m.o.i. of 100 was employed. After 48h, the cells were examined for the presence of β-galactosidase by FDG staining (a), and GFP by fluorescence microscopy (original magnification 20X) (b). A representative of three independent experiments is shown.

Human skin fibroblasts

Human dermal fibroblasts express αV integrins and MHC class I but only low levels of the CAR receptor (Panetti T.S. et al. 1995; Gailit J. and Clark A.R. 1996; Gailit J. et al. 1997; Hidaka C. et al. 1999). Concerning their infection efficiency, however, there has been a large discrepancy between different studies; the amount of virus required for 100% infection ranges from a multiplicity of infection of 10 to 1000 (Hashimoto Y. et al. 1997; Bond M. et al. 1998; Teramoto S. et al. 1997). Using a method developed by my colleagues, I was able to infect HSF with high efficiency at a m.o.i. of 50-100 (Bondeson J. et al. 2000). As shown in figure 3.6 by using an adenovirus expressing GFP, a m.o.i. of 100 was sufficient to transduce almost 100% of HSF (figure 3.6). This viral titre was used in all subsequent studies.

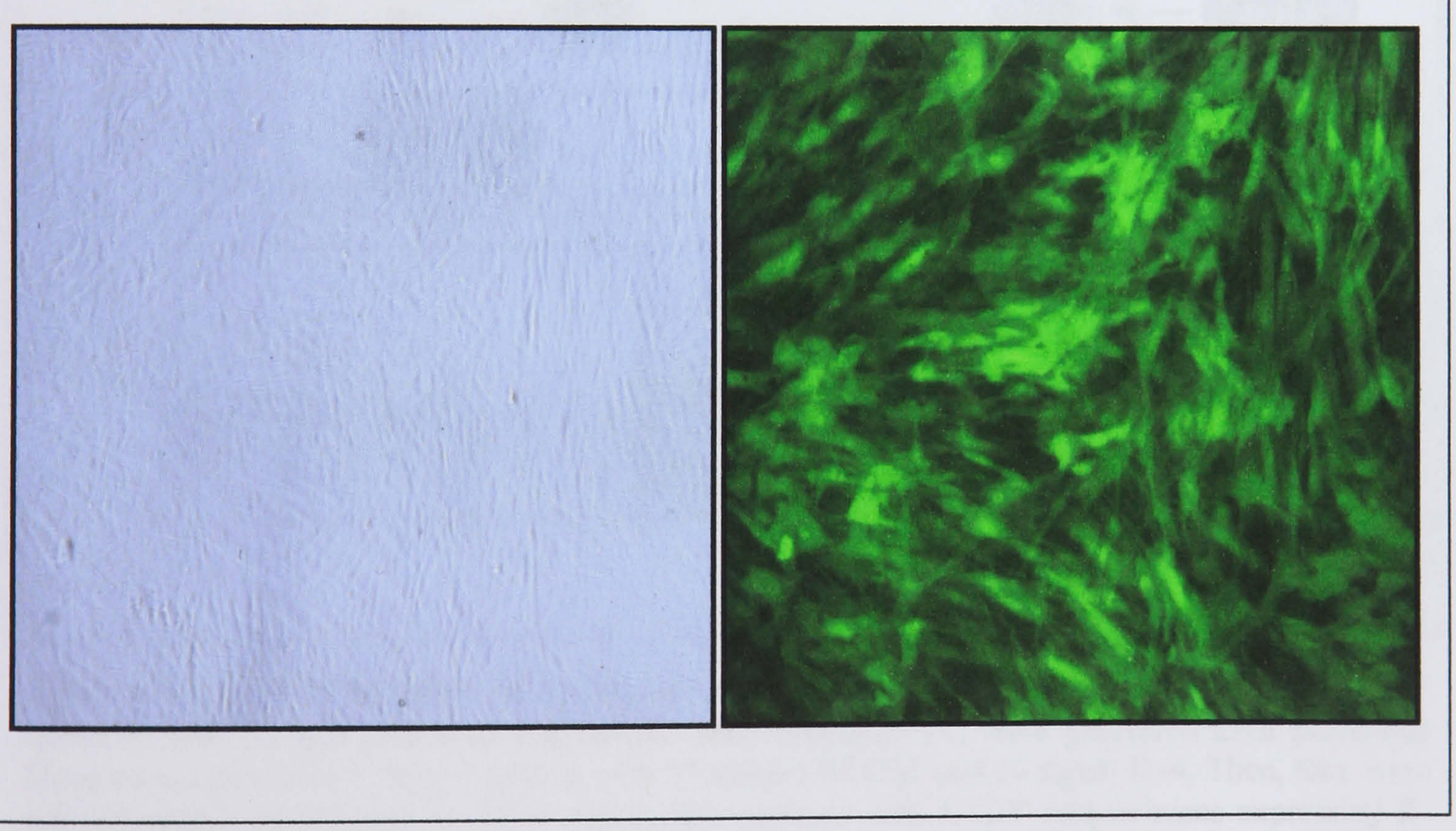


Figure 3.6: Efficient adenoviral gene transfer into HSF is achieved at a m.o.i. of 100. Human skin fibroblasts were cultured in 5% FCS 1% p/s DMEM. Then, they were infected for 2h in serum-free medium with Ad0(GFP), an $E1^-E3^-$ adenovirus carrying the gene of GFP. A m.o.i. of 100 was used. After 48h, the cells were examined for the presence of GFP by fluorescence microscopy (original magnification 20X) and photographed. Representative phase-contrast and fluorescent pictures of five independent experiments are shown.

3.3. Dominant negative IKK2 inhibits the allogeneic MLR

Using this efficient adenoviral gene transfer technique, I overexpressed IκBα and IKK2 into human immature DC in order to study their role in DC antigen-presenting function. In agreement with the findings of others (Yoshimura S. et al. 2000; Oyama T. et al. 1998), IκBα overexpression abrogated the MLR of immature DC (figure 3.8). At the same time, IκBα overexpression inhibited NF-κB nuclear translocation in response to LPS, thus confirming the importance of NF-κB in antigen presentation and LPS-induced DC activation (figure 3.7).

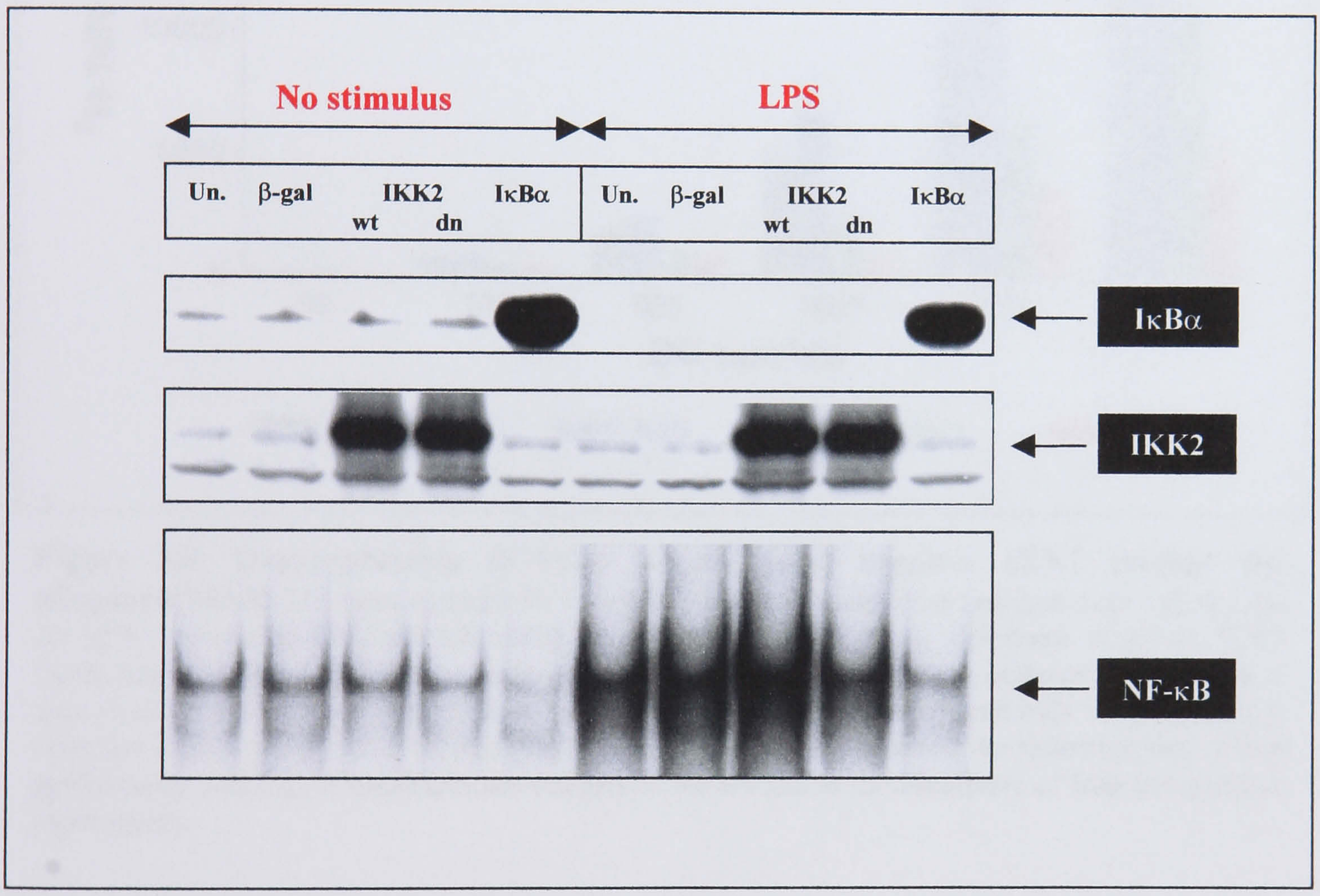


Figure 3.7: Overexpression of IκBα but not dominant negative IKK2 inhibits LPS-induced NF-κB activation in immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (β-gal), wild-type (wt) and dominant negative (dn) forms of IKK2, or IκBα (IκBα). A m.o.i. of 100 was used. After 48 h, cells were stimulated for 1 h with 100 ng/ml LPS and were subsequently lysed. Their cytosolic and nuclear extracts collected and examined for the presence of cytosolic IKK2 or IκBα, and for the presence of nuclear NF-κB DNA-binding activity by EMSA. A representative of three independent experiments is shown.

Overexpression of dominant-negative IKK2, on the other hand, had no effect on LPS-induced NF-κB activation (figure 3.7) but did inhibit the allogeneic MLR induced by immature DC (figure 3.8).

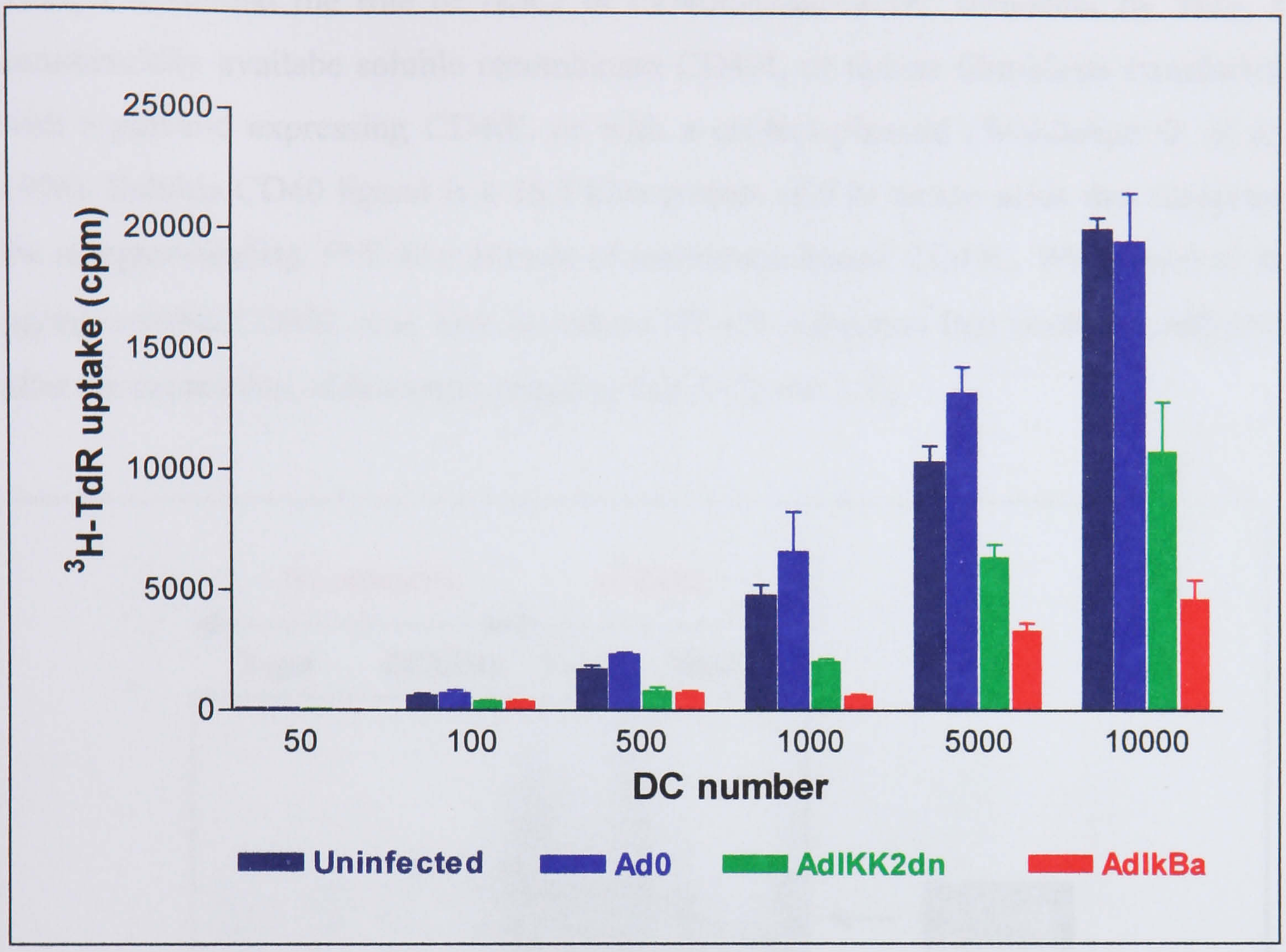


Figure 3.8: Overexpression of IκBα or dominant negative IKK2 inhibits the allogeneic MLR. DC were cultured for 5 days with GM-CSF and IL-4 and then were infected for 2h with replication-deficient adenoviral constructs overexpressing dominant negative IKK2 (AdIKK2dn), IκBα (AdIκBα) or having no insert (Ad0). DC were then cultured for a further 2 days in complete medium. After 2 days, graded numbers of DC were cultured with 10^5 allogeneic T cells for 5 days, pulsed with tritiated thymidine at day 5 and harvested the following day. Mean proliferation (±SEM) of quadruplicate cultures is shown and is representative of four independent experiments.

3.4. Dominant negative IKK2 blocks CD40L- but not LPS-induced DC NF-κB activation and cytokine production

These findings suggested that LPS-induced and allogeneic T cells-induced DC activation differ in their requirement for IKK2. One of the major signals that are involved in the allogeneic MLR and the T cell-induced DC activation is CD40L-mediated stimulation of CD40. In DC, CD40 stimulation induces the production of large amounts of IL-12, the up-regulation of antigen-presenting and costimulatory

molecules, and the enhancement of T cell-stimulating ability (Cella M. et al. 1996; Koch F. et al. 1996).

Thus, I examined the role of IKK2 in CD40L-induced DC activation by using a commercially availabe soluble recombinant CD40L or mouse fibroblasts transfected with a plasmid expressing CD40L or with a control plasmid (Wohlleben G. et al. 1996). Soluble CD40 ligand is a 16.3 kDa protein of 149 amino acids that comprise the receptor-binding TNF-like domain of membrane-bound CD40L. When used at 30 μg/ml, soluble CD40L was able to induce NF-κB activation that could be inhibited after the expression of dominant negative IKK2 (figure 3.9).

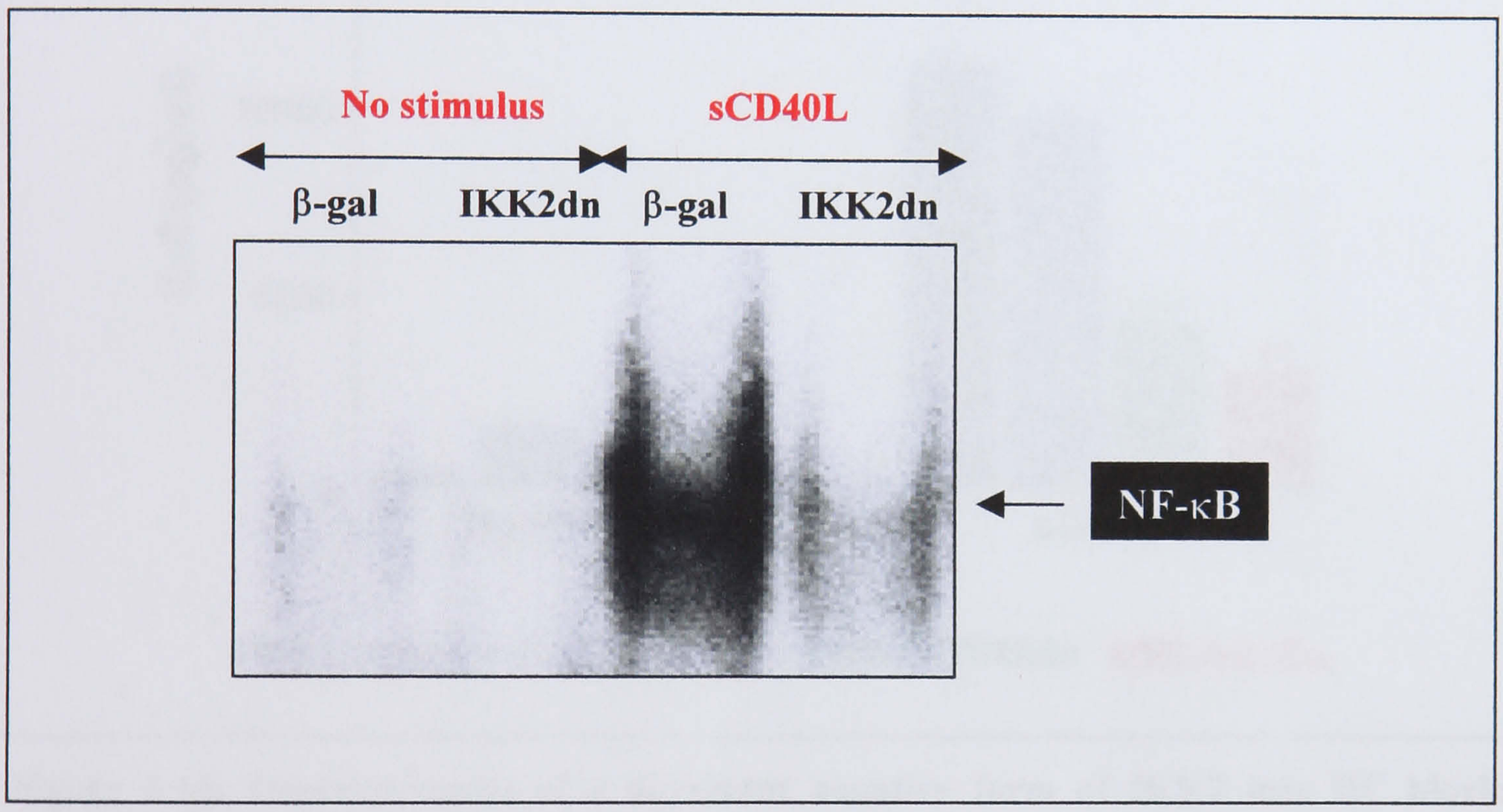


Figure 3.9: Overexpression of dominant negative IKK2 inhibits CD40 ligand-induced NF-κB activation in immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal) or a dominant negative form of IKK2 (AdIKK2dn). A m.o.i. of 100 was used. After 48 h, cells were stimulated for 1 h with 50 μg/ml of soluble CD40L and were subsequently lysed. Their nuclear extracts were collected and examined for the presence of nuclear NF-κB DNA-binding activity by EMSA. A representative of two independent experiments is shown.

Similarly, soluble CD40L could induce IL-6 and IL-8 production that was again inhibited by dominant negative IKK2 (figure 3.10).

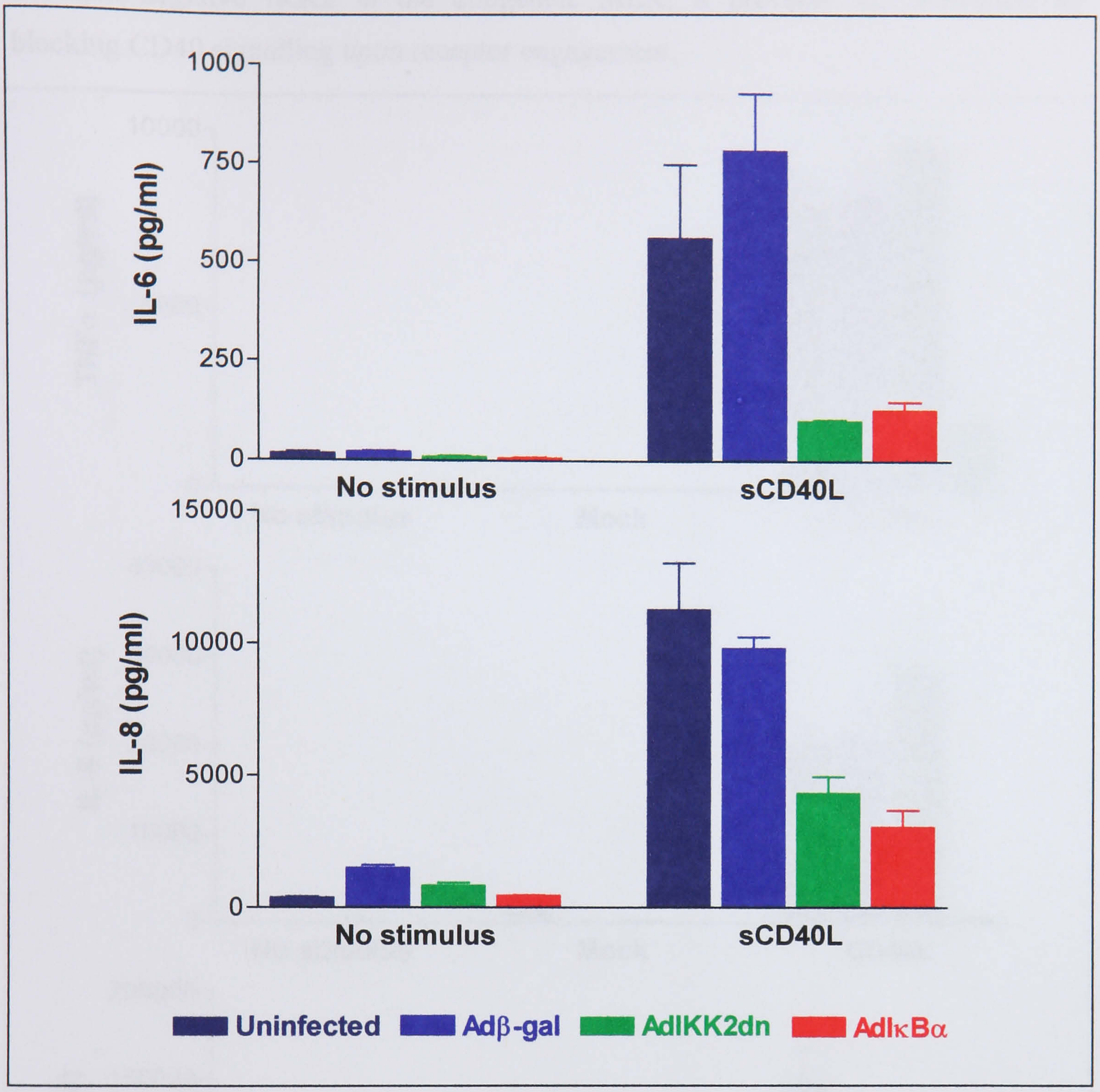


Figure 3.10: Overexpression of a dominant negative form of IKK2 into DC blocks soluble CD40 ligand-induced cytokine production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), dominant negative IKK2 (AdIKK2dn) or IκBα (AdIκBα). After 2 days of further culture, 30 μg/ml of soluble CD40L (sCD40L) were used to stimulate DC for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of two independent experiments.

CD40L-transfected cells, on the other hand, were more potent in inducing DC cytokine production. Thus, they induced higher levels of IL-6 and IL-8 as well as TNFα that could be inhibited by expressing of a dominant negative form of IKK2 (figure 3.11). These observations suggested that IKK2 is necessary for CD40 signalling, and provide a possible mechanism for part of the inhibitory effect of

dominant-negative IKK2 in the allogeneic MLR; it prevents DC activation by blocking CD40 signalling upon receptor engagement.

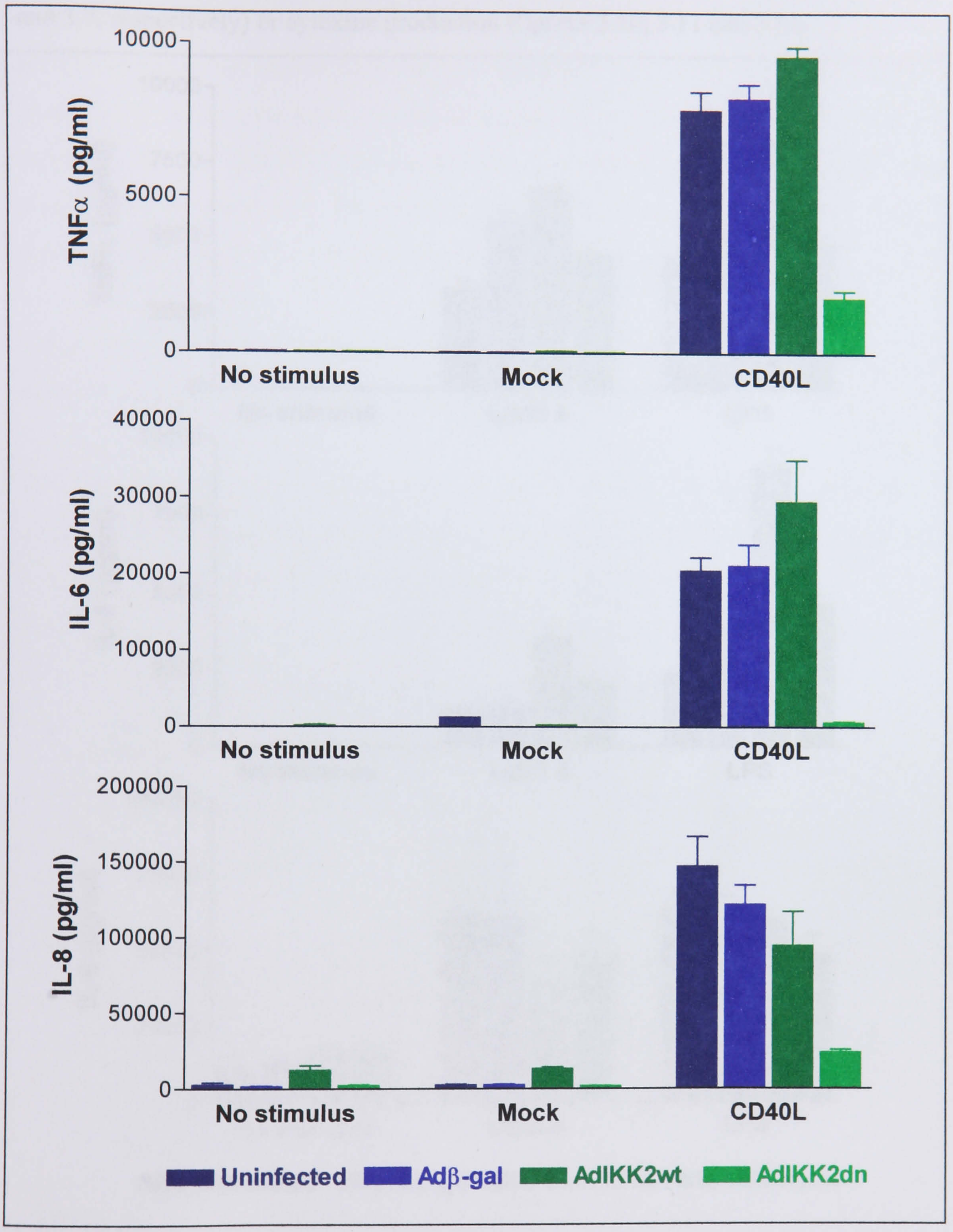


Figure 3.11: Overexpression of a dominant negative form of IKK2 into DC blocks membrane-bound CD40 ligand-induced cytokine production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), and wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn). After 2 days of further culture, $1x10^5$ mock- or CD40L-transfected mouse fibroblasts were used to stimulate DC for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of four independent experiments.

This requirement of IKK2 seems to be specific for CD40L-induced downstream signalling as it inhibits CD40L- but not LPS-induced NF-κB activation (figures 3.9 and 3.7, respectively) or cytokine production (figures 3.10, 3.11 and 3.12).

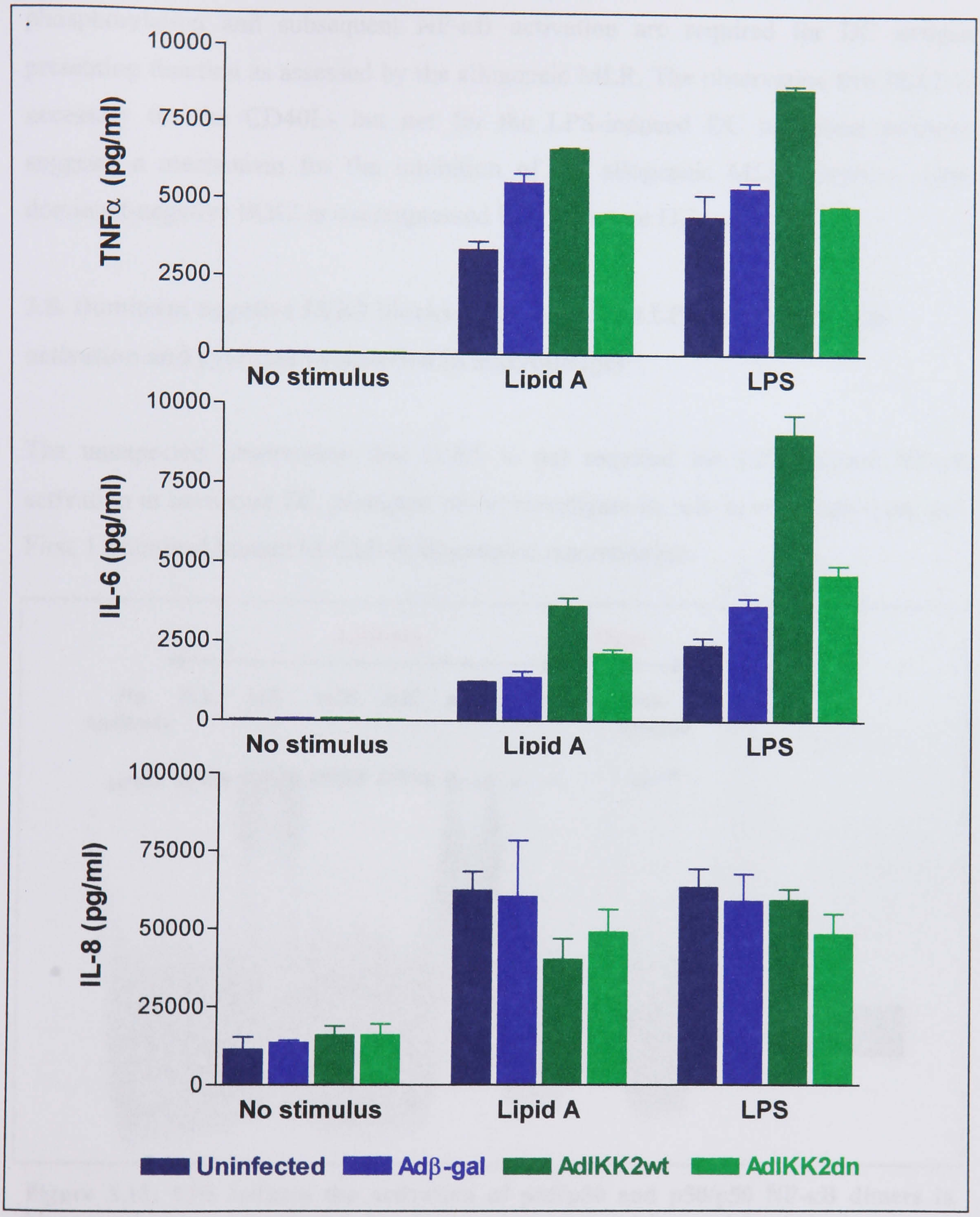


Figure 3.12: Overexpression of a dominant negative form of IKK2 into DC does not block LPS- or lipid A-induced cytokine production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), and wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn, respectively). After 2 days of further culture, 1 μg/ml of Lipid A or 100 ng/ml of LPS were used to stimulate DC for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of four independent experiments.

At the same time, overexpression of wild-type IKK2 has no effect on the allogeneic MLR or CD40L-induced cytokine production whereas it does synergize with LPS to superinduce TNFα and IL-6. In summary, these findings suggest that IKK2 phosphorylation and subsequent NF-κB activation are required for DC antigen presenting function as assessed by the allogeneic MLR. The observation that IKK2 is necessary for the CD40L- but not for the LPS-induced DC activation pathway suggests a mechanism for the inhibition of the allogeneic MLR observed when dominant-negative IKK2 is overexpressed into immature DC.

3.5. Dominant negative IKK2 blocks CD40L- but not LPS-induced NF-κB activation and cytokine production in macrophages

The unexpected observation that IKK2 is not required for LPS-induced NF-κB activation in immature DC prompted me to investigate its role in other cell types too. First, I examined human M-CSF-differentiated macrophages.

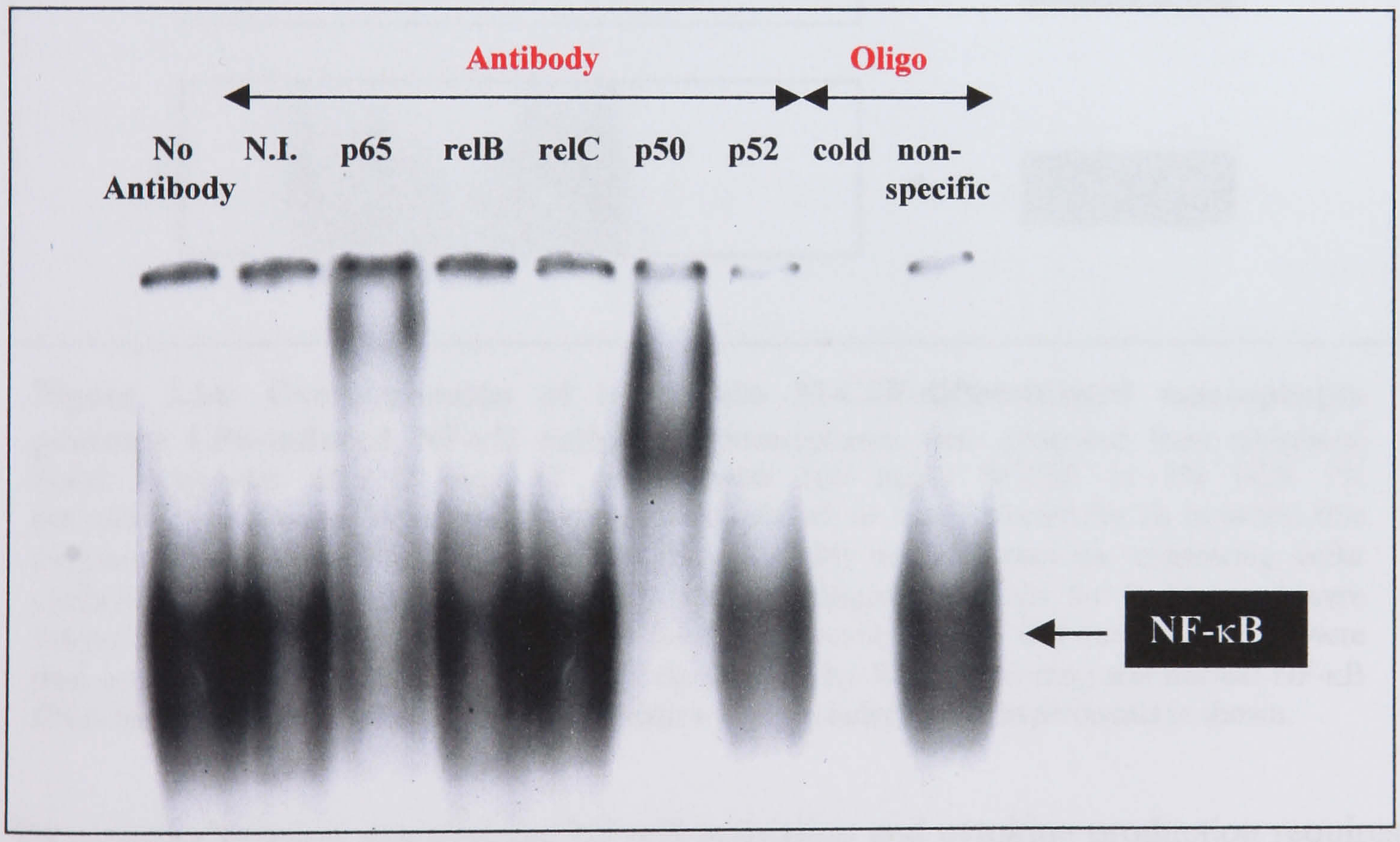


Figure 3.13: LPS induces the activation of p65/p50 and p50/p50 NF-κB dimers in human primary macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. They were then stimulated for 45 min with 10 ng/ml, and nuclear extracts were collected and examined for the activity of NF-κB by EMSA. Addition of anti- NF-κB subunit-specific antibodies were used to determine the presence of specific NF-κB members. The specificity of the band was confirmed by using a 100X excess of an unlabelled (cold) NF-κB oligonucleotide or a AP-1 (non-specific) unlabelled oligonucleotide to deplete the signal.

In this cell type, LPS induces IκB degradation and NF-κB activation that involves p65/p50 and p50/p50 dimers as detected by electrophoretic mobility shift assay (figure 3.13). In addition, LPS induces the production of various cytokines including TNFα, IL-6 and IL-8 (Bondeson J. et al. 1999).

Overexpression of IκBα into human macrophages by adenoviral gene transfer leads to inhibition of LPS-induced IκBα degradation and subsequent NF-κB nuclear translocation (figure 3.14). Using this technique, my colleagues have recently shown that TNFα, IL-6 and IL-8, produced in response to LPS, are NF-κB-dependent (Foxwell B.M.J. et al. 1998; Bondenson J. et al. 1999). This effect was not due to loss of cells through apoptosis or other causes (Foxwell B.M.J. et al. 1998).

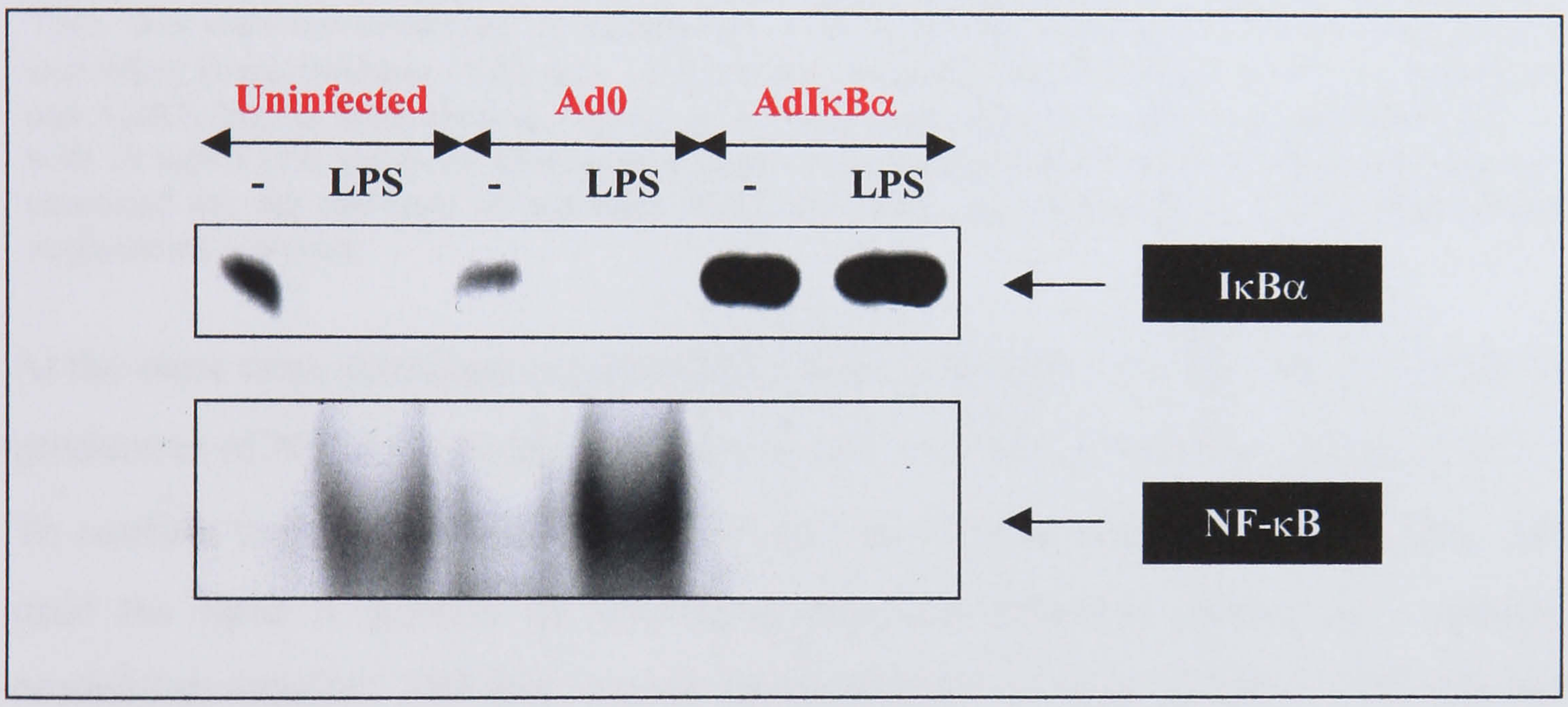


Figure 3.14: Overexpression of IκBα into M-CSF-differentiated macrophages prevents LPS-induced NF-κB activation. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or were infected for 2h in serum-free medium with a control adenovirus without insert (Ad0) or an adenovirus expressing IκBα (AdIκBα). Cells were cultured in 5% FCS 1% penicillin/streptomycin for 2 days and were subsequently stimulated with 10 ng/ml LPS for 1 h. Cytosolic extracts and nuclear extracts were then collected and examined for cytosolic levels of IκBα by Western blotting and nuclear NF-κB DNA-binding activity by EMSA. A representative of three independent experiments is shown.

To examine whether macrophage NF-κB activation and cytokine production requires IκB-inducing kinase 2 (IKK2), dominant-negative (inhibitory) and wild-type forms of IKK2 were overexpressed in M-CSF-differentiated macrophages (figure 3.15). It was found that dominant negative IKK2 did not inhibit the LPS-induced degradation of IκBα (figure 3.15).

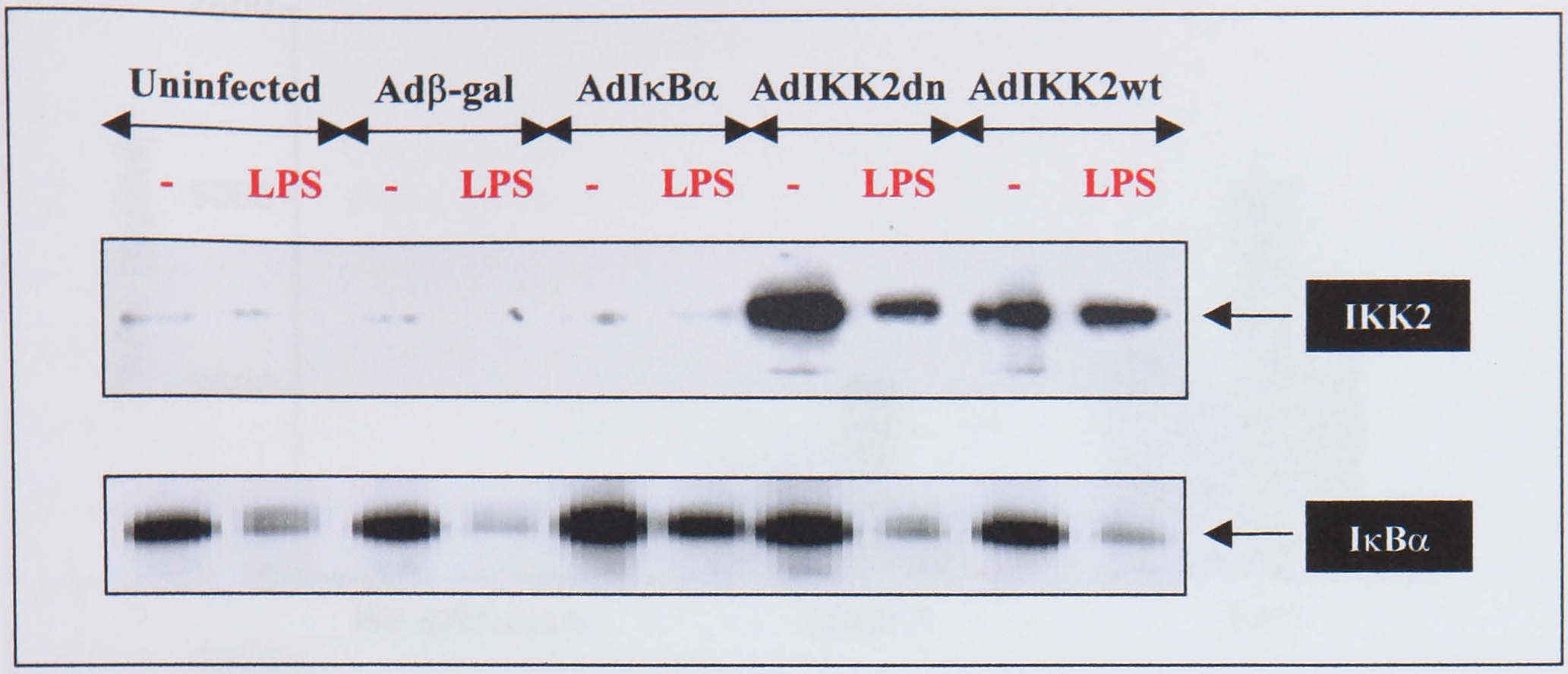


Figure 3.15: Overexpression of dominant negative IKK2 does not inhibit LPS-induced NF-κB activation in human M-CSF-differentiated macrophages. Human macrophages were generated from peripheral blood monocytes after 2-3 days of culture with 100 ng/ml M-CSF. Then, they were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), wild-type and dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn), or IκBα (IκBα). A m.o.i. of 100 was used. After 48 h, cells were stimulated for 1 h with 10 ng/ml LPS and were subsequently lysed. Their cytosolic and nuclear extracts collected and examined for the presence of cytosolic IKK2 and IκBα. A representative of two independent experiments is shown.

At the same time, dominant negative IKK2 expression did not inhibit the LPS-induced production of NF-κB-dependent cytokines such as TNFα, IL-6 and IL-8 (figure 3.15). To confirm that LPS signalling does not use IKK2 to activate NF-κB, we have also used the lipid A portion of LPS as a stimulus. Efficient macrophage cytokine production required 100-fold higher concentrations of lipid A than LPS but still induced IKK2-independent macrophage cytokine production (figure 3.16).

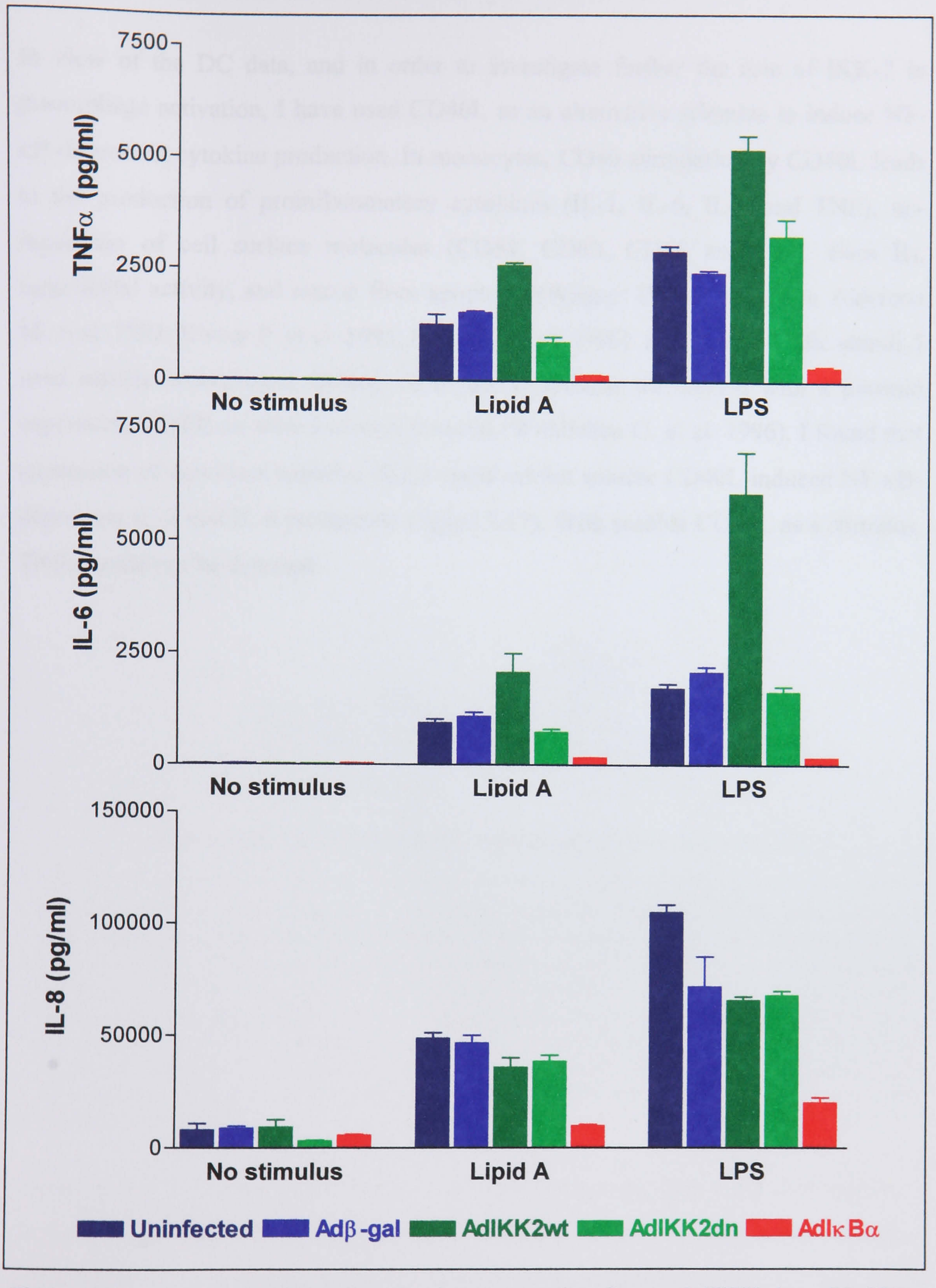


Figure 3.16: Overexpression of a dominant negative form of IKK2 into M-CSF-differentiated macrophages does not block LPS- or lipid A-induced cytokine production. M-CSF-differentiated macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF. Cells were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn, respectively), or IκBα (AdIκBα). After 2 days of further culture, 1 μg/ml of Lipid A or 10 ng/ml of LPS were used to stimulate macrophages for 24h. Supernatants were collected and assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of four independent experiments.

In view of the DC data, and in order to investigate further the role of IKK-2 in macrophage activation, I have used CD40L as an alternative stimulus to induce NF-κB-dependent cytokine production. In monocytes, CD40 stimulation by CD40L leads to the production of proinflammatory cytokines (IL-1, IL-6, IL-8 and TNF), up-regulation of cell surface molecules (CD54, CD80, CD86 and HLA class II), tumoricidal activity, and rescue from apoptosis (Wagner D.H. et al. 1994; Alderson M. et al. 1993; Kiener P. et al. 1995; Suttles J. et al. 1996). As CD40-specific stmuli, I used soluble recombinant CD40L or mouse fibroblasts transfected with a plasmid expressing CD40L or with a control plasmid (Wohlleben G. et al. 1996). I found that expression of dominant negative IKK2 could inhibit soluble CD40L-induced NF-κB-dependent IL-6 and IL-8 production (figure 3.17). With soluble CD40L as a stimulus, TNFα could not be detected.

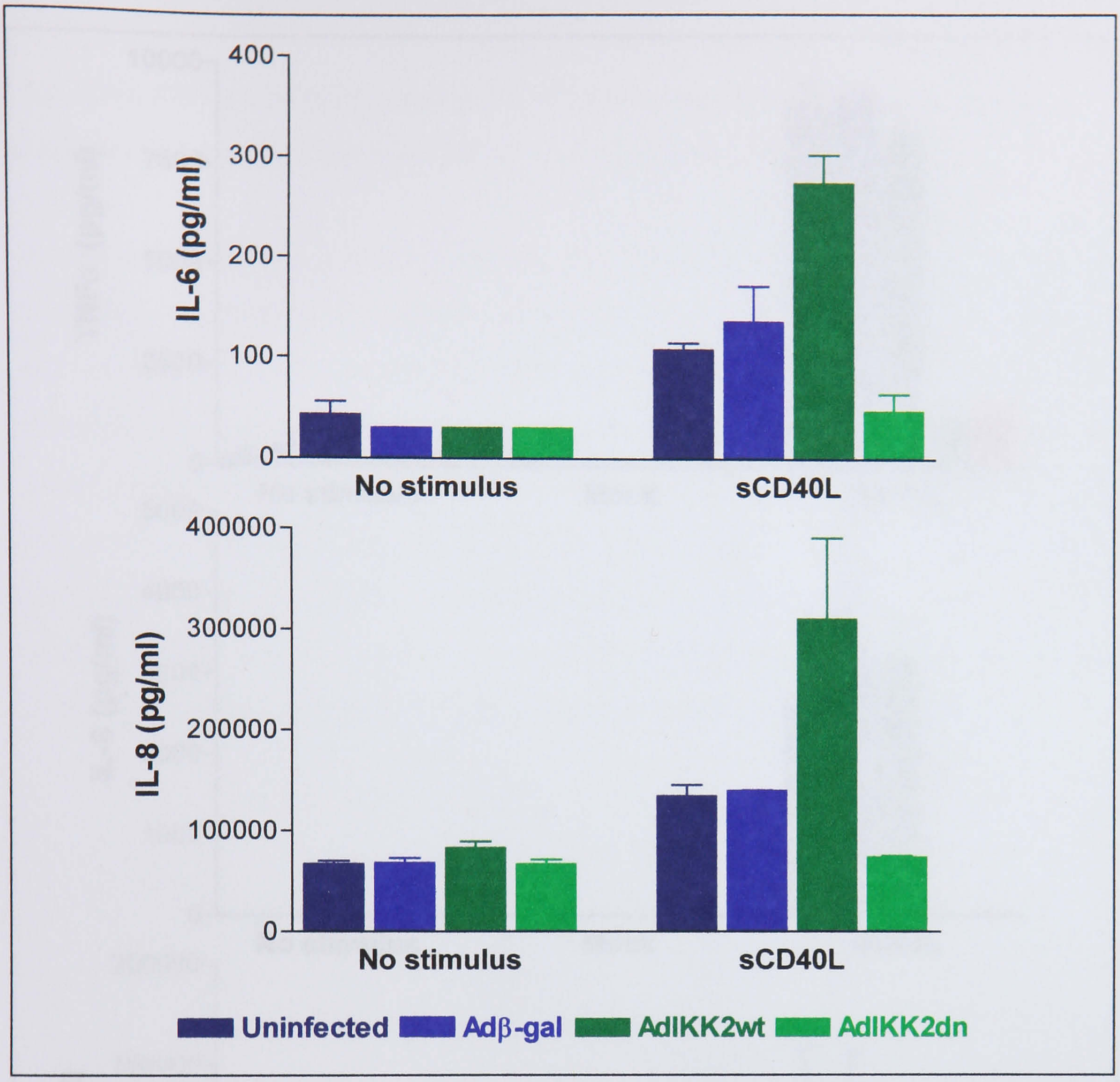


Figure 3.17: Overexpression of a dominant negative form of IKK2 into M-CSF-differentiated macrophages blocks soluble CD40L-induced cytokine production. M-CSF-differentiated macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF. Cells were left uninfected, or infected for 2h in serum-free medium with $E1^-$ $E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn, respectively). After 2 days of further culture, 30 μg/ml of soluble CD40L were used to stimulate $1x10^5$ macrophages for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of two independent experiments.

When, however, CD40L-transfected mouse fibroblasts were used as a CD40 stimulus, TNFα could be induced in addition to IL-6 and IL-8. Whether this is due to other CD40-independent signals provided or to their ability to present trimeric CD40L more efficiently is not clear. Certainly, the higher levels of IL-6 and IL-8 produced by CD40L-transfected fibroblasts support the latter possibility. Nevertheless, the expression of dominant negative IKK2 could abrogate CD40L-induced NF-κB-dependent cytokine production in both systems (figure 3.18).

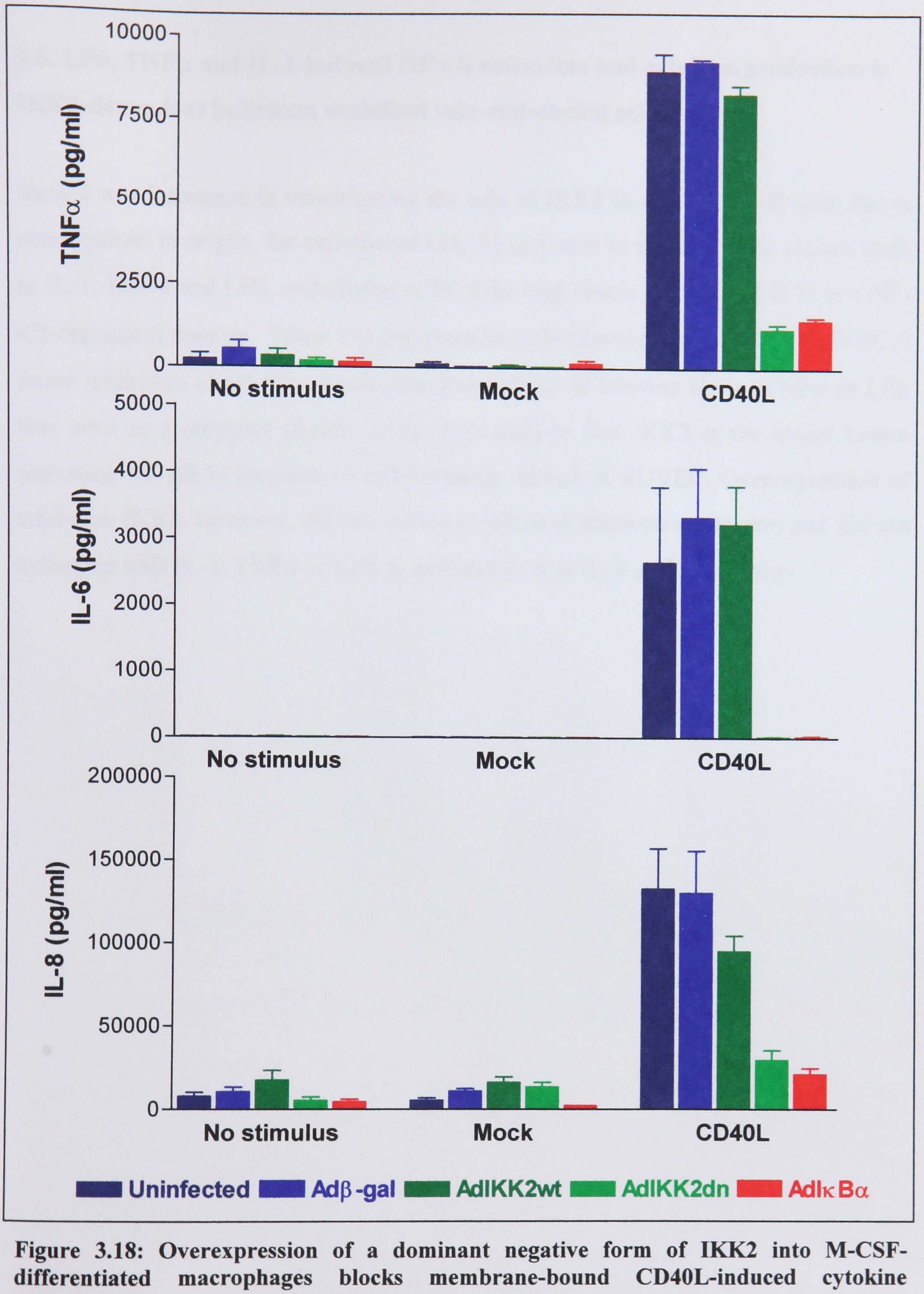


Figure 3.18: Overexpression of a dominant negative form of IKK2 into M-CSF-differentiated macrophages blocks membrane-bound CD40L-induced cytokine production. M-CSF-differentiated macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF. Cells were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn, respectively), or IκBα (AdIκBα). After 2 days of further culture, $1x10^5$ mock (control) and CD40L-transfected mouse fibroblasts were used to stimulate $1x10^5$ macrophages for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of four independent experiments.

3.6. LPS, TNFα and IL-1-induced NF-κB activation and cytokine production is IKK2-dependent in human umbilical vein endothelial cells

Next, I was interested in investigating the role of IKK2 in a primary cell type that is non-myeloid in origin, the endothelial cell. In response to inflammatory signals such as IL-1, TNFα and LPS, endothelial cells make high levels of IL-6 and IL-8 in a NF-κB-dependent manner. When I overexpressed dominant-negative IKK2 in HUVEC, I found inhibition of cytokine production irrespective of whether IL-1α, TNFα or LPS was used as a stimulus (figure 3.19). This implies that IKK2 is the major kinase activating NF-κB in response to inflammatory stimuli in HUVEC. Overexpression of wild-type IKK2, however, did not induce cytokine production on its own and did not synergize with IL-1, TNFα or LPS to increase IL-6 or IL-8 in this cell type.

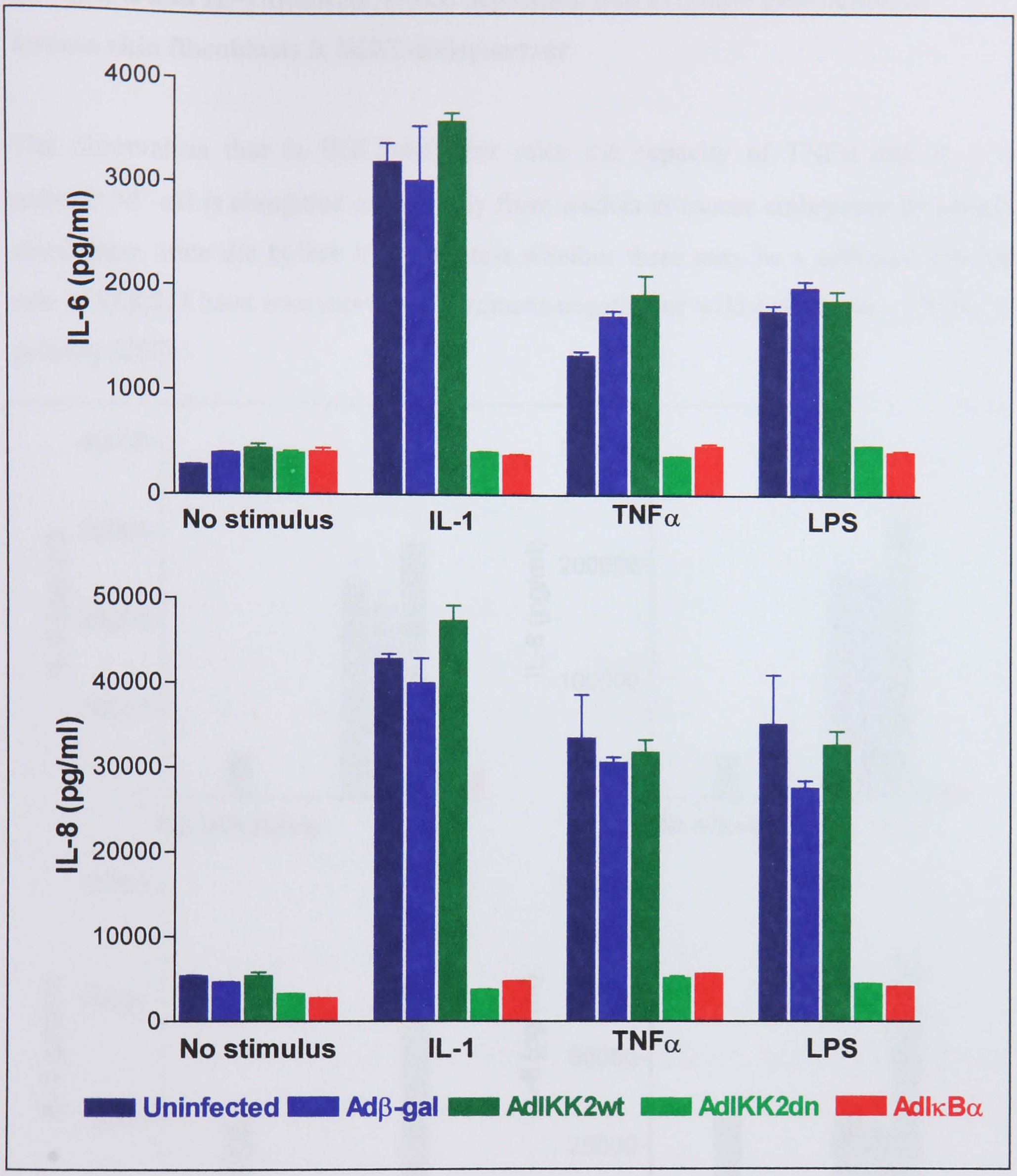


Figure 3.19: Overexpression of a dominant negative form of IKK2 into HUVEC inhibits IL-1-, TNFα- and LPS-induced cytokine production. HUVEC were cultured in 10% FCS 10% NBS 1% p/s RPMI. Cells were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn, respectively), or IκBα (AdIκBα). After 1-2 days of further culture, cells were stimulated with 20 ng/ml IL-1, 20 ng/ml TNFα or 1 μg/ml of LPS for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of five independent experiments.

3.7. TNFα and IL-1-induced NF-κB activation and cytokine production in human skin fibroblasts is IKK2-independent

The observation that in IKK2-deficient mice the capacity of TNFα and IL-1 to activate NF-κB is abrogated comes only from studies in mouse embryonic fibroblasts since these mice die before birth. To test whether there may be a cell-type specific role of IKK2, I have overexpressed dominant-negative or wild-type forms of IKK2 in primary HSF.

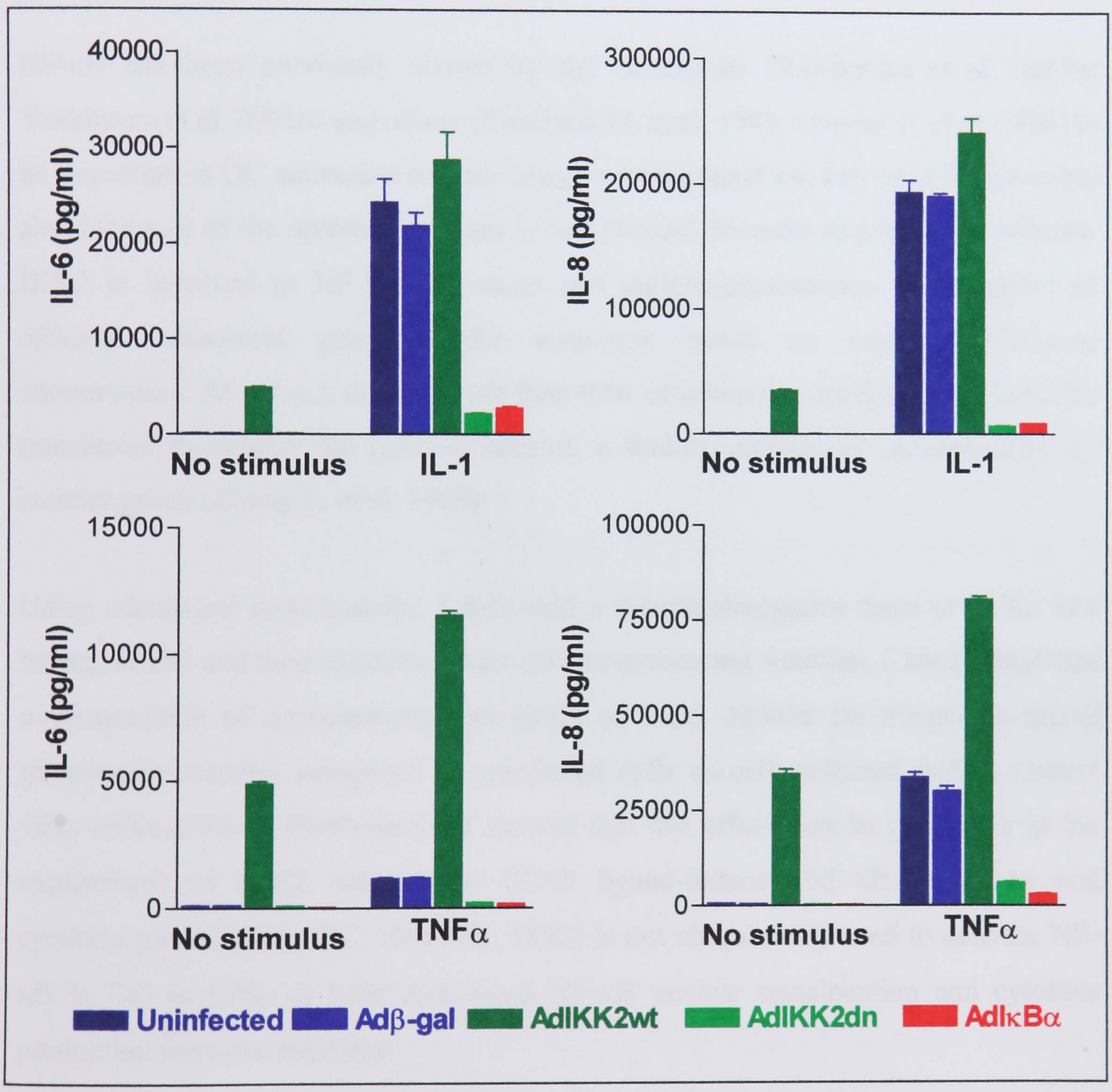


Figure 3.20: Overexpression of a dominant negative form of IKK2 into HSF inhibits IL-1- and TNFα-induced cytokine production. Human skin fibroblasts were cultured in 5% FCS 1% p/s DMEM. Cells were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), wild-type or dominant negative forms of IKK2 (AdIKK2wt and AdIKK2dn, respectively), or IκBα (AdIκBα). A m.o.i. of 100 was used. After 1-2 days of further culture, cells were stimulated with 20 ng/ml IL-1 or 20 ng/ml TNFα for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of five independent experiments.

I found that dominant-negative IKK2 and IκBα completely inhibited TNFα and IL-1-induced IL-6 and IL-8 production (figure 3.20). Overexpression of wild-type IKK2, on the other hand, induced IL-6 and IL-8 production on its own and synergized with TNFα but not IL-1 to superinduce cytokine production. Thus, IKK2 has an essential role in NF-κB-dependent cytokine production in HSF and HUVEC but not in macrophages or DC.

3.8. Discussion

NF-κB has been previously shown by my colleagues (Yoshimura et al. 2001a; Yoshimura et al. 2001b) and others (Rescigno M. et al. 1998; Oyama T. et al. 1998) to be important in DC activation and antigen-presenting function, but no evidence exists about the role of the upstream kinases in that process. In order to investigate whether IKK2 is involved in NF-κB activation and antigen-presentation, I developed an efficient adenoviral gene transfer technique based on replication-deficient adenoviruses. At a m.o.i. of 100, more than 95% of immature dendritic cells could be transfected to express the gene of interest, a finding reproduced independently by another group (Zhong L. et al. 1999).

Using adenoviral gene transfer, I delivered a dominant-negative form of IKK2 into immature DC and then examined their antigen-presenting function. I have found that overexpression of dominant-negative IKK2 or IκBα inhibits the allogeneic mixed lymphocyte reaction compared to uninfected cells or cells infected with a control virus without insert. Furthermore, I showed that this effect may be partly due to the requirement of IKK2 activity for CD40 ligand-induced NF-κB activation and cytokine production in DC. However, IKK2 is not ubiquitously used to activate NF-κB in DC as LPS- or lipid A-induced NF-κB nuclear translocation and cytokine production were not inhibited.

The finding of signal-specific utilization of IKK2 in DC was unexpected and in contrast to the current 'paradigm' of universal utilization of IKK2 for IL-1, TNFα, LPS or other inflammatory signalling processes (Karin M. and Delhase M. 2000b). . Numerous *in vitro* studies have recently addressed the question of the involvement of

IKK2 in signalling pathways induced by IL-1 and TNF. It was found that expression of dominant negative forms of IKK2 could block IL-1-induced NF-κB activation in 293 human embryonic kidney cells (Regnier C.H. et al. 1997; Woronicz J.D. et al. 1997; Delhase M. et al. 1999; Pomerantz J.L. and Baltimore D. 1999), HeLa human epithelial cells (Regnier C.H. et al. 1997; Mercurio F. et al. 1997; Delhase M. et al. 1999) human alveolar epithelial carcinoma cells (C.C. Chen et al. 2000), and primary mouse osteoclast-like cells (Miyazaki T. et al. 2000). Similarly, expression of dominant negative IKK2 could block TNFα-induced NF-κB activation in 293 human embryonic kidney cells (Regnier C.H. et al. 1997; Woronicz J.D. et al. 1997; Delhase M. et al. 1999; Pomerantz J.L. and Baltimore D. 1999; Inohara N. et al. 2000; Tojima Y. et al. 2000), HeLa human epithelial cells (Regnier C.H. et al. 1997; Mercurio F. et al. 1997; Zandi E. 1997; Delhase M. et al. 1999), THP-1 human monocytic cells (Fischer C. et al. 1999) and primary fibroblast-like synoviocytes (Aupperle K.R. et al. 1999).

In contrast to IL-1 and TNFα, fewer studies have investigated the role of IκB kinases in LPS signalling. These suggested that the IKK complex is a target of LPS signalling (O'Connell M.A. et al. 1998; Hawiger J. et al. 1999; Fischer C. et al. 1999), and the generation of IKKγ-deficient mice confirmed that and further demonstrated that the IKK complex is necessary for LPS-induced NF-κB activation (Rudolph D. et al. 2000). The similarity of TLR4, the proposed LPS receptor, with the IL-1 receptor in intracellular signalling events has lead to the assumption that IKK2 is required for LPS signalling too (Anderson K.V. 2000). Two recent studies in THP-1 human monocytic cells have examined the role of IKK1 and IKK2 in LPS signal transduction. The first found that a dominant negative form of IKK2 but not IKK1 inhibits the LPS-induced κB-dependent transcription by 30-45% (Connell M.A. et al. 1998). The second demonstrated that inhibitory forms of either IKK1 or IKK2 could inhibit LPS-induced NF-κB activity by around 30%, and that this increased to 50-60% when both constructs were transfected together to completely abrogate the activity of the IKK complex (Fischer C. et al. 1999). In real terms, this inhibitory effect may be even smaller, as instead of using empty vectors to obtain control levels of LPS-induced NF-κB activity, this study used vectors overexpressing wild-type forms of IKK1 and IKK2. This may further increase the inhibition observed as I found that at

least wild-type IKK2 synergizes with LPS to superinduce NF-κB-dependent cytokine production in primary human macrophages. Although both studies concluded that IKK2 plays an important role in LPS signal transduction, their data provided a hint that the pathway of LPS-induced NF-κB activation may be more complex than previously thought.

The fact that LPS-induced NF-κB activation was not inhibited by dominant negative IKK2 in human immature DC, prompted me to investigate further the role of IKK2 in other cell types involved in inflammatory responses. Using a very reliable system of human primary cells and an efficient adenoviral gene transfer technique, I found that IKK2 is not required for LPS-induced IκBα degradation and NF-κB activation in human M-CSF-differentiated macrophages. At the same time, IKK2 is not required for the expression of the endogenous NF-κB-dependent genes of TNFα, IL-6 or IL-8, as the expression of a dominant negative form of IKK2 could not block that. In contrast, IKK2 is essential for the production of TNFα, IL-6 and IL-8 when CD40 ligand is used as a stimulus. These results parallel those obtained in DC and suggest that the regulation of NF-κB by IKK2 may be similar in myeloid cells. In addition, these results are to my knowledge the first ones to demonstrate that CD40 signals via IKK2 to activate NF-κB.

When I used HUVEC, another primary cell type involved in leukocyte extravasation during an inflammatory response (Karmann K. et al. 1996), dominant negative IKK2 could inhibit the production of the NF-κB-dependent cytokines IL-6 and IL-8 induced by LPS as well as by IL-1 or TNFα. The degree of inhibition paralleled that observed after IκBα overexpression, suggesting that IKK2 is the major kinase involved in NF-κB activation in these cells. This finding has been confirmed by another study just published (Oitzinger W. et al. 2001), and suggested that the requirement of IKK2 for LPS signalling in different cell types differs. Whether the requirement of IKK2 for TNFα and IL-1 signalling also differs in human macrophages and DC has not been addressed in this study but preliminary data of currently ongoing experiments imply that this is not the case.

Finally, of substantial interest has been the study of HSF. Dominant-negative IKK2 inhibits both TNFα and IL-1-induced cytokine production and this is comparable to IκBα overexpression, the natural inhibitor of NF-κB. Thus, in fibroblasts IKK2 seems to be absolutely required for NF-κB activation, as previously shown from studies in the IKK2-deficient mice (Li Q et al. 1999; Tanaka M et al. 1999; Li Z.W. et al. 1999). IKK2 is required for NF-κB activation that takes place during the embryonic liver development and also for TNFα and IL-1-induced activation of NF-κB in *Ikk2*$^{-/-}$ embryonic fibroblasts, and is weakly compensated by other kinases. In contrast, studies in IKK1-deficient mice have shown that IKK1 is involved in NF-κB activation in dermal and skeletal development but is not required for TNFα-, IL-1- or LPS-induced NF-κB activation in *Ikk1*$^{-/-}$ embryonic fibroblasts, primary keratinocytes or liver tissue (Hu Y. et al. 1999; Takeda K. et al. 1999).

In summary, this study shows that the requirement of IKK2 for NF-κB activation and NF-κB-dependent cytokine production is both cell type- and stimulus-specific, and demonstrates the importance of studying signalling pathways in the most relevant cell types as well as in primary cells, rather than transformed cell lines. Even then, the physiological significance of scientific observations should be interpreted with caution and should be tested in diseased tissue and disease models whenever possible.

In the light of our studies, the question that arises is whether dominant-negative forms of IKK1, or IKK1 and IKK2, can block LPS-induced NF-κB activation in human macrophages and immature DC. It will be interesting to investigate whether additional kinases such as p90^{rsk}, PKR (dsRNA-dependent kinase), casein kinase II and the catalytic subunit of DNA-dependent kinase that have also been shown to phosphorylate IκB (Schouten G.J. et al. 1997; Kumar A. et al. 1994; Li S. and Sedivy J.M. 1993; Liu L. et al. 1998) also play a role. Recently, a new homologue of IκB-kinases termed, IKKi (IKKε), was identified which shares 30% overall identity with IKK1 or IKK2. IKKi phosphorylates IκBα preferentially at Ser36 and stimulates an NF-κB reporter gene (Shimada T. et al. 1999). However, although IKKi mRNA was strongly induced by LPS in a mouse macrophage cell line, its kinase activity was not regulated by any known NF-κB stimuli. Subsequently, it was shown by another group

that IKKi is important for PMA- and T cell receptor- but not TNFα- or IL-1-induced NF-κB activation in HeLa cells (Peters R.T. et al. 2000). Nonetheless, this study highlights the possibility that other previously unidentified kinases may also play a role in NF-κB activation in certain systems.

The findings presented in this chapter provide novel targets for therapeutic intervention. Activation of endothelial cells by the inflammatory cytokines TNFα or IL-1 triggers the up-regulation of adhesion molecules (CD40, CD62E, CD106 and CD54) and chemokines (IL-8, MCP-1, MIP-1α) and promotes the extravasation and accumulation of leukocytes at the sites of inflammation (Karmann K. et al. 1995; Karmann K. et al. 1996). Hence, prevention of TNFα and IL-1 signalling by inactivation of IKK2 would be a good strategy for blocking endothelial cell activation and increased cell extravasation during undesirable inflammatory responses.

DC play a major role in tolerance (Forster I. and Lieberam I. 1996; Kurts et al. 1997), transplant rejection (Larsen C.P. et al. 1990; Fu F. et al. 1996) and certain stages of autoimmunity (Ludewig B. et al. 1999; Drakesmith H. et al. 2000). The observation that inhibition of IKK2 activity inhibits the allogeneic MLR, a model of allograft rejection and antigen-presenting function, suggests that IKK2 may be a good target for the suppression of inappropriate antigen presentation and initiation of inflammatory responses. The recent finding that inhibition of the NF-κB activity of DC tolerizes T cells (Yoshimura S. et al. 2001b), suggests that inhibition of IKK2 activity may also be beneficial in inducing tolerance. Blocking IKK2 rather than NF-κB, on the other hand, may have potential advantages clinically because of the multiple roles that NF-κB has in physiological processes such as homeostasis and immunity (Karin M. and Delhase M. 2000b). One such advantage comes from the ability of inhibitory forms of IKK2 to prevent efficient DC antigen presentation without compromising the response of macrophages and DC to bacterial endotoxin.

The effects of IKK2 inhibition in dendritic cells may be partly due to the prevention of CD40 signalling. CD40 signalling in antigen-presenting cells results in the up-regulation of antigen-presenting and costimulatory molecules that are essential for the activation rather than the induction of anergy of T cells. Administration of anti-

CD40L monoclonal antibodies has been shown to prevent both acute and chronic graft versus host disease (Durie F.H. et al. 1994). This has been confirmed in a number of models including pancreatic islets allografts (Parker D.C. et al. 1995), cardiac allografts (Larsen C.P. et al. 1996), renal allografts (Kirk A.D. et al. 1997) and xenogeneic skin transplants (Larsen C.P. et al. 1996).

CD40-CD40L interactions, on the other hand, are very important in a number of other processes of adaptive immunity. Engagement of CD40 by its ligand in B cells results in immunoglobulin isotype switching (Rousset F. et al. 1991; Spriggs M.K. et al. 1992; Jumper M.D. et al. 1994), cell proliferation and rescue from apoptosis (Lane P. et al. 1992; Lederman S. et al. 1992; Holder M.J. et al. 1993). In monocytes, CD40 ligand leads to the production of proinflammatory cytokines (IL-1, IL-6, IL-8 and TNF), up-regulation of cell surface molecules (CD54, CD80, CD86 and HLA class II), tumoricidal activity, and rescue from apoptosis (Wagner D.H. et al. 1994; Alderson M. et al. 1993; Kiener P. et al. 1995; Suttles J. et al. 1996). Inhibiting CD40-CD40L interactions by administration of blocking antibodies has been shown to prevent disease in murine models of lupus nephritis (Mohan C. et al. 1995), diabetes (Balasa B. et al. 1997), oophoritis (Griggs N.D. et al. 1996), inflammatory bowel disease (Stuber E. et al. 1996), atherosclerosis (Mach F. et al. 1998), collagen-induced arthritis (Durie F.H. et al. 1993) and experimental autoimmune encephalomyelitis (EAE) (Gerritse K. et al. 1996).

However, treatment of established disease with neutralizing antibodies against CD40/CD40L has yielded more contrasting effects; although beneficial in established EAE and experimental autoimmune thyroiditis (Carayanniotis G. et al. 1997), it was not effective in progressive diabetes (Balasa B. et al. 1997) and collagen-induced arthritis (Mauri C. et al. 2000). This may be explained by a recent report showing that at least in later stages of collagen-induced arthritis, the CD40-CD40L interaction plays an important role in inducing regulatory T cells important for the control of the disease (Mauri C. et al. 2000). This finding, in conjunction with the requirement of CD40-CD40L interactions for protective immunity against infection (Campbell K.A. et al. 1996; Soong L. et al. 1996; Kamanaka M. et al. 1996; Wiley J.A. and Harmsen A.G. 1995), suggests that therapeutic interventions to prevent CD40-CD40L interactions will need to be applied with care.

CHAPTER 4

NF-κB-INDUCING KINASE DEFINES A REDUNDANT BUT POTENT PATHWAY OF DENDRITIC CELL ACTIVATION

4.1. Introduction

NF-κB transcription factors regulate an exceptionally large number of genes in response to stressful situations requiring rapid reprogramming of gene expression such as infections or inflammatory responses. Under normal circumstances, NF-κB is sequestered in the cytoplasm in an inactive form complexed with inhibitor of NF-κB (IκB) proteins. Its activation does not depend on new protein synthesis but requires the phosphorylation, ubiquitination and subsequent degradation of IκB by the 26S proteasome (Chen Z. et al. 1995; Scherer D. et al. 1995; Traenckner E.B. et al. 1995). This exposes a nuclear translocation sequence of the NF-κB dimer, which translocates into the nucleus to regulate gene expression (Beg A.A. et al. 1993; Brown K. et al. 1993; Henkel T. et al. 1993; Mellits K.A. et al. 1993). The main kinases responsible for IκB phosphorylation and consequent NF-κB nuclear translocation were recently described to be IKK1 and IKK2 (DiDonato J.A. et al. 1997; Mercurio F. et al. 1997; Regnier C.H. et al. 1997). They are believed to be the major point of convergence of various inflammatory signals such as LPS, CD40L, TNFα, and IL-1 (reviewed by Karin M. and Ben-Neriah Y. 2000).

Upstream of IκB kinases, several proteins that belong to the family of mitogen-activated protein kinase kinase kinases (MAP3Ks) have been proposed to activate the IKK complex. Thus, overexpression of either NF-κB-inducing kinase (NIK), MEKK1 (MAPK/ERK kinase kinase 1), MEKK2 or MEKK3 (Lee F.S. et al. 1998; Nemoto S. et al. 1998; Zhao Q. and Lee F.S. 1999) leads to activation of IKKs and NF-κB reporter genes. These MAP3Ks, act upstream of the IKK complex as kinase inactive versions of IKK1 or IKK2 inhibit NF-κB activation. NIK and MEKK-1 specifically phosphorylate both IKK1 and IKK2 in vitro although NIK has been proposed to display greater specificity for IKK1, and MEKK1 for IKK2 (Regnier C.H. et al. 1997; Woronicz J.D. et al. 1997; Lee F.S. et al. 1998; Ling L. et al. 1998; Nakano H. et al. 1998; Nemoto S. et al 1998).

MAP3K-like kinases have also been proposed to connect NIK and MEKK1 to receptor complexes. First, transforming growth factor β activated kinase 1 (TAK1) together with its activator TAK1-binding protein 1 (TAB1) may link TRAF-6 to the

NIK/IKK cascade to mediate NF-κB activation by IL-1 (Ninomiya-Tsuji J. et al. 1999). Second, TANK-binding kinase 1 (TBK1), a novel kinase related to IKK1 or IKK2 and 48% identical to IKKi, has been shown to mediate TRAF-2, -5,-6 and TANK-induced NF-κB activation (Pomerantz J.L. and Baltimore D. 1999). TBK1 forms a complex with TANK and TRAF-2 that functions upstream of NIK and the IKK complex, but it seems that TBK1 is not required for the activation of NF-κB by TNFα, IL-1 or CD40.

In dendritic cells (DC), NF-κB is required for efficient antigen-presenting function in both human and mouse cells (Rescigno M. et al. 1998; Yoshimura S. et al. 2001a; Oyama T. et al. 1998; Burkly L. et al. 1995), but very little is known about the upstream kinases involved in its activation. In the previous chapter of this thesis, I have shown that IKK2 is involved in that process, as inhibition of its activity blocks DC antigen presentation. This may be partly due to the major role IKK2 has in CD40 ligand-induced NF-κB activation.

In this chapter, I investigated the role of NIK in DC antigen-presenting function. I will present data showing that NIK defines a redundant but potent pathway of DC activation.

4.2. Overexpression of a dominant negative form of NIK in DC does not inhibit the allogeneic MLR

NIK has been proposed to be the main kinase upstream of the IKK complex that leads to NF-κB activation. To examine its role in DC antigen-presenting function, I used adenoviral gene transfer to express a dominant negative form of NIK (table 2.6). I found that this had no effect in the allogeneic mixed lymphocyte reaction (MLR), suggesting that NIK may have a redundant role in NF-κB and consequent DC activation (figure 4.1).

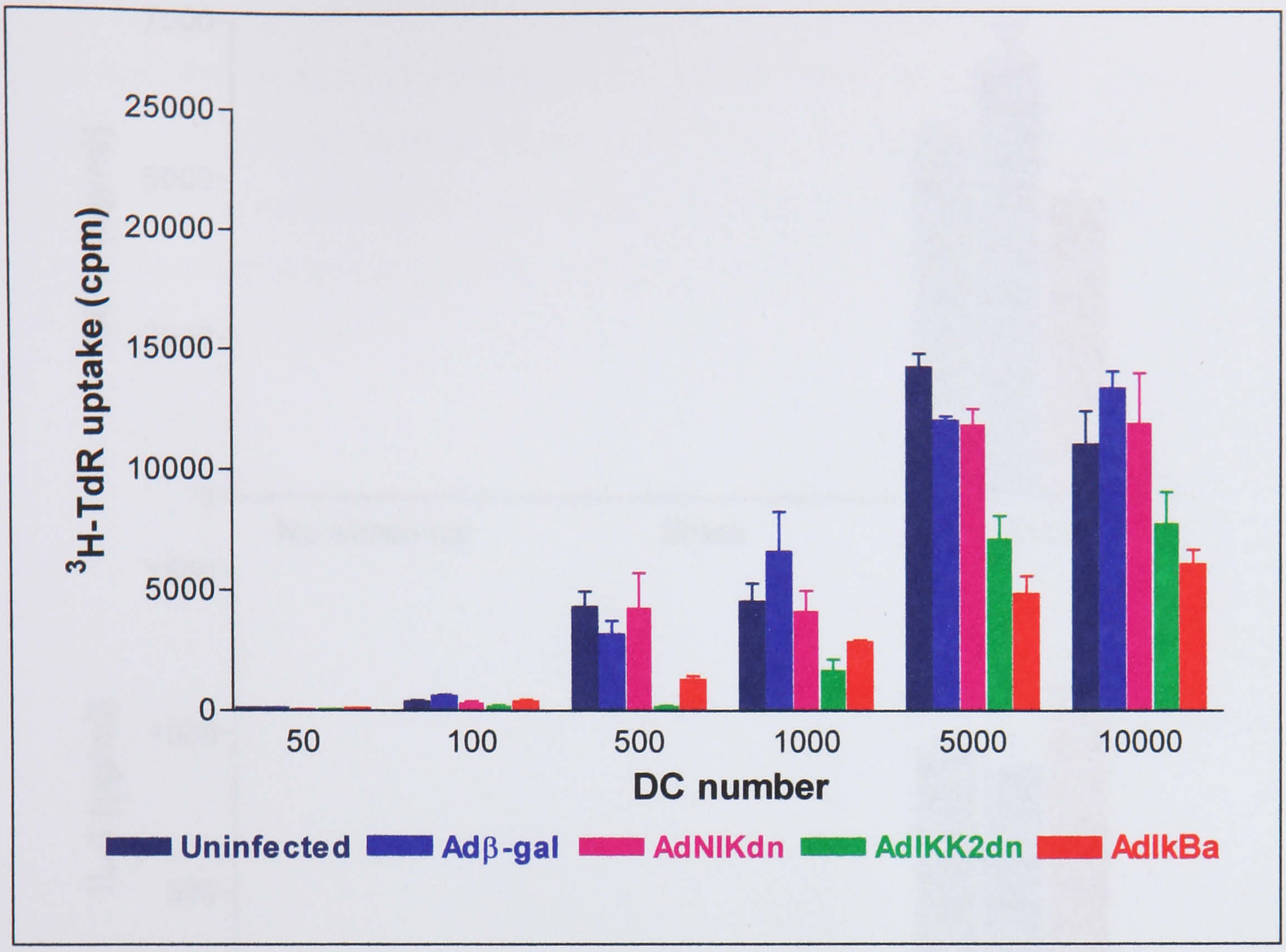


Figure 4.1: Overexpression of dominant negative NIK does not inhibit the allogeneic MLR. DC were cultured for 5 days with GM-CSF and IL-4 and then were infected for 2h with replication-deficient adenoviral constructs overexpressing β-galactosidase (Adβ-gal), dominant negative NIK (AdNIKdn), dominant negative IKK2 (AdIKK2dn) or IκBα (IκBα). DC were then cultured for a further 2 days in complete medium. After 2 days, graded numbers of DC were cultured with 10^5 allogeneic T cells for 5 days, pulsed with tritiated thymidine at day 5 and harvested the following day. Mean proliferation (±SEM) of quadruplicate cultures is shown and is a representative of four independent experiments.

4.3. A dominant negative form of NIK in DC does not block CD40 ligand- or LPS-induced cytokine production

Next, I examined whether NIK was involved in DC activation induced by CD40 ligand, a major signal thought to be involved in DC-T interactions (reviewed by Grewal I.S. and Flavell R.A. 1998). Expression of dominant negative NIK, however, did not inhibit the CD40L-induced production of TNFα, IL-6 or IL-8, whereas expression of dominant negative IKK2 did (figure 4.2).

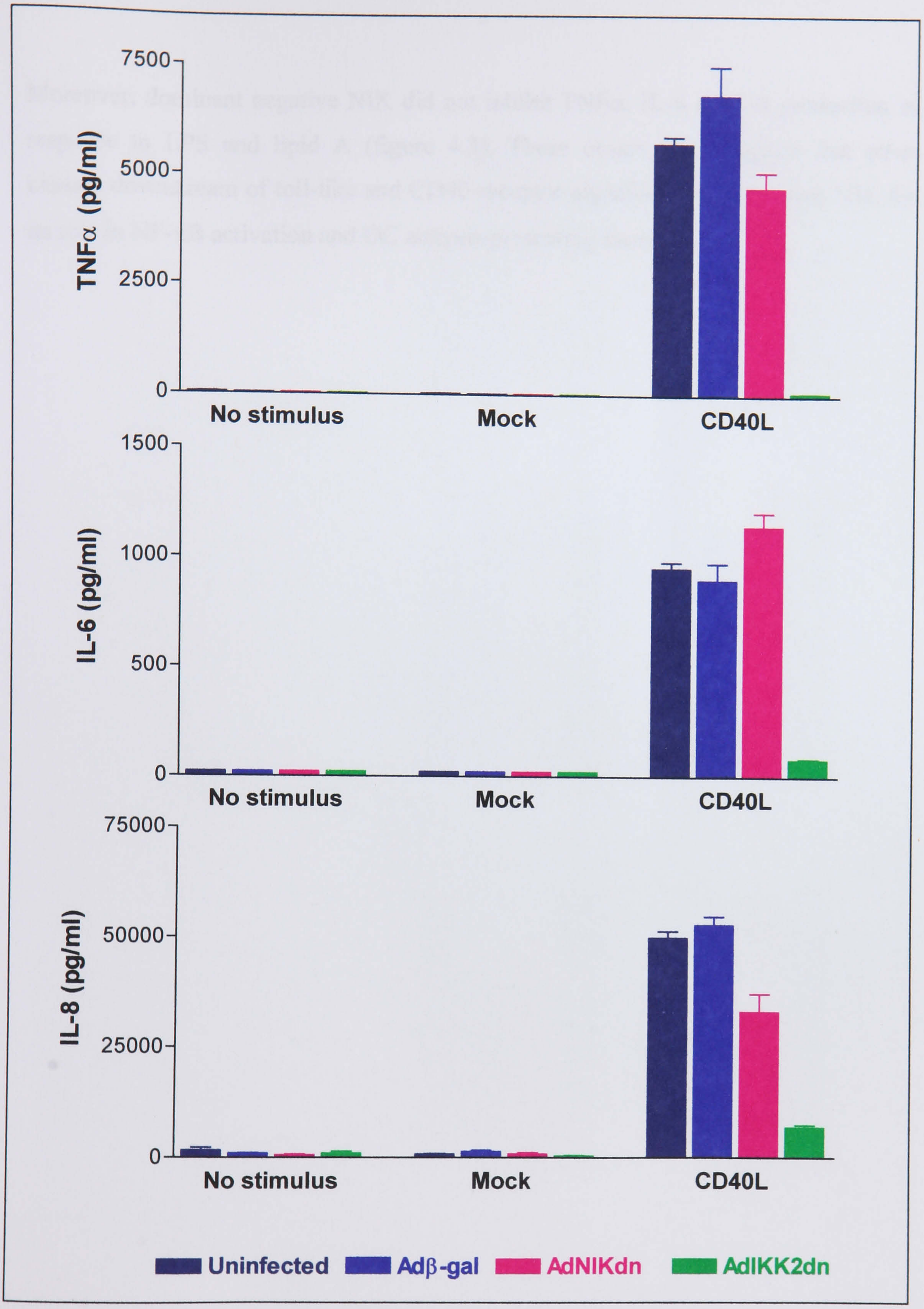


Figure 4.2: Overexpression of a dominant negative form of NIK into DC does not block CD40 ligand-induced cytokine production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), a dominant negative form of NIK (AdNIKdn) or a dominant negative form of IKK2 (IKK2dn). After 2 days of further culture, $1x10^5$ CD40L or control mock transfectants were used to stimulate DC for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is a representative of four independent experiments.

Moreover, dominant negative NIK did not inhibit TNFα, IL-6 or IL-8 production in response to LPS and lipid A (figure 4.3). These observations suggest that other kinases downstream of toll-like and CD40-receptor signalling, can substitute NIK for its role in NF-κB activation and DC antigen-presenting function.

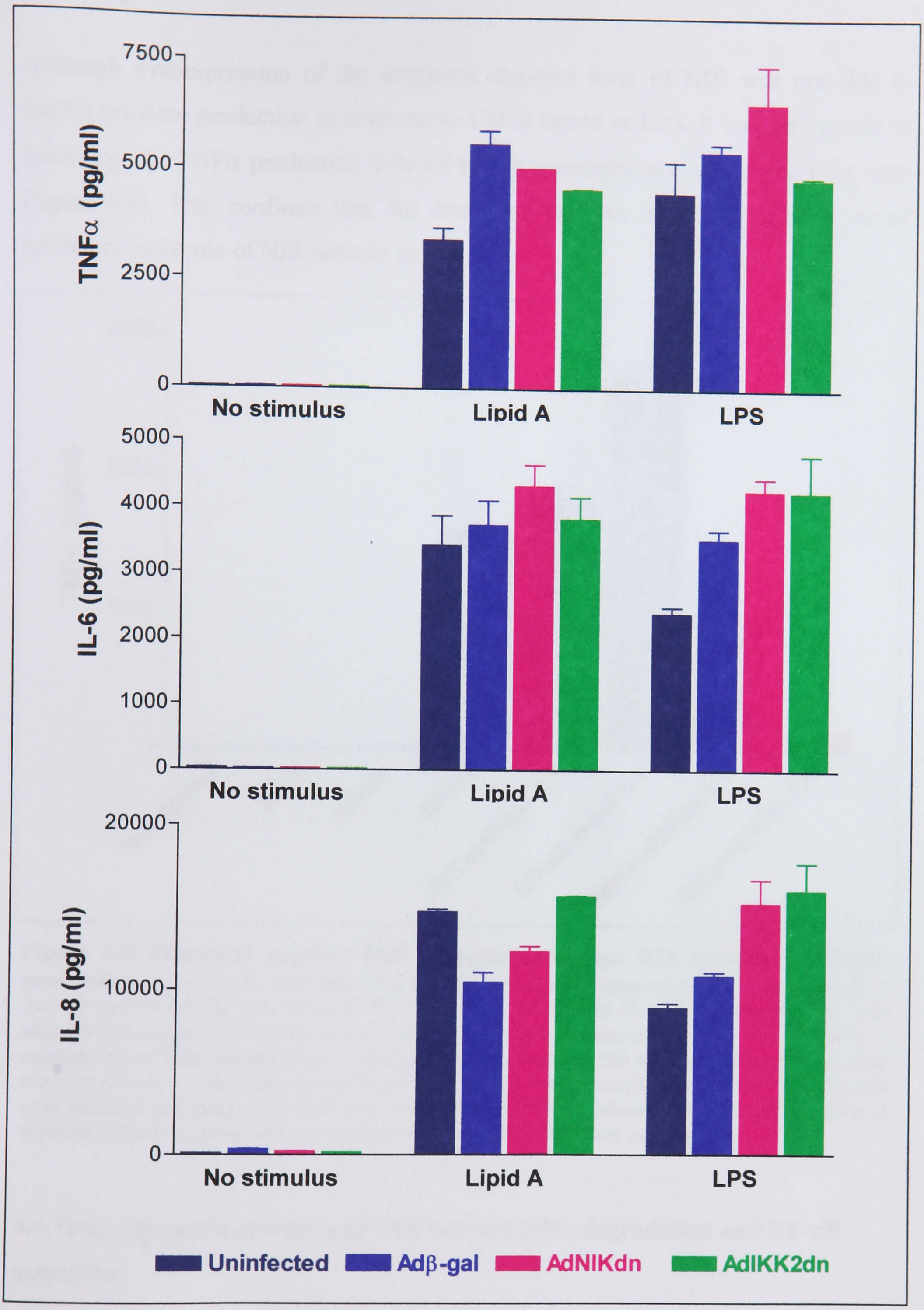


Figure 4.3: Overexpression of a dominant negative form of NIK into DC does not block LPS- or lipid A-induced cytokine production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected, or infected for 2h in serum-free medium with $E1^-E3^-$ adenoviruses expressing β-galactosidase (Adβ-gal), a dominant negative form of NIK (AdNIKdn) or a dominant negative form of IKK2 (IKK2dn). After 2 days of further culture, 1 μg/ml of Lipid A or 100 ng/ml of LPS were used to stimulate DC for 24h. Supernatants were collected were assayed by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is a representative of four independent experiments.

Although overexpression of the dominant negative form of NIK was not able to inhibit cytokine production in response to CD40 ligand or LPS, it was very potent in inhibiting DC TNFα production induced by the overexpression of its wild-type form (figure 4.4). This confirms that the dominant negative form of NIK acts as an inhibitory molecule of NIK activity in DC.

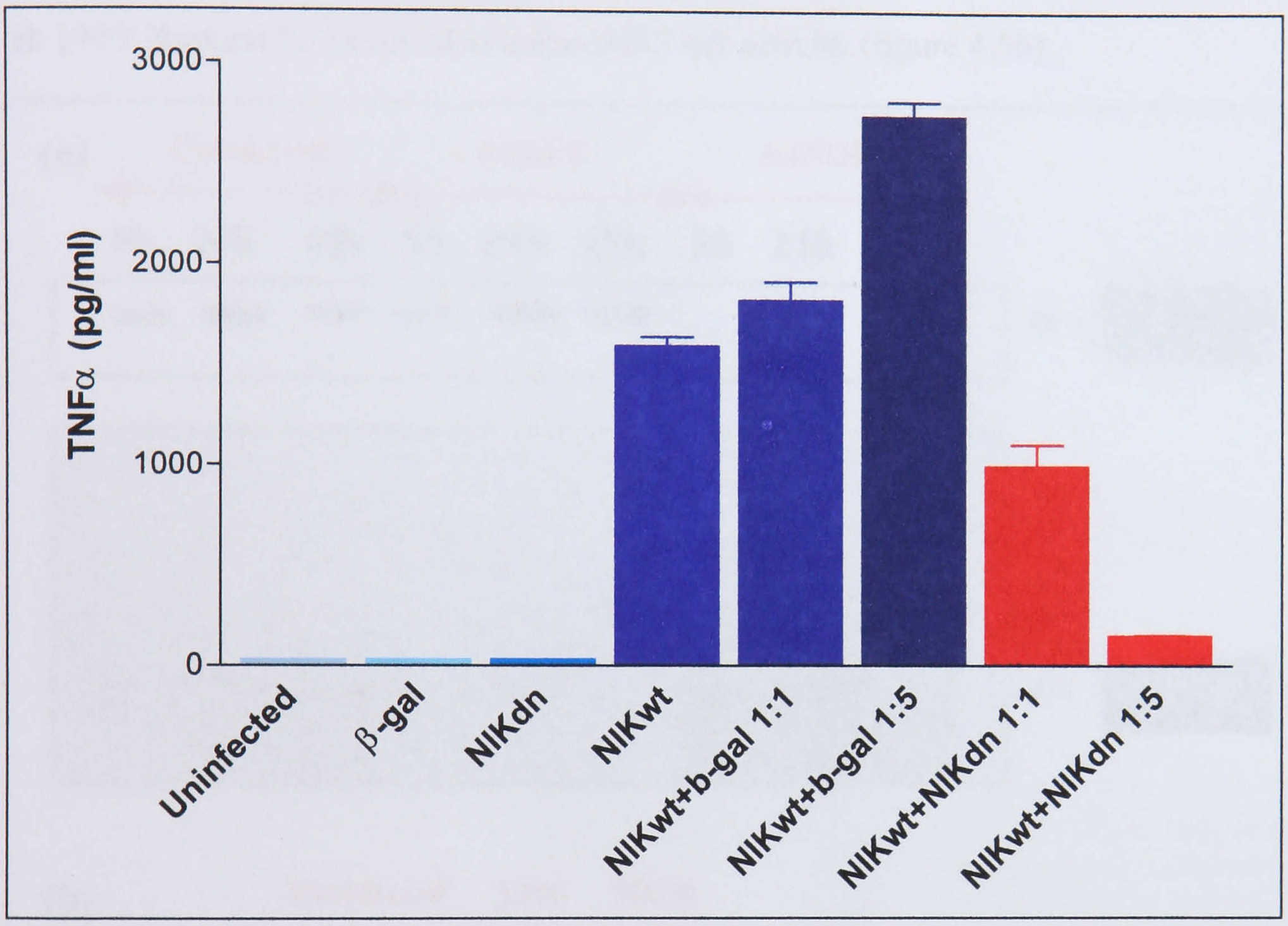


Figure 4.4: Dominant negative NIK abrogates wild-type NIK-induced DC TNFα production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal), wild-type or dominant negative forms of NIK, or combinations of these adenoviruses. A multiplicity of infection of 100 was used for each virus when used at a ratio of 1. Cells were then cultured for another 1 day in complete medium and supernatants were collected and assayed by ELISA for the presence of TNFα. Mean TNFα production (±SD) of triplicate cultures is shown and is a representative of two independent experiments.

4.4. Overexpression of wild-type NIK induces IκBα degradation and NF-κB activation

NIK has been shown to be a potent activator of IKK1 and IKK2 *in vitro* (Regnier C.H. et al. 1997; Woronicz J.D. et al. 1997). Although I found that inhibition of NIK activity has no effect in DC antigen-presenting function, I wanted to investigate whether induction of NIK activity could enhance it. In order to test my hypothesis, I

decided to overexpress the wild-type form of NF-κB-inducing kinase (NIK) into immature DC. Overexpression of wild-type NIK resulted in prolonged IκBα degradation that can be seen after 6h and 24h of adenoviral gene transfer into immature DC but not after 48h (figure 4.5). Strong NF-κB activation was also induced at 6h and continued to increase up to 48h (figure 4.5a). Similarly, LPS or monocyte-conditioned medium (MCM), two known activators of DC (Sallusto F. et al. 1995; Romani N. 1996), also induced NF-κB activity (figure 4.5b).

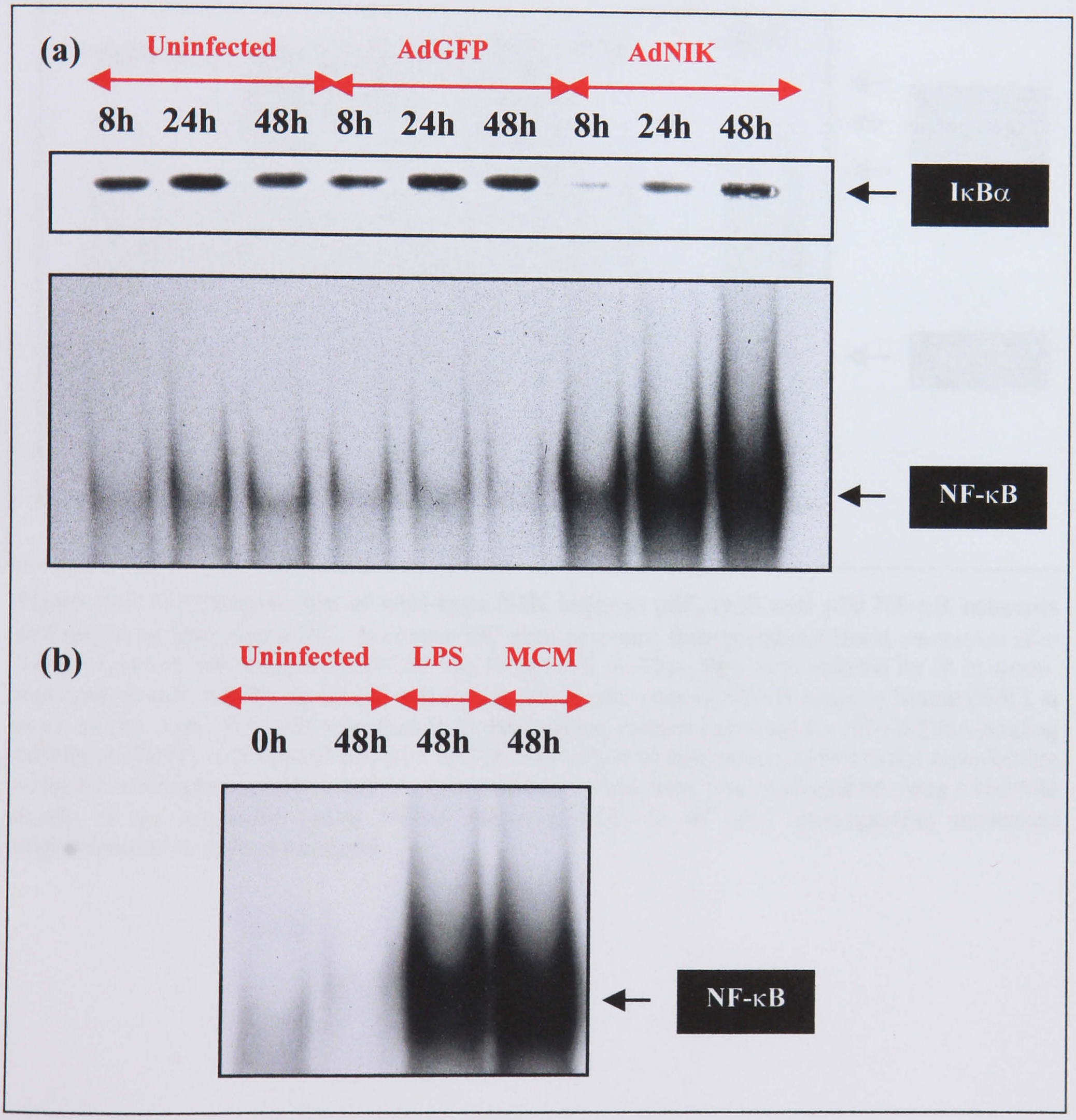


Figure 4.5: Overexpression of NIK into immature DC induces high levels of NF-κB that are comparable to these induced by LPS or MCM. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected for 2h in serum-free medium with AdGFP or AdNIK, two $E1^-$ $E3^-$ adenoviruses carrying the gene of green fluorescent protein (GFP) or NF-κB-inducing kinase (NIK), respectively. An m.o.i. of 100 was used. Some uninfected dendritic cells were also cultured in the presence of 100 ng/ml LPS or 50% v/v MCM. After 8, 24 or 48h, cells were lysed and their cytosolic extracts were examined for the presence of IκBα by western blotting (a), whereas their nuclear extracts for NF-κB DNA-binding activity by EMSA (a and b).

Electrophoretic mobility shift assays show that the NF-κB activity induced by NIK consists of the p65, relB and p50 subunits (figure 4.6) that have been proposed to be present in high levels in the nucleus of mature/activated DC (Granelli-Piperno A. et al. 1995).

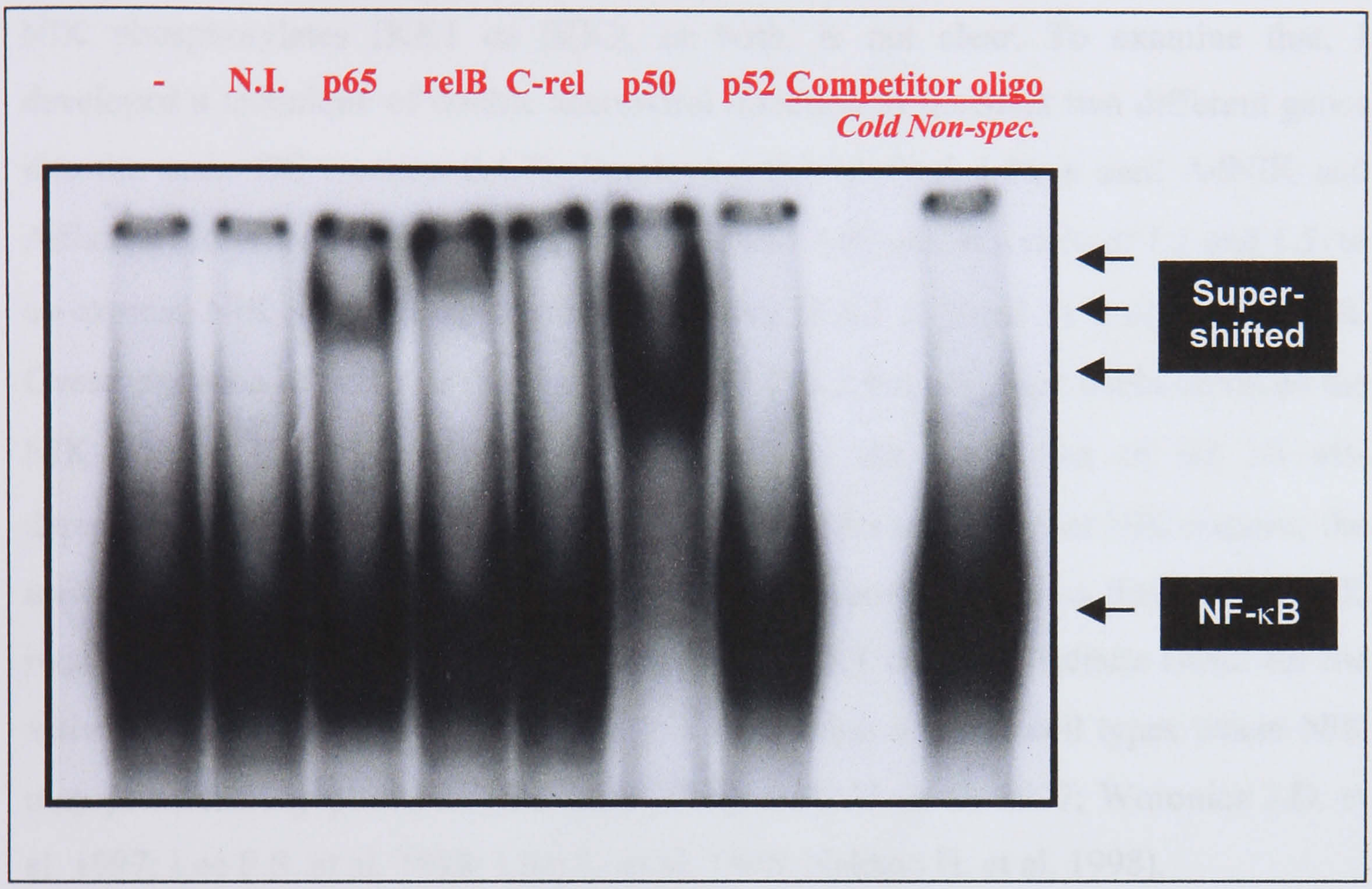


Figure 4.6: Overexpression of wild-type NIK induces p65, relB and p50 NF-κB subunits activation in immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were infected for 2h in serum-free medium with AdNIK, an $E1^-E3^-$ adenovirus carrying the gene of NF-κB-inducing kinase (NIK), at m.o.i. of 100. After 48h, cells were lysed and their nuclear extracts examined for NF-κB DNA-binding activity by EMSA. Addition of anti-p50, anti-p65 and anti-relB antibodies resulted in the supershifting of the NF-κB-oligonucleotide complex. The specificity of the band was confirmed by using a 100-fold excess of an unlabelled (cold) NF-κB oligonucleotide or an AP-1 (non-specific) unlabelled oligonucleotide to deplete the signal.

4.5. Wild-type NIK-induced NF-κB-activation requires IKK2 activation and IκBα degradation

NIK has been shown to act through the IKK complex to activate NF-κB, but whether NIK phosphorylates IKK1 or IKK2, or both, is not clear. To examine that, I developed a technique of double adenoviral infection to transfect two different genes into the same DC (section 2.4.7). Employing this method, I have used AdNIK and AdIκBα, AdNIK and AdIKK2dn, or AdNIK and Adβ-gal, at a ratio of 1:1 and 1:5, to co-express NIK with IκBα, dominant negative IKK2 or β-gal as a control protein. Overexpression of IκBα or dominant negative IKK2 but not β-gal could abrogate the NIK-induced NF-κB activation as measured by the production of the NF-κB-dependent cytokines TNFα and IL-6 (figure 4.7). This suggests that NIK requires the activation of of IKK2 upstream for its action. Moreover, it shows that in DC, NIK requires the phosphorylation of IKK2, and that IKK1 cannot substitute IKK2 for the activation of NF-κB. This is different to the situation in other cell types where NIK may preferentially phosphorylate IKK1 (Regnier C.H. et al. 1997; Woronicz J.D. et al. 1997; Lee F.S. et al. 1998; Ling L. et al. 1998; Nakano H. et al. 1998).

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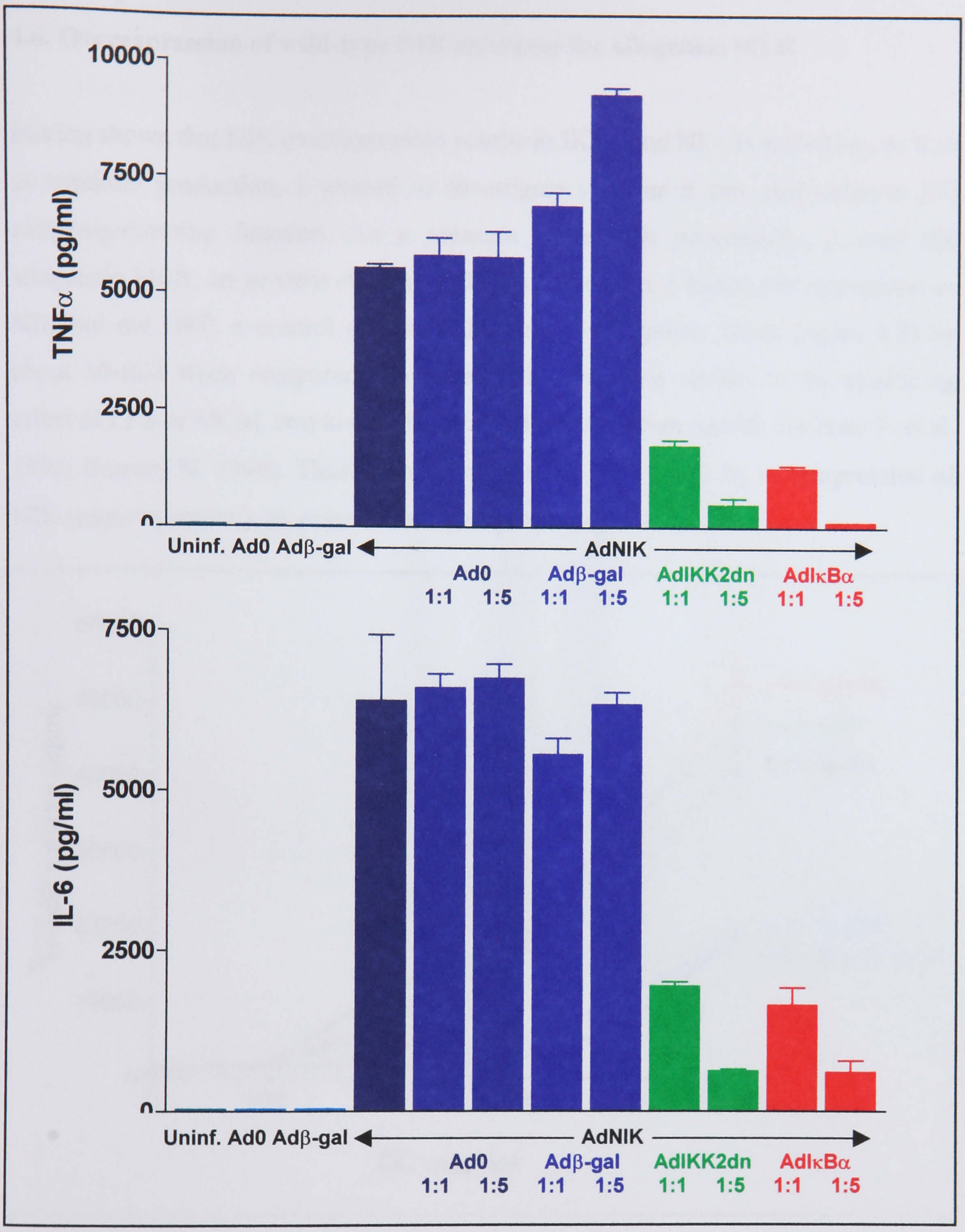


Figure 4.7: Expression of dominant negative IKK2 or IκBα inhibit NIK-induced DC TNFα and IL-6 production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were infected for 2h in serum-free medium with an adenovirus without insert (Ad0), adenoviruses expressing β-galactosidase (Adβ-gal), wild-type NIK (AdNIK), dominant negative IKK2 (AdIKK2dn), IκBα (AdIκBα), or combinations of them. A multiplicity of infection of 100 was used for each virus when used at a ratio of 1. Cells were then cultured for another 1 day in 5% FCS 1% penicillin/streptomycin and supernatants were collected and assayed by ELISA for the presence of TNFα. Mean cytokine production (±SD) of triplicate cultures is shown and is a representative of three independent experiments.

4.6. Overexpression of wild-type NIK enhances the allogeneic MLR

Having shown that NIK overexpression results in IKK2 and NF-κB activation, as well as cytokine production, I wanted to investigate whether it can also enhance DC antigen-presenting function. As a measure of antigen presentation, I used the allogeneic MLR, an *in vitro* system of allograft rejection. I found that expression of NIK but not GFP, a control protein, enhances the allogeneic MLR (figure 4.8) by about 10-fold when compared to control cells. This was similar to the enhancing effect of LPS or MCM, two known DC activation/maturation signals (Sallusto F. et al. 1995; Romani N. 1996). Thus, activation of IKK2 and NF-κB by overexpression of NIK seems to potently increase DC antigen-presenting ability.

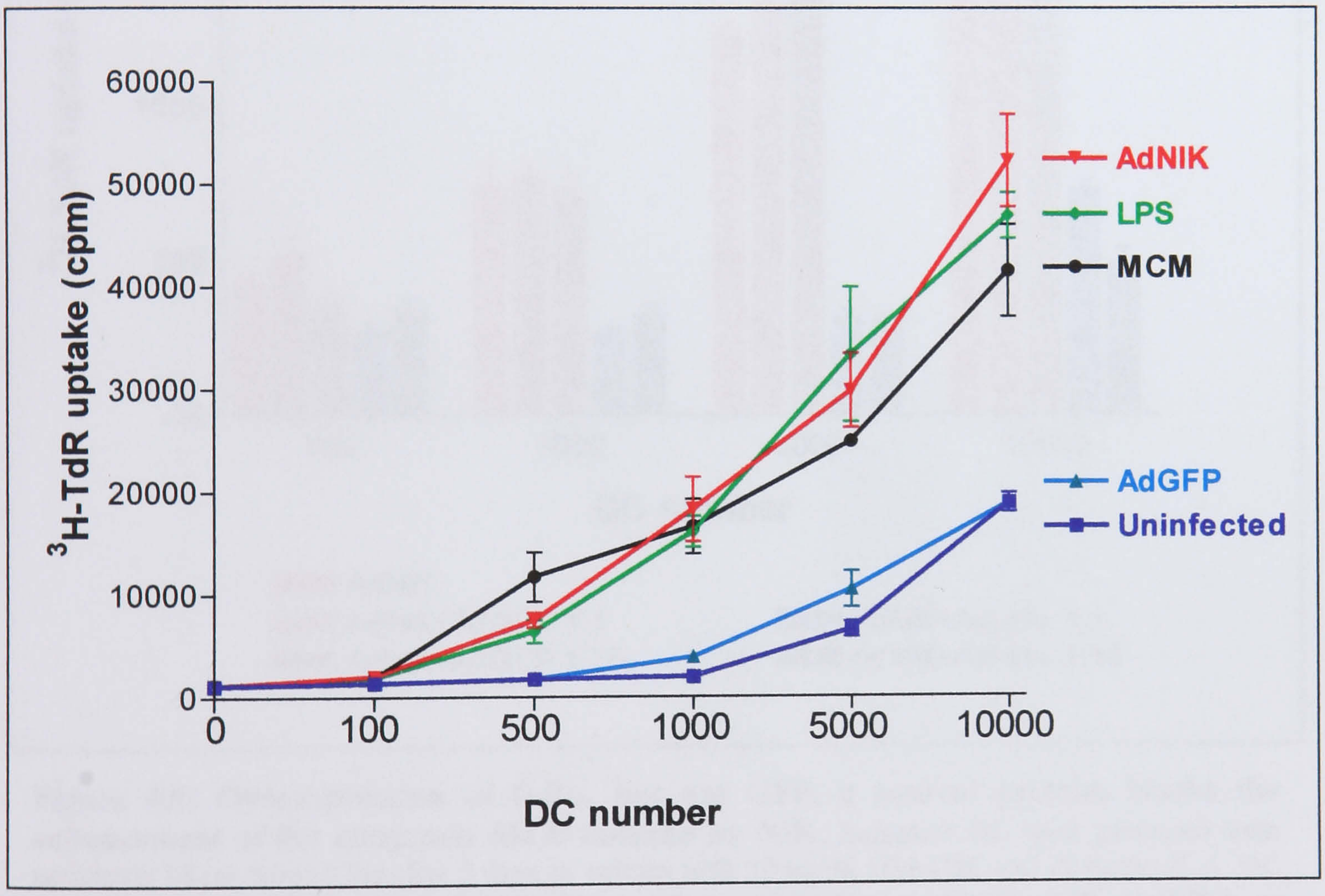


Figure 4.8: Overexpression of wild-type NIK enhances the allogeneic MLR. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. In (a), DC were infected for 2h in serum-free medium with an adenovirus expressing NIK (AdNIK) or a control adenovirus without insert (Ad0). Some cells were left uninfected and cultured in the presence of LPS or MCM. After 2 days, the DC were plated in graded doses with $1x10^5$ purified, allogeneic T cells in quadruplicate in 96-well flat-bottom microtitre plates. Proliferation was determined by pulsing the cultures overnight at day 5 with tritiated thymidine. Mean proliferation (±SEM) is shown on a logarithmic scale, and is a representative of four independent experiments.

Using the method of double adenoviral infection described in 4.3, I have used AdNIK and AdIκBα or Adβ-gal at a ratio of 1:1 and 1:10 to co-overexpress NIK with IκBα or a control protein, GFP. Overexpression of IκBα but not GFP could abrogate the NIK-mediated enhancement of DC antigen-presenting function in the allogeneic MLR even at the 1:1 ratio, thus confirming that wild-type NIK requires the activation of NF-κB for its adjuvant effect (figure 4.9).

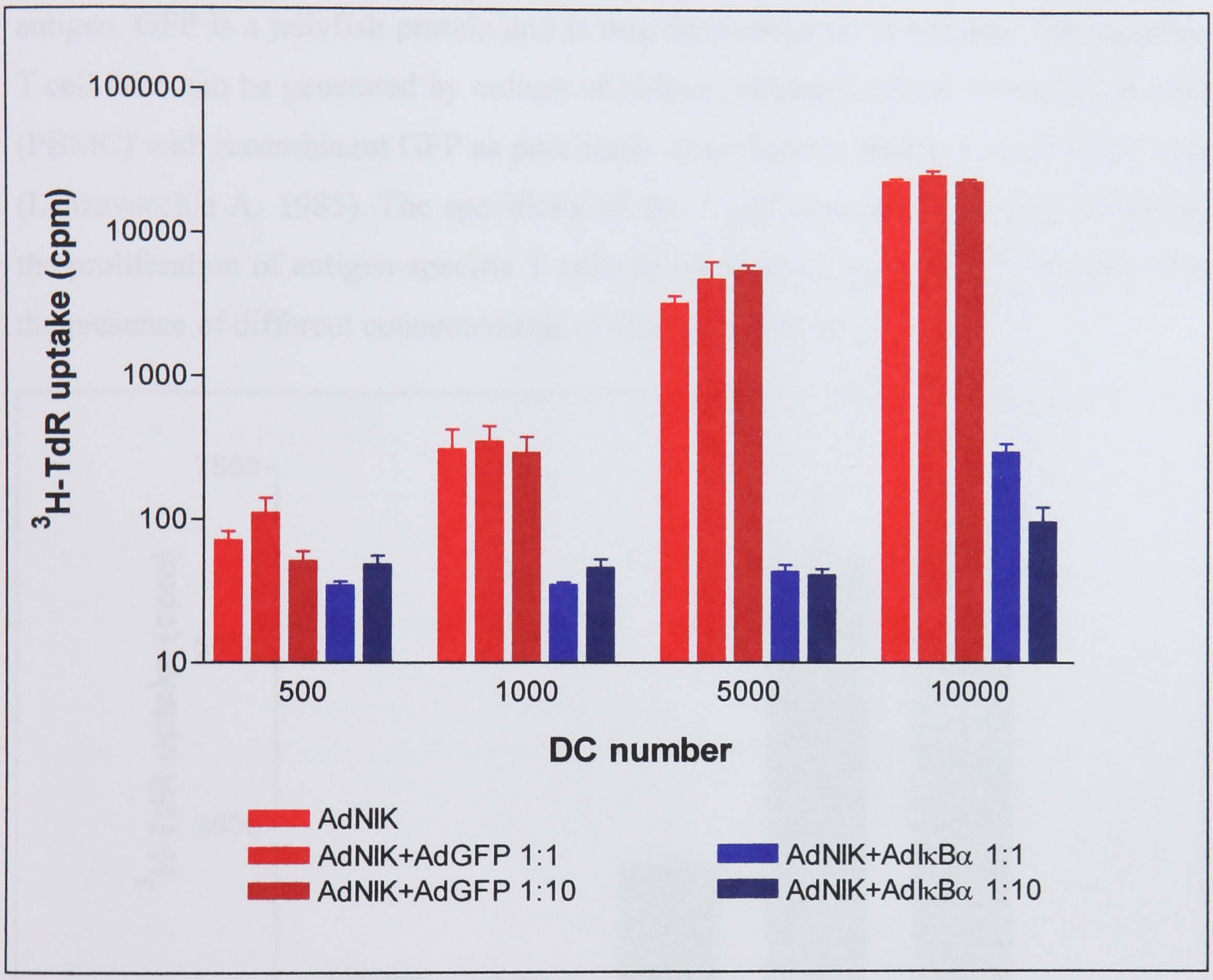


Figure 4.9: Overexpression of IκBα, but not GFP, a control protein, blocks the enhancement of the allogeneic MLR induced by NIK. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were infected for 2h in serum-free medium with an adenovirus expressing NIK (AdNIK), or combinations of AdNIK with AdGFP or AdIκBα, two adenoviruses expressing GFP, a control protein, or IκBα, respectively. After 2 days, the DC were plated in graded doses with $1x10^5$ purified, allogeneic T cells in 96-well flat-bottom microtitre plates. Proliferation was determined by pulsing the cultures overnight at day 5 with tritiated thymidine. Mean proliferation (±SEM) of quadruplicate cultures is shown on a logarithmic scale and is representative of three independent experiments.

4.7. Adenoviral gene transfer can be used to deliver antigen to dendritic cells

Having found that the wild-type form of NIK can enhance DC antigen-presenting function in the allogeneic MLR when delivered by adenoviral gene transfer, I wanted to examine whether adenoviral gene transfer could also be used to deliver antigen to dendritic cells as previously suggested (Brossart P. et al. 1997). Due to the incorporation of GFP into our adenoviral vectors, I decided to use GFP as a prototype antigen. GFP is a jellyfish protein and is thus immunogenic in humans. GFP-specific T cell lines can be generated by culture of human peripheral blood mononuclear cells (PBMC) with recombinant GFP as previously described for tetanus toxoid T cell lines (Lanzavecchia A. 1985). The specificity of the T cell lines was tested by measuring the proliferation of antigen-specific T cells in response to irradiated PBMC pulsed in the presence of different concentrations of GFP (figure 4.10).

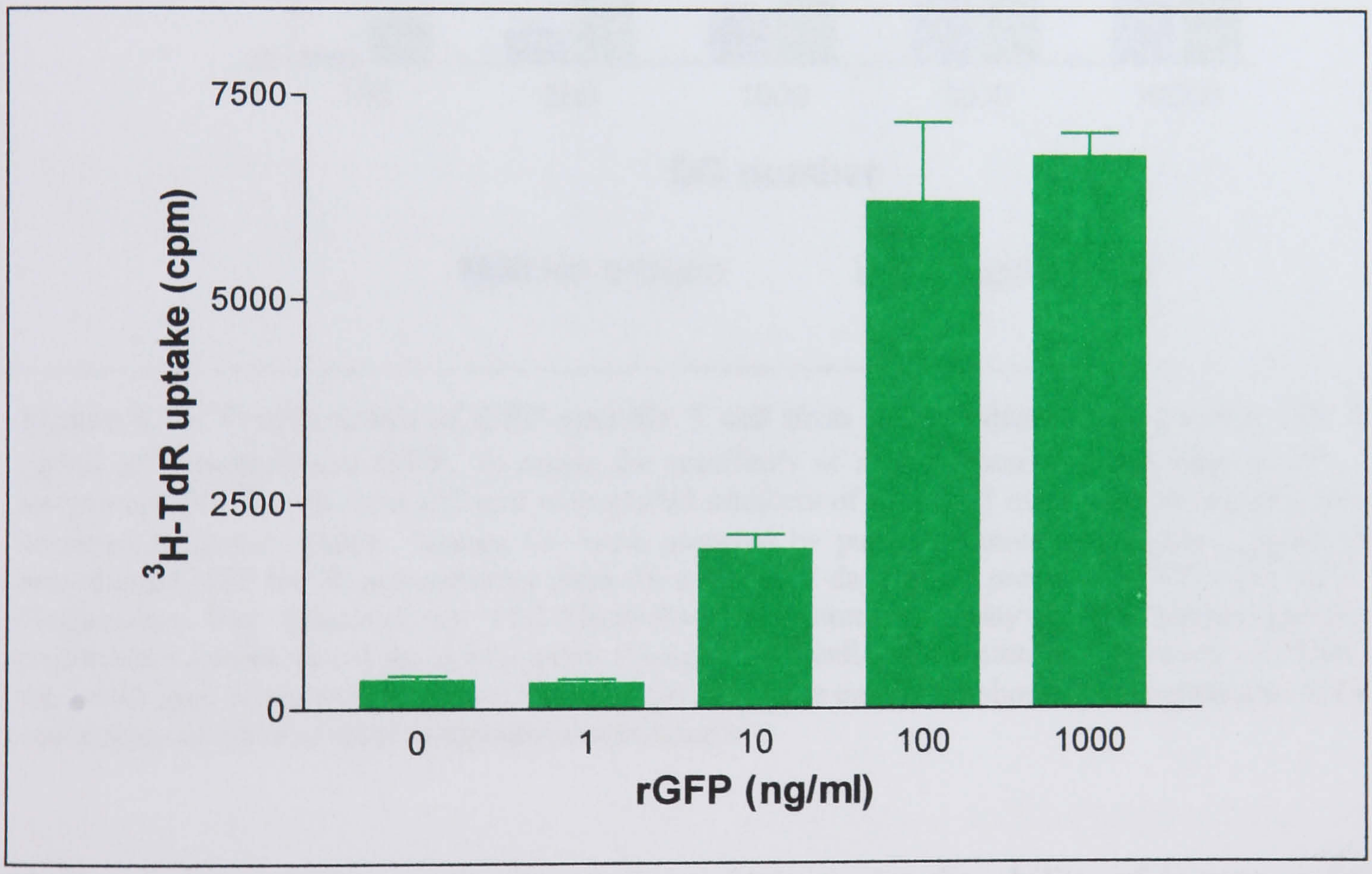


Figure 4.10: Proliferation of GFP-specific T cell lines in response to irradiated PBMC and increasing doses of recombinant GFP. To assess the specificity of antigen-specific T cell lines, $1x10^5$ of antigen-specific T cells were cultured with $1x10^5$ autologous irradiated PBMC and various concentrations of antigen in 96-well flat-bottomed microtiter plates. After 3 days, cells were pulsed with [^{3}H]Thymidine overnight and harvested the following day. Background T cell proliferation in the absence of PBMC was <100 cpm. Mean proliferation (±SEM) of quadruplicate cultures is shown on a logarithmic scale, and is representative of three independent experiments.

Similarly, the specificity of the T cell lines was confirmed by measuring T cell proliferation in response to immature DC pulsed with recombinant GFP and then activated further by LPS for 2 days (figure 4.11).

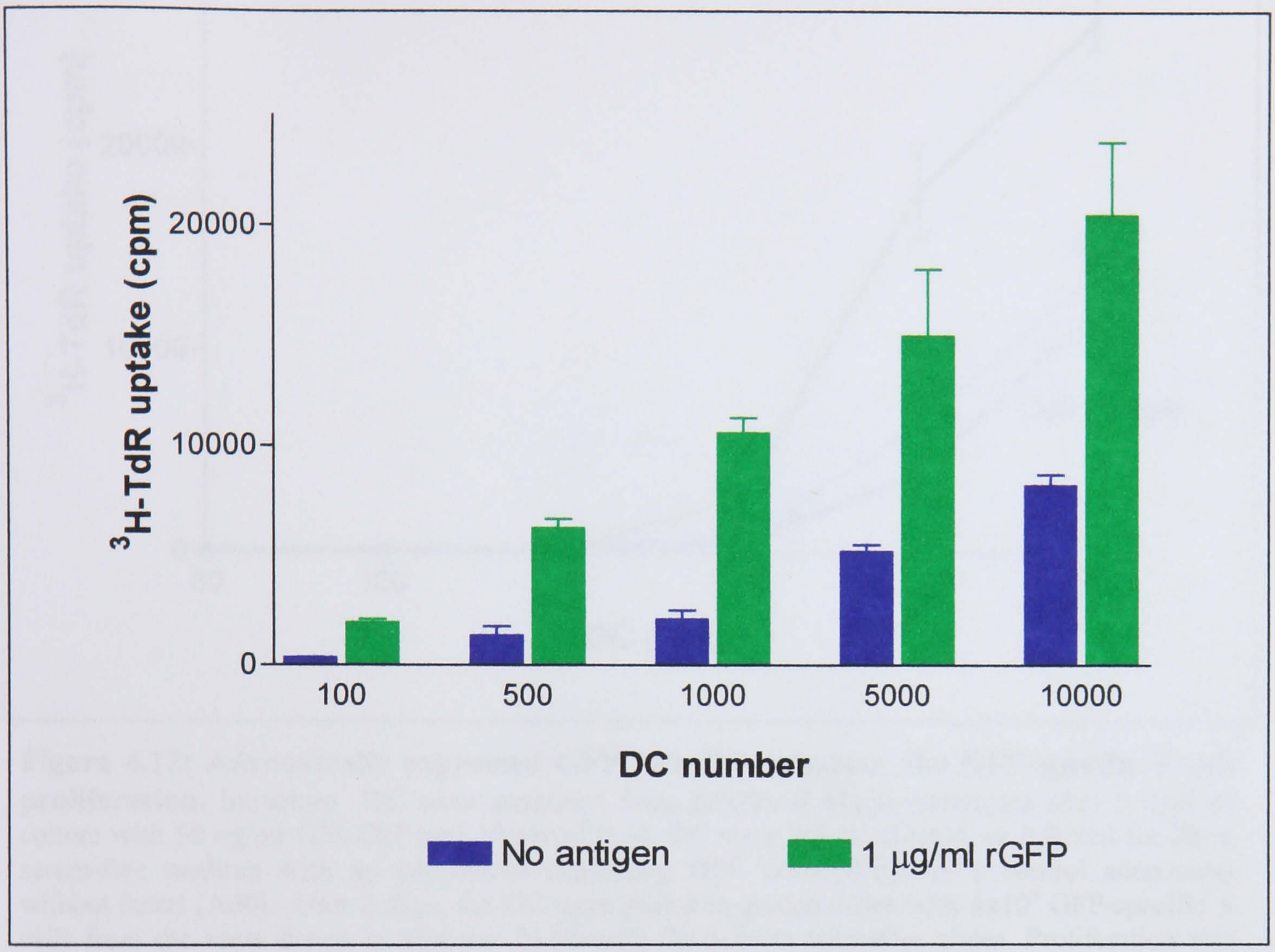


Figure 4.11: Proliferation of GFP-specific T cell lines in response to DC pulsed with 2 μg/ml of recombinant GFP. To assess the specificity of antigen-specific T cell lines, $4x10^4$ of antigen-specific T cells were cultured with graded numbers of irradiated mature DC in 96-well flat-bottomed microtiter plates. Mature DC were prepared by pulsing immature DC with 2 μg/ml of recombinant GFP for 2h and culturing them for a further 2 days in the presence of 100 ng/ml LPS. Proliferation was measured by [^{3}H]-Thymidine incorporation at day 3. All antigen-specific proliferation assays were done in triplicates. Background T cell proliferation in the absence of PBMC was <100 cpm. Mean proliferation (±SEM) of quadruplicate cultures is shown on a logarithmic scale and is representative of three independent experiments.

Having defined GFP-specific T cell lines, I examined the ability of immature DC infected with an adenovirus overexpressing GFP (AdGFP) or an adenovirus without insert as a control (Ad0) to stimulate proliferation of antigen-specific T cells. I found that only DC modified with the GFP gene, but not control uninfected or Ad0-infected DC, induce significant T cell proliferation (figure 4.12). At high DC numbers, some background T cell proliferation could also be induced by control cells too, as reported previously (Inaba K. and Steinman R. 1989).

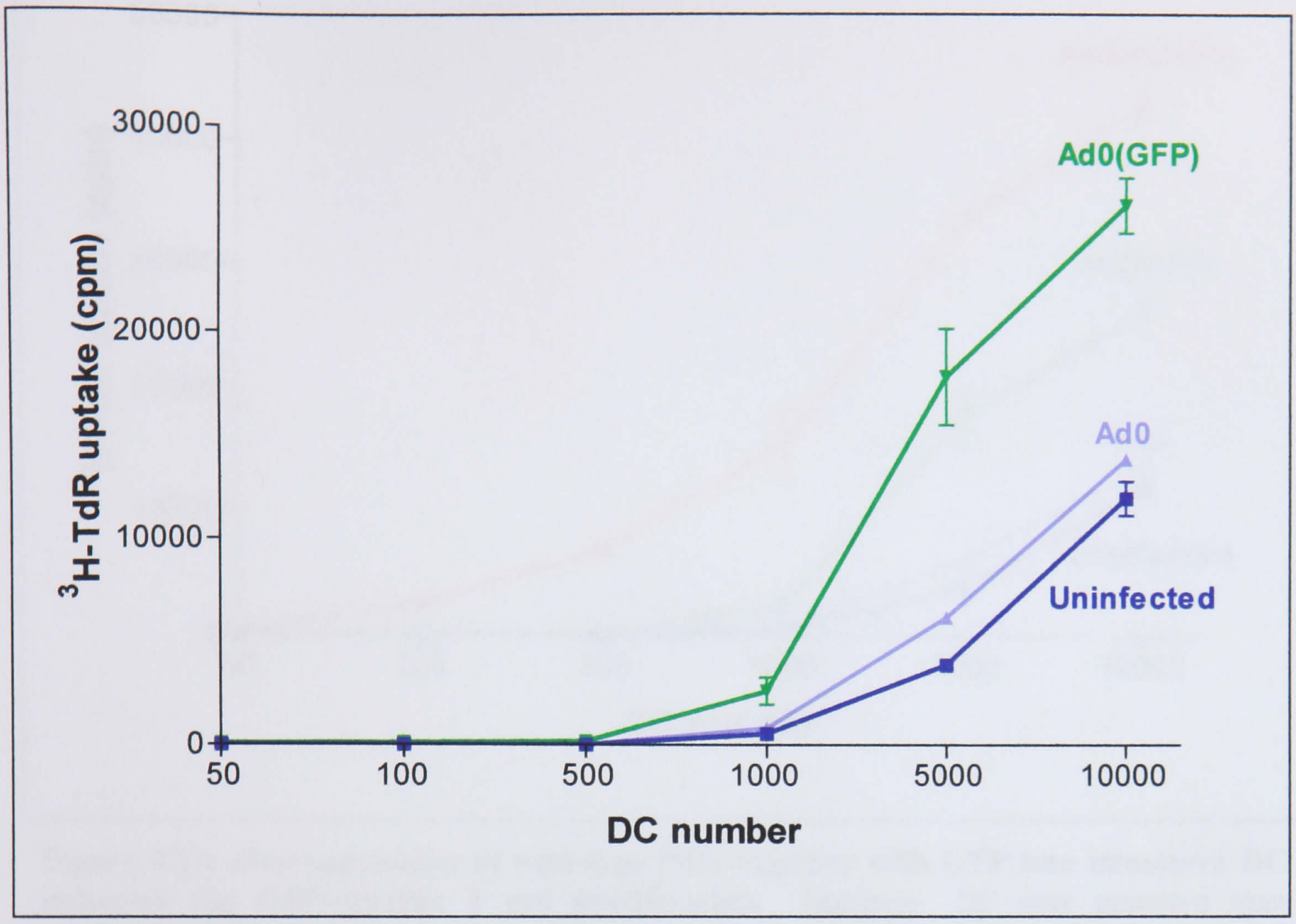


Figure 4.12: Adenovirally expressed GFP into DC enhances the GFP-specific T cell proliferation. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected, or infected for 2h in serum-free medium with an adenovirus expressing GFP [Ad0(GFP)], or a control adenovirus without insert [Ad0]. After 2 days, the DC were plated in graded doses with $4x10^5$ GFP-specific T cells from the same donor in triplicate in 96-well flat-bottom microtitre plates. Proliferation was determined by pulsing the cultures overnight at day 2 and with thymidine. Background T cell proliferation in the absence of PBMC was <100 cpm. Mean proliferation (±SEM) of quadruplicate cultures is shown and is representative of three independent experiments.

4.8. Administration of antigen together with wild-type NIK into dendritic cells enhances antigen-specific T cell proliferation

Having shown that adenoviral gene transfer can be used to deliver antigen to dendritic cells, the next question I wanted to answer was whether incorporation of both wild-type NIK and antigen in the same vector could enhance antigen-specific T cell proliferation by using NIK to activate DC. Indeed, NIK could very potently enhance T cell proliferation several fold (figure 4.13). These findings suggest that incorporation of the NIK gene into DNA vectors may be a novel potent adjuvant for recombinant DNA vaccines.

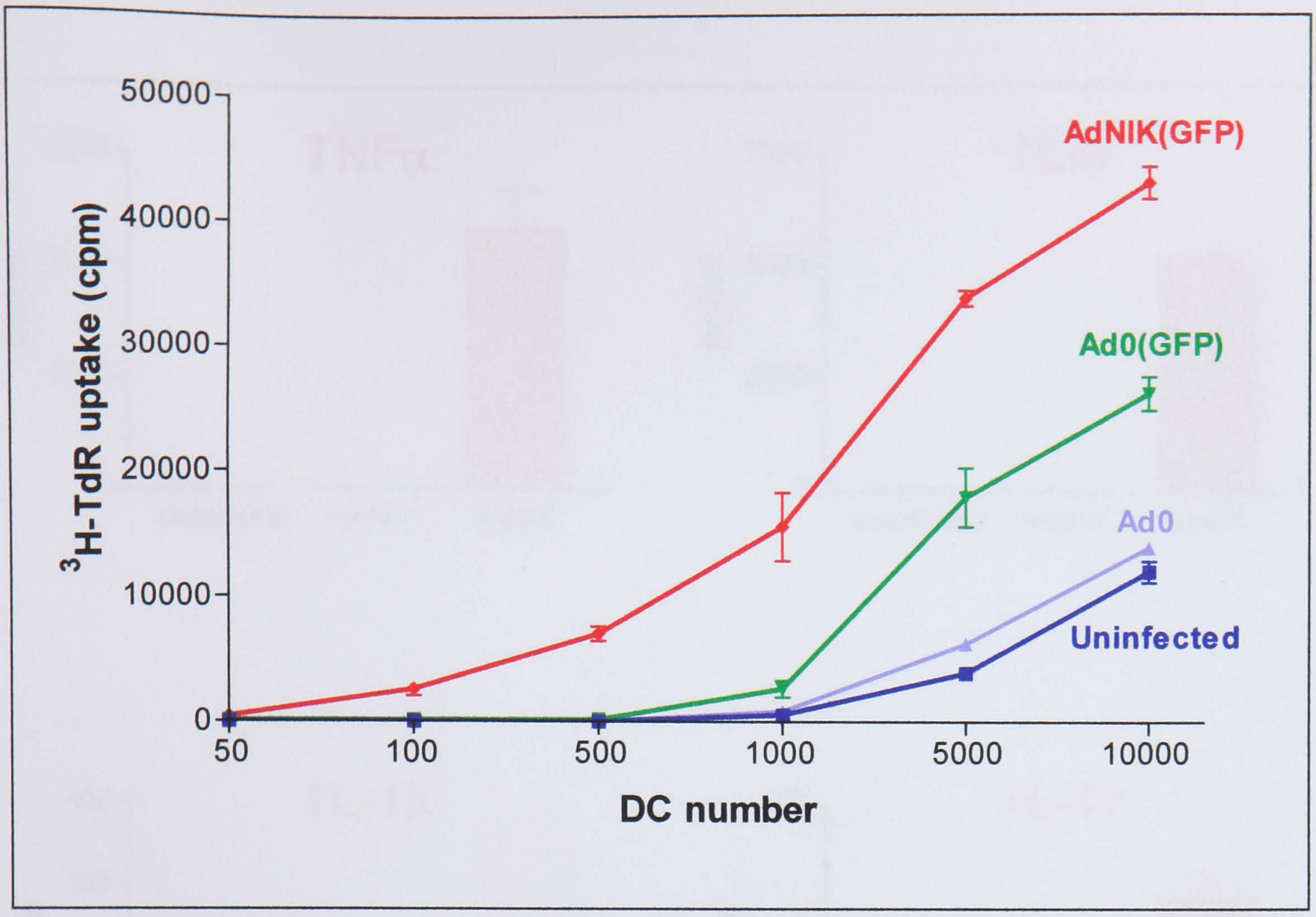


Figure 4.13: Overexpression of wild-type NIK together with GFP into immature DC enhances the GFP-specific T cell proliferation. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC left uninfected, or were infected for 2h in serum-free medium with an adenovirus expressing GFP [Ad0(GFP)], NIK together with GFP [AdNIK(GFP)], or a control adenovirus without insert [Ad0]. After 2 days, the DC were plated in graded doses with $4x10^5$ GFP-specific T cells from the same donor in triplicate in 96-well flat-bottom microtitre plates. Proliferation was determined by pulsing the cultures overnight at day 2 and with thymidine. Background T cell proliferation in the absence of PBMC was <100 cpm. A representative experiment of five from two different donors is shown.

4.9. Overexpression of wild-type NIK induces cytokine and chemokine production

To understand how NF-κB activation, induced by NIK overexpression, enhances DC antigen-presenting function, I investigated the expression of cytokines and chemokines. I found that NIK overexpression induces the release of high levels of cytokines such as TNFα, IL-6, IL-12, IL-18 but not IL-1β (figure 4.14), and chemokines such as RANTES, MIP-1α, MCP-1, MCP-3 and IL-8 (figure 4.15). The coordinated production of cytokines and chemokines may partly account for the adjuvant effect of NIK. The reason of the differential induction of IL-18 but not IL-1β after NIK overexpression is not clear, but could be due to a defect of immature monocyte-derived DC to produce IL-1β.

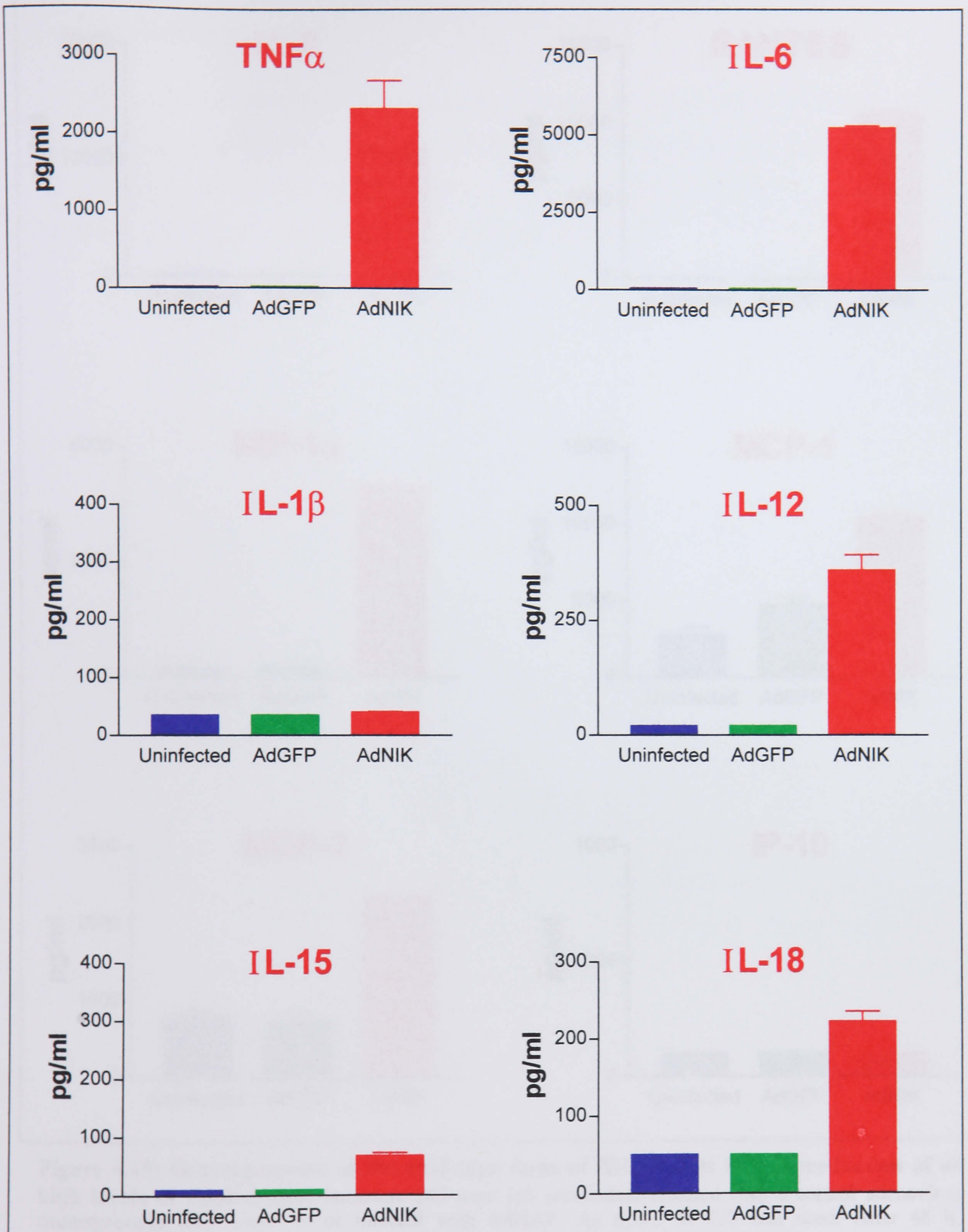


Figure 4.14: Overexpression of the wild-type form of NIK results in the production of of high levels of cytokines. Immature DC were left uninfected, infected with a control adenovirus overexpressing GFP (AdGFP) or infected with AdNAP. An m.o.i. of 100 was used. After 48 h, supernatants were collected and assayed by ELISA for TNFα, IL-1β, IL-6, IL-12, IL-15 and IL-18. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

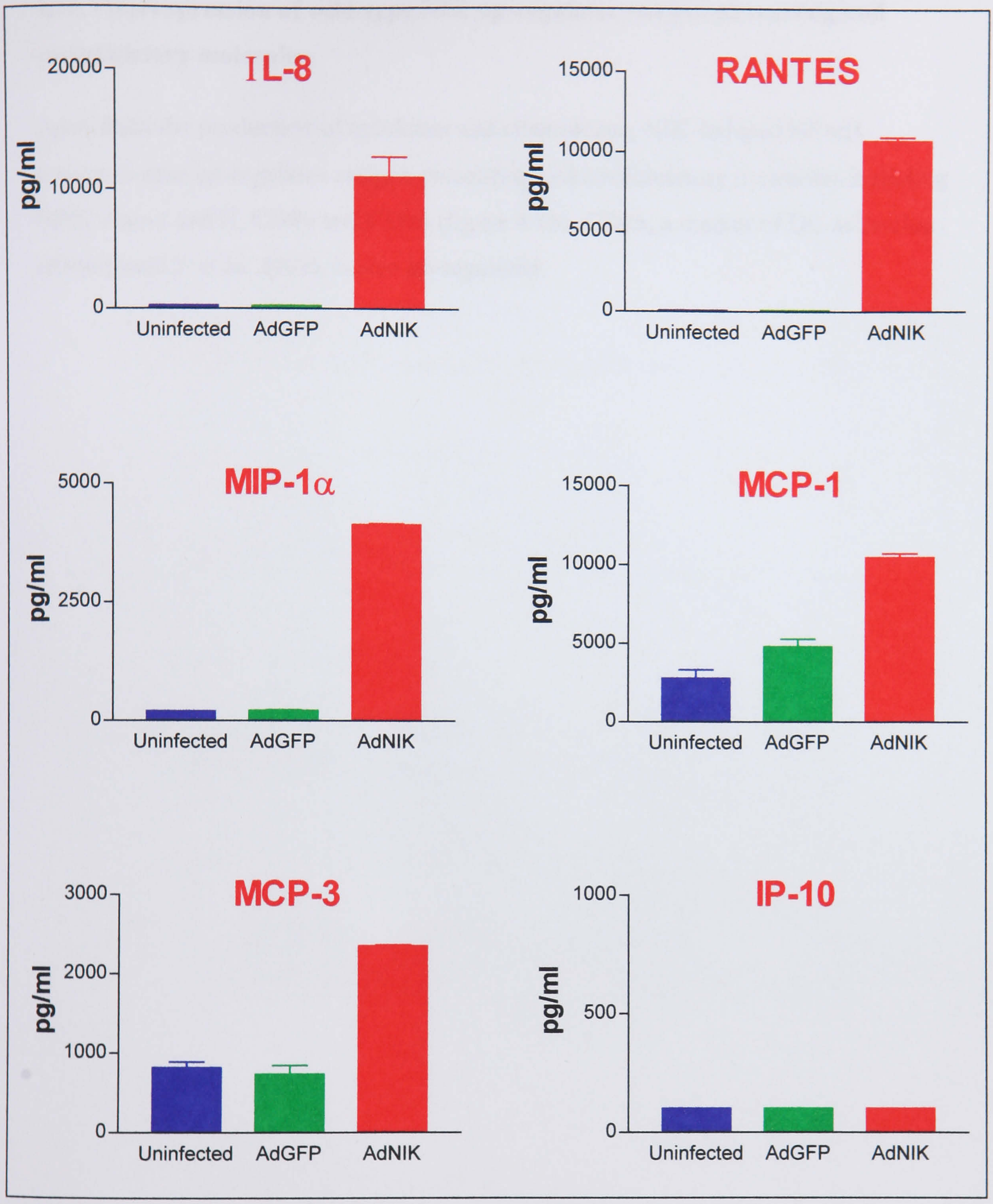


Figure 4.15: Overexpression of the wild-type form of NIK results in the production of of high levels of chemokines. Immature DC were left uninfected, infected with a control adenovirus overexpressing GFP (AdGFP) or infected with AdNAP. An m.o.i. of 100 was used. After 48 h, supernatants were collected and assayed by ELISA for IL-8, RANTES, MIP-1α, MCP-1, MCP-3 and IP-10. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

4.10. Overexpression of wild-type NIK up-regulates antigen-presenting and costimulatory molecules

Apart from the production of cytokines and chemokines, NIK-induced NF-κB activation also up-regulates antigen-presenting and costimulatory molecules including MHC class I and II, CD80 and CD86 (figure 4.16). CD25, a marker of DC activation (Banchereau J. et al. 2000), is also up-regulated.

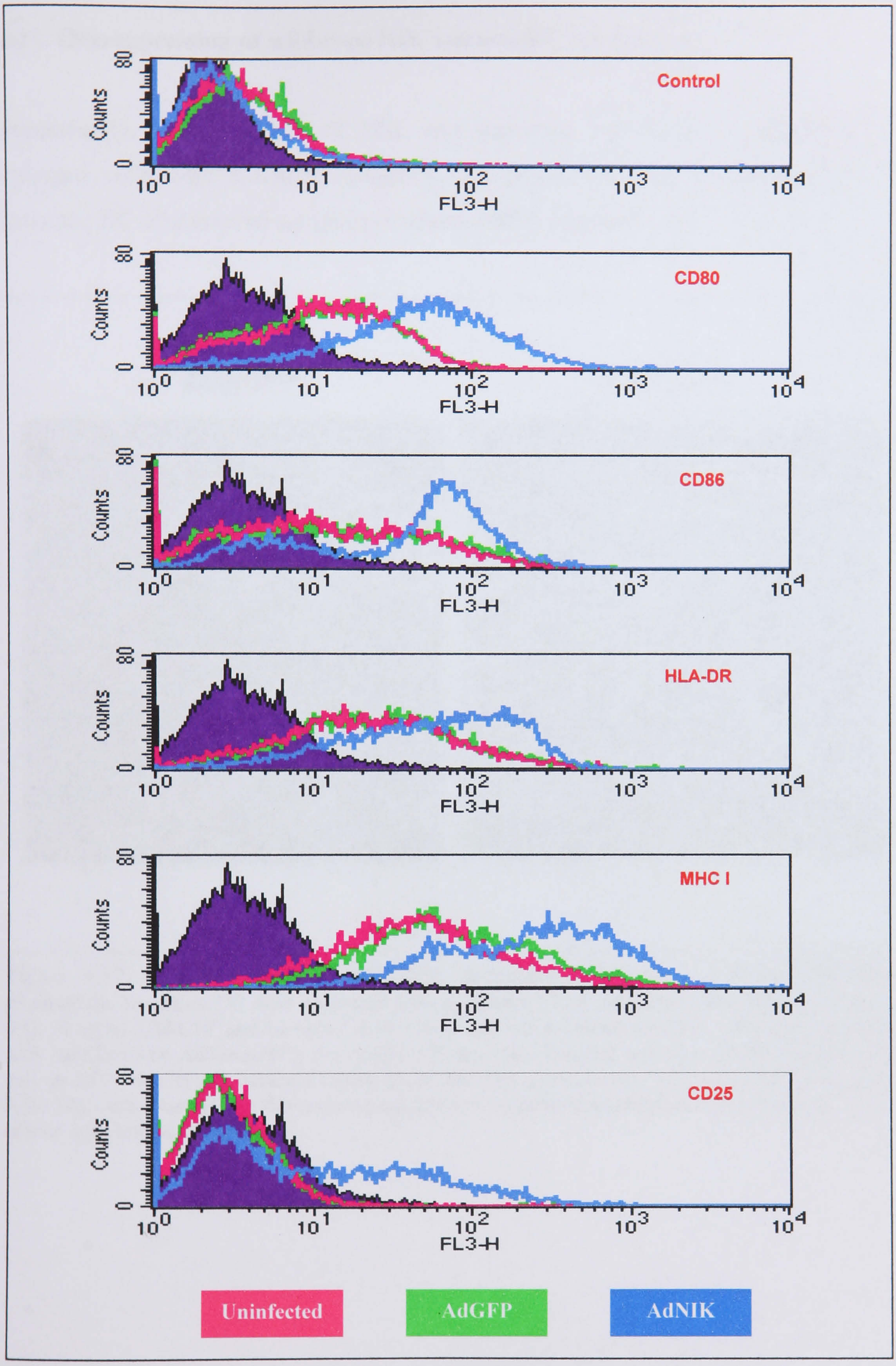


Figure 4.16: Overexpression of NIK up-regulates DC antigen-presenting and costimulatory molecules. Briefly, immature DC were infected with AdGFP or AdNIK, or left uninfected. After 2 days, they were stained for cell surface expression of CD80, CD86, HLA-DR, MHC class I and CD25, or a control antibody. Fluorescence was measured by FACS. A representative of three independent experiments is shown.

4.11. Overexpression of wild-type NIK induces DC morphology

Interestingly, overexpression of NIK into immature DC induces a phenotype of enlarged cells with extended dendrites and protrusions that is characteristic of activated DC of increased antigen-presenting ability (figure 4.17).

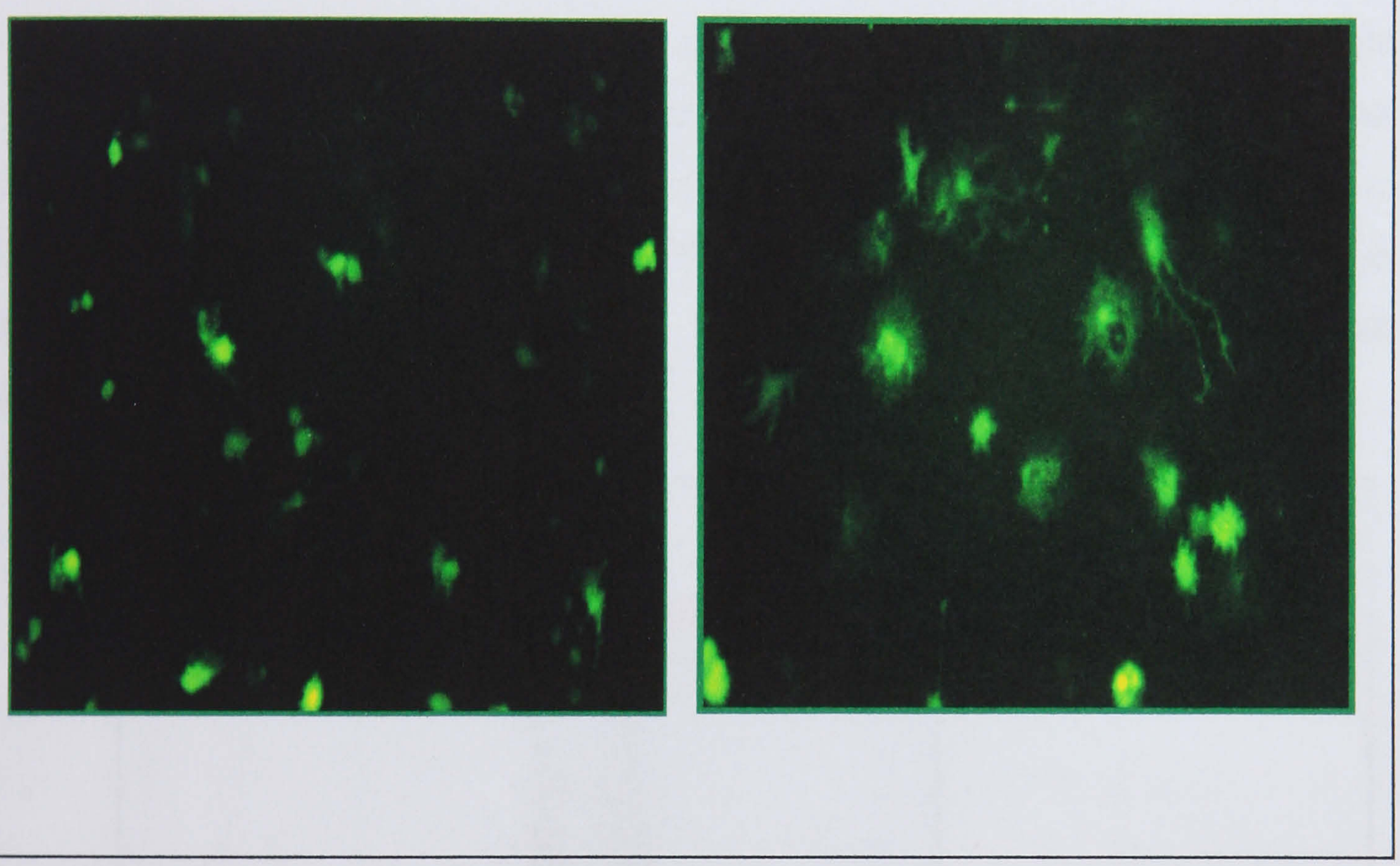


Figure 4.17: Overexpression of NIK into immature DC induces a mature DC-like phenotype. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were infected for 2h in serum-free medium with Ad0(GFP) or AdNIK(GFP), two $E1^-E3^-$ adenoviruses carrying the gene of GFP [Ad0(GFP)] only or GFP with NF-κB-inducing kinase [AdNIK(GFP)], respectively. An m.o.i. of 100 was used. After 24h, cells examined by fluorescence microscopy (original magnification 20X). A representative of four experiments is shown.

4.12. Other wild-type NF-κB-activating molecules fail to activate DC

Apart from NIK many other MAP3K-like molecules have been proposed to activate NF-κB. To test whether other NF-κB-activating proteins could also induce DC activation, I used adenoviral gene transfer to overexpress wild-type forms of IKK2, NIK, MEKK1 and MyD88. However, I found that overexpression of NIK only, but not the other proteins, was able to induce NF-κB activation and cytokine production (figure 4.18). This may be explained by the fact that wild-type forms of these kinases are not constitutively active and require an activation signal from upsteam to become activated. In many cell types such as HSF, this signal is present and simple overexpression of IKK2 or MyD88, for example, results in NF-κB activation (chapters 3 and 5). In DC, however, this signal seems to be absent. With respect to NIK, there may be a specific signal that results in its activation, or simple overexpression of NIK may result in its autophosphorylation and consequent activation.

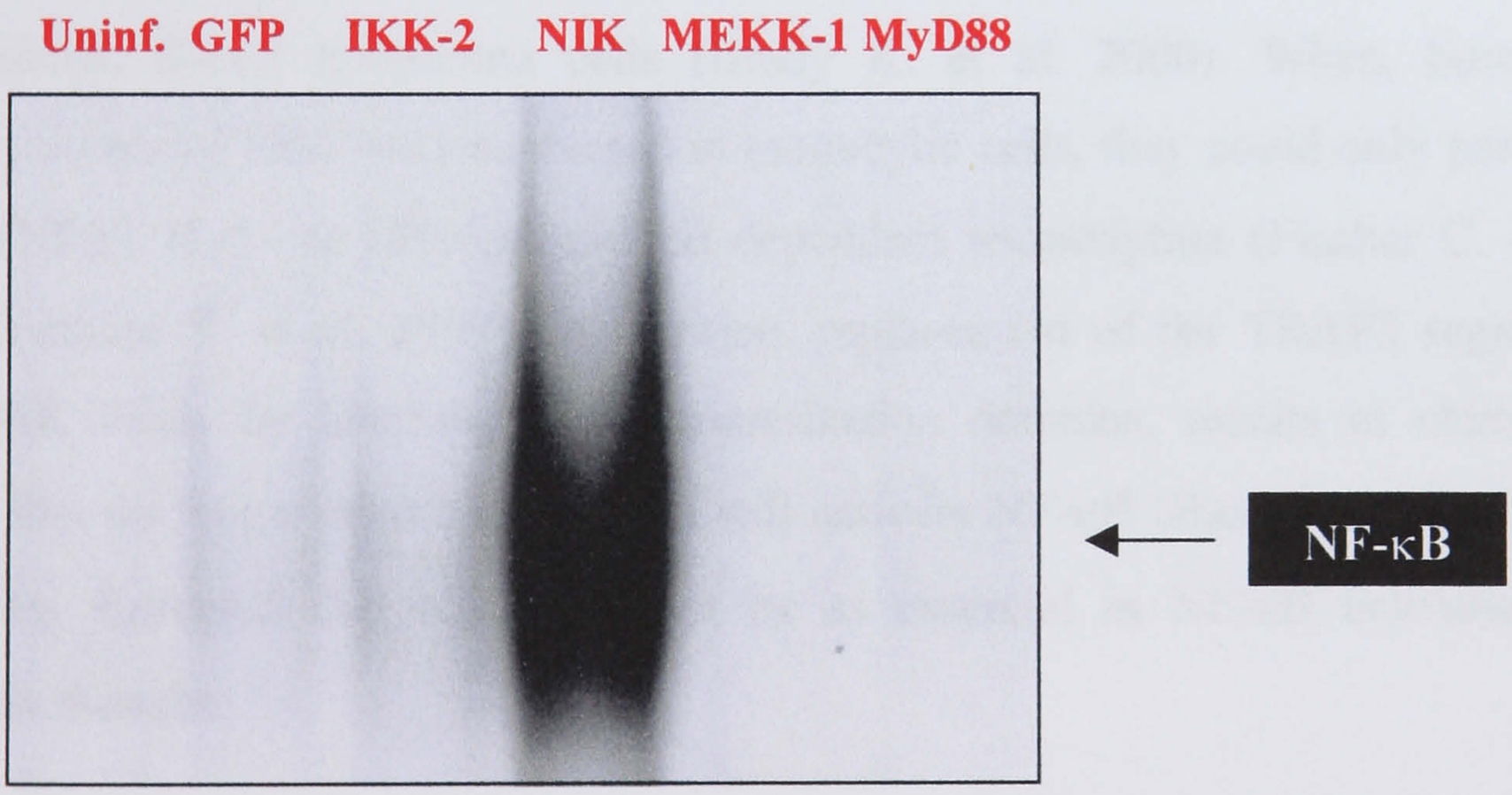


Figure 4.18: Overexpression of NIK only but not wild-type forms of IKK-2, MEKK-1 and MyD88 induces NF-κB activation in immature dendritic cells. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected for 2h in serum-free medium with AdGFP, AdIKK2, AdNIK, AdMEKK1 and AdMyD88, $E1^{-}E3^{-}$ adenoviruses carrying the gene of GFP or wild-type forms of IKK2, NIK, MEKK1 and MyD88, respectively. An m.o.i. of 100 was used. After 24h, cells were lysed and their nuclear extracts examined for NF-κB DNA-binding activity by EMSA.

4.13. Discussion

NF-κB-inducing kinase (NIK) is the most potent activator of the IKK complex known to date. This serine/threonine kinase was originally identified by means of its association with TNF receptor associated factor 2 (TRAF2) and was found to potently activate NF-κB when overexpressed (Malinin N.L. et al. 1997). Expression of kinase-defective forms of NIK were shown to block TNFα-induced NF-κB activation in various cell types including 293 human embryonic kidney cells (Malinin N.L. et al. 1997; Song H.Y. et al. 1997; Cohen L. et al. 1998), human dermal microvessel endothelial cells (Zhang F.X. et al. 1999) and NCI-H292 human alveolar epithelial carcinoma cells (Chen C.C. et al. 2000)

Similarly, kinase-defective forms of NIK were found to inhibit IL-1-induced NF-κB activation in 293 human embryonic kidney cells (Malinin N.L. et al. 1997; Li X. et al. 2000), human dermal microvessel endothelial cells (Zhang F.X. et al. 1999) and A549 human epithelial carcinoma alveolar cells, as well as CD40L-induced NF-κB activation in 293 human embryonic kidney cells (Tsukamoto N. et al. 1999) and BJAB human B-cell lymphoma cells (Brady K. et al. 2000). When, however, inhibitory forms of NIK were expressed in monocytic cells, they could only partially inhibit TNFα-, IL-1- or LPS-induced κB-dependent transcription (Fischer C. et al. 1999; Nasuhara Y. et al. 1999). In addition, replacement of the TRAF2 region to which NIK binds by heterologous oligomerization domains, results in chimaeric proteins that no longer bind NIK but can still activate NF-κB (Baud V. et al. 1999). These data indicated that NIK may not be as essential in NF-κB activation as previously thought.

Further support for that came from the analysis of the alymphoplasia (*aly*) mutation of mice. Affected mice fail to develop lymphoid organs and exhibit a phenotype similar to that of mice that are doubly deficient in TNFα and lymphotoxin α or mice that are deficient in the lymphotoxin β receptor (Shinkura R. et al. 1999; Eugster H.P. et al. 1996). Positional cloning identified a point mutation in the *NIK* locus as the mutant allele that occurs in the TRAF-interacting region of NIK and affects its activation but not its catalytic ability or its ability to activate NF-κB after overexpression (Shinkura

R. et al. 1999). Despite the NIK mutation though, mice exhibited similar TNF-mediated endotoxin shock after generalized LPS administration (Shinkura R. et al. 1999), and mouse embryonic fibroblasts could still up-regulate vascular cell adhesion molecule 1 (VCAM-1) expression after stimulation with TNFα (Matsumoto M. et al. 1999). These observations suggested that NIK was not a critical element in the LPS or TNFα pathway to NF-κB activation.

In this chapter, I investigated the role of NIK in DC activation and antigen-presenting ability. As a tool, I used adenoviral gene transfer to efficiently deliver wild-type and dominant negative forms of NIK into immature DC, circumventing in that way one of the major obstacles of studying signalling pathways in this cell type (Zhong L. et al. 1999). Interestingly, when dominant negative NIK was expressed into immature DC, it did not block the allogeneic MLR. At the same time, it did not inhibit the CD40L- or LPS-induced TNFα, IL-6 or IL-8 production. These findings suggested that NIK does not play a major role in NF-κB activation in human immature DC, and were soon confirmed by another study in the *aly/aly* mice. That study demonstrated that the *aly/aly* mutation has no effect on CD40L- or TNFα-induced IκBα phosphorylation, CD40L-induced IL-12 production or antigen presentation when dendritic cells were used (Garceau N. et al. 2000). When, however, B cells were examined, the *aly/aly* mutation could prevent CD40L- or LPS-induced B cell proliferation and antibody production, as well as up-regulation of the cell-surface markers CD80, CD86, MHC class II and CD23. At the same time, it could block the phosphorylation of IκBα in response to CD40L and LPS but not PMA and ionomycin (Garceau N. et al. 2000), suggesting that the role of NIK as a major mediator of NF-κB activation may be lineage-restricted.

Next, I examined the effects of wild-type NIK overexpression in immature DC. I found that wild-type NIK induced IκBα degradation, NF-κB activation and NF-κB-dependent cytokine production, that could be blocked by the expression of a kinase-defective form of IKK2. This suggested that wild-type NIK needs to activate IKK2 in order to exert its NF-κB-activating action. Whether this is physiologically relevant or is an artifact of overexpression, and whether IKK1 is also required for NIK-induced NF-κB activation is not clear. Nevertheless, NIK provides a potent way of activating

IKK2, a kinase shown in chapter 3 to be of major importance in DC antigen-presenting function.

The ability of NIK to activate IKK2 and NF-κB in immature dendritic cells simply by overexpression, and in the absence of further stimulation, prompted me to investigate whether NIK could be used to up-regulate DC antigen presentation. Indeed, overexpression of NIK could significantly enhance DC antigen-presenting function in the allogeneic mixed lymphocyte reaction (MLR) and the antigen-specific T cell proliferation, two *in vitro* models of DC function. In the allogeneic MLR, the enhancement of DC antigen presentation was comparable to that observed when LPS or monocyte-conditioned medium (Sallusto F. et al. 1995; Romani N. 1996) are used as DC activators. In the antigen-specific T cell proliferation, the vector-encoded green fluorescent protein (GFP) was used as a prototype antigen. Incorporation of NIK together with antigen in the same vector greatly enhanced antigen-specific T cell proliferation.

To understand further the mechanisms by which NIK overexpression enhances DC antigen presentation, I analysed the expression of cytokines, chemokines and costimulatory molecules. As expected, NIK overexpression resulted in the activation of p65, relB and p50 NF-κB subunits. This induced the production of high levels of cytokines (TNFα, IL-6, IL-12 and IL-18) and chemokines (RANTES, MIP1α, IL-8, MCP-1, MCP-3) , and the up-regulation of antigen-presenting (HLA-DR, HLA-A,B,C) and costimulatory (CD80, CD86) molecules. The fact that the promoters of many immune and inflammatory genes including TNFα, IL-6, IL-12, IL-18, RANTES, MIP1α, HLA-DR, HLA-A,B,C, CD80 and CD86 contain NF-κB DNA-binding sites (Barnes P.J. and Karin M. 1997; Gobin S.J. et al. 1998; Brown A.M. et al. 1994; Zhao J. et al. 1996; Li J. et al. 1999), suggests that the immune system has evolved a way of coordinating dendritic cell antigen presentation through the activation of the transcription factor NF-κB. Thus, NF-κB activation induced by NIK overexpression in immature DC enhances their antigen-presenting function through the **coordinated** production of cytokines and chemokines and the up-regulation of antigen-presenting and costimulatory molecules. This is the converse of the inhibition of antigen-presenting cell function of mature DC seen by Yoshimura et al. (2001)

after IκBα overexpression. This effect is so strong that can even be visualized morphologically as NIK overexpression induces a mature DC-like appearance *in vitro* with an increased cell size and the induction of extended dendrites and protrusions.

Overexpression of the wild-type form of NIK induces IκBα degradation and NF-κB activation. When other known NF-κB-activating proteins such as the wild-type forms of IKK2, MEKK1 and MyD88 were tested, they were not able to activate NF-κB in immature dendritic cells. When, however, IKK2 and MyD88 were tested in human skin fibroblasts (chapters 3 and 5), they were equally potent in activating NF-κB and NF-κB-dependent cytokine production. As these proteins are not constitutively active, but require upsteam signals for their activation, these findings suggest that in immature dendritic cells these signals may be absent or more specific for NIK. Or, simple overexpression of NIK, but not the other proteins, is sufficient to result in its autophosphorylation and activation. This leaves open the possibility that the effect of DC activation observed is not specific to NIK, and that overexpression of constitutively active forms of IKK2, MEKK1 or MyD88 may be equally potent with NIK to enhance DC antigen-presenting function

The observations made in this study have some important clinical implications. First, the finding that NIK is not required for CD40L- or LPS-induced NF-κB activation in DC but is in B cells (Garceau N. et al. 2000) suggests that inhibition of NIK activity may constitute a novel strategy of specifically blocking B cell activation. Signalling through CD40 induces isotype switching, B cell proliferation and rescue from apoptosis. In particular, CD40-induced NF-κB activation, plays a key role B cell isotype switching from IgM to IgE (reviewed by Grewal I.S. and Flavell R.A. 1998). Because excessive CD40L-induced B cell activation and isotype switching to IgE are involved in allergic reactions, inhibitors of NIK activity may be useful way of controlling these unwanted responses.

Second , activation of NF-κB through overexpression of NIK and probably other NF-κB-activating proteins or constitutively active forms of these molecules provide a potent way of enhancing DC antigen-presenting function. The implications of this may be very important in vaccinology.

For vaccines to be effective, antigens contained in the vaccine mixture must be acquired and displayed by dendritic cells (Hart D.N. et al. 1997; Cella M. et al. 1997; Banchereau J. and Steinman R.N. 1998). This process is significantly improved when antigens are administered in combination with adjuvants, immunostimulatory agents that can enhance DC antigen-presenting function. My findings presented in this chapter suggest that NIK and probably other intracellular NF-κB-activating signalling molecules may define a novel family of potent adjuvants. This may be of particular importance in DNA vaccine preparations where there is a strong need to increase immunogenicity of vector-encoded antigens in humans (reviewed by Gurunathan S. 2000). Although many ways of increasing immunogenicity have been tested to date, including the incorporation of cytokines, chemokines, costimulatory molecules, or the combination of cytokines and chemokines, into the same DNA vector, success has been limited (Gurunathan S. et al. 2000). Evidence presented in this chapter suggests that the incorporation of NIK may be a superior way of improving immunogenicity of DNA vaccines as it activates the natural pathway involved in antigen presentation coordinating the production of cytokines and chemokines, and the up-regulation of antigen-presenting and costimulatory molecules.

CHAPTER 5

A PARADOXICAL EFFECT AFTER INHIBITION OF MyD88 ACTIVITY IN HUMAN MACROPHAGES AND DENDRITIC CELLS

5.1. Introduction

Innate immunity enables an organism to respond rapidly to invading microorganisms. It provides a first line of defense against pathogens and it determines the type of the adaptive immunity by modulating the activation of adaptive antigen recognition receptors and by inducing costimulatory molecules and cytokines (Fearon D.T. and Locksley R.M. 1996). The innate immune system uses receptor proteins that can recognize microbial pathogens by the molecular patterns they display. This strategy has been conserved throughout evolution, probably due to two main advantages: It prevents the generation of microbial escape mutants and it allows a limited number of germline-encoded receptors to recognize a great variety of molecular structures present in pathogens (Hoffmann J.A. et al. 1999). Central to the recognition of these molecular structures are toll-like receptors (TLR), a family of phylogenetically conserved proteins present in species ranging from insects to humans.

In mammals, an expanding family of receptors homologous to Drosophila Toll has been described (Rock F.L. et al. 1998; Takeuchi O. et al. 1999; Du X. et al. 2000). This TLR family comprises at the moment nine TLRs (TLR1-9) as well as the eight IL-1 receptor family members (IL-1RI, IL-1RacP, IL-18R, IL-18RacP, T1/ST-2, IL-1Rrp2 and IL-1RAPL) (reviewed by O'Neill L.A.J. and Dinarello C.A. 2000). TLR display remarkable specificity for different ligands. Thus, TLR4 is required for the response to lipopolysaccharide found in the outer membranes of gram-negative bacteria (Poltorak A. et al. 1998; Qureshi S.T. et al. 1999). TLR2 mediates the response to microbial lipoproteins such as peptidodoglycan and lipoteichoic acid , and yeast carbohydrates (Underhill D.M. et al. 1999; Takeuchi O. et al. 1999), whereas TLR9 has been shown to recognize and respond to undermethylated CpG motifs found in bacterial DNA (Hemmi H. et al. 2000). More recently, TLRs have been shown to form functional pairs that can change their specificity or influence their signalling ability. Thus, TLR2 alone recognizes lipopeptide of gram-positive bacteria, whereas it needs to pair with TLR6 to recognize peptidoglycan (Ozinsky A et al. 2000). Pairing of TLR2 with TLR1, on the other hand, results in the recognition of soluble factors released by Neisseria meningitis (Wyllie D.H. et al. 2000).

Although the extracellular portions of TLRs are relatively divergent, their cytoplasmic portions are conserved. They contain a well-defined region known as the toll domain, which is also found in the cytoplasmic portion of the IL-1 receptor family members (O'Neill L.A.J. and Greene C. 1998). This lead to the suggestion that TLRs and IL-1 receptor family members use an analogous framework of signalling molecules; upon ligand binging; they recruit the adaptor molecule MyD88 through homotypic interactions with a toll domain that MyD88 contains in its C-terminus (Wesche H. et al. 1997; Burns K. et al. 1998). MyD88, in turn, recruits IRAK and IRAK-2 through its death domain in the N-terminus that results in their autophosphorylation and activation (Wesche H. et al. 1997; Burns K. et al. 1998). At this moment, the pathway bifurcates and results in the activation of the TNF receptor-associated factor 6 (TRAF-6) and MAP kinases (Muzio M. et al. 1998). TRAF-6 subsequently activates NF-κB probably through the IKK complex and the kinases TAB-1 and TAK-1 (Ninomiya-Tsuji J. et al. 1999), or through ECSIT and MEKK-1 (Kopp E. et al. 1999).

MyD88 is believed to be central to TLR signalling. MyD88 has been proposed to be essential for IL-1 signalling in 293 human embryonic kidney cells (Burns K. et al. 1998; Burns K. et al. 2000), mouse NK and T cells (Adachi O. et al. 1998), HepG2 human hepatoma cells (Jensen L.E. et al. 2000) as well as LPS signalling in 293 human embryonic kidney cells (Yang R.B. et al. 1999), human gingival fibroblasts (Wang P.L. et al. 2000), mouse embryonic fibroblasts and B cells (Kawai T. et al. 1999), human dermal endothelial cells (Zhang F.X. et al. 1999) and THP-1 human monocytic cells (Zhang F.X. et al. 1999). Although, however, inhibition of MyD88 activity was shown to completely abrogate downstream signalling in response to IL-1 or LPS in numerous cell types, the construction of MyD88-deficient mice suggested that the pathway is more complex than previously thought. Although MyD88-deficient mice display defects in NF-κB activation in response to the structurally similar cytokines IL-1 and IL-18 in T cells (Adachi O et al. 1998) and are highly resistant to LPS-induced shock and cytokine production, LPS-induced MAPK and NF-κB activation in peritoneal macrophages remains almost normal (Kawai T et al. 1999). This suggested that other mechanisms can bypass the requirement of MyD88

for downstream TLR signalling, and that the resistence of MyD88-deficient mice to LPS-induced shock cannot be attributed simply to inhibition of TLR signalling.

The findings from the MyD88-deficient mice implied that MyD88 may have a different role in different cell types. To address that, I investigated in this chapter the role of MyD88 in IL-1R/TLR signalling in various human primary cells. As a tool, I used adenoviral gene transfer to efficiently express wild-type and dominant negative forms of MyD88 and examine the effect this has on MAPK phosphorylation, NF-κB activation and NF-κB-dependent cytokine production. I first looked at the role of MyD88 in fibroblasts and endothelial cells, and then extended the study to human macrophages and DC. I will provide evidence for a novel inhibitory function that MyD88 has, that is specific for macrophages and DC, but not for the other cell types tested.

5.2. Overexpression of dominant negative MyD88 blocks IL-1 but not TNFα signalling in human skin fibroblasts

Previous studies have suggested that human gingival fibroblasts and mouse embryonic fibroblasts signal through TLRs in response to LPS and IL-1 in a MyD88-dependent manner (Wang P.L. et al. 2000; Kawai T. et al. 1999). To examine whether MyD88 is also important in IL-1R/TLR signalling in human skin fibroblasts (HSF), I used adenoviral gene transfer to express wild-type and dominant negative forms of MyD88 (MyD88wt and MyD88dn, respectively). At a multiplicity of infection of 100, high levels of the MyD88 forms could be detected in HSF (figure 5.1). Expression of MyD88dn could block IL-1- but not TNFα-induced NF-κB activation, as anticipated (figure 5.1). MyD88wt, on the other hand, could induce NF-κB activation in the absence of further stimulation. LPS was not a good stimulus for this cell-type and was not used further.

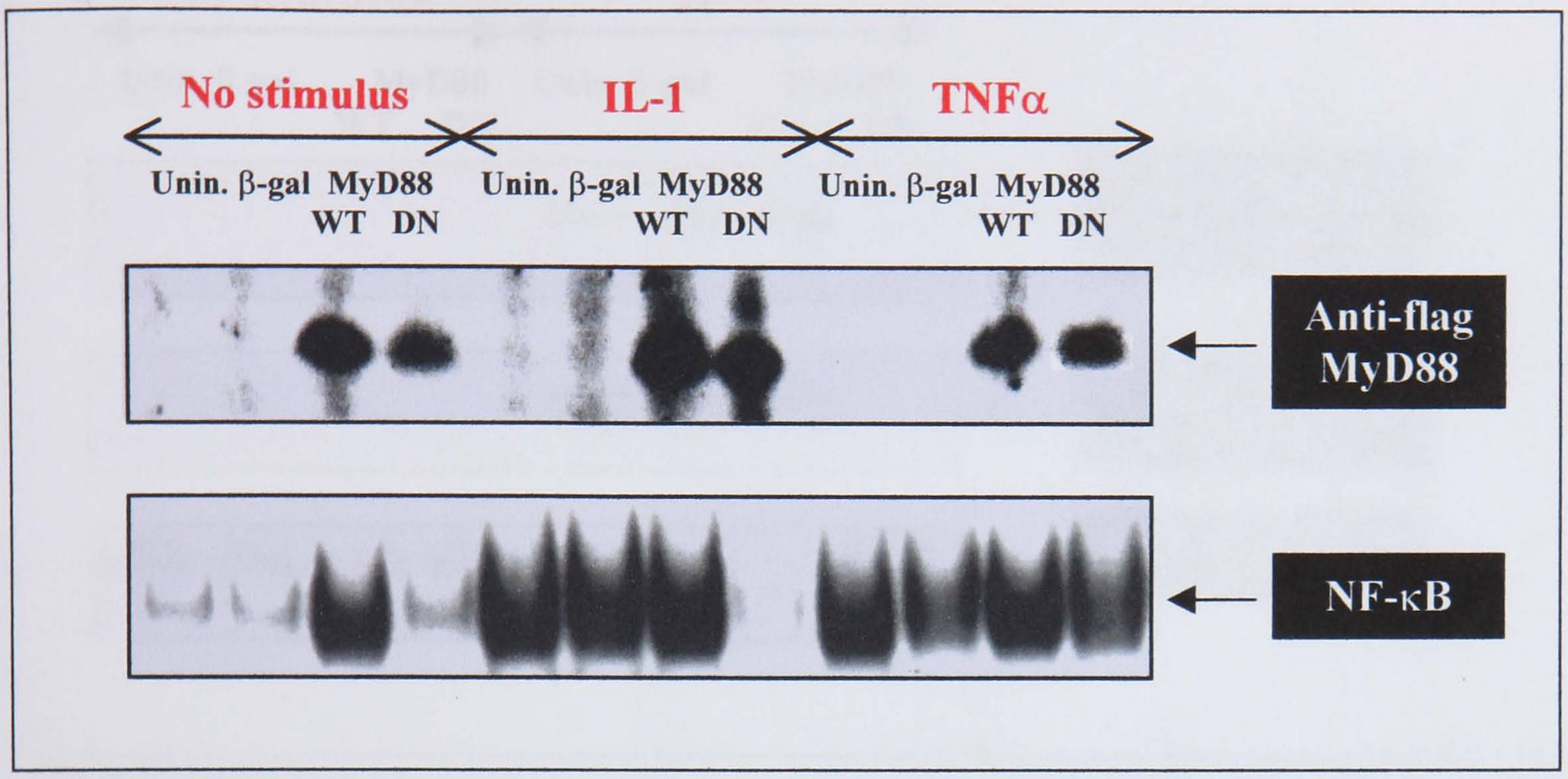


Figure 5.1: Overexpression of dominant negative MyD88 blocks IL-1- but not TNFα-induced NF-κB activation in HSF. HSF were plated at $3x10^6$ cells/plate in 10 cm tissue culture plates in 5% FCS 1% penicillin/streptomycin DMEM. After overnight incubation, they were infected for 2h with adenoviruses overexpressing β-galactosidase, MyD88wt or MyD88dn, in serum-free DMEM. A multiplicity of infection of 100 was used. Then cells were cultured in 5% FCS DMEM for another 1 day and stimulated with IL-1 or TNFα for 45 min. Cytosolic and nuclear extracts were then collected and assayed for MyD88 and NF-κB activity, respectively. A representative of three independent experiments is shown.

Expression of MyD88dn could also block IL-1-induced p38 and p42/44 MAPK activation in these cells whereas MyD88wt had no effect (figure 5.2).

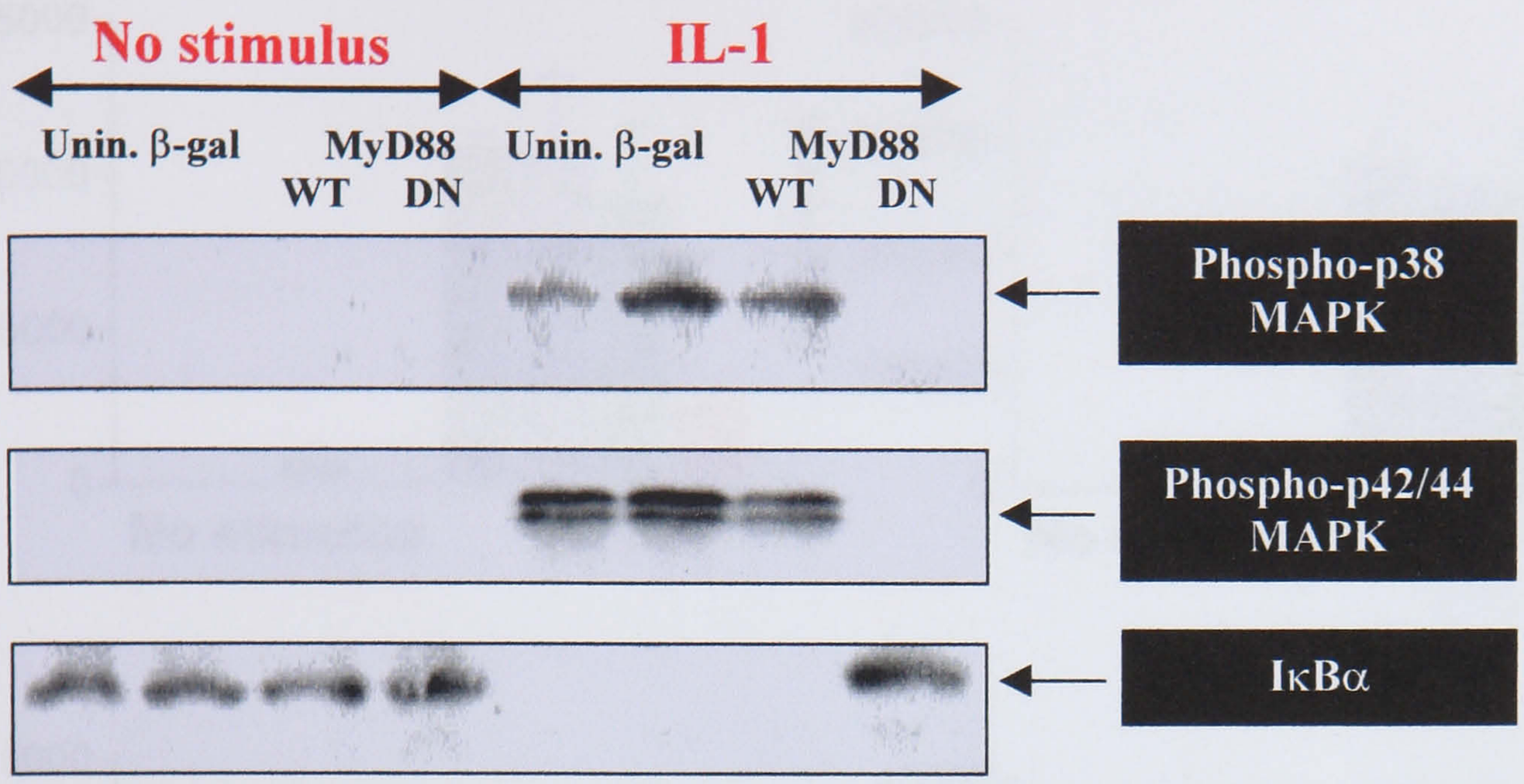


Figure 5.2: Dominant negative MyD88 blocks p38 and p42/44 MAPK activation, and IκBα degradation in HSF. HSF were plated at $3x10^6$ cells/plate in 10 cm tissue culture plates in 5% FCS 1% penicillin/streptomycin DMEM. After overnight incubation, they were infected for 2h with adenoviruses overexpressing β-galactosidase and MyD88wt or MyD88dn in serum-free DMEM. A m.o.i. of 100 was used. Then, cells were cultured in 5% FCS DMEM for another 1 day and stimulated with IL-1 for 30 min. Cytosolic extracts were then collected and assayed for the presence of phosphorylated p38 and p42/44 MAPK or total IκBα by western blotting. The same blot, stripped and reprobed two times, is shown and is a representative of three independent experiments.

The inhibitory effect of MyD88dn on MAPK and NF-κB activation was reflected in the levels of cytokines produced. MyD88dn thus blocked the IL-6 and IL-8 production in response to IL-1, but not TNFα (figure 5.3). In contrast, MyD88wt induced IL-6 and IL-8 production in the absence of additional stimulation, although, it had no effect in IL-1- or TNFα-induced NF-κB activation and cytokine production (figure 5.3).

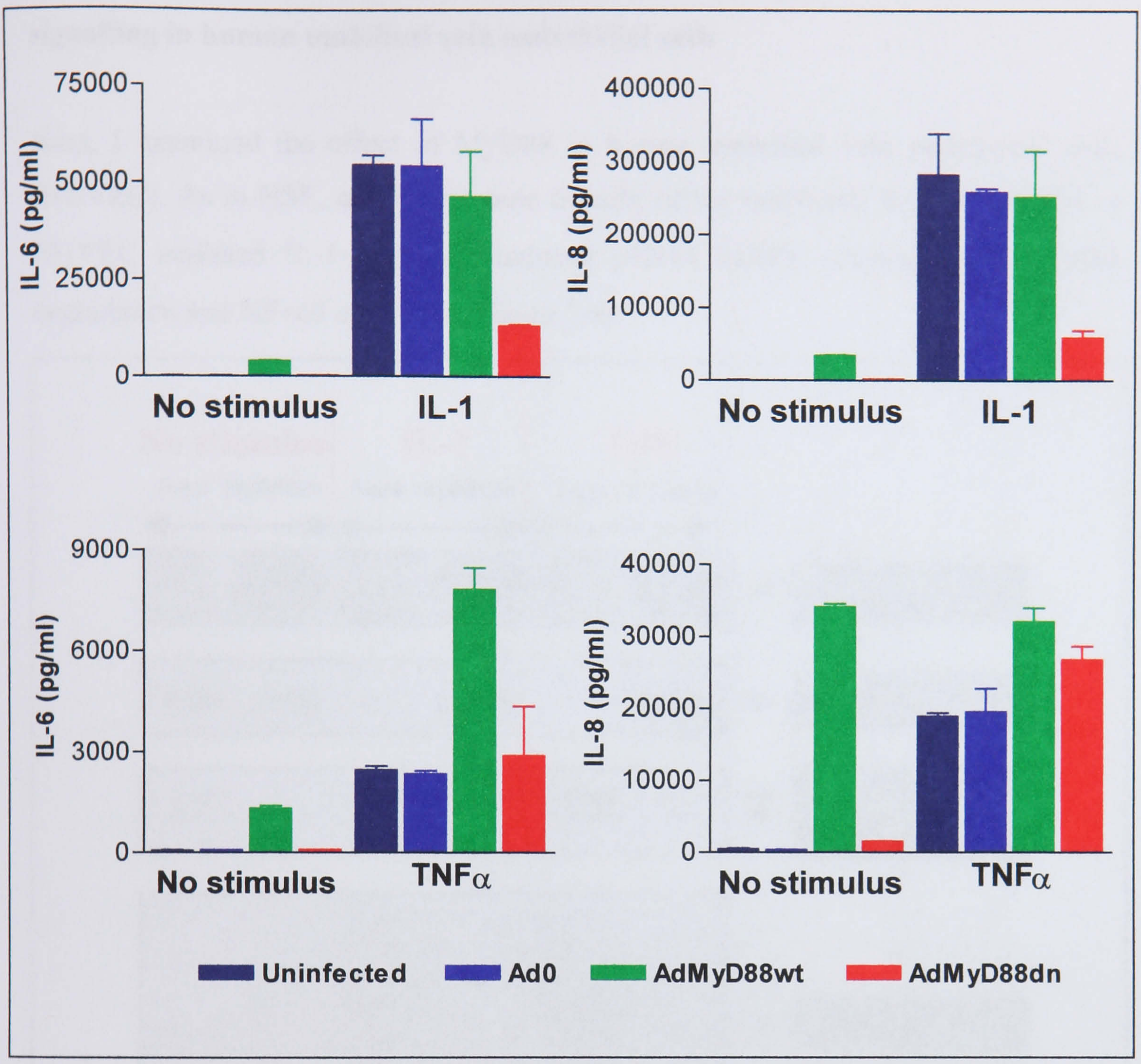


Figure 5.3: Overexpression of dominant negative MyD88 in HSF inhibits IL-1- but not TNFα-induced IL-6 and IL-8 production. HSF were plated at $1x10^5$ cells/plate in 24-well tissue culture plates in 5% FCS 1% penicillin/streptomycin DMEM. After overnight incubation, they were infected for 2h with a control adenovirus without insert (Ad0) or adenoviruses overexpressing MyD88wt (AdMyD88wt) and MyD88dn (AdMyD88dn), in serum-free DMEM. A m.o.i. of 100 was used. Then, cells were cultured in 5% FCS DMEM for another 1 day and stimulated with IL-1 or TNFα for. After 24h, supernatants were collected and assayed for the presence of IL-6 and IL-8 by ELISA. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of five independent experiments

These findings show that MyD88 activity is specifically required for IL-1R- but not TNF-R signalling in HSF.

5.3. Overexpression of dominant negative MyD88 blocks IL-1 but not TNFα signalling in human umbilical vein endothelial cells

Next, I examined the effect of MyD88 in human umbilical vein endothelial cells (HUVEC). As in HSF, adenoviral gene transfer of the inhibitory form of MyD88 in HUVEC inhibited IL-1- and LPS-induced p42/44 MAPK phosphorylation, IκBα degradation and NF-κB activation (figure 5.4).

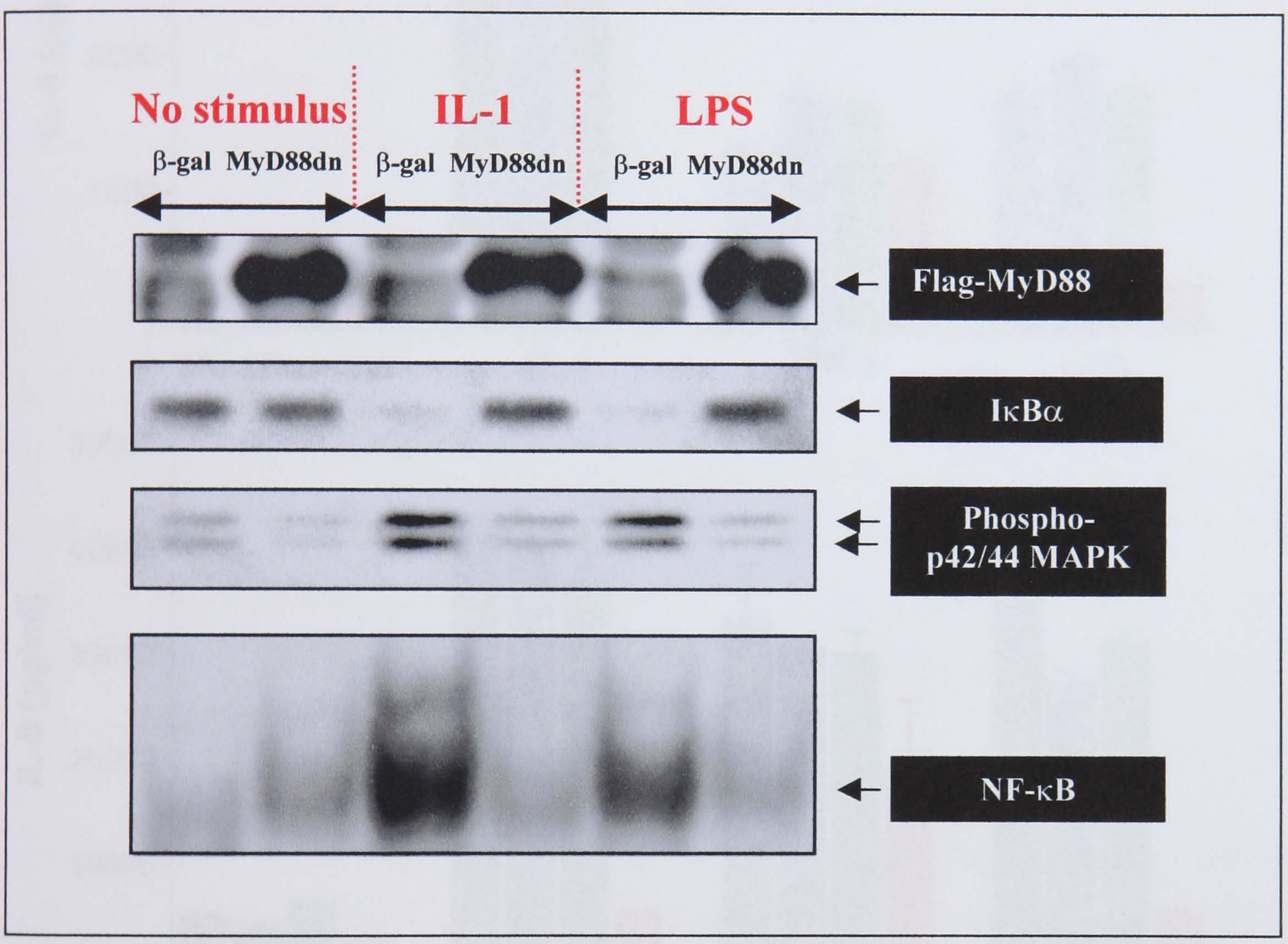


Figure 5.4: Dominant negative MyD88 blocks p42/44 MAPK phosphorylation, IκBα degradation and NF-κB activation in HUVEC in response to IL-1 and LPS. HUVEC were plated at $3x10^6$ cells/plate in T75 tissue culture flasks in 10% FCS 10% FNS 1% heparin and 1% penicillin/streptomycin RPMI. After overnight incubation, they were infected for 2h with adenoviruses overexpressing β-galactosidase, MyD88wt or MyD88dn, in serum-free RPMI. A multiplicity of infection of 100 was used. Then cells were cultured in complete RPMI for another 1 day and stimulated with 20 ng/ml IL-1 or 1μg/ml LPS for 30 min. Cytosolic and nuclear extracts were collected and assayed for the presence of MyD88dn, IκBα, phosphorylated p42/44 MAPK and nuclear NF-κB DNA-binding activity. A representative experiment of three is shown.

When I examined the effect of MyD88dn overexpression on HUVEC cytokine production, I found that it could also block IL-1- and LPS-induced IL-6- and IL-8 production, whereas it had no effect on TNFα-induced IL-6 or IL-8 (figure 5.5). In

contrast to HSF, however, MyD88wt could not by itself induce NF-κB activation or cytokine production.

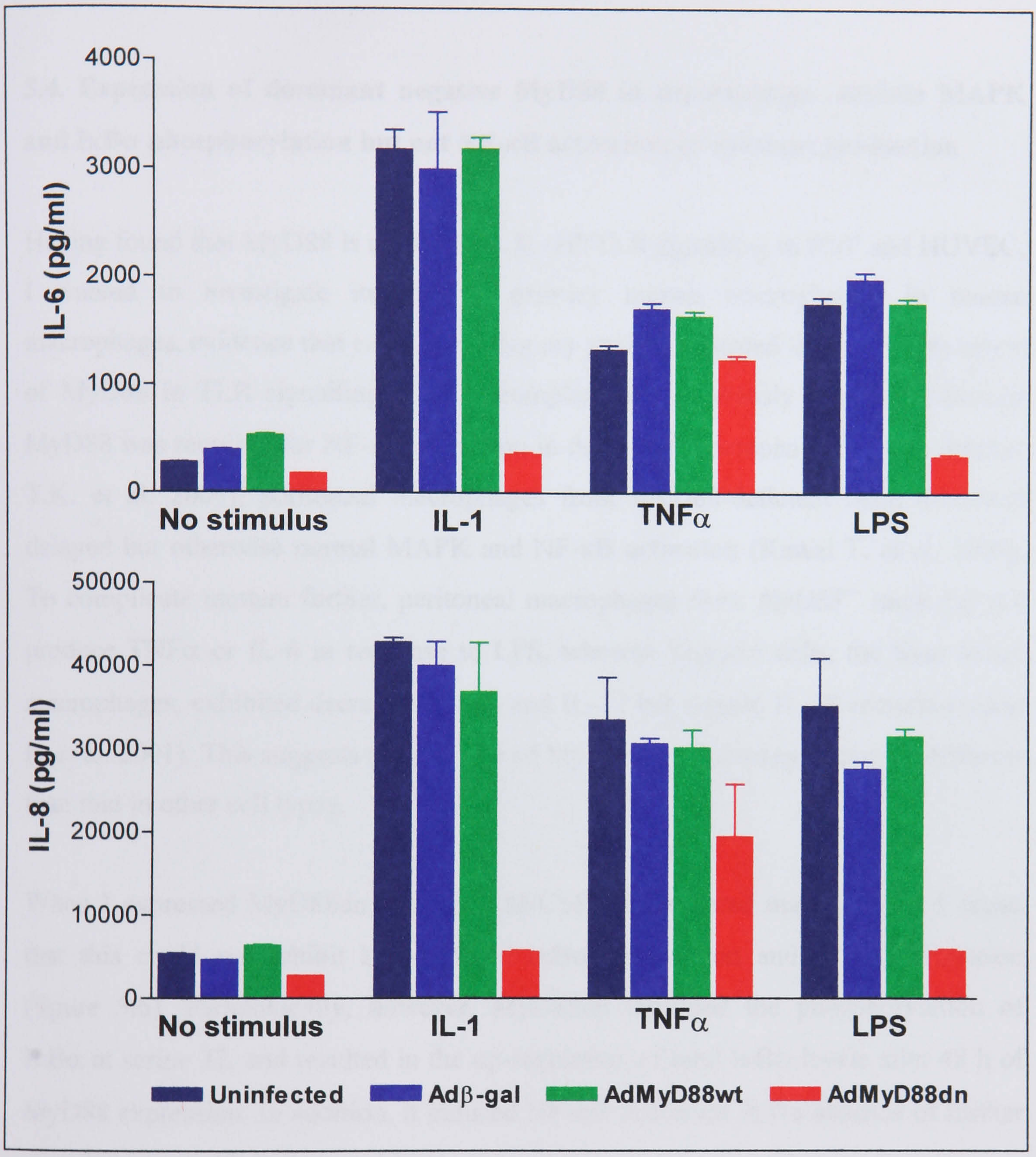


Figure 5.5: Overexpression of dominant negative MyD88 in HUVEC blocks cytokine production in response to IL-1 and LPS but not TNFα. HUVEC were left uninfected, or infected for 2h in serum-free medium with a control adenovirus expressing β-galactosidase (Adβ-gal), or adenoviruses overexpressing MyD88wt or MyD88dn (AdMyD88wt and AdMyD88dn, respectively). After 24h, cells were stimulated with 20 ng/ml IL-1, 20 ng/ml TNFα or 1 μg/ml LPS and supernatants were collected after another 24h. The levels of IL-6 and IL-8 were then assayed by ELISA. Mean cytokine production (±SD) of duplicate cultures is shown and is representative of five independent experiments

These findings suggest again that MyD88 activity is required for efficient IL-1R and TLR signalling and its inhibition completely abrogates IL-1- or LPS-induced HUVEC activation.

5.4. Expression of dominant negative MyD88 in macrophages inhibits MAPK and IκBα phosphorylation but not NF-κB activation or cytokine production

Having found that MyD88 is required for IL-1R/TLR signalling in HSF and HUVEC, I wanted to investigate its role in primary human macrophages. In mouse macrophages, evidence that came up during my studies indicated that the involvement of MyD88 in TLR signalling is more complex than previously thought. Although MyD88 was required for NF-κB activation in the RAW macrophage cell line (Means T.K. et al. 2001), peritoneal macrophages from MyD88-deficient mice exhibited delayed but otherwise normal MAPK and NF-κB activation (Kawai T. et al. 1999). To complicate matters further, peritoneal macrophages from *MyD88*$^{-/-}$ mice did not produce TNFα or IL-6 in response to LPS, whereas Kuppfer cells, the liver tissue macrophages, exhibited decreased IL-1β and IL-12 but normal IL-18 secretion (Seki E. et al. 2001). This suggests that the role of MyD88 in macrophages may be different than that in other cell types.

When I expressed MyD88dn in human M-CSF-differentiated macrophages, I found that this could not inhibit LPS-induced IκBα degradation and NF-κB activation (figure 5.6). Paradoxically, however, MyD88dn inhibited the phosphorylation of IκBα at serine 32, and resulted in the up-regulation of total IκBα levels after 48 h of MyD88 expression. In addition, it induced NF-κB activation in the absence of further stimulation, an effect discussed later.

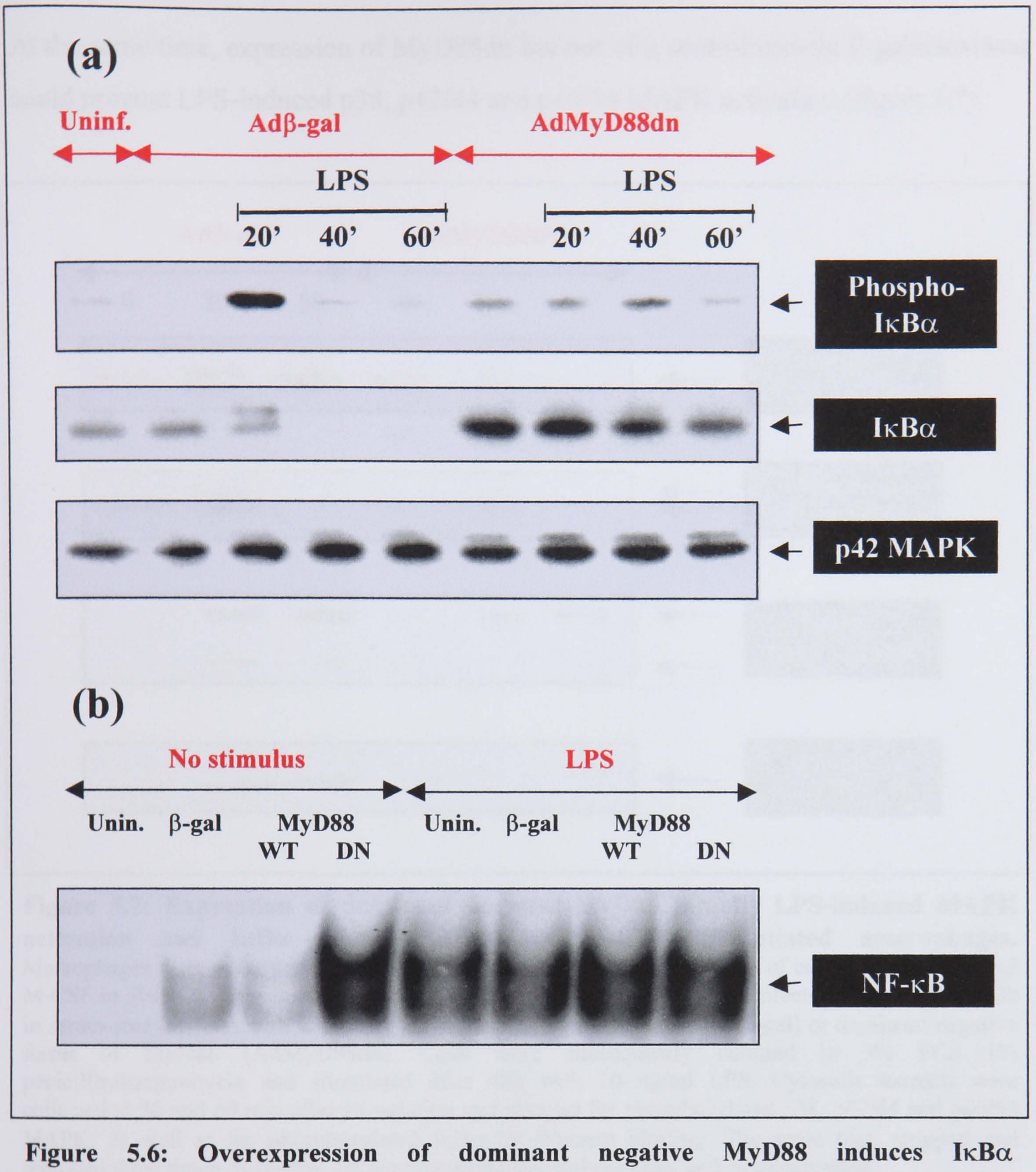


Figure 5.6: Overexpression of dominant negative MyD88 induces IκBα phosphorylation and degradation in M-CSF-differentiated macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal), MyD88wt (AdMyD88wt) or MyD88dn (AdMyD88dn). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and after 48h they were stimulated with 10 ng/ml LPS for 1h. Cytosolic and nuclear extracts were collected and assayed in (a) for normal or phosphorylated IκBα, or total p42 MAPK by Western blotting, and in (b) for NF-κB DNA-binding activity. A representative of three independent experiments is shown, although the NF-κB DNA-binding activity induced by MyD88dn (figure b) is more pronounced in this experiment.

At the same time, expression of MyD88dn but not of a control protein β-galactosidase could prevent LPS-induced p38, p42/44 and p46/54 MAPK activation (figure 5.7).

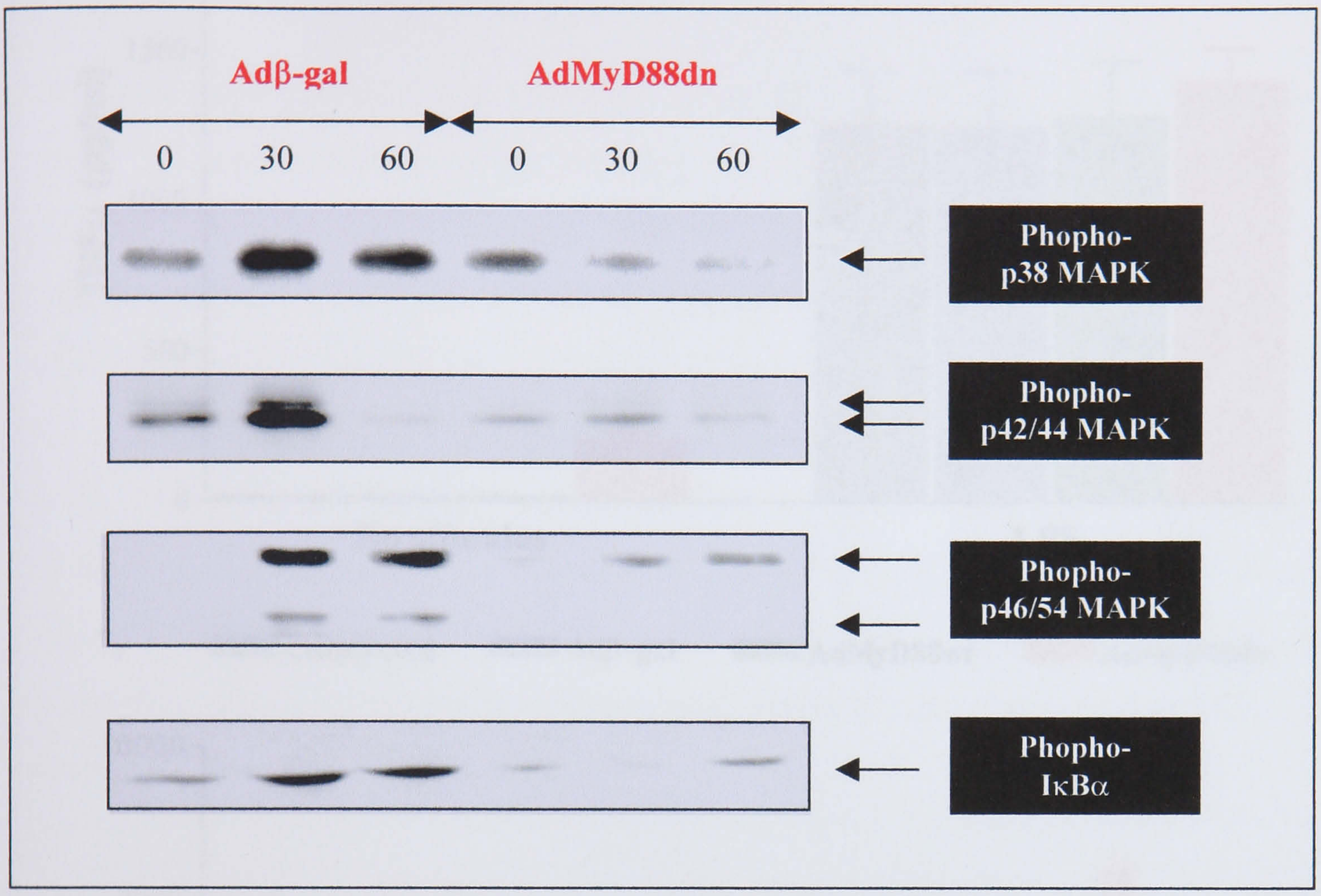


Figure 5.7: Expression of dominant negative MyD88 inhibits LPS-induced MAPK activation and IκBα phosphorylation in M-CSF-differentiated macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal) or dominant negative forms of MyD88 (AdMyD88dn). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and stimulated after 48h with 10 ng/ml LPS. Cytosolic extracts were collected at 30 and 60 min after stimulation and assayed for phosphorylated p38, p42/44 and p46/54 MAPK, as well as for phosphorylated IκBα by Western blotting. The same blot, stripped and reprobed three times, is shown. This is representative of three independent experiments.

Although MyD88dn was able to inhibit MAPK and IκBα phosphorylation in response to LPS, it could not block the production of TNFα (figure 5.8). This effect was independent of the LPS concentration used, and correlated with the inability of MyD88dn to inhibit LPS-induced NF-κB activation.

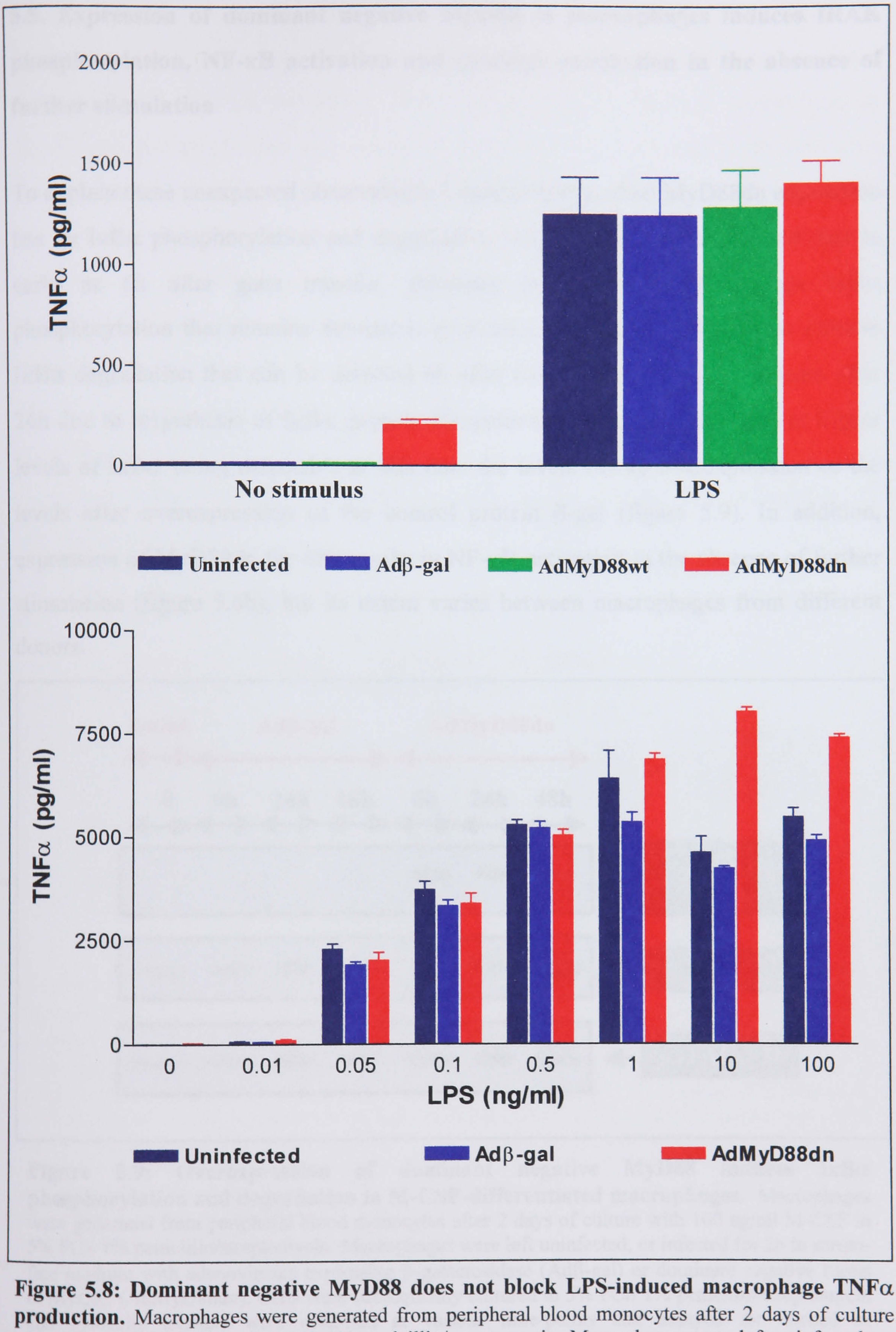


Figure 5.8: Dominant negative MyD88 does not block LPS-induced macrophage TNFα production. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal) or MyD88dn. Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and stimulated after 48h with various LPS concentrations. After another 24h, supernatants were collected and measured for TNFα production. Mean TNFα production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

5.5. Expression of dominant negative MyD88 in macrophages induces IRAK phosphorylation, NF-κB activation and cytokine production in the absence of further stimulation

To explain these unexpected observations, I examined the effect MyD88dn expression has on IκBα phosphorylation and degradation over the 48h period. I found that as early as 6h after gene transfer, dominant negative MyD88 induces IκBα phosphorylation that remains detectable even after 48h (figure 5.9). It also induces IκBα degradation that can be detected 6h after overexpression but disappears after 24h due to resynthesis of IκBα protein. Resynthesis continues and results at higher levels of IκBα being detectable at 48h than the levels before overexpression or the levels after overexpression of the control protein β-gal (figure 5.9). In addition, expression of MyD88dn for 48h results in NF-κB activation in the absence of further stimulation (figure 5.6b), but its extent varies between macrophages from different donors.

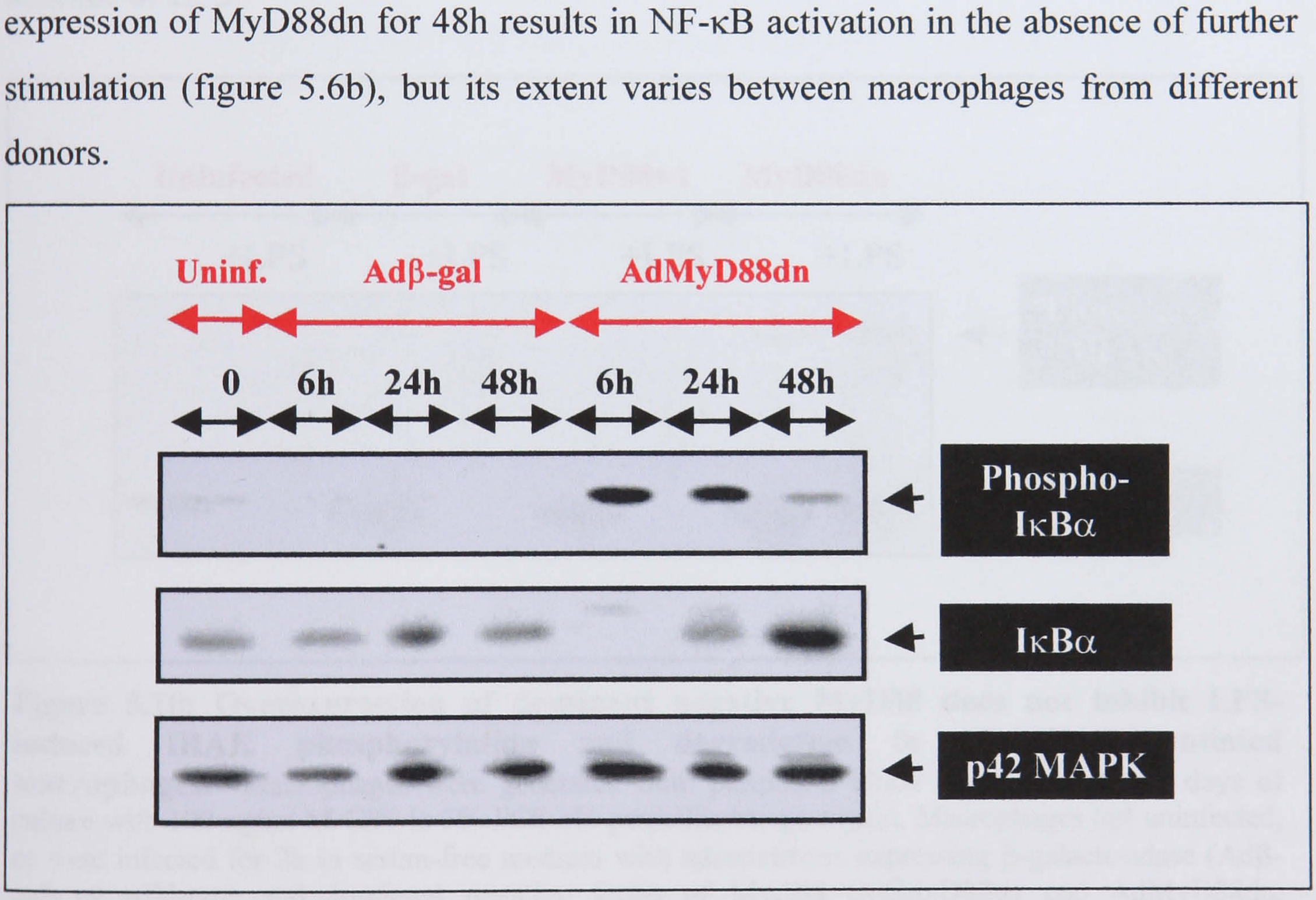


Figure 5.9: Overexpression of dominant negative MyD88 induces IκBα phosphorylation and degradation in M-CSF-differentiated macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal) or dominant negative forms of MyD88 (AdMyD88dn). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and cytosolic extracts were collected at various time-points and assayed for normal or phosphorylated IκBα by Western blotting. A representative of two independent experiments is shown.

MyD88 has been proposed to be involved in the activation of IRAK, a major kinase involved in IL-1R/TLR signalling. Within minutes of IL-1R/TLR ligand-binding, IRAK gets phosphorylated and degraded. Degradation occurs within minutes after stimulation, and then IRAK gets resynthesized (Li L. et al. 2000). In the light of the previous findings showing that MyD88dn results in the phosphorylation of IκBα, I examined the activation of IRAK. Unexpectedly, I found that the expression of MyD88dn in macrophages induces IRAK phosphorylation and activation in the absence of further stimulation (figure 5.10). IRAK degradation was not detected after 48h of expression of MyD88dn, but is probably occurring at earlier timepoints. On the other hand, MyD88dn did not prevent LPS-induced IRAK degradation when compared to the IRAK levels present in MyD88dn-expressing macrophages in the absence of LPS.

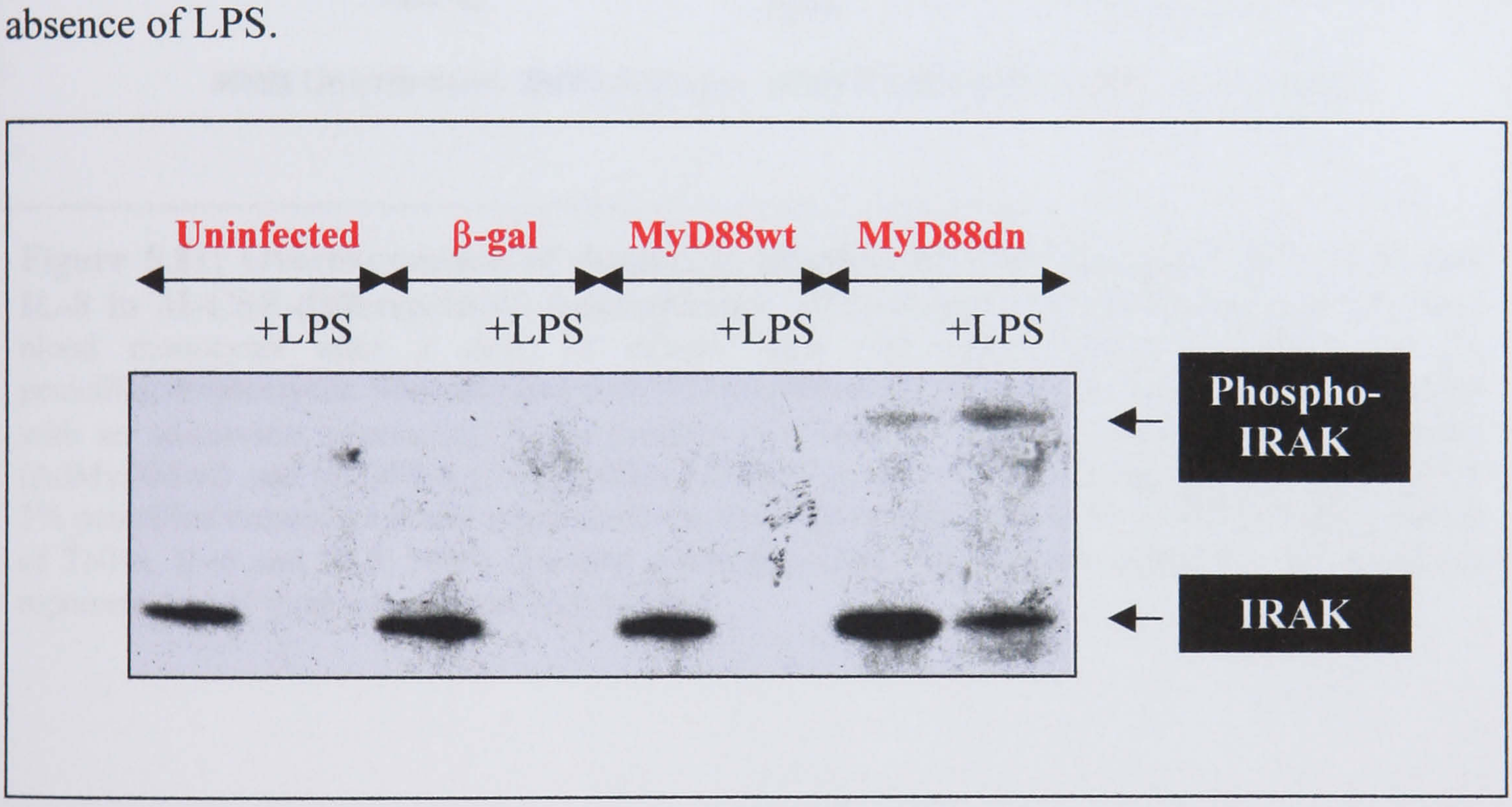


Figure 5.10: Overexpression of dominant negative MyD88 does not inhibit LPS-induced IRAK phosphorylation and degradation in M-CSF-differentiated macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages left uninfected, or were infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal) or wild-type and dominant negative forms of MyD88 (AdMyD88wt and AdMyD88dn, respectively). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and stimulated after 48h with 10 ng/ml LPS. Total cell extracts were collected at 0 and 5 min after stimulation and assayed for the presence of IRAK by immunoprecipitation and Western blotting. A representative of three independent experiments is shown.

Moreover, expression of MyD88dn rather than MyD88wt in macrophages induces IL-6 and IL-8 production as well as low levels of TNFα (figure 5.12).

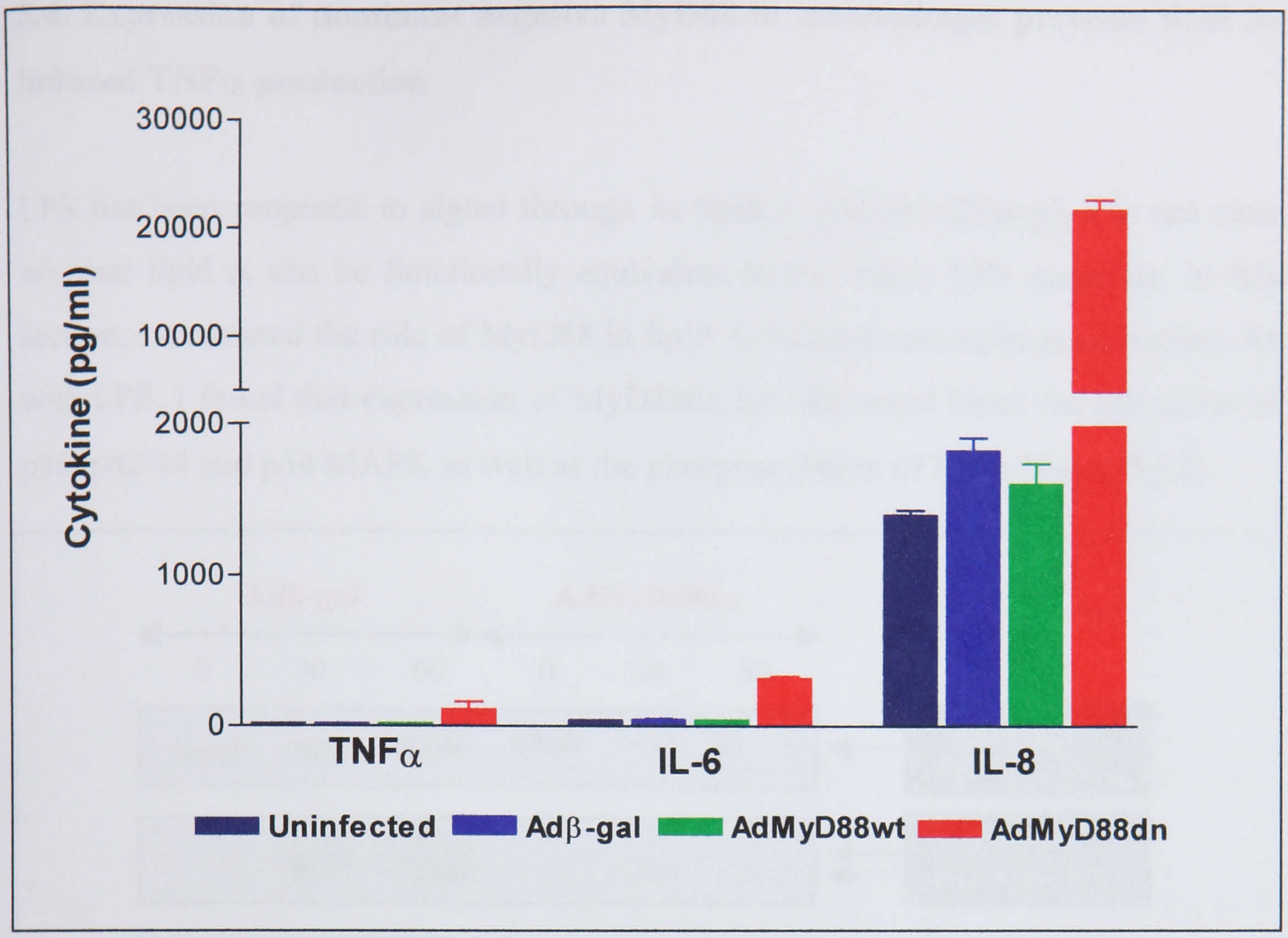


Figure 5.11: Overexpression of dominant negative MyD88 induces TNFα, IL-6 and IL-8 in M-CSF-differentiated macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with an adenovirus expressing β-galactosidase (Adβ-gal) or adenoviruses expressing MyD88wt (AdMyD88wt) and MyD88dn (AdMyD88dn). Cells were then cultured for another 1 day in 5% FCS 1% penicillin/streptomycin and supernatants were collected and assayed by ELISA for the presence of TNFα, IL-6 and IL-8. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

These findings suggest that inhibition of MyD88 activity in M-CSF-differentiated macrophages results in the activation of the IL-1R/TLR pathway in the absence of further stimulation. It induces the phosphorylation of IRAK, the degradation of IκBα and the spontaneous release of TNFα, IL-6 and IL-8. This provides an explanation for the paradoxical observations made before. The higher levels, thus, of total IκBα observed after 48h of expression of dominant negative MyD88 compared to control cells, may be due to the stimulatory effect that MyD88dn has by itself, that results in resynthesis of IκBα. Newly resynthesized IκBα, however, does not bind NF-κB well and may not be able to inhibit NF-κB nuclear translocation.

5.6. Expression of dominant negative MyD88 in macrophages prevents lipid A-induced TNFα production

LPS has been proposed to signal through its lipid A portion, although it is not clear whether lipid A can be functionally equivalent to the whole LPS molecule. In this section, I examined the role of MyD88 in lipid A-induced macrophage activation. As with LPS, I found that expression of MyD88dn for 48h could block the activation of p38, p42/44 and p54 MAPK as well as the phosphorylation of IκBα (figure 5.12).

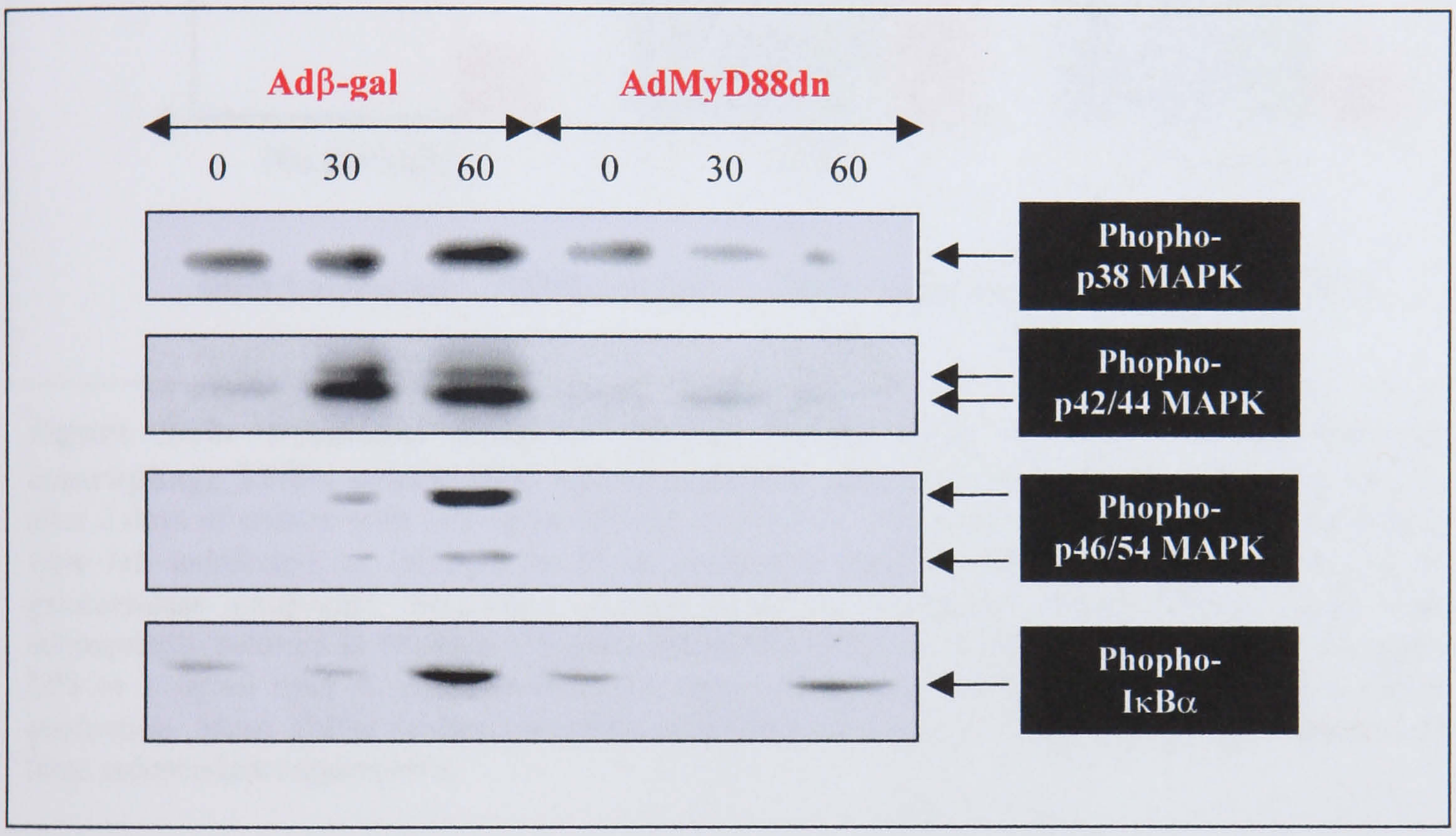


Figure 5.12: Overexpression of dominant negative MyD88 inhibits lipid A-induced MAPK activation and IκBα phosphorylation in M-CSF-differentiated macrophages. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal) or MyD88dn (AdMyD88dn). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and stimulated after 48h with 1 μg/ml lipid A. Cytosolic extracts were collected at 30 and 60 min after stimulation, adjusted for equal protein amounts, and assayed for phosphorylated p38, p42/44 and p46/54 MAPK, as well as for phosphorylated IκBα by Western blotting. The same western, stripped and reprobed three times, is shown. This was a representative of three independent experiments.

Surprisingly, however, the expression of MyD88dn could inhibit lipid A-induced macrophage TNFα production (figure 5.13). As LPS-induced TNFα production was not inhibited, this suggested that LPS and lipid A are not functionally equivalent with respect to TLR signalling.

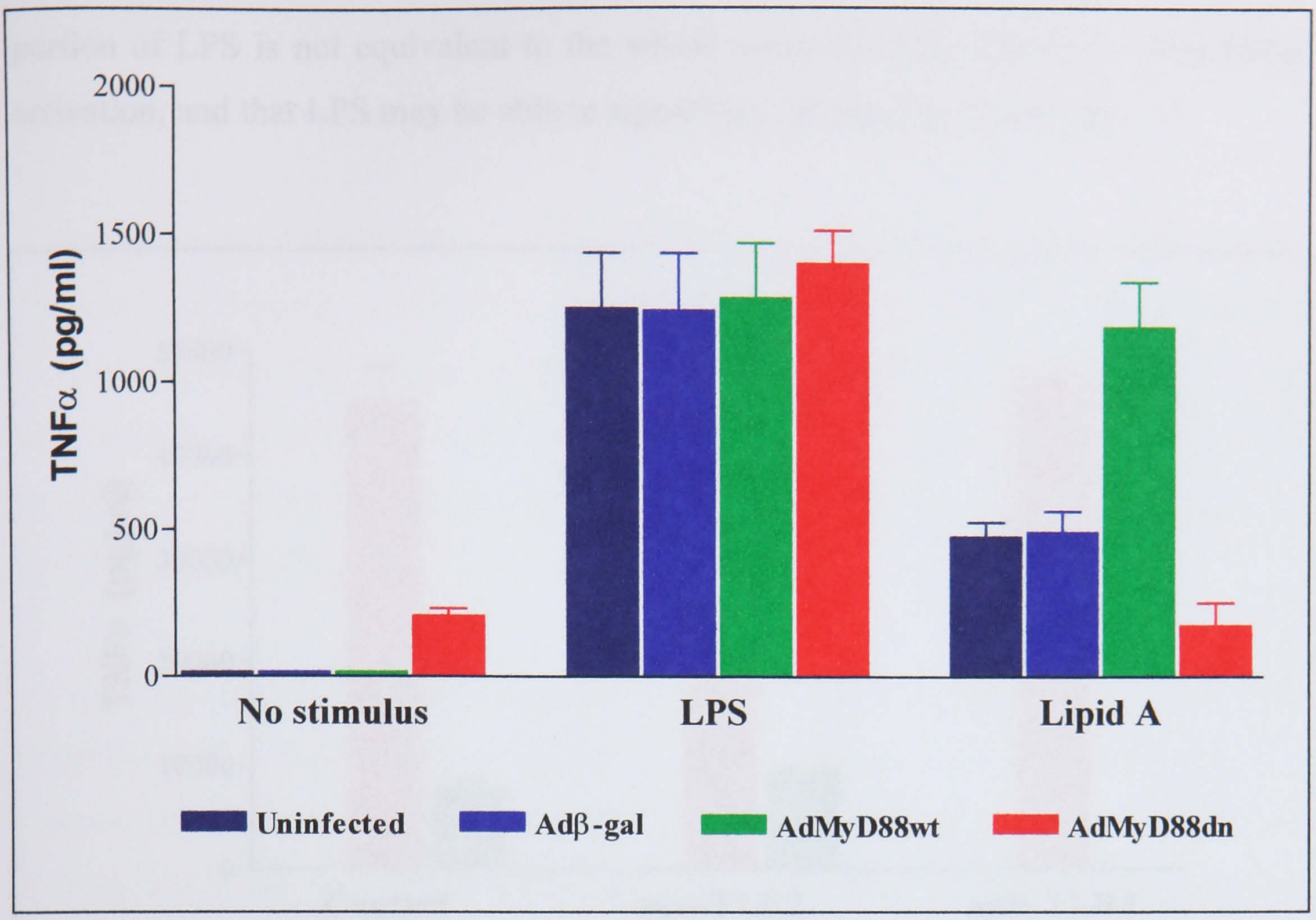


Figure 5.13: Dominant negative MyD88 blocks lipid A- but not LPS-induced macrophage TNFα production. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Macrophages were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal), MyD88wt (AdMyD88wt) or MyD88dn (AdMyD88dn). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and stimulated after 48h with 10 ng/ml LPS or 1 μg/ml lipid A. After another 24h, supernatants were collected and measured for TNFα production. Mean TNFα production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

My colleague Dr. Stonehouse investigated the involvement of TLRs in LPS and lipid A signalling. He found that blockade of TLR4 through an anti-TLR4 neutralizing antibody could prevent lipid A but not LPS-induced TNFα production (figure 5.14). Salmonella and E. coli LPS have been shown to activate mouse macrophages through TLR4 (Tapping R.I. et al. 2000) but this has been suggested to require pure LPS preparations because otherwise a mixture of contaminants can result in signalling through TLR2 too. To exclude the possibility of bacterial contaminants resulting in a TLR-4-independent signal, the commercially available S.typhimirium LPS was repurified by using the protocol previously demonstrated to yield LPS of high purity that acts through TLR4 only (Hirschfeld M. et al. 2000). When used in our studies, however, it induced TNFα production that could not be blocked by TLR4 neutralizing antibodies (Dr. Stonehouse, personnal communication), suggesting that the lipid A

portion of LPS is not equivalent to the whole molecule with respect to macrophage activation, and that LPS may be able to signal through other receptors too.

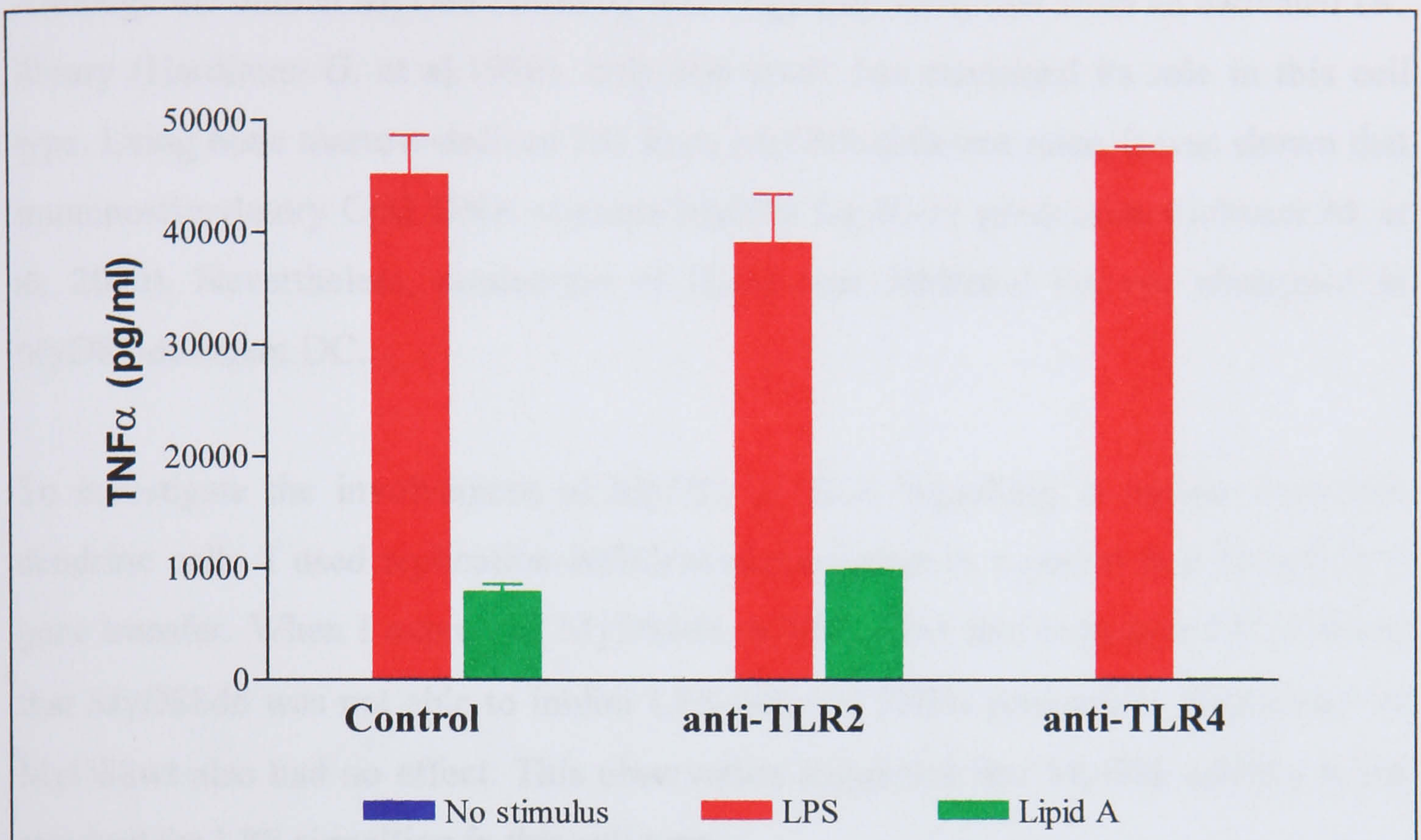


Figure 5.14: Anti-TLR4 neutralizing antibodies block lipid A- but not LPS-induced macrophage TNFα production. Macrophages were generated from peripheral blood monocytes after 2 days of culture with 100 ng/ml M-CSF in 5% FCS 1% penicillin/streptomycin. Anti-TLR2 or anti-TLR4 antibodies were then added, and cells were subsequently stimulated with 10 ng/ml LPS or 100 ng/ml of lipid A. After another 24h, supernatants were collected and assayed for the presence of TNFα. Mean TNFα production (±SD) of triplicate cultures is shown and is representative of three independent experiments. This figure is the work of Dr. T.Stonehouse.

In summary, these data demonstrate that MyD88dn can prevent the TLR4-specific signal, lipid A, from activating macrophages. The unexpected observation, however, that expression of MyD88dn activates macrophages by itself, suggests that this may not be a direct effect. As activation of macrophages can be observed within 6h of MyD88dn expression, and its inhibitory effect in lipid A activation is observed after 48h, it may be that this is due to a mechanism of desensitization taking place to already stimulated cells. This leaves open the possibility that lipid A, a weaker signal, cannot bypass these downregulatory mechanisms taking place, whereas LPS , a stronger signal, can.

5.7. MyD88 is not required for LPS signalling in immature DC

Although the human MyD88 homolog was originally identified from an activated DC library (Hardiman G. et al.1996), only one study has examined its role in this cell type. Using bone marrow-derived DC from MyD88-deficient mice, it was shown that immunostimulatory CpG DNA requires MyD88 for IL-12 production (Schnare M. et al. 2000). Nevertheless, production of IL-12 was inhibited but not abrogated in MyD88-deficient DC.

To investigate the involvement of MyD88 in TLR signalling in human immature dendritic cells, I used replication-deficient adenoviruses as a potent tool for efficient gene transfer. When I expressed MyD88dn or MyD88wt into immature DC, I found that MyD88dn was not able to inhibit LPS-induced TNFα production. Expression of MyD88wt also had no effect. This observation suggested that MyD88 activity is not required for LPS signalling in this cell type.

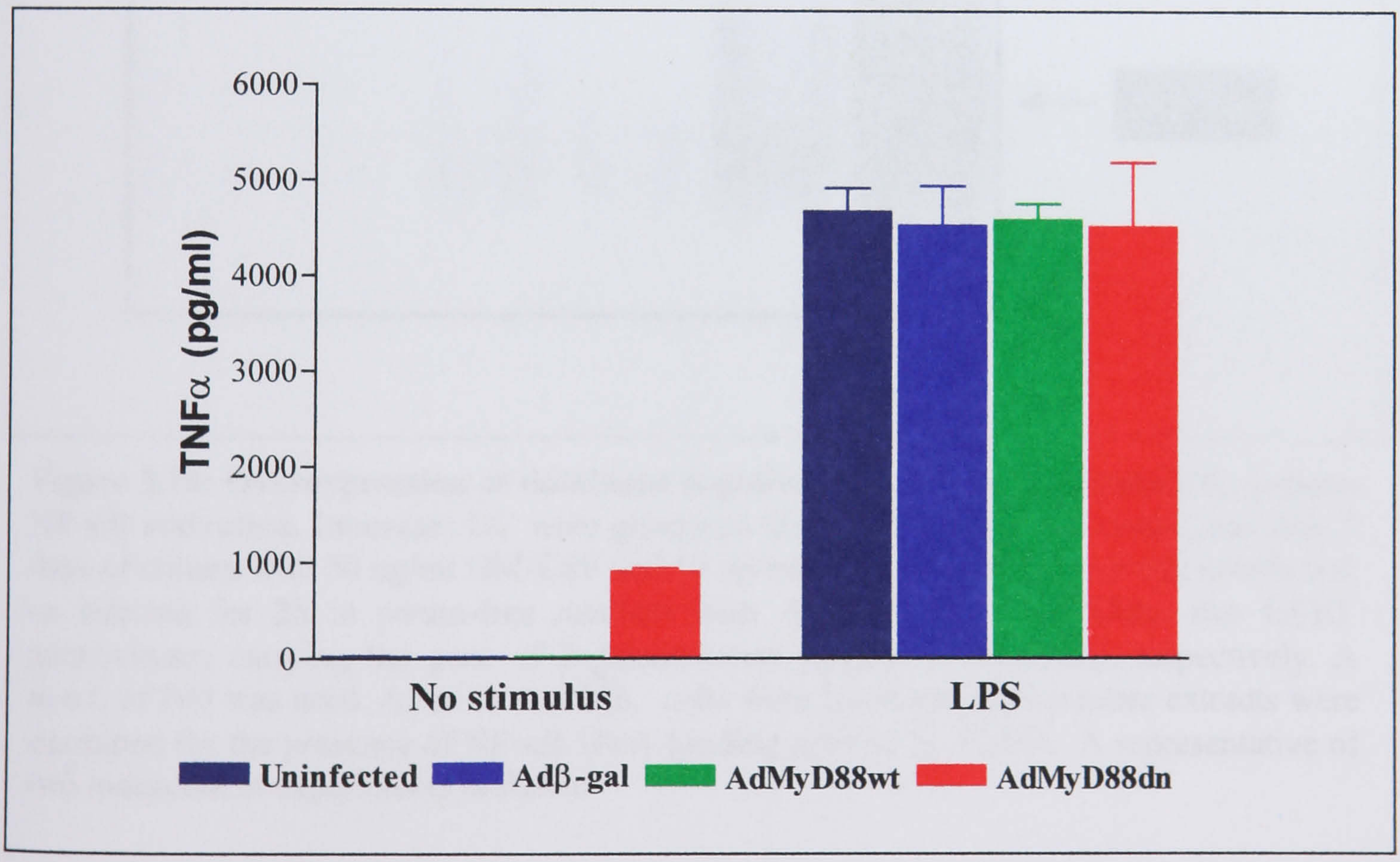


Figure 5.15: Dominant negative MyD88 does not block LPS-induced immature DC TNFα production. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in 5% FCS 1% penicillin/streptomycin. DC were left uninfected, or infected for 2h in serum-free medium with adenoviruses expressing β-galactosidase (Adβ-gal), MyD88wt (AdMyD88wt) or MyD88dn (AdMyD88dn). Cells were subsequently cultured in 5% FCS 1% penicillin/streptomycin and stimulated after 48h with 100 ng/ml of LPS. After another 24h, supernatants were collected and measured for TNFα production. Mean TNFα production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

5.8. Inhbition of MyD88 activity in immature dendritic cells results in NF-κB activation, cytokine production and up-regulation of costimulatory molecules

The finding that MyD88dn could result in the activation of a TLR-like pathway in macrophages in the absence of further stimulation, prompted me to investigate whether it could do the same in immature DC, another myeloid-derived cell type. Interestingly, I found that the inhibitory form of MyD88 was able to induce NF-κB activation that was detectable 6h and 24h after expression of the transgene (figure 5.16).

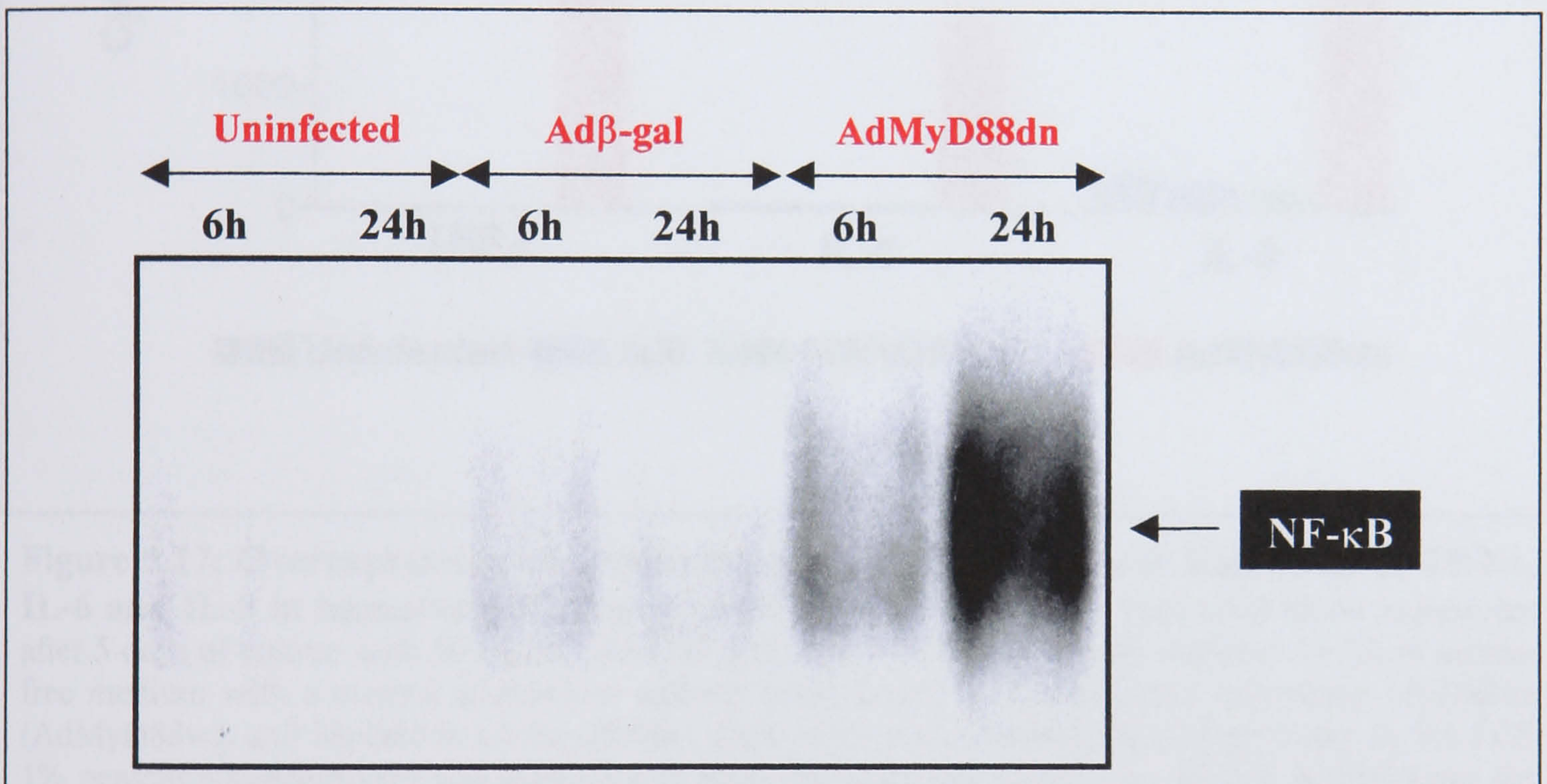


Figure 5.16: Overexpression of dominant negative MyD88 into immature DC induces NF-κB activation. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected for 2h in serum-free medium with Adβ-gal or AdMyD88dn, two $E1^-E3^-$ adenoviruses carrying the gene of β-galactosidase (β-gal) or MyD88dn, respectively. A m.o.i. of 100 was used. After 6h and 24h, cells were lysed and their nuclear extracts were examined for the presence of NF-κB DNA-binding activity by EMSA. A representative of two independent experiments is shown.

This resulted in high levels of TNFα, IL-6 and IL-8 that could be detected 24h after expression of MyD88dn (figure 5.17).

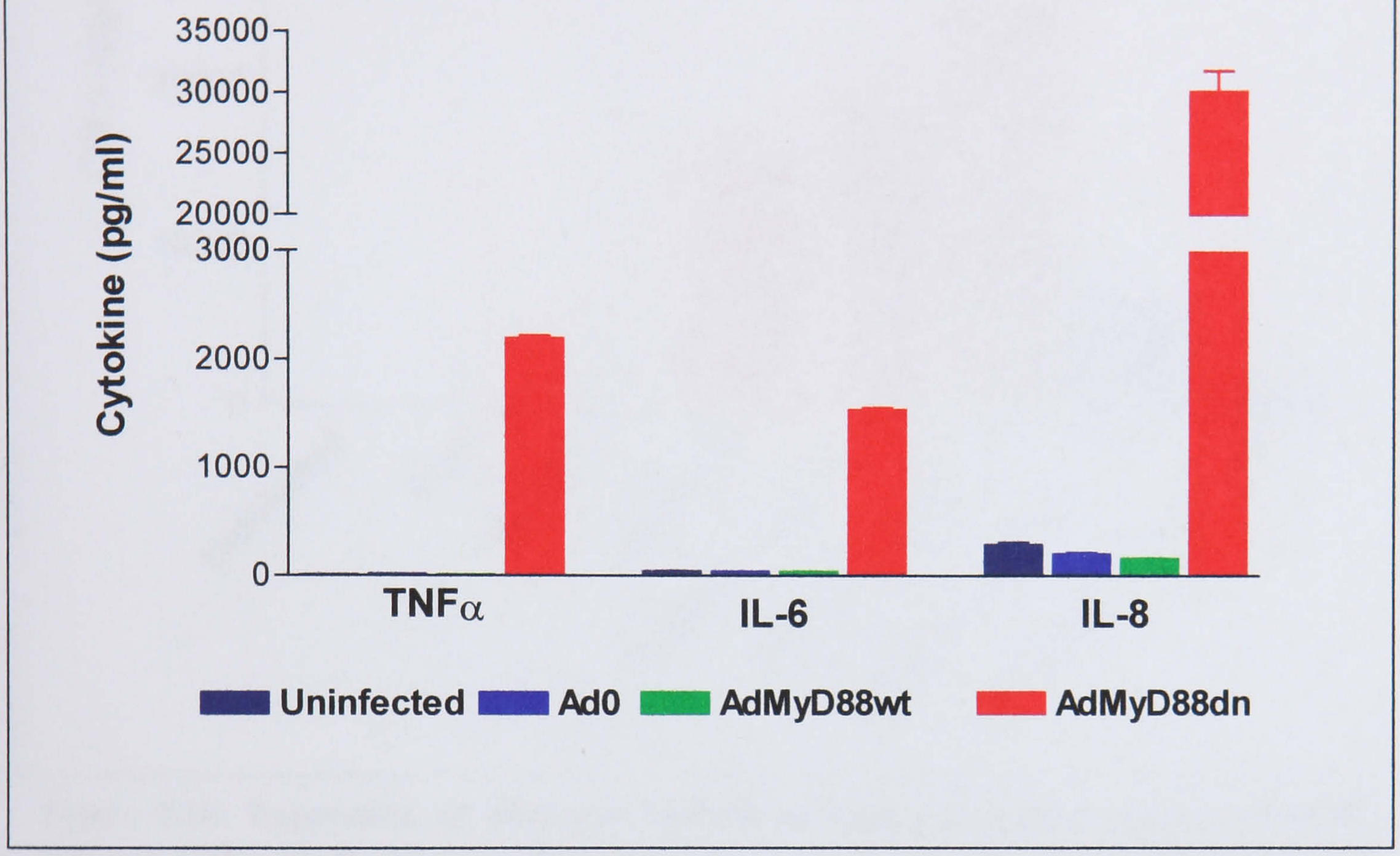


Figure 5.17: Overexpression of dominant negative MyD88 induces high levels of TNFα, IL-6 and IL-8 in immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were infected for 2h in serum-free medium with a control adenovirus without insert (Ad0) or adenoviruses expressing MyD88wt (AdMyD88wt) and MyD88dn (AdMyd88dn). Cells were then cultured for another 1 day in 5% FCS 1% penicillin/streptomycin and supernatants were collected and assayed by ELISA in triplicates for the presence of TNFα, IL-6 and IL-8. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of five independent experiments.

Using the technique of double adenoviral infection described in section 2.4.7, I found that the activation of DC by MyD88dn effect could be abrogated by expression of MyD88wt together with MyD88dn. Thus, DC activation seems to be MyD88dn-specific, resulting from the inhibition of MyD88 activity (figure 5.18).

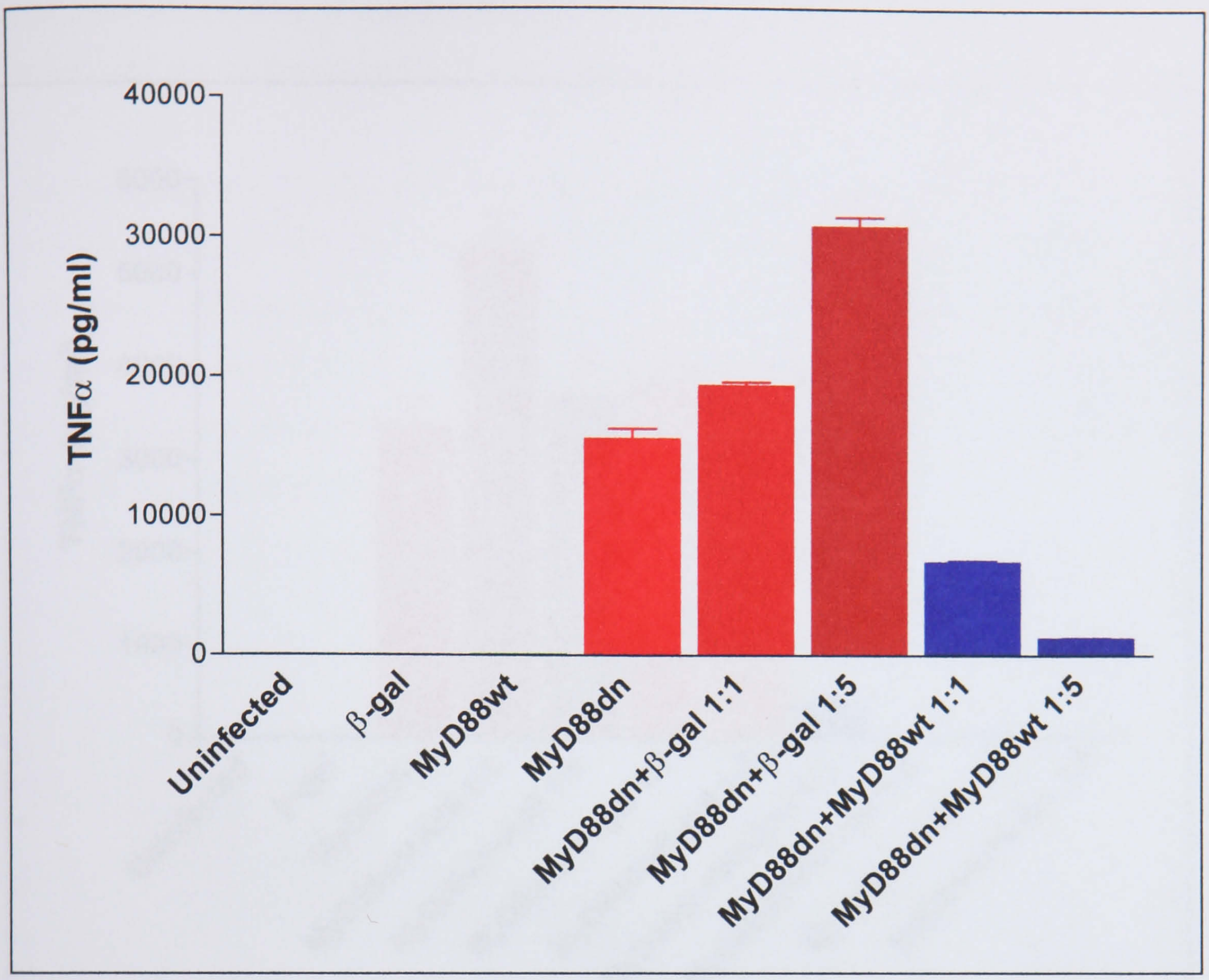


Figure 5.18: Expression of wild-type MyD88 abrogates dominant negative-MyD88-induced TNFα production im immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were infected for 2h in serum-free medium with an adenovirus expressing β-galactosidase (β-gal), adenoviruses expressing MyD88wt or MyD88dn, or combinations of them. A multiplicity of infection of 100 was used for each virus when used at a ratio of 1. Cells were then cultured for another 1 day in 5% FCS 1% penicillin/streptomycin and supernatants were collected and assayed by ELISA for the presence of TNFα. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

Using the same technique, I also investigated the involvement of IKK2 and IκBα in MyD88dn-induced DC activation. I found that overexpression of IκBα or a dominant-negative form of IKK2 in immature DC could inhibit MyD88dn-induced TNFα production (figure 5.19). This observation supports the idea that MyD88dn initiates the normal IL-1R/TLR pathway that requires the activation of IKK2, the phosphorylation of IκB and the nuclear translocation of NF-κB (Krutzik S.R. et al. 2001).

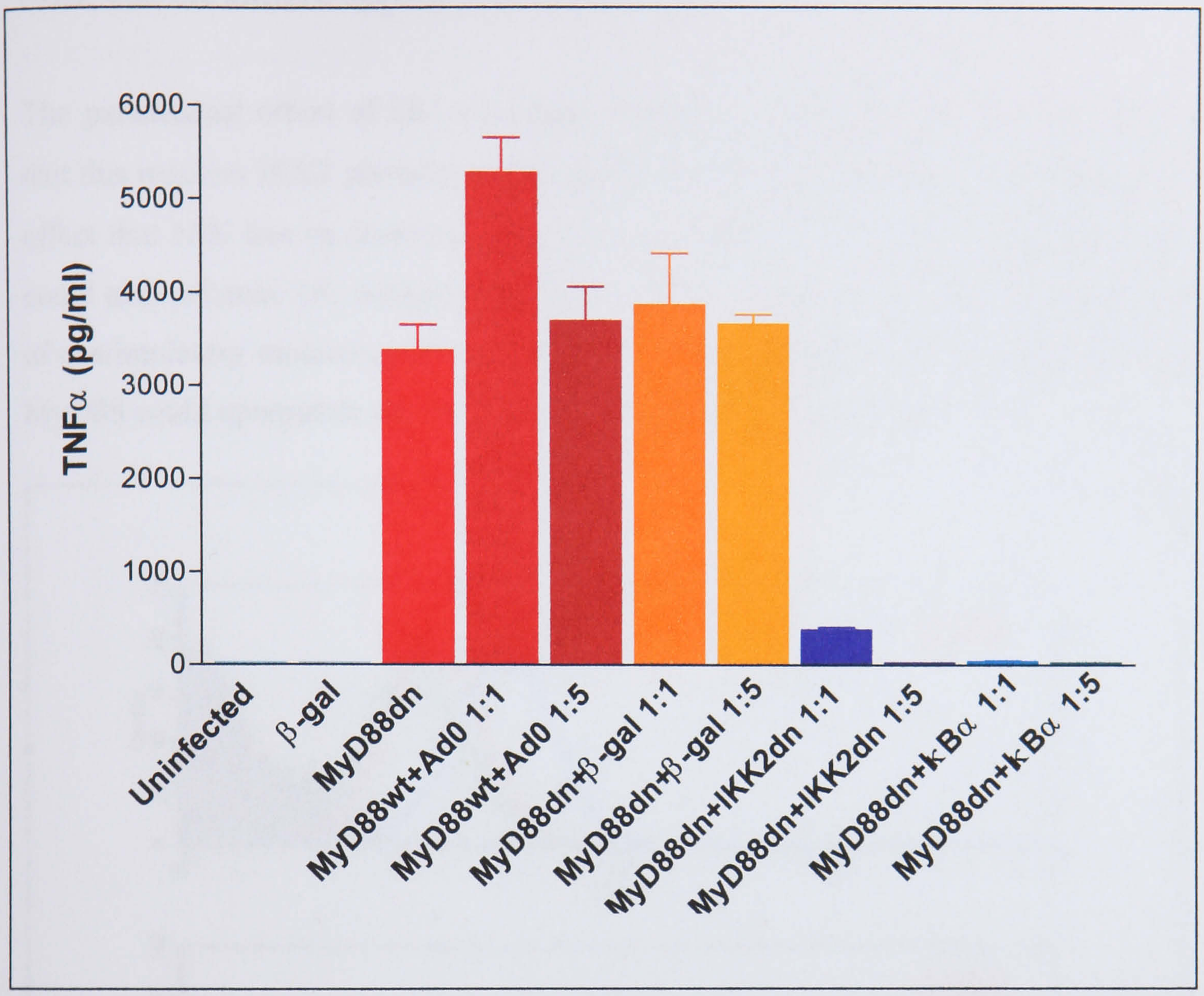


Figure 5.19: Expression of dominant negative IKK2 or IκBα inhibits dominant negative-MyD88-induced TNFα production in immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were infected for 2h in serum-free medium with an adenovirus without insert (Ad0), adenoviruses expressing β-galactosidase (β-gal), MyD88dn, a dominant negative form of IKK2 (IKK2dn), IκBα, or combinations of them. A multiplicity of infection of 100 was used for each virus when used at a ratio of 1. Cells were then cultured for another 1 day in 5% FCS 1% penicillin/streptomycin and supernatants were collected and assayed by ELISA for the presence of TNFα. Mean cytokine production (±SD) of triplicate cultures is shown and is representative of three independent experiments.

5.9. Overexpression of dominant negative MyD88 in DC enhances the allogeneic MLR and the antigen-specific T cell proliferation

The paradoxical effect of DC activation induced by MyD88dn and the observation that this requires IKK2 phosphorylation and IκBα degradation was reminiscent of the effect that NIK has on immature DC. This prompted me to investigate whether this could also enhance DC antigen-presenting function. First, I examined the expression of costimulatory molecules and I found that overexpression of the inhibitory form of MyD88 could upregulate the expression of CD80 and CD86 (figure 5.20).

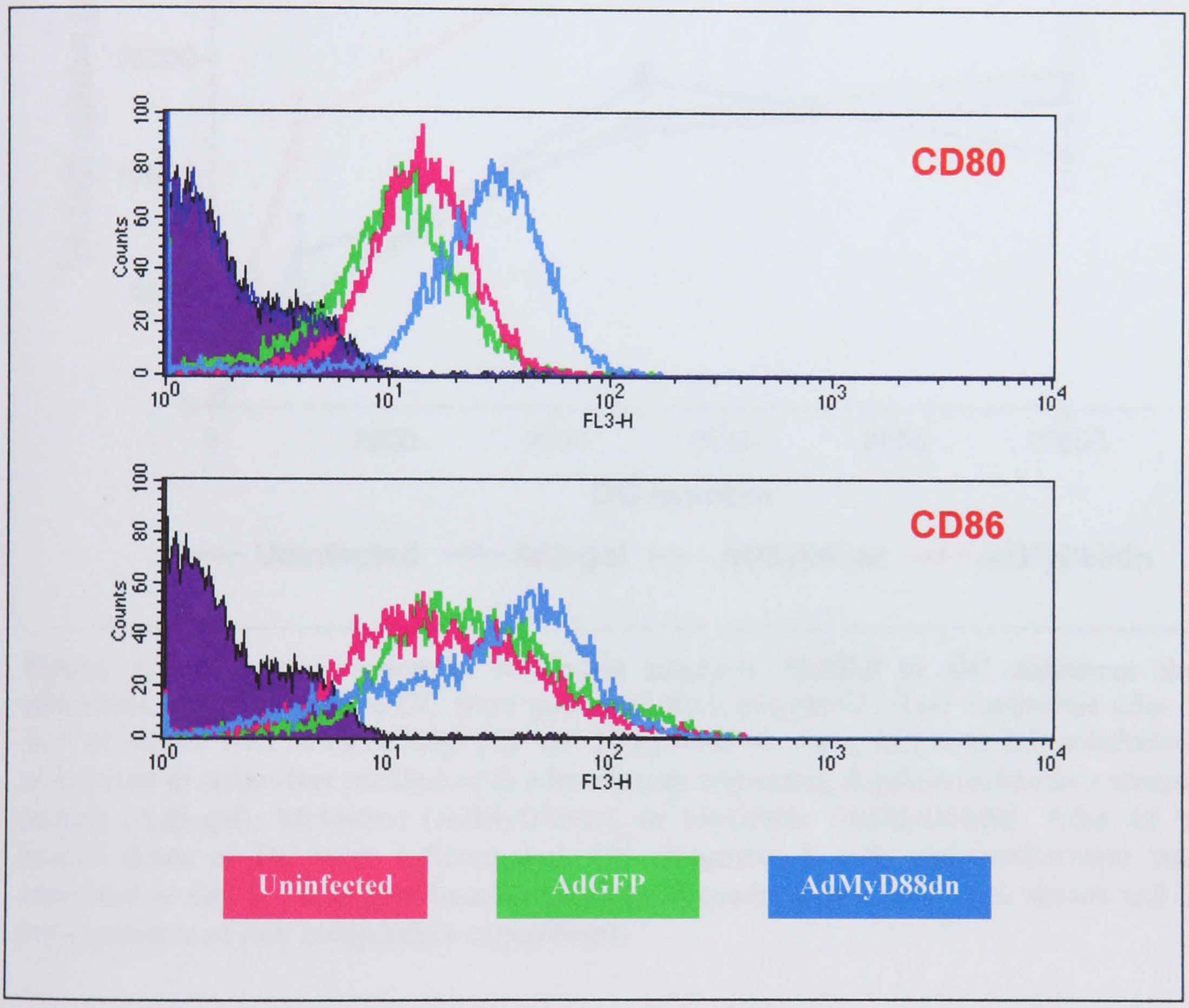


Figure 5.20: Overexpression of dominant negative MyD88 up-regulates the expression of the costimulatory molecules CD80 and CD86 in immature DC. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. DC were left uninfected or infected for 2h in serum-free medium with an adenovirus expressing GFP (AdGFP) or MyD88dn. A multiplicity of infection of 100 was used. Cells were then cultured in 5% FCS 1% penicillin/streptomycin, and after 2 days they were collected and examined for the expression of CD80 and CD86 by FACS staining. A representative of two independent experiments is shown.

Next, I examined the effect of MyD88dn in the allogeneic MLR and I found that its overexpression for 2 days in immature DC could enhance allogeneic T cell proliferation compared to uninfected DC or DC expressing β-galactosidase as a control protein (figure 5.21). Expression of MyD88wt, on the other hand, had no effect.

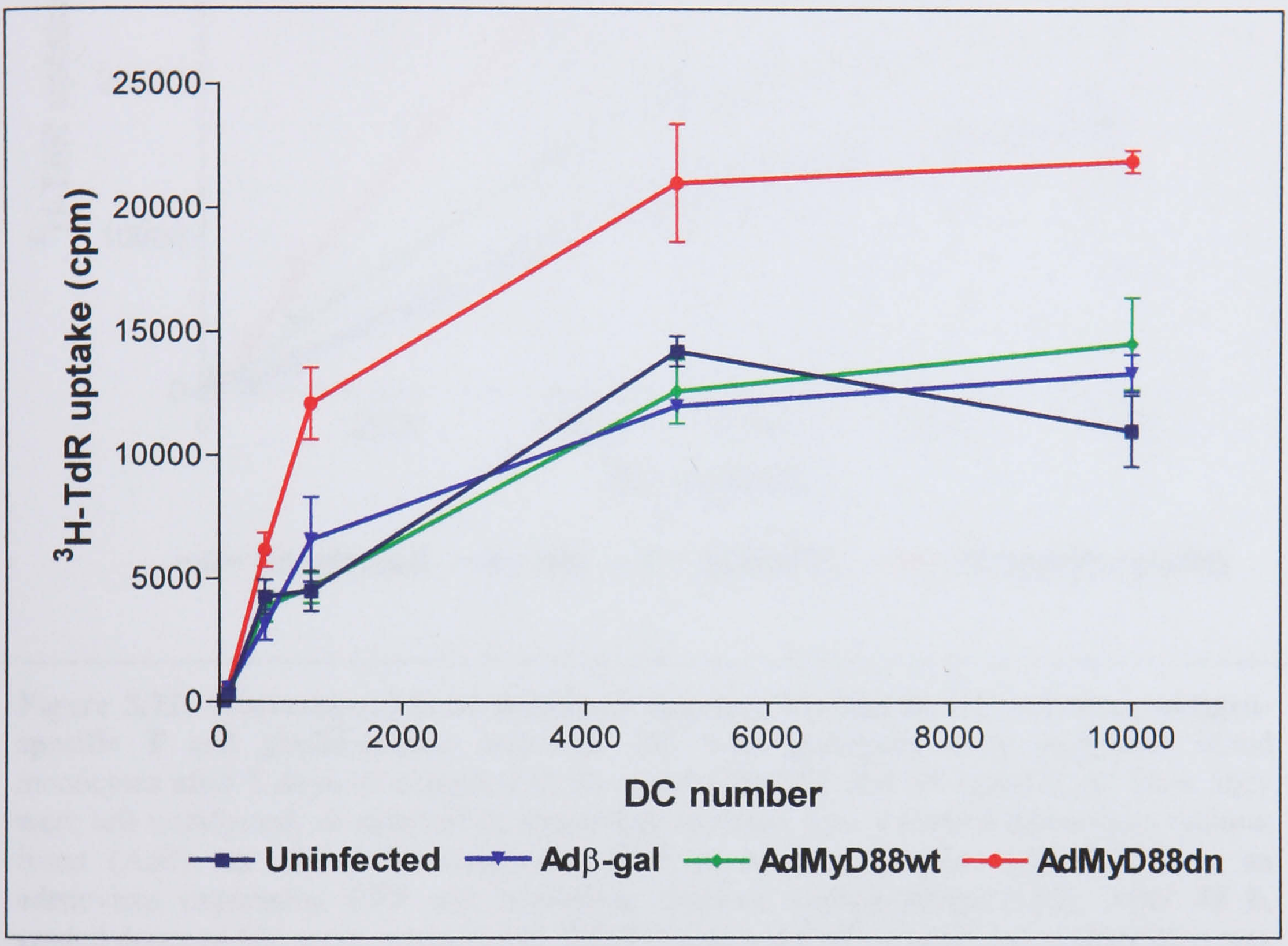


Figure 5.21: Overexpression of dominant negative MyD88 in DC enhances the allogeneic MLR. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected in serum-free medium with adenoviruses expressing β-galactosidase as a control protein (Adβ-gal), MyD88wt (AdMyD88wt), or MyD88dn (AdMyD88dn). After 48 h, graded doses of DC were cultured with 10^5 allogeneic T cells and proliferation was measured at day 5. Mean proliferation (±SEM) of quadriplicate cultures is shown and is representative of four independent experiments.

In addition, MyD88dn could also enhance the antigen-specific T cell proliferation. Using the same model of antigen-specific T cell proliferation, with recombinant GFP as a model antigen (chapter 4), I found that MyD88dn expression could enhance GFP-specific T cell proliferation (figure 5.22). These findings suggested that MyD88dn potently enhances DC antigen-presenting function.

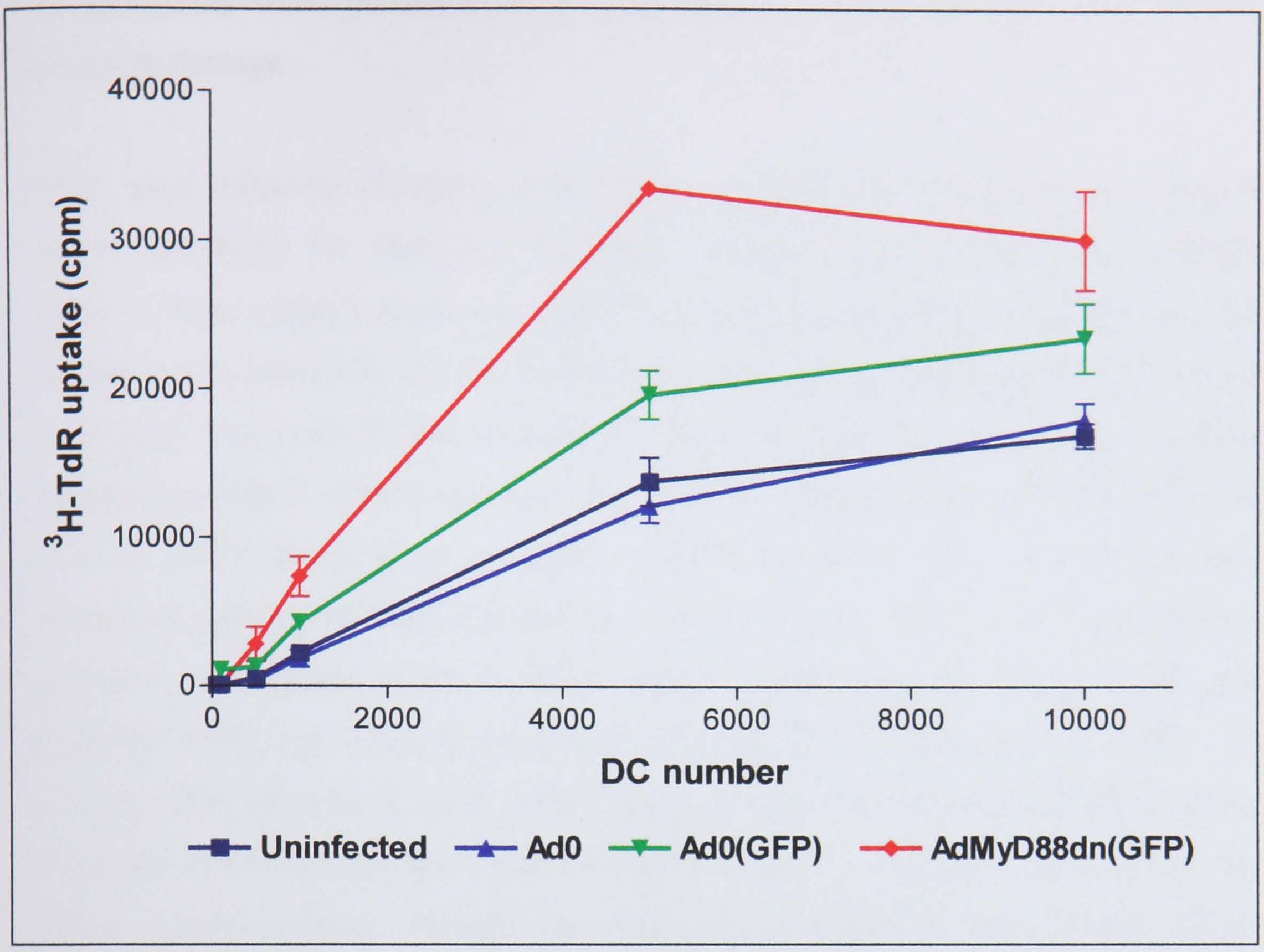


Figure 5.22: Overexpression of dominant negative MyD88 in DC enhances antigen-specific T cell proliferation. Immature DC were generated from peripheral blood monocytes after 5 days of culture with 50 ng/ml GM-CSF and 10 ng/ml IL-4. Then, they were left uninfected, or infected in serum-free medium with a control adenovirus without insert (Ad0), an adenovirus expressing GFP as a model antigen [Ad0(GFP], or an adenovirus expressing GFP and MyD88dn together [AdMyD88dn(GFP)]. After 48 h, graded doses of DC were cultured with $2x10^4$ antigen-specific T cells and proliferation was measured at day 3. Mean proliferation (±SEM) of quadriplicate cultures is shown and is representative of four independent experiments from two unrelated donors.

5.10. Discussion

The innate immune system has a dual role in host defense. First, it provides a direct and immediate line of defense against pathogens, and second, it directs the adaptive immune system to generate the appropriate response mechanisms. Central to the recognition of invading organisms are TLRs, a family of phylogenetically conserved mediators present in species from insects to humans. These receptors recognize numerous molecular patterns of pathogens ranging from bacterial LPS, lipoproteins and undermethylated DNA, to yeast carbohydrates, and initiate an inflammatory response. Understanding the mechanisms by which TLRs signal inside the cell after recognizing foreign structures at the cell surface is imperative in order to intervene

therapeutically when needed to induce appropriate immune responses or to prevent extensive damage.

TLRs have relatively divergent extracellular portions that have evolved to confer unique specificity for particular molecular structures. Their cytoplasmic portions, however, have a highly conserved region known as the toll domain. The fact that this domain is also shared by the IL-1R family members, suggested that TLR may signal in a similar way to the IL-1R. Binding of IL-1 to the IL-1R type I is known to induce aggregation with IL-1RacP and recruitment of the adaptor protein MyD88 (Wesche H. et al. 1997; Burns K. et al. 1998). MyD88 associates with the IL-1 receptors through its carboxy-terminal toll domain (Muzio M. et al. 1997). IL-1 is also known to induce recruitment of the IL-1R-associated serine/threonine kinase IRAK and probably IRAK-2 or IRAK-M too (Muzio M. et al. 1997; Wesche H. et al. 1997; Cao Z. et al. 1996; Wesche H. et al. 1999). These contain amino-terminal death domains (DD) that associate with the N-terminal death domain of MyD88. Then, IRAK gets highly phosphorylated, through an unknown mechanism, that results in its dissociation from the IL-1 receptors and its association with TRAF6 (Yamin T.T. and Miller D.K. 1997; Cao Z. et al. 1996). Subsequently, TRAF6 activates the Jun N-terminal kinase (JNK or p54 MAPK) and NF-κB (Song H.Y. et al. 1997; Baud V. et al. 1999).

Similarly, TLRs have being shown to employ an analogous framework of molecules comprising MyD88, IRAK and TRAF6 to signal downstream (Yang R.B. et al. 1998; Yang R.B. et al. 1999; Swantek J.L. et al. 2000; Kawai T. et al. 1999; Zhang F.X. et al. 1999). Thus, studies in 293 human embryonic kidney cells or endothelial cells have shown that MyD88, IRAK and TRAF6 are absolutely required for MAPK and NF-κB activation in response to IL-1 or LPS. The situation in macrophages, however, may be more complex as the construction of MyD88-deficient mice suggested. MyD88-deficient mice display defects in NF-κB activation in response to the structurally similar cytokines IL-1 and IL-18 (Adachi O. et al. 1998) and are highly resistant to LPS-induced shock and cytokine production. Despite that, LPS-induced MAPK and NF-κB activation in MyD88-deficient macrophages appear to be slightly delayed but otherwise normal (Kawai T. et al. 1999), implying that at least in

knockout mice other mechanisms may compensate for the absence of MyD88 in TLR signalling. Similarly, IRAK-deficient macrophages also display delayed but otherwise normal MAPK and NF-κB activation, suggesting either that IRAK-2 and IRAK-M can compensate for the absence of IRAK, or that other mechanisms can bypass IRAK to activate the cell (Kanakaraj P. et al. 1998; Swantek J.L. et al. 1999). In contrast, macrophages from TRAF6-deficient mice show a complete loss of NF-κB activation in response to LPS stimulation (Lomaga M.A. et al. 1999). The conclusion from these studies is that LPS is able to induce MAPK and NF-κB activation in mouse macrophages in a MyD88/IRAK-independent but TRAF6-dependent way.

In this study, I examined the role of MyD88 in primary human macrophages and DC. I used replication-deficient adenoviruses as a tool of efficient gene transfer and MyD88wt or MyD88dn as specific activators or inhibitors of MyD88 activity, respectively. The dominant negative/inhibitory form of MyD88 has a point mutation at position 56 and a deletion in its death domain that prevents DD-DD interactions (see table 2.6). Expression of MyD88dn in HSF or HUVEC prevents IL-1- or LPS-induced MAPK and NF-κB activation as well as cytokine production, a finding that is in agreement with previous studies and suggests that our dominant negative form is indeed acting as an inhibitory molecule. On the other hand, overexpression of MyD88wt could induce NF-κB activation in HSF but not HUVEC, demonstrating that the intracellular signalling pathways that take place in these cell types are different.

When, however, I tested the effect of MyD88dn in human macrophages, the results were unexpected. MyD88dn rather than MyD88wt induced macrophage TNFα, IL-6 and IL-8 production in the absence of additional stimulation. Its overexpression induced IRAK and p38 MAPK phosphorylation, as well as NF-κB activation. Examination of the cytosolic levels of IκBα showed that it becomes phosphorylated and degraded after 6h of MyD88dn overexpression. After 24h, resynthesis is occurring and continues up to 48h, whereas phosphorylation of IκBα is still taking place and is detectable even after 48h. At the same time, NF-κB gets activated and can be measured 24h after overexpression. These data provide evidence for a possible role of MyD88 in the generation of an inhibitory signal that prevents macrophage activation. Interestingly, if this inhibitory role of MyD88 exists, it occurs early in the

IL-1R/TLR pathway and prevents ligand-independent IRAK phosphorylation and activation.

Subsequently, I examined the effect of MyD88 in LPS-induced macrophage activation. Expression of MyD88dn was able to prevent MAPK and IκBα phosphorylation in response to LPS, but not TNFα production. These results resembled the tolerance events induced in peritoneal macrophages after pretreatment with LPS (Medvedev A.E. et al. 2000) where LPS-tolerant cells failed to phosphorylate p38, p42/44 and p54 MAPK, and exhibited delayed IκBα degradation and NF-κB activation. The only difference was that LPS-tolerant macrophages had decreased TNFα production too, whereas MyD88dn-expressing macrophages had not. In addition, another study reported that cross-tolerance between different TLR can occur, as TLR2-mediated activation of macrophages by mycoplasmal lipoproteins inhibits the response of macrophages to a second LPS challenge and vice versa (Sato S. et al. 2000). Taken together, these findings suggested that the inhibitory effect that MyD88dn has on MAPK and IκBα phosphorylation may not be due to a direct effect in TLR signalling.

When, however, I used the lipid A portion of LPS as a TLR ligand, I found that MyD88dn could inhibit both MAPK and IκBα phosphorylation, and LPS-induced TNFα production. When my colleagues tested the specificity of the commercially available LPS and lipid A preparations, they found that neutralizing antibodies to TLR4 could block only lipid A-induced TNFα production. Repurification of the commercially available LPS to get rid of other bacterial proteins such as lipoproteins and peptidoglycans that may be contaminating the preparation and result in a TLR4-independent signal, did not alter this result (Dr. Stonehouse T., unpublished observation). This suggested that LPS and its lipid A portion are not equivalent with respect to macrophage activation, and that LPS may be using a TLR-independent pathway too. This would explain why MyD88dn-tolerant macrophages can block lipid A, a TLR4-specific signal, but not LPS, although it still would leave doubt whether this is due to MyD88dn blocking TLR signalling directly or indirectly through tolerance induction. A similar mechanism of macrophage tolerance could account for the paradoxical data of MyD88-deficient macrophages where LPS-induced cytokine

production is inhibited but MAPK and NF-κB activation are only delayed. Recently, Nod1 and Nod2, the human homologs of the plant disease-resistant R genes, have been proposed to act as LPS receptors too (Inohara N. et al. 2001). These are cytosolic molecules, contain leucine-rich repeats and were shown to bind LPS and activate NF-κB in 293 human embryonic kidney cells in a TLR4- and MyD88-independent manner. Whether these molecules also sense bacterial LPS is not clear. But with at least 20 Nod1-like sequences in the human genome database (Yang R.B. et al. 1998), these receptors may turn out to be another family of major importance in immunity.

The expression of MyD88dn in DC resulted in the unexpected outcome of activation rather than inhibition as well. This phenomenon was similar to that observed in macrophages but was more pronounced. Thus, it resulted in the activation of NF-κB and the production of high levels of TNFα, IL-6 and IL-8 that could be inhibited by the simultaneous expression of MyD88wt. This prompted me to investigate whether inhibition of MyD88 in DC could enhance their antigen-presenting function. Indeed, expression of MyD88dn could increase the allogeneic MLR as well as the antigen-specific T cell proliferation, suggesting that blockade of MyD88 activity has a potent adjuvant effect in DC.

It is not clear how the expression of MyD88dn can result in the activation of human primary macrophages and immature DC. Undoubtedly, the expression of this MyD88 form that has its death domain disrupted by a point mutation and a 53 amino acid deletion, allows a TLR-like pathway with IRAK and IKK2 activation, and NF-κB nuclear translocation to take place. This could be due to an artifact of the high levels of expression of MyD88dn disrupting the stoichiometry of intracellular signalling complexes involved in TLR signalling, Or, it could be due to MyD88 playing an inhibitory role early in the IL-1/TLR signalling pathway. The observations that the expression of high levels of MyD88wt has no effect in macrophages or DC, and that MyD88wt can inhibit the effect seen after expression of MyD88dn, suggest that an inhibitory role of MyD88 may be a more plausible explanation.

According to that, MyD88 prevents the phosphorylation of IRAK, either by blocking IRAK autophosphorylation or by interfering with the binding of a crucial signalling

molecule such as a second kinase (Li X. et al. 1999). Thus, inhibition of MyD88 activity, by expression of MyD88dn, allows IRAK phosphorylation to take place and the IL-1R/TLR pathway to become activated in a ligand-independent manner. This is reminiscent of the silencer of death domains (SODD), a protein found to be associated with the death domains of TNF-R1 and death receptor-3 (DR3). SODD prevents signalling from these DD-containing receptors, and is only released in the presence of ligand binding (Jiang Y. et al. 1999). Similarly, MyD88 may be associated with a "silencer" protein that prevents IL-1R/TLR signalling.

Support for an inhibitory pathway preventing IRAK phosphorylation comes from the recent identification of Tollip, a novel component of the IL-1 pathway (Burns K. et al. 2000). Tollip is present in a complex with IRAK, and is recruited in the IL-1RI complex only after binding of IL-1 to its receptor. The recruitment of Tollip/IRAK complexes to the activated receptor complex occurs through the association of Tollip with IL-1RacP, and results in the rapid dissociation of Tollip. This is required for the phosphorylation of IRAK and the activation of JNK and NF-κB, as overexpression of Tollip prevents this from happening. These findings lead to the suggestion that Tollip may act by silencing a component or components of the IL-1-signalling cascade such as IRAK. MyD88, on the other hand, is not required for that process but seems to be also recruited to the IL-1R independently of IRAK-receptor association. Although it was proposed that IRAK may get phosphorylated through DD-DD interactions with MyD88, Burns and colleagues found evidence that less IRAK associates with the IL-1R in cells overexpressing MyD88 than in cells that do not. This finding suggests that MyD88 may be preventing the recruitment of IRAK to the IL-1R complex in the absence of ligand-binding. As the Burns study was done in 293 human embryonic kidney cells, this effect may be more pronounced in other cell types such as macrophages and DC. In Drosophila, the Toll receptor also has an inhibitory domain localised in its C-terminal 60 residues that may block ligand-independent Toll signalling by impeding the catalytic activity of Pelle, an IRAK homologue (Shen B. and Manley J.L. 1998). The fact that this region is not conserved in other members of the IL-1 family suggests that mammals may have evolved other mechanisms of a similar inhibitory function. Our study together with that of Burns suggests that such

an inhibitory pathway seems to be present in certain cell types and to prevent IRAK phosphorylation by utilising Tollip and/or MyD88.

The question thus arising is why this inhibitory role of MyD88 is not universal but is restricted only to certain cell types. Recently, it has been reported that TLRs interact with each other forming functional pairs (Ozinsky A. et al. 2000). This increases the degree of specificity of the TLRs, with TLR2 homodimers recognizing bacterial lipoprotein and TLR2-TLR6 heterodimers recognizing bacterial peptidoglycan. Functional cooperation between TLRs also occurs in their cytoplasmic domains. Thus, and in the absence of additional stimulation, dimerization of the cytoplasmic domain of TLR1, TLR2 or TLR6 does not induce NF-κB activation or TNFα production in CHO cells or RAW-TT10 macrophages, respectively, whereas dimerization of the cytoplasmic domain of TLR4 does (Ozinsky A. et al. 2000). In contrast, heterodimerization of the cytoplasmic domains of TLR1 with TLR2 or TLR2 with TLR6 but not TLR1 with TLR6 induces NF-κB activation and TNFα production (Ozinsky A et al. 2000). This observation clearly shows that the homologous cytoplasmic domains of TLRs are not functionally equivalent with respect to signalling, and may be recruiting different complexes in the presence or absence of ligand binding. Functional interactions of TLRs have been proposed by another study too, showing that TLR2 cooperates with TLR6 to mediate the response to phenol-soluble modulin, a factor secreted by Staphylococcus epidermidis (Hajjar AM et al. 2001). Interestingly, in this study TLR6 could increase the response of TLR2 to phenol-soluble modulin, whereas TLR1 could inhibit that response. This observation shows the complexity of TLR interactions, and introduces the idea that apart from increasing specificity and response to pathogens, functional cooperation of TLRs may also exert a negative regulatory effect.

All these studies indicate how important the particular repertoire and the relative abundance of TLR homo- and heterodimers that each cell expresses are, and provide possible explanations for my data. Thus, in macrophages and DC the complex interactions of TLRs may result in a state that is able to activate the IL-1R/TLR signalling pathway in the absence of ligand binding. What prevents that from happening is MyD88 that induces an inhibitory signal, and inhibition of MyD88

activity in these cell types allows this activation process to proceed. HSF and HUVEC have combinations of TLRs that may not be in a state of ligand-independent TLR signalling, and inhibition of MyD88 activity would not activate these cells. On the contrary, in some TLR dimers, MyD88 could be required for TLR signalling for some unknown reason. Support for different roles of MyD88 in IL-1R/TLR family members comes from the recent studies in MyD88-deficient mice where IRAK, MAPK and NF-κB activation is completely abrogated when the signal comes through the IL-1R and IL-18R but not when it comes through TLR4. Whether there are other MyD88-like molecules that associate with different IL-1/TLR members and account for differences in downstream signalling is not known. Although the precise mechanisms by which MyD88 exerts its inhibitory function are not yet understood, my data clearly demonstrate that the role of MyD88 in IL-1/TLR signalling is much more complex than previously thought. Actual studies aimed at identifying the molecules with which MyD88 interacts in HSF, HUVEC, macrophages and DC are required to explain the paradoxical effects of MyD88 inhibition observed.

The benefits of understanding how IL-1R/TLR family members signal are multiple. Cytokines such as IL-1 and IL-18 play a crucial role in inflammation as they induce the production of various proinflammatory proteins including cycloxygenase, adhesion molecules, cytokines, chemokines, tissue-degrading enzymes and acute-phase proteins such as serum amyloid A (O'Neill L.A.J. and Dinarello C.A. 2000). TLRs, on the other hand, play a crucial role in the host immune response to a wide range of pathogens by initiating and regulating the innate and adaptive parts of the immune system. Recent evidence suggests that TLRs may be more widely involved in immune and inflammatory functions than previously thought, as heat shock protein 60 and EDA-containing fibronectin fragments are capable of activating TLR4 and inducing LPS-like responses (Ohashi K. et al. 2000; Okamura Y. et al. 2001). Thus, the unwinding of the complex mechanisms involved in IL-1R/TLR signalling, and the complex interactions between different TLRs may provide important clues about the regulation of inflammation and may result in novel therapeutic targets for a wide range of pathological conditions including septic shock and chronic inflammatory diseases. The findings presented in this chapter argue that MyD88 is unlikely to be a valid target for the development of novel anti-inflammatory drugs. Moreover, the

observation that blockade of MyD88 activity specifically activates macrophages and DC, and potently enhances DC antigen-presenting function, suggests that mutated inhibitory forms of MyD88 or other molecules that inhibit MyD88 activity may act as novel adjuvants that could be useful in the design of more convenient molecular vaccines.

CHAPTER 6

NIK ENHANCES THE IMMUNE RESPONSE AGAINST A VECTOR-ENCODED ANTIGEN IN VIVO AND SKEWS IT TOWARDS A T_{H1} PROFILE

6.1. Introduction

Vaccination was originally developed by Jenner and Pasteur in order to confer protection against smallpox and cholera (Jenner E. 1778; Pasteur L. 1880), and is the most cost effective and efficacious medical intervention that has taken place to date. It takes advantage of the specificity and inducibility of the immune system to prevent disease, and has resulted in the eradication or virtual elimination of various human diseases including smallpox. Traditional vaccines are diverse, and may either consist of live attenuated pathogens, whole inactivated organisms, or inactivated toxins. Although the most effective vaccines by far are live attenuated microorganisms and viruses, or sometimes closely related non-pathogenic microorganisms, several drawbacks have limited their use against more challenging diseases such as hepatitis C and AIDS. First, live attenuated vaccines carry some risk of revertion to a more virulent phenotype and may also be potentially lethal in immunosuppressed or immunodeficient individuals. Second, whole inactivated vaccines (e.g. Bordetella pertussis) contain reactogenic components such as toxins, that can still cause undesirable side effects. Third, some pathogens (e.g. hepatitis B, hepatitis C, and human papillomavirus) are difficult or even impossible to grow in culture, forbiding the preparation of a vaccine against them.

With the advent of DNA technology, several new approaches to vaccine development have emerged that may offer significant advantages over traditional approaches. These new approaches include the use of recombinant protein subunits, synthetic peptides, and plasmid DNA, and have benefited from the continuously increasing understanding of infectious disease pathogenesis. Thus, the identification of antigens associated with various aspects of the life cycle of the infectious agent such as its entry, replication and progression, has provided novel targets against which the immune response should be directed. In addition, the identification of cellular antigens that are associated primarily with tumour cells has introduced the idea of using vaccination to specifically kill tumour cells, a process termed tumour immunotherapy. Although less toxic, immunization with purified or recombinant antigens alone usually elicits poor or weak immunity, whereas traditional vaccines contain many heterogeneous antigens, some of which can activate a

broader spectrum of T cells or function as immunostimulatory molecules. To increase their immunogenicity, antigens need to be administered together with adjuvants.

Adjuvants were originally described by Ramon as "substances used in combination with a specific antigen that produce more immunity than the antigen alone" (Ramon G. 1924). A very wide range of materials have been evaluated for their use as adjuvants (Singh M. and O'Hagan D. 1999) but despite that, only aluminium-based mineral salts have obtained approval for their use in humans by the US Food & Drug Administration (Rockville, MD). Most of the other adjuvants tested in clinical trials were found to be potent but too toxic for routine clinical use in healthy individuals. If, however, adjuvants are aimed to be used in patients with life-threatening situations such as cancer, toxicity becomes less of an issue.

Most conventional adjuvants support the generation of some types of immune responses but fail to induce other important arms of the immune response that may be required for the generation of long-lasting immunity. The fact that the great majority of the adjuvants tested have been discovered empirically and their mechanism of action is poorly understood, has hampered the rational design of novel adjuvant compounds with reduced toxicity and increased efficiency. However, this situation is now changing due to the progress made in the understanding of the immune system, and attempts are underway to design more sophisticated adjuvants that specifically act in the regulation of the immune response.

According to the current concepts, a productive immune response is defined by the generation of clonally expanded antigen-specific T and/or B cells. This requires presentation of the antigen to specific TCRs on naïve T cells or BCRs on B cells. Central to that mechanism are dendritic cells, the most potent antigen presenting cells identified to date. Immature dendritic cells are highly specialized in the uptake and processing of antigens and reside in the peripheral tissues from which they traffic to lymphoid organs where they present antigen to T cells (Banchereau J. and Steinman R.M. 1998). In the presence of an inflammatory stimulus in the periphery, this rate of migration of DC to

lymphoid organs is greatly increased, as is their ability to present antigen to T cells and induce their proliferation and activation. T cells, in turn, activate B cells and antibody responses, and/or differentiate to effector cells to clear the invading organism. The potency of DC in initiating immune responses has been shown in various systems where antigen-loaded dendritic cells could prime resting T cells in mice and elicit protective antiviral and antitumour immunity (Mayordomo J.I. et al. 1995; Fields R. et al. 1998; Su H. et al. 1998; Ludewig B. et al. 1998; Song W. et al. 1997; Specht J.M. et al. 1997). In humans, immunization with antigen-pulsed DC has also been reported (Nestle F.O. et al. 1998). This suggests that for immunization purposes, the antigen should be targeted to dendritic cells and that the adjuvant preparation should aim to activate DC and increase their antigen-presenting ability.

I have previously shown (chapter 4) that activation of the NF-κB pathway by overexpression of the wild-type form of NF-κB-inducing kinase (NIK) enhances DC antigen-presenting function through a mechanism involving the production of cytokines and chemokines, and the up-regulation of antigen-presenting MHC and costimulatory molecules. In this chapter, I examined the effect of NF-κB activation via overexpression of NIK in the induction of immune responses in vivo. The observation that NIK cDNA is sufficient to activate DC suggested that its major use as an adjuvant may be in genetic vaccines.

Several types of genetic vaccines exist, including viral, bacterial or naked DNA vaccines. Viral or bacterial vectors that invade the cytoplasm of cells are routinely used in experiment protocols of vaccination. These include adenoviruses, vaccinia viruses, Salmonella, *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or *Listeria monocytogenes*, and offer the advantage of introducing antigens directly into the antigen-presenting cells (Panicali D. et al. 1983; Morin J.E. et al. 1987; Dietrich G. et al. 1999). Their disadvantages, however, include the potential to cause disease in humans, especially immunocompromised individuals, and the production of neutralizing antibody responses to the vector, that may render further immunizations ineffective. Naked DNA vectors, on the other hand, consist of plasmid expression vectors that allow repeated

immunizations to be effective, and seem to be safer than viral vectors, although plasmid integration into the genome could theoretically mutate or disrupt host genes (Tang D.C. et al. 1992; Ulmer J.B. et al. 1992; Fynan E.F. et al. 1993; Donnelly J.J. et al. 1995; Dittmer U. et al. 1998).

For the purpose of my study to determine whether NIK activation of DC would provide adjuvant action, I devised a system of DNA vaccination by using replication-deficient adenoviruses as DNA delivery vehicles. As antigen, it was convenient to use green fluorescent protein (GFP), a jellyfish protein already incorporated into our adenoviral vectors that has been previously found to be immunogenic in animals (Stripecke R. et al. 1999). Previous studies performed by others to assess the potential of replication-deficient adenoviruses as vaccine vehicles have used the bacterial protein β-galactosidase (β-gal) as a model antigen. As an experimental host, I decided to use BALB/c mice, a strain that is genetically skewed to practice T_{H2} responses and is commonly used for vaccine studies. As a route of immunization, I decided to use subcutaneous immunization that targets skin DC. Thus, the purpose of my experiment was to examine whether incorporation of the NIK gene into the same viral vector that encodes the prototype antigen GFP, could enhance the immune response against that antigen, and whether it could skew it to a T_{H1} profile. Comparisons of that with traditional animal adjuvants such as complete Freund's adjuvant (CFA) were also included.

There are several ways of measuring the immune response against a specific antigen. Commonly, antibody responses that are indicative of B cell responses (De Franco A.L. 1987), and lymph node cell proliferation responses that are indicative of T cell responses in animals (Alkan S.S. 1978), are evaluated. The presence of cytotoxic responses is tested by measuring the ability of cytotoxic T lymphocytes to kill target cells, whereas cell-mediated immune responses are examined by measuring the proliferation of lymph node cells and the delayed-type hypersensitivity reaction (DTH). To test whether immunization is protective against disease, the challenge of the organism with the disease-causing infectious agent or cancer cells needs to be used, and disease progression assessed. Unfortunately, this study is far from complete, and the data presented in this chapter are

limited to the examination of B and T cell responses only. Nevertheless, this study will provide evidence that the incorporation of NIK and hence probably other NF-κB-activating intracellular signalling molecules into DNA, and may be a novel potent way of enhancing and skewing the immune response towards T_{H1}-type immunity, the type of immunity needed for efficient protection against viruses, various parasites and cancer.

6.2. Overexpression of human NIK activates mouse macrophages and induces a DC-like phenotype

Our adenoviral construct expresses the wild-type form of the human NIK. NIK is highly conserved between human and mouse with 85% identity in the amino acid sequence (Shinkura R. et al. 1999). To confirm that human NIK can activate mouse macrophages, I have used adenoviral gene transfer to overexpress it into starch-elicited macrophages. As expected, overexpression of NIK is sufficient to induce TNFα production (figure 6.1). Addition of LPS synergizes with NIK to superinduce TNFα (figure 6.1).

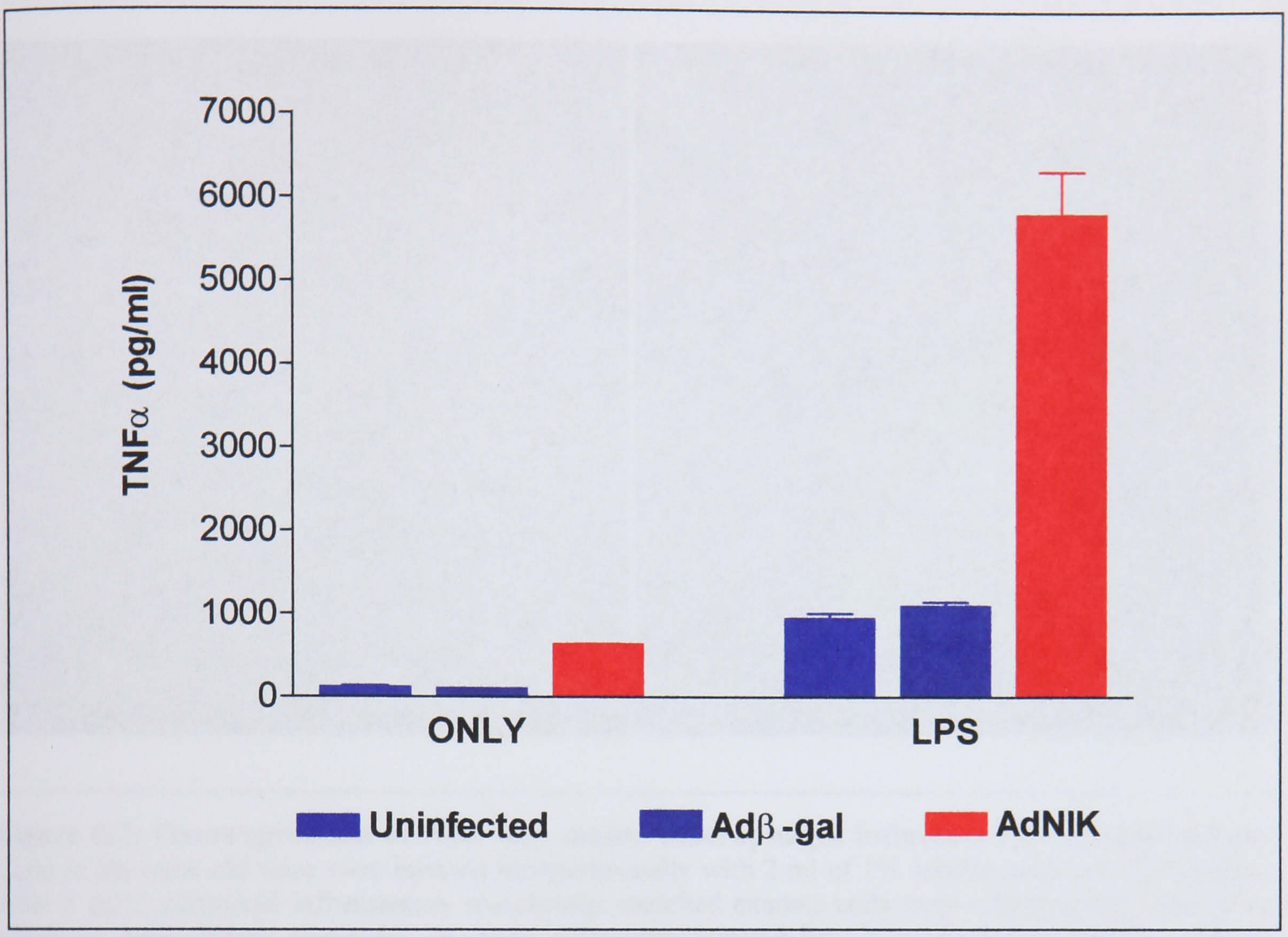


Figure 6.1: Overexpression of human NIK into mouse macrophages induces TNFα production and enhances TNFα production induced by LPS. Eight to ten week old mice were injected intraperitoneally with 2 ml of 1% soluble potato starch solution. After 5 days, peritoneal inflammatory macrophage-enriched exudate cells were collected and allowed to adhere to tissue culture plates for 2h. Then, they were left uninfected or infected for 2h in serum-free medium with an adenovirus expressing the control protein β-galactosidase (Adβ-gal) or wild-type NIK (AdNIK). Cells were subsequently cultured in DMEM containing 5% FCS, and stimulated with 10 μg/ml LPS. After a further 24h, supernatants were collected and assayed for the presence of TNFα. Mean TNFα levels (±SD) of triplicate cultures from a representative of four experiments is shown.

The incorporation of GFP in the adenoviral vector allows the careful examination of the cells by fluorescence microscopy. Interestingly, overexpression of NIK into mouse macrophages not only induces their activation, but also induces their differentiation from a round to a dendritic cell-like phenotype with extended dendrites and protrusions (figure 6.2). The cells increase in size, with a subpopulation of them seemingly increasing significantly more in size more than the rest (figure 6.2).

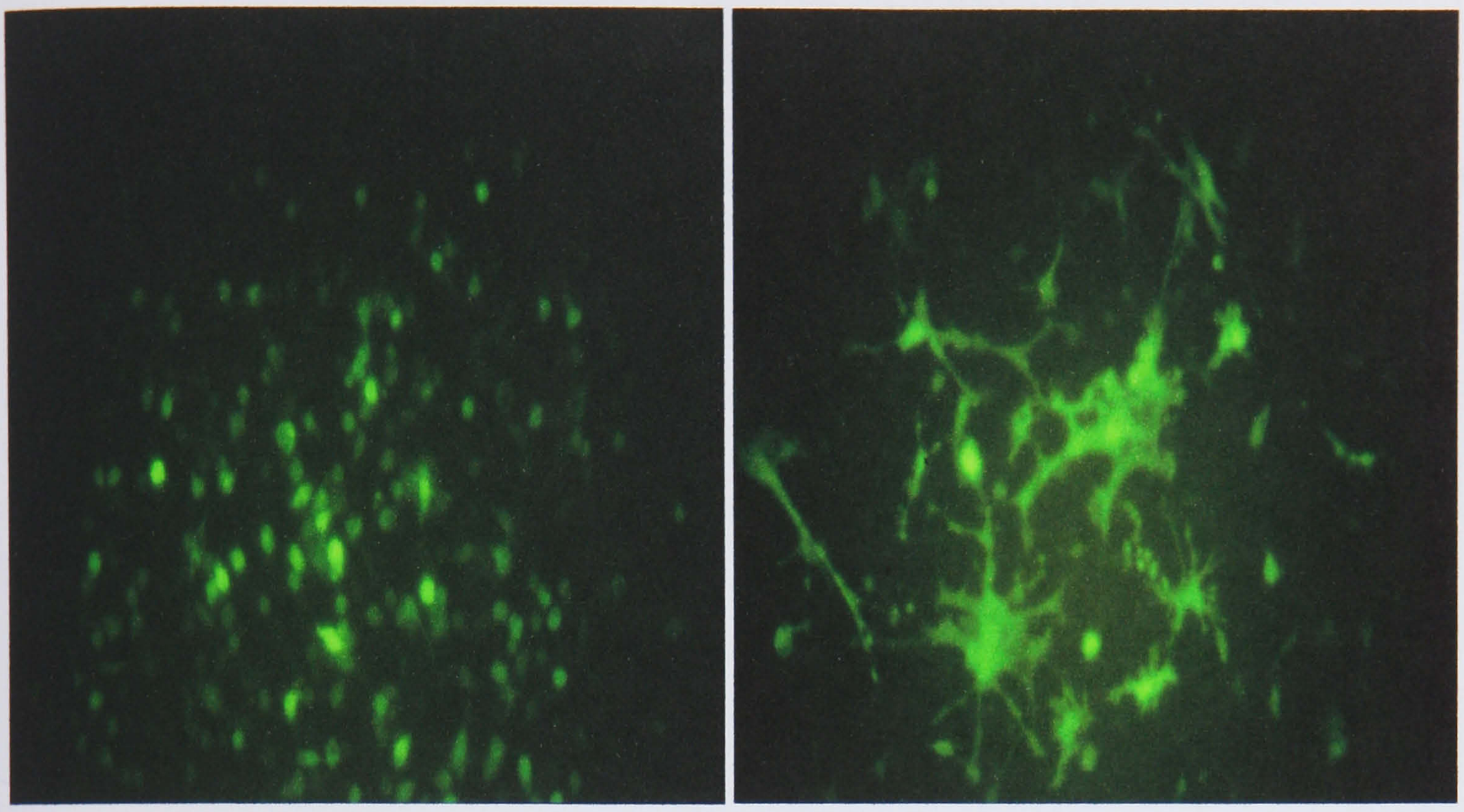


Figure 6.2: Overexpression of NIK into mouse macrophages induces a DC-like phenotype. Eight to ten week old mice were injected intraperitoneally with 2 ml of 1% soluble potato starch solution. After 5 days, peritoneal inflammatory macrophage-enriched exudate cells were collected and allowed to adhere to tissue culture plates for 2h. Then, cells were infected for 2h in serum-free medium with an adenovirus expressing expressing GFP [Ad0(GFP)] alone or GFP together with NIK [AdNIK(GFP)]. Cells were subsequently cultured in DMEM containing 5% FCS for 24h, and examined by fluorescence microscopy (original magnification X20). A representative of four experiments is shown.

6.3. Immunization with recombinant GFP and complete Freund's adjuvant induces strong humoral responses

A recent study has shown that the jellyfish *Aequorea victoria* protein GFP induces a strong immune response that results in the lysis of GFP-expressing leukaemic cells in BALB/c mice (Stripecke R. et al. 1999). As GFP is incorporated into our adenoviral vectors, I decided to use this protein antigen for immunization studies. To confirm the study by Stripecke and colleagues, BALB/c mice were immunized with 20 μg of recombinant GFP emulsified in CFA, and GFP-specific antibody responses were measured 14 days after immunization. A strong antibody response against GFP was detected in immunized animals consisting of high IgG and negligible IgM levels (figure

6.3), but the relative contribution of IgG and IgM antibodues to the total antibody production could not be deduced from this assay.

When I examined the IgG isotype profile of the GFP-specific antibody response, I found that it contained high IgG1 and low IgG2a levels (figure 6.3). This suggested that immunization with recombinant GFP in CFA induces mainly T_{H2} responses in BALB/c mice, as IgG2a antibody levels correlate with T_{H1} and IgG1 antibody levels with T_{H2} profiles (Mosmann T.R. and Coffman R.L. 1989).

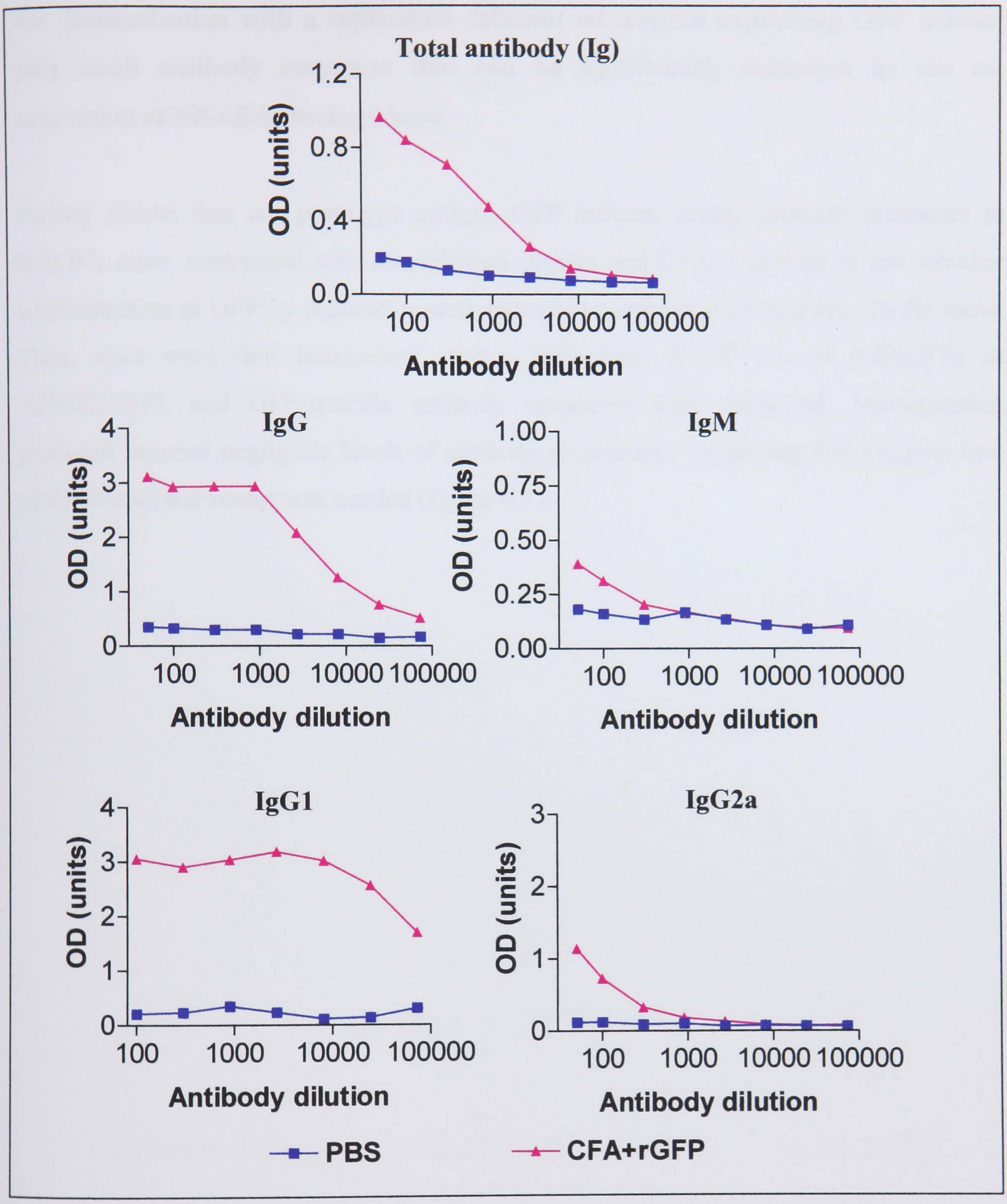


Figure 6.3: Immunization of BALB/c mice with recombinant GFP in CFA induces anti-GFP antibody responses. Groups of five BALB/c mice (eight to ten weeks old) were vaccinated by the subcutaneous route with PBS or 20 μg/ml of recombinant GFP emulsified with CFA at a 1:1 ratio. A total volume of 100 μl/mouse was injected at the base of the tail. After 14 days, mice were tail-bled and serum antibody levels of each mouse were assayed separately in triplicates by ELISA by using a colorimetric assay. Titration curves showing the mean absorbance of each group are shown and are a representative of two independent experiments.

6.4. Immunization with a replication-deficient adenovirus expressing GFP induces only weak antibody responses that can be significantly enhanced by the co-expression of NF-κB-inducing kinase

Having shown that our prototype antigen GFP induces strong antibody responses in BALB/c mice immunized with recombinant protein and CFA, I wanted to test whether administration of GFP by replication-deficient adenoviral vectors could also do the same. Thus, mice were first immunized with a low dose of 10^6 pfu of Ad0(GFP) or AdNIK(GFP), and GFP-specific antibody responses were measured. Immunization produced induced negligible levels of antibody production, suggesting that a higher titre of the adenoviral vector was needed (figure 6.4).

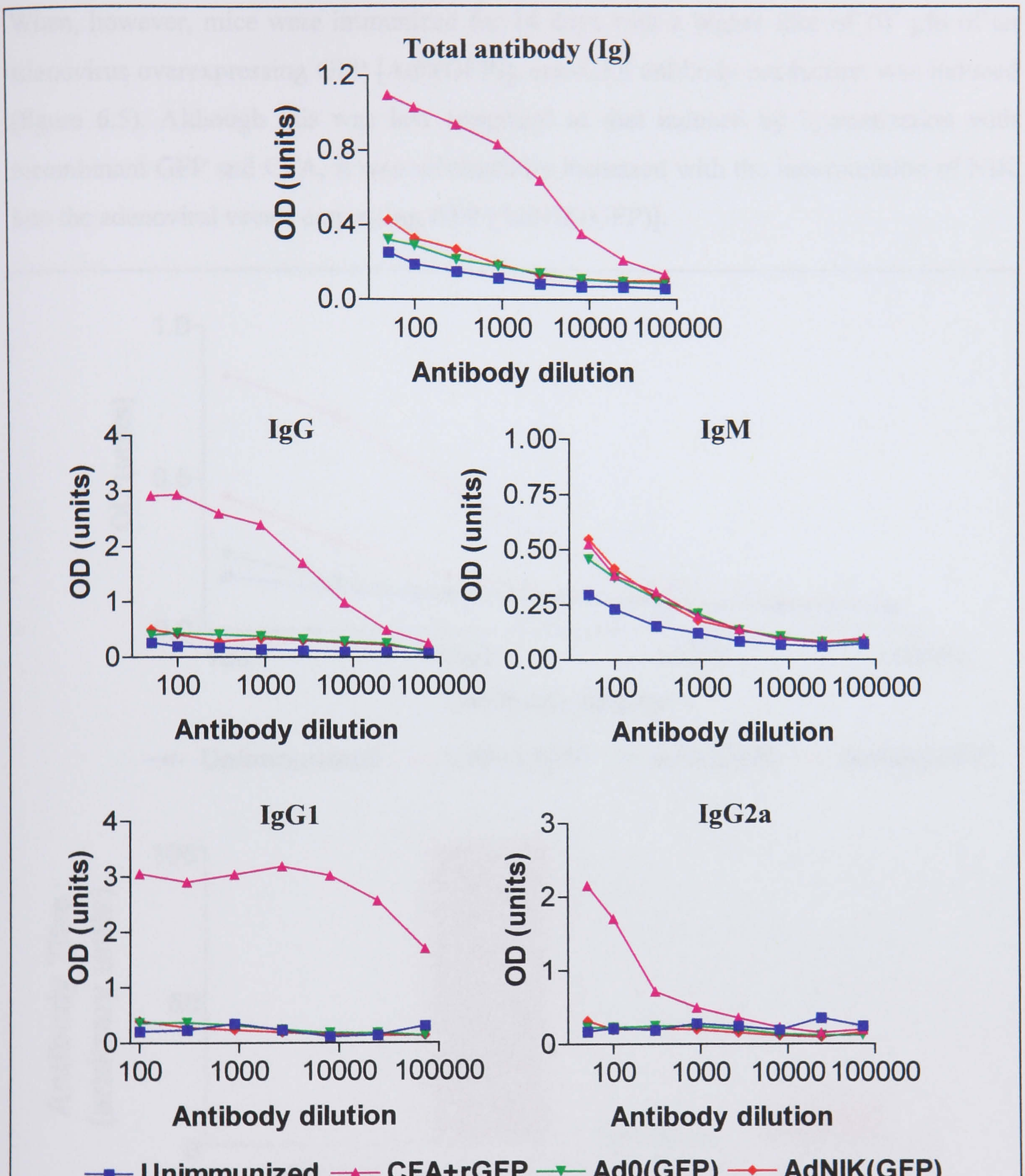


Figure 6.4: Immunization with 10^6 i.u. of Ad0(GFP) or AdNIK(GFP) fails to induce anti-GFP antibody production. Groups of five BALB/c mice (eight to ten weeks old) were vaccinated by the subcutaneous route with PBS or 20 μg/ml of recombinant GFP emulsified with CFA at a 1:1 ratio. A total volume of 100 μl/mouse was injected at the base of the tail. After 14 days, mice were tail-bled and serum antibody levels of each mouse were assayed separately in triplicates by ELISA by using a colorimetric assay. Titration curves showing the mean absorbance of each group are shown and are a representative of two independent experiments.

When, however, mice were immunized for 14 days with a higher titre of 10^7 pfu of an adenovirus overexpressing GFP [Ad0(GFP)], anti-GFP antibody production was induced (figure 6.5). Although this was low compared to that induced by immunization with recombinant GFP and CFA, it was substantially increased with the incorporation of NIK into the adenoviral vector expressing GFP [AdNIK(GFP)].

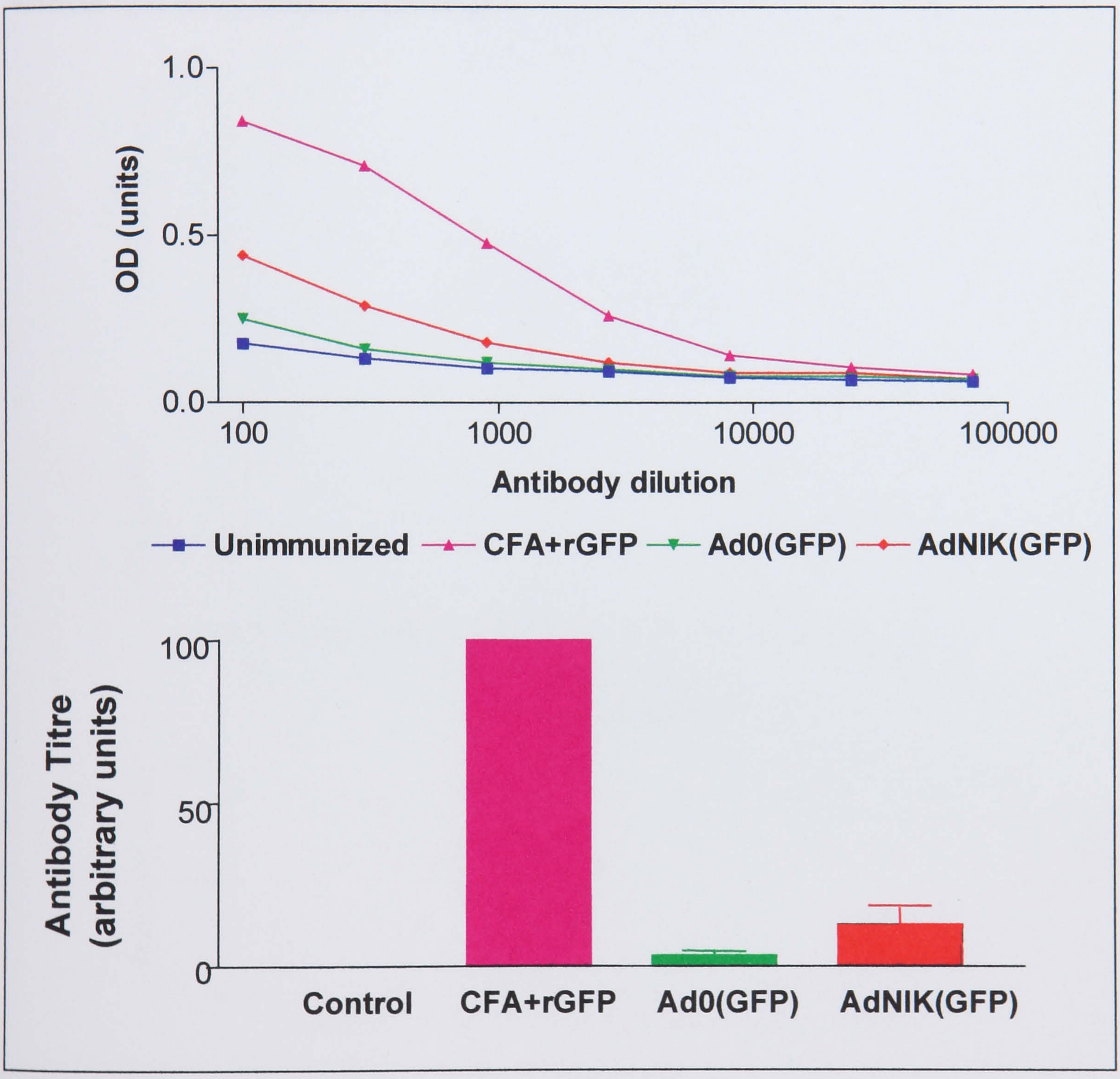


Figure 6.5: Immunization with 10^7 pfu of AdNIK(GFP) increases Ad0(GFP)-induced anti-GFP antibody production. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with PBS, 20 μg of recombinant GFP emulsified with CFA at a 1:1 ratio, or 10^6 pfu of recombinant adenoviruses expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 μl/mouse was injected at the base of the tail. After 14 days, mice were tail-bled and serum antibody levels of each mouse separately were assayed in triplicates by ELISA by using a colorimetric assay. At the top, a titration curve showing the mean absorbance of each group is shown. At the bottom, results are expressed as mean arbitrary antibody units (±SEM) of 5 mice/group and are representative of two independent experiments. Arbitrary antibody titres were calculated as decribed in section 2.7.4.

The antibody levels induced by immunization with Ad0(GFP) and AdNIK(GFP) consisted mainly of the IgG isotype with only low levels of IgM being detectable (figure 6.6). IgG levels were increased when NIK was incorporated into the adenoviral vector, whereas IgM levels remained unchanged.

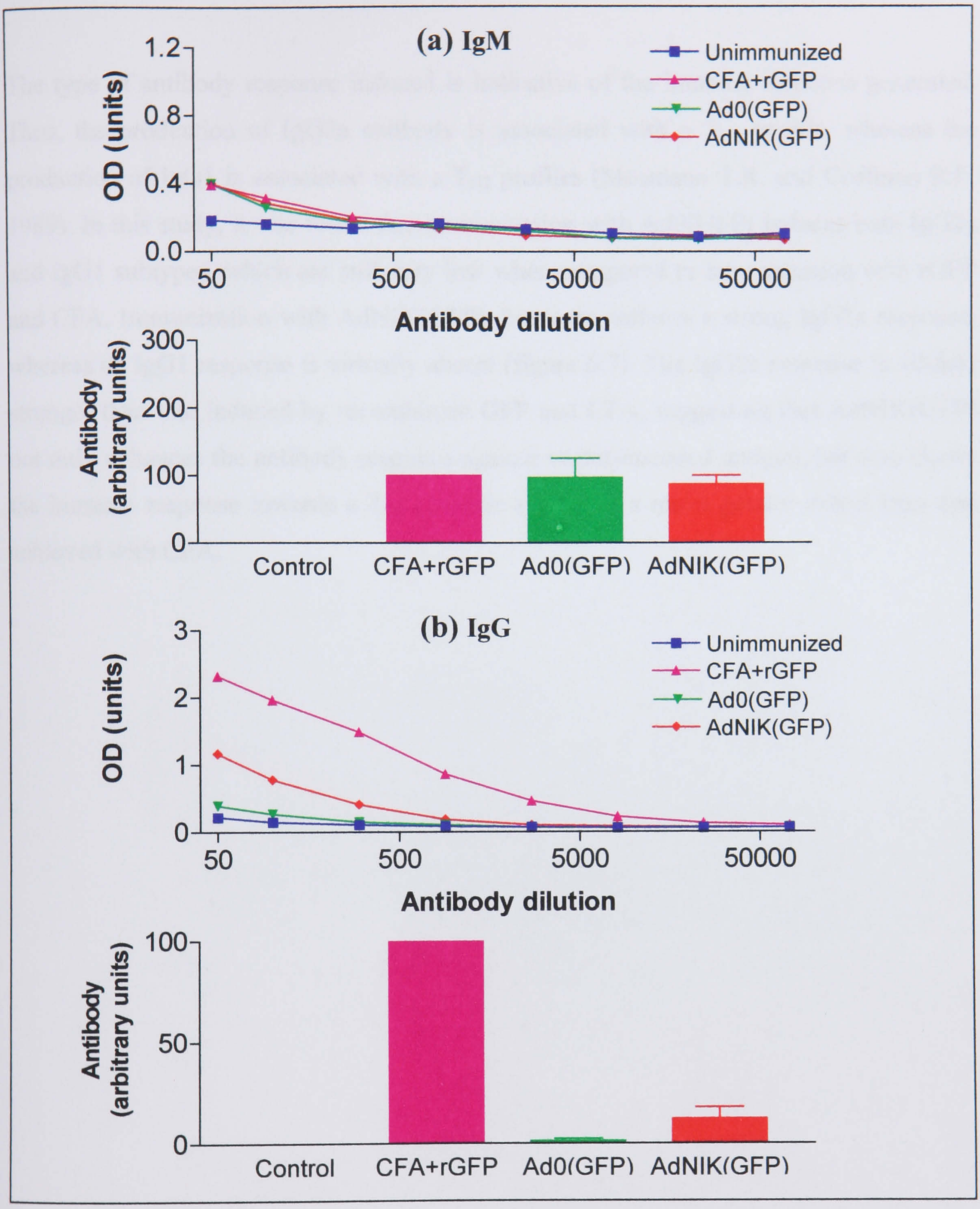


Figure 6.6: Immunization with 10^7 pfu of AdNIK(GFP) induces IgG anti-GFP antibody production. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with PBS, 20 μg of recombinant GFP emulsified with CFA at a 1:1 ratio, or 10^7 pfu of recombinant adenoviruses expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 μl/mouse was injected at the base of the tail. After 14 days, mice were tail-bled and serum antibody levels of each mouse separately were assayed for IgM (a) and IgG (b) levels in triplicates by ELISA by using a colorimetric assay. At the top, a titration curve showing the mean absorbance of each group is shown. At the bottom, results are expressed as mean arbitrary antibody units (±SEM) of 5 mice/group and are representative of two independent experiments. Arbitrary antibody titres were calculated as decribed in section 2.7.4.

The type of antibody response induced is indicative of the immune response generated. Thus, the production of IgG2a antibody is associated with a T_{H1} profile, whereas the production of IgG1 is associated with a T_{H2} profiles (Mosmann T.R. and Coffman R.L. 1989). In this study, it was found that immunization with Ad0(GFP) induces both IgG2a and IgG1 subtypes, which are still very low when compared to immunization with rGFP and CFA. Immunization with AdNIK(GFP), however, induces a strong IgG2a response, whereas an IgG1 response is virtually absent (figure 6.7). The IgG2a response is 10-fold stronger than that induced by recombinant GFP and CFA, suggesting that AdNIK(GFP) not only enhances the antibody response against vector-encoded antigen, but also skews the immune response towards a T_{H1} cytokine profile to a much greater extent than that achieved with CFA.

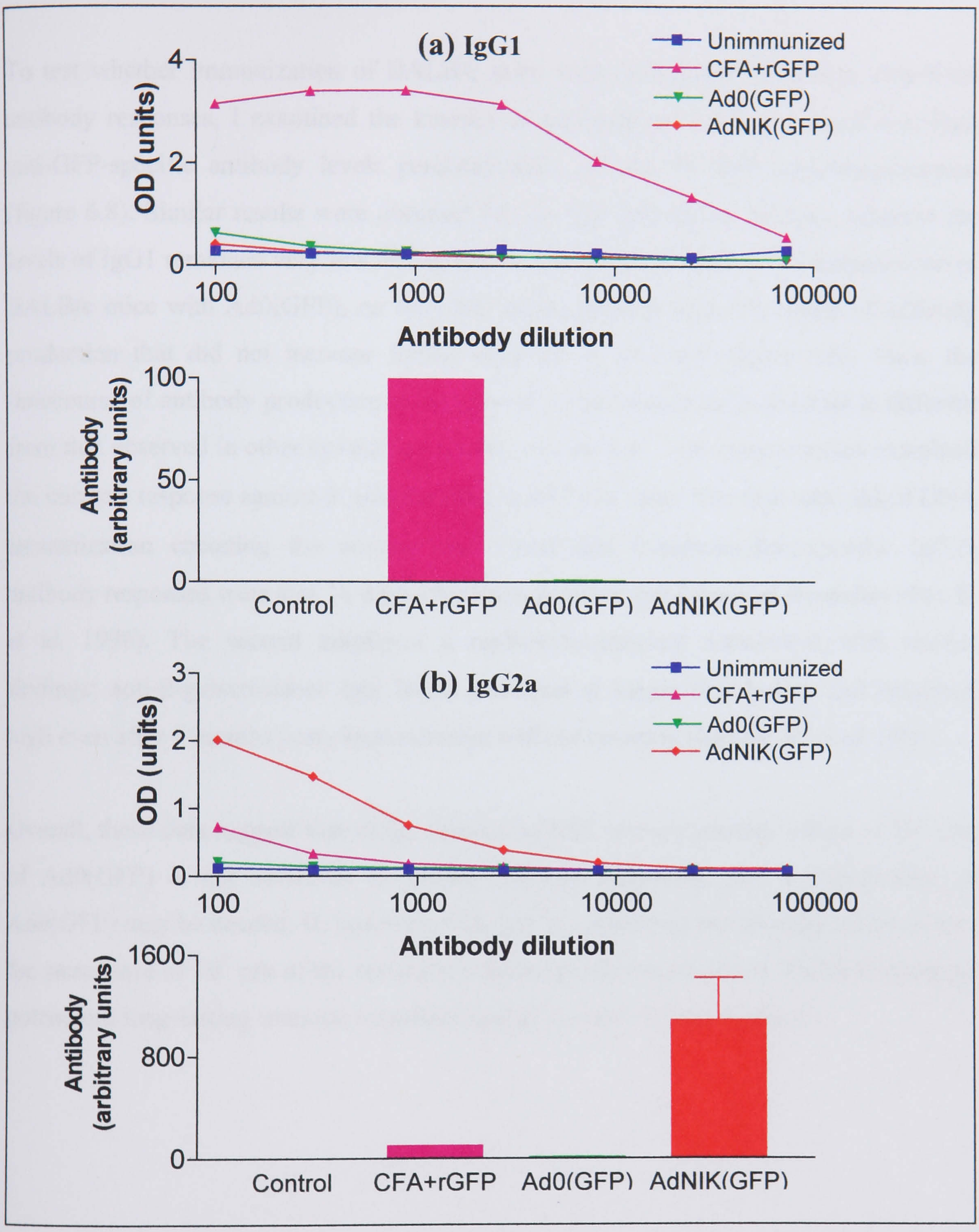


Figure 6.7: Immunization with 10^7 pfu of AdNIK(GFP) induces high IgG2a but low IgG1 anti-GFP antibody production. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with PBS, 20 μg of recombinant GFP emulsified with CFA at a 1:1 ratio, or 10^7 pfu of recombinant adenoviruses expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 μl/mouse was injected at the base of the tail. After 14 days, mice were tail-bled and serum antibody levels of each mouse separately were assayed for IgG1 (a) and IgG2a (b) levels in triplicates by ELISA by using a colorimetric assay. At the top, a titration curve showing the mean absorbance of each group is shown. At the bottom, results are expressed as mean arbitrary antibody units (±SEM) of 5 mice/group and are representative of two independent experiments. Arbitrary antibody titres were calculated as decribed in section 2.7.4.

To test whether immunization of BALB/c mice with AdNIK(GFP) induces long-lived antibody responses, I examined the kinetics of antibody production. I found that high anti-GFP-specific antibody levels persisted after 28 and 56 days post-immunization (figure 6.8). Similar results were obtained for the IgG and IgG2a isotypes, whereas the levels of IgG1 remained very low throughout all this period (figure 6.8). Immunization of BALB/c mice with Ad0(GFP), on the other hand, induced very low levels of antibody production that did not increase further after 28 or 56 days (figure 6.8). Thus, the timecourse of antibody production in our system of adenoviral immunization is different from that observed in other systems of genetic vaccination. Two recent studies examined the immune response against β-galactosidase in BALB/c mice. The first used naked DNA immunization encoding the antigen and found that β-galactosidase-specific IgG2a antibody responses were low 14 days after immunization and increased thereafter (Raz E. et al. 1996). The second employed a replication-deficient adenovirus with similar findings; anti-β-galactosidase IgG levels increased at longer time-points and remained high even after 6 months post- immunization without boosting (Juillard V. et al. 1995).

Overall, these data suggest that in the absence of NIK overexpression, a dose of 10^7 pfu. of Ad0(GFP) is not sufficient to induce antibody responses, and a higher dose of Ad0(GFP) may be needed. If, however, NIK gets incorporated into the adenoviral vector, the same dose of 10^7 pfu of the replication-deficient adenoviral vector is able to generate potent and long-lasting immune responses against a vector-encoded antigen.

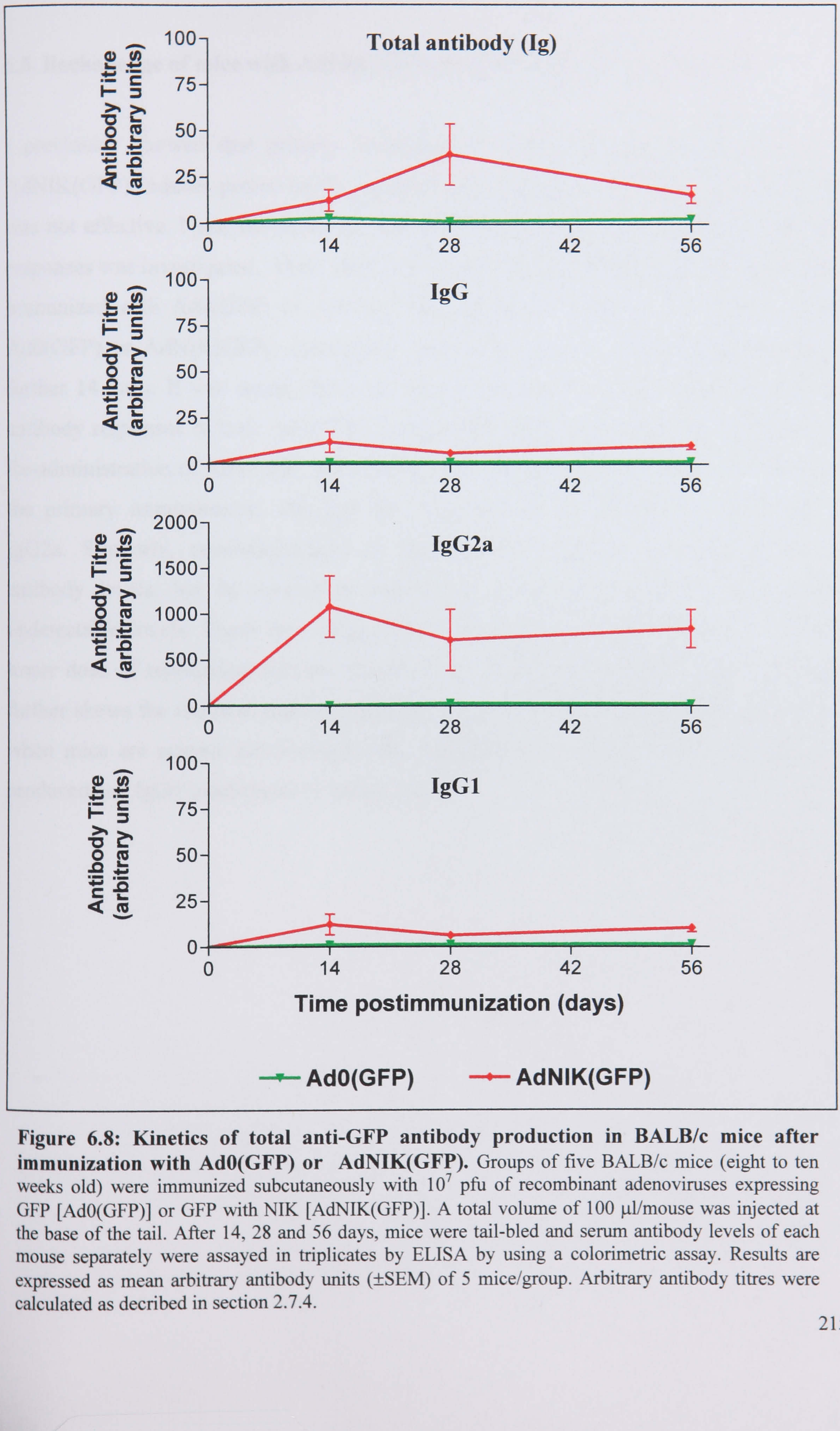


Figure 6.8: Kinetics of total anti-GFP antibody production in BALB/c mice after immunization with Ad0(GFP) or AdNIK(GFP). Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with 10^7 pfu of recombinant adenoviruses expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 µl/mouse was injected at the base of the tail. After 14, 28 and 56 days, mice were tail-bled and serum antibody levels of each mouse separately were assayed in triplicates by ELISA by using a colorimetric assay. Results are expressed as mean arbitrary antibody units (±SEM) of 5 mice/group. Arbitrary antibody titres were calculated as decribed in section 2.7.4.

6.5. Rechallenge of mice with AdNIK(GFP) boosts IgG2a and T_{H1} responses

I previously showed that primary immunization of BALB/c mice with 10^7 pfu of AdNIK(GFP) induces potent IgG2a antibody responses whereas at that titre Ad0(GFP) was not effective. Next, the ability of AdNIK(GFP) or Ad0(GFP) to boost the antibody responses was investigated. Thus, 56 days after the primary immunization, BALB/c mice immunized with Ad0(GFP) or AdNIK(GFP) received a boosting dose of 10^6 pfu of Ad0(GFP) or AdNIK(GFP), respectively, and antibody levels were measured after a further 14 days. It was found that a secondary immunization was capable of boosting antibody responses in both Ad0(GFP)- and AdNIK(GFP)-immunized mice (figure 6.9). Re-administration of Ad0(GFP) increases Ig, IgG, IgG2a and IgG1 isotypes compared to the primary immunization, although the most pronounced increase was observed in IgG2a. Similarly, re-administration of AdNIK(GFP) increases Ig, IgG and IgG2a antibody levels, but in contrast to Ad0(GFP), it decreases IgG1 levels to almost undetectable levels. These data suggest that a secondary immunization with a 10-fold lower dose of replication-deficient adenoviruses effectively boosts antibody levels and further skews the immune response towards a T_{h1} profile. This is especially pronounced when mice are primed and boosted with AdNIK(GFP), as higher levels of IgG2a are produced and IgG1 levels become undetectable.

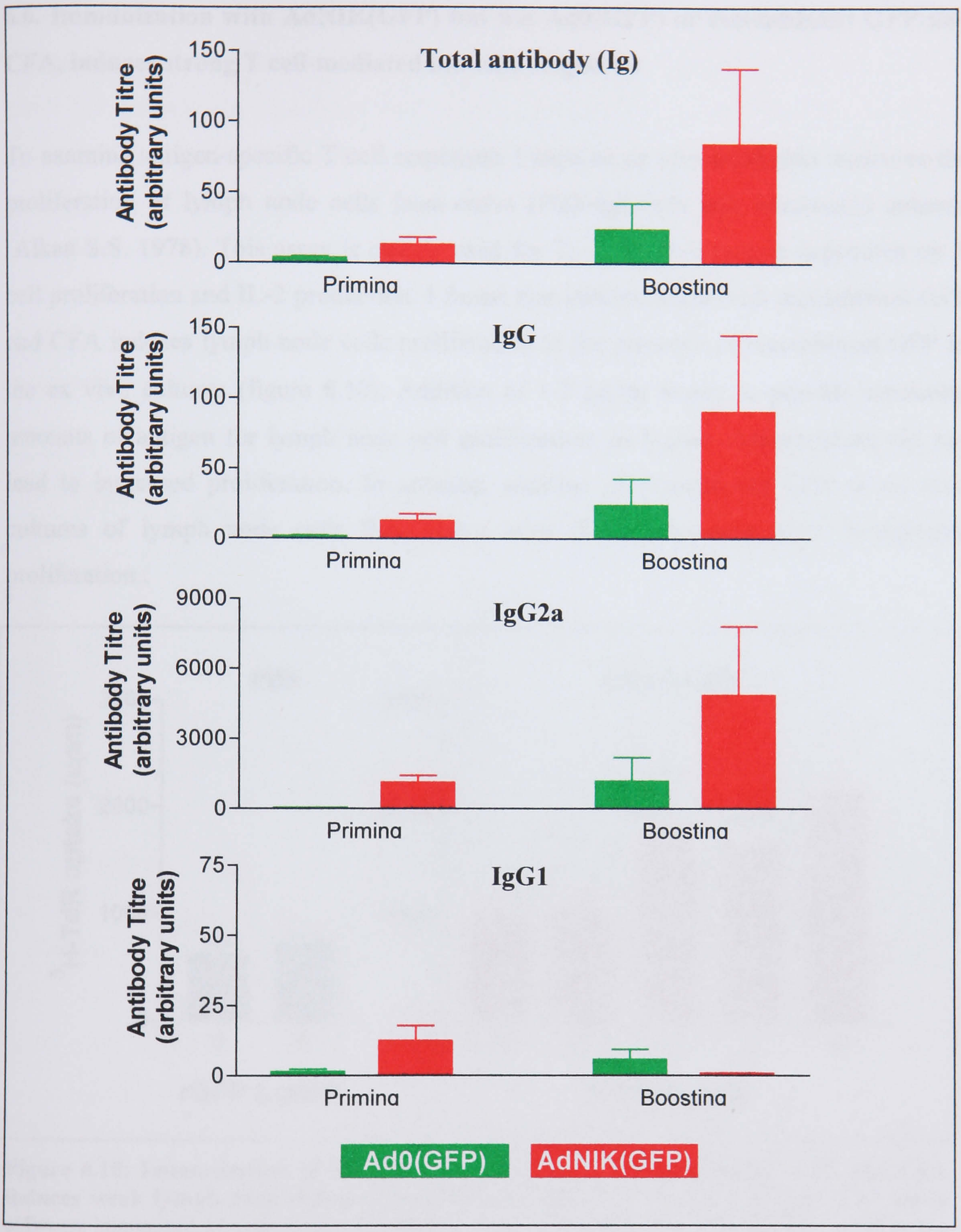


Figure 6.9: A second immunization with either Ad0(GFP) or AdNIK(GFP) boosts total and IgG2a antibody responses. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with 10^7 pfu of recombinant adenoviruses expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 μl/mouse was injected at the base of the tail. After 14 days, mice were tail-bled and serum antibody levels of each mouse separately were assayed in triplicates by ELISA. After 56 days, mice received a boosting dose of 10^6 pfu of the same adenovirus that they were primed, and antibody levels measured again after another 14 days. Mean arbitrary antibody units (±SEM) of 5 mice/group are shown.

6.6. Immunization with AdNIK(GFP) but not Ad0(GFP) or recombinant GFP and CFA, induces strong T cell-mediated immune responses

To examine antigen-specific T cell responses, I used an *ex vivo* assay that measures the proliferation of lymph node cells from naïve (PBS-injected) and immunized animals (Alkan S.S. 1978). This assay is chiefly used for T_{H1} responses as it is dependent on T cell proliferation and IL-2 production. I found that immunization with recombinant GFP and CFA induces lymph node cells proliferation in the presence of recombinant GFP in the ex vivo cultures (figure 6.10). Addition of 1-2 μg/ml seems to provide saturating amounts of antigen for lymph node cell proliferation, as higher concentrations did not lead to increased proliferation. In contrast, addition of recombinant GFP to ex vivo cultures of lymph node cells from naïve mice did not increase their background proliferation..

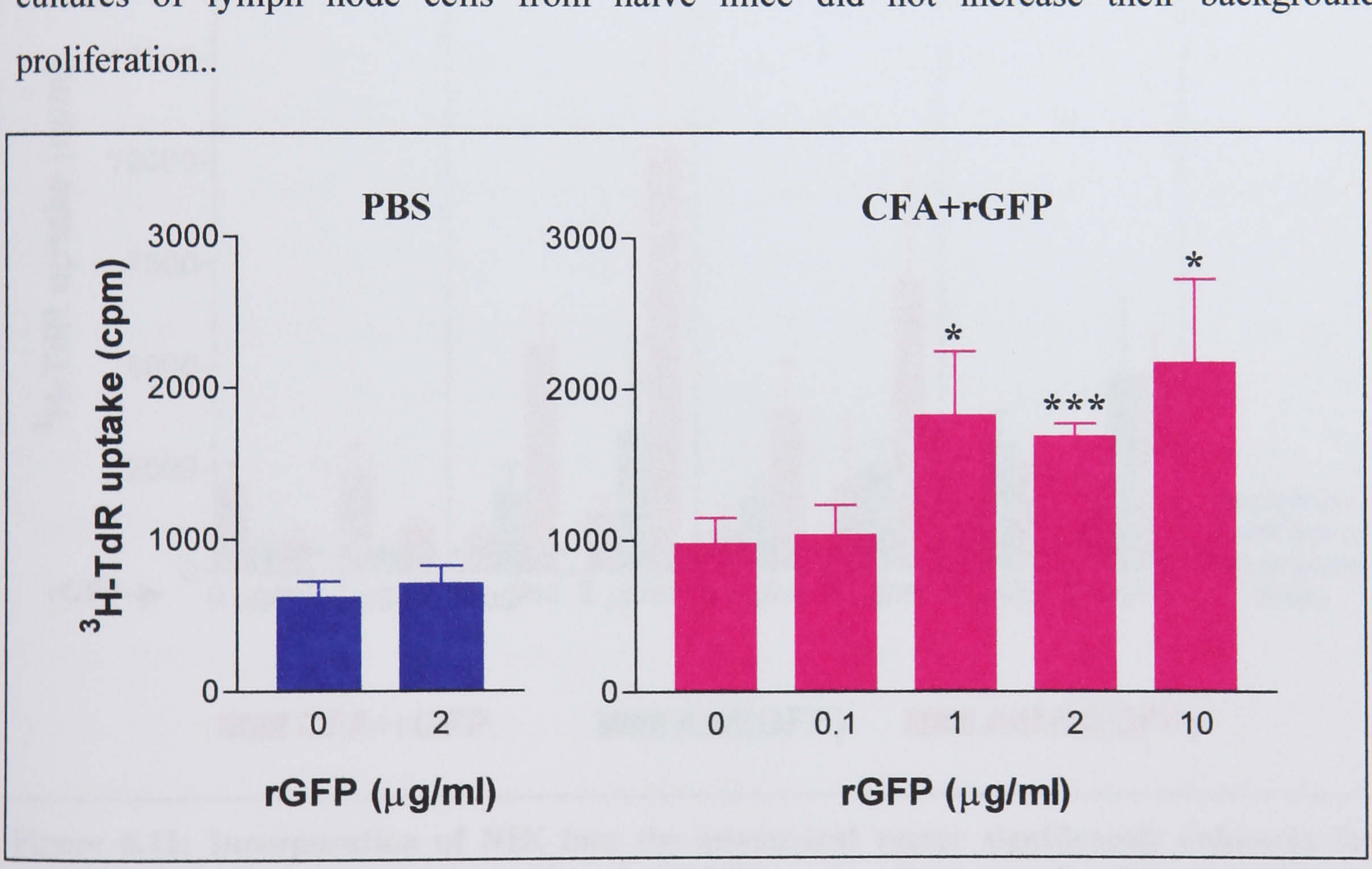


Figure 6.10: Immunization of BALB/c mice for 7 days with recombinant GFP and CFA induces weak lymph node cells proliferation. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with PBS or 20 μg of recombinant GFP emulsified with CFA at a 1:1 ratio. A total volume of 100 μl/mouse was injected at the base of the tail. After 7 days, mice were sacrificed, inguinal lymph nodes excised and cells cultured as single-cell suspensions in the presence or absence of recombinant GFP. Cells from each mouse were cultured separately in triplicates. Proliferation was measured after 72h by incorporation of tritiated thymidine. Mean proliferation (±SEM) of 5 mice/group is shown and is representative of two independent experiments. An unpaired students t-test (two-tailed) was used to compare groups with the background proliferation of lymph node cells from PBS control mice (* $p<0.05$, *** $p<0.001$).

These results show that immunization of BALB/c mice with recombinant GFP in CFA induces a low level of lymph node cell proliferation when measured after 7 days.. This is weak but statistically significant ($p<0.05$), a finding that echoes the T_{H2} profile of antibody responses observed after immunization with recombinant protein and CFA

Immunization of BALB/c mice with 10^7 infectious units of a replication-deficient adenovirus expressing GFP [Ad0(GFP)] also induced lymph node cells proliferation (figure 6.11) that was enhanced when NIK was incorporated into the adenoviral vector [AdNIK(GFP)].

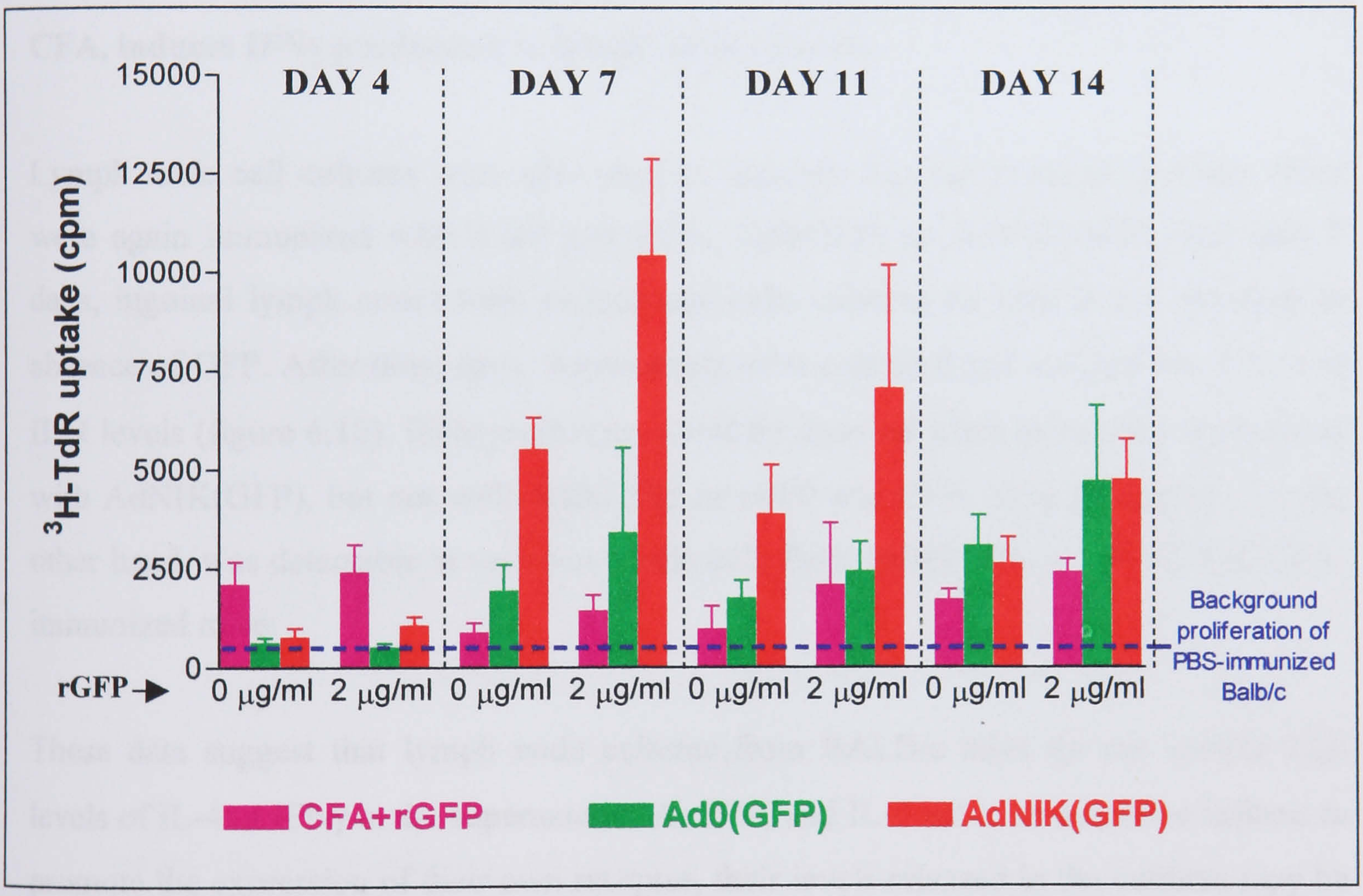


Figure 6.11: Incorporation of NIK into the adenoviral vector significantly enhances the proliferation of lymph node cells of BALB/c mice immunized with recombinant adenoviruses. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with PBS, 20 μg of recombinant GFP emulsified with CFA at a 1:1 ratio, or 10^7 pfu of recombinant adenoviruses expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 μl/mouse was injected at the base of the tail. After 7 days, mice were sacrificed, inguinal lymph nodes excised and cells cultured as signle-cell suspensions in triplicates in the presence or absence of recombinant GFP. Proliferation was measured at day three by incorporation of tritiated thymidine. Mean proliferation (±SEM) of 5 mice/group is shown and is representative of two independent experiments.

Strong proliferative responses were observed even without the addition of antigen in the ex vivo cultures, suggesting that there the T cells are already in an activated state or that there is already antigen present in the lymph node cells presented by APCs. Immunization with AdNIK(GFP) enhanced lymph node cells proliferation 4-fold when compared to Ad0(GFP) and 5-fold when compared to CFA and rGFP, indicating that NIK overexpression has powerful adjuvant properties. Strong T cell-mediated immune responses correlate with T_{H1} cytokine profiles and IgG2a antibody responses that AdNIK(GFP) was shown to induce very efficiently in section 6.4.

6.7. Immunization with AdNIK(GFP) but not Ad0(GFP) or recombinant GFP and CFA, induces IFNγ production in lymph node cultures

Lymph node cell cultures were also used to measure T_{H1}/T_{H2} cytokine profiles. Mice were again immunized with rGFP and CFA, Ad0(GFP) or AdNIK(GFP), and after 7 days, inguinal lymph nodes were excised and cells cultured ex vivo in the presence or absence of GFP. After three days, supernatants were collected and assayed for IFNγ and IL-4 levels (figure 6.12). IFNγ production could be detected when mice were immunized with AdNIK(GFP), but not with Ad0(GFP) or rGFP and CFA. IL-4 production, on the other hand, was detectable at very low levels only from Ad0(GFP)- and rGFP and CFA-immunized mice.

These data suggest that lymph node cultures from BALB/c mice do not secrete high levels of IL-4 or IFNγ in the supernatants. As IFNγ and IL-4 act in an autocrine fashion to promote the expression of their own receptor, their levels released in the cultures may be rapidly consumed (Mosmann T.R. and Coffman R.L. 1989). Thus, more sensitive methods including intracellular FACS staining may be a better way of examining their cytokine profile. Nevertheless, the observation that only immunization with AdNIK(GFP) results in detectable IFNγ levels in ex vivo lymph node cultures, is consistent with the strong lymph node cell proliferative responses and the high IgG2a levels that it induces.

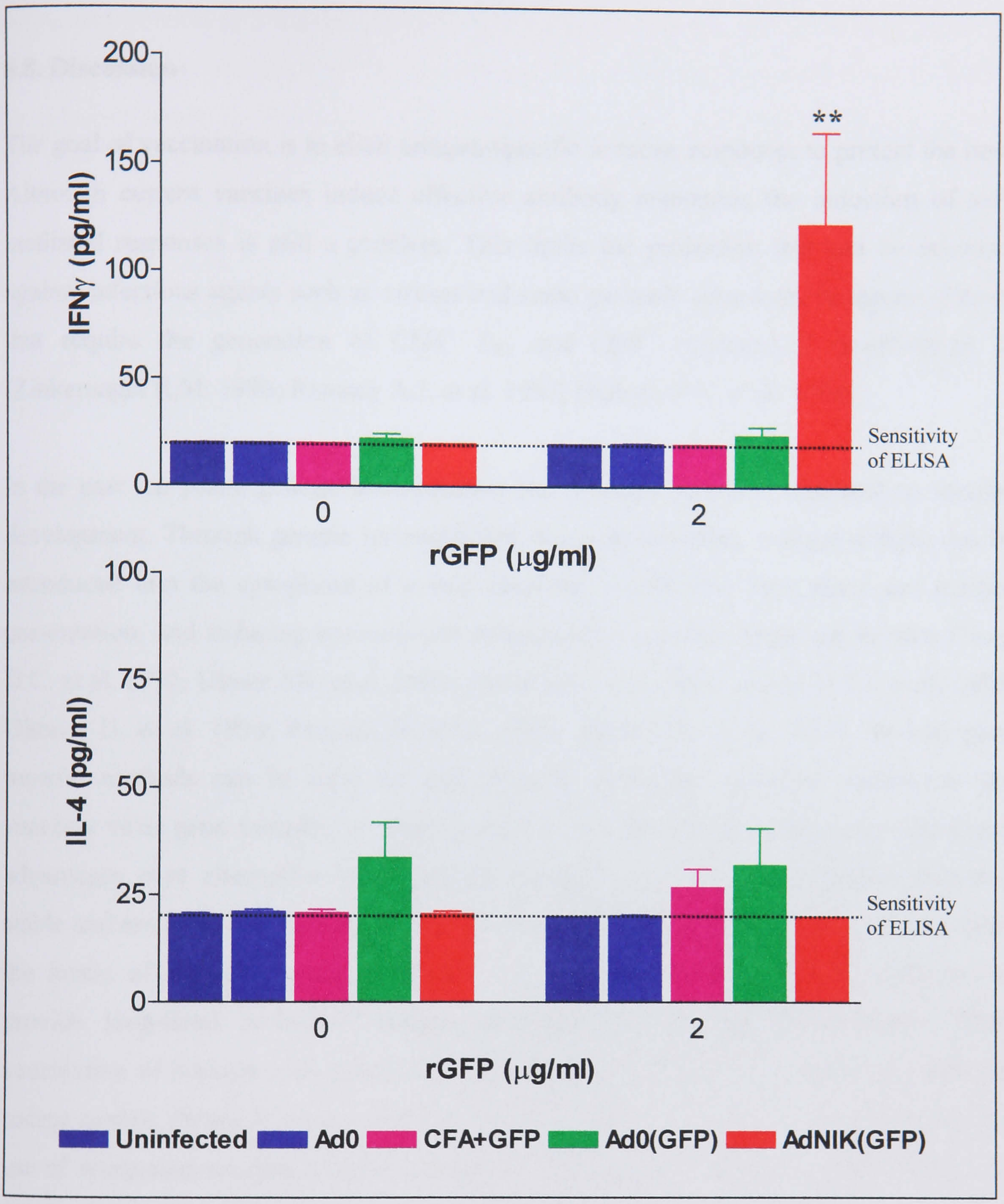


Figure 6.12: Immunization of mice with 10^7 pfu of AdNIK(GFP) but not Ad0(GFP) or rGFP and CFA induces IFNγ production in ex vivo cultures of lymph node cells. Groups of five BALB/c mice (eight to ten weeks old) were immunized subcutaneously with PBS, 20 μg of recombinant GFP emulsified with CFA at a 1:1 ratio, or 10^7 pfu of recombinant adenoviruses without insert (Ad0), or expressing GFP [Ad0(GFP)] or GFP with NIK [AdNIK(GFP)]. A total volume of 100 μl/mouse was injected at the base of the tail. After 7 days, mice were sacrificed, inguinal lymph nodes excised and cells cultured as signle-cell suspensions in triplicates in the presence or absence of recombinant GFP. After 3 days, supernatants were collected and assayed for the presence of IFNγ and IL-4 by ELISA. Mean cytokine production (±SEM) of 5 mice/group is shown and is representative of two independent experiments. Statistical analysis was performed by using a one-way ANOVA Dunnet's multiple comparisons test. Cytokine production by PBS-immunized mice was used as a control group. (**$p<0.01$)

6.8. Discussion

The goal of vaccination is to elicit antigen-specific immune responses to protect the host. Although current vaccines induce effective antibody responses, the induction of cell-mediated responses is still a problem. This limits the protection that can be achieved against infectious agents such as viruses and some parasitic infections, or against cancer, that require the generation of $CD4^+$ T_{H1} and $CD8^+$ cytotoxic T lymphocytes or (Zinkernagel R.M. 1996; Ramsay A.J. et al. 1993; Doherty P.C. et al. 1984).

In the past ten years, genetic immunization has emerged as a new approach to vaccine development. Through genetic immunization, the gene encoding a target antigen can be introduced into the cytoplasm of a cell, resulting in effective processing and antigen presentation, and inducing humoral and cell-mediated immune responses in vivo (Tang D.C. et al. 1992; Ulmer J.B. et al. 1992; Fynan E.F. et al. 1993; Donnelly J.J. et al. 1995; Dittmer U. et al. 1998; Panicali D. et al. 1983; Morin J.E. et al. 1987). Several gene transfer methods can be used for that purpose, including retroviral, adenoviral and vaccinia virus gene transfer, or direct injection of naked DNA. They offer significant advantages over alternative immunization strategies, as they are replication-deficient, stable and are relatively easy to prepare. However, and despite encouraging early results, the levels of specific immunity induced by these vectors has not been sufficient to provide long-lived protection against challenge with pathogenic organisms. Thus, vaccination of humans with naked DNA has been disappointing in comparison with the rodent models (Wang R. et al. 1998; Le T.P. et al. 2000; Calarota S. et al. 1998), and the use of attenuated vaccinia viruses has shown little efficacy (Seder R.A. and Hill A.V.S. 2000). This prompted investigators to try to optimize the immunogenicity of genetic vaccines themselves or use priming/boosting immunization strategies with naked DNA and viral vaccines to enhance immunity.

To optimize genetic vaccines in humans, most approaches have focused on improving immunogenicity of vector-encoded antigens. The intrinsic immunogenicity of naked DNA vaccines is mainly due to undermethylated CpG motifs, specific nucleotide

sequences of viral or bacterial genes found within the plasmid, that have been shown in human and mice to stimulate the immune system, inducing T_{H1} and cytotoxic $CD8^+$ T lymphocyte responses (Cho H.J. et al. 2000; Cowdery J.S. et al. 1996; Klinman D.M. et al. 1996; Sato Y. et al. 1996). To enhance or skew the immune responses generated by DNA vaccination, several groups have introduced various cytokine, chemokine, costimulatory molecules, or combinations of them to the DNA backbone. These studies and their effects on humoral and cellular immune responses have been recently reviewed by Gurunathan and colleagues (table 6.1). Although promising, their efficacy and safety in humans remains questionable.

Live virus vectors, on the other hand, generate stronger cellular immune responses than do DNA vaccines in small animals. But if poxviruses and adenoviruses are used in humans or other animals with pre-existing immunity against the viral vectors, their efficacy dramatically decreases. Pre-existing immunity reduces the expression of the transgene by destroying cells expressing the transgene and by diminishing the ability of the virus to deliver the transgene (Yang Y. et al. 1994; Kuriyama S. et al. 1998). To circumvent this problem, less immunogenic vectors such as adeno-associated virus, lentivirus or gutless adenovirus are being tested. At the same time, different methods of viral delivery look promising (Siemens D.R. 2001).

Recently, a novel strategy involving a heterologous prime-boost immunization has been shown to be helpful. It makes use of naked DNA vaccines for priming and recombinant viral vectors encoding the same foreign antigens for boosting the immune response. This approach has been demonstrated to be effective in several infectious diseases in mice and in primates, leading to substantial enhancement of T_{H1} and cytotoxic T lymphocyte responses (Schneider J. et al. 1998; Schneider J. et al. 1999; Gilbert S.C. et al. 1999). As viral vectors, several poxviruses, such as modified vaccinia virus Ankara (MVA) and fowlpox, as well as replication-defective adenoviruses have this capacity to boost a primed cytotoxic T lymphocyte response substantially (Kent S.J. et al. 1998; Hanke T. et al. 1999; Rothel J.S. et al. 1997). This approach is now under clinical trials in malaria and HIV.

Cytokine	Antibody	Cellular response	CTL
IL-1	↑IgG ↑IgG2a	↑proliferation ↑IFNγ	↑CTL
IL-2	↑IgG ↑IgG2a	↑proliferation ↑IFNγ	↑CTL
IL-4	↑IgG ↑IgG1	↑proliferation ↓DTH ↑IL-4	
IL-5	↑IgG	±proliferation ↑IFNγ	
IL-7	↑IgG2a ↑IgG1	↑IFNγ	
IL-8		↑Neutrophils ↓DTH	
IL-10	↑IgG ↓IgG2a	↓DTH ↓proliferation	
IL-12	↑IgG2a IgG1? IgG?	↑DTH ↑proliferation ↑IFNγ	
IL-15	IgG?	±↑proliferation	↑CTL
IL-18	↑IgG	↑proliferation	↑CTL
TNF	↑IgG	↑proliferation	↑CTL
GM-CSF	↑IgG ↑IgG2a ↑IgG1	↑proliferation ↑IFNγ ↑IL-4	↑CTL
TGF-β	↑IgG1	↓DTH ↓proliferation ↓cytokines	
IFNγ	↑IgG2a ?IgG?	?Proliferation ↑IFNγ ↓IL-5	↑CTL
CD80			↑CTL
CD86		↑DTH ↑Proliferation	↑CTL
CD40L	↑IgG ↑IgG2a ↑IgG1	↑IFNγ	↑CTL
CD54		↑proliferation ↑IFNγ	↑CTL
LFA-3		↑proliferation ↑IFNγ	↑CTL
CTLA4	↑IgG ↑IgG1/IgG2a	↑proliferation	

Table 6.1: Incorporation of cytokines/chemokines and costimulatory molecules as a way of enhancing or regulating immunity induced by DNA vaccines (adapted from Gurunathan S. et al. 2000)

In chapter 4, I showed that expression of the wild-type form of NF-κB-inducing kinase (NIK) into immature dendritic cells enhances their antigen-presenting function. It induces the activation of p65, relB and p50 NF-κB subunits, and it coordinates the up-regulation of cytokines, chemokines, MHC antigen-presenting and costimulatory molecules. As genetic immunization has been shown to work through the direct or indirect transfection of dendritic cells (Corr M. et al. 1996; Doe B. et al. 1996; Condon C. et al. 1996; Raz E. et al. 1994; Albert M.L. et al. 1998), the most potent antigen-presenting cells, I wanted to test whether incorporation of the gene encoding NIK could increase the immunogenicity and potency of DNA vaccines. For that purpose, I devised a model of genetic immunization against a model antigen green fluorescent protein (GFP) by replication-deficient adenoviral vectors to compare humoral and cell-mediated immune responses. Adenoviruses expressing GFP alone [Ad0(GFP)], or GFP together with NIK as an adjuvant [AdNIK(GFP)] were used.

First, I examined whether the jellyfish protein GFP used as a model antigen is immunogenic in BALB/c mice. I found that administration of recombinant GFP in CFA subcutaneously induces a strong humoral immune response against the antigen that can be measured after 14 days and that consists of high IgG1 but low IgG2a levels. At the same time, immunization with GFP in CFA induces only weak antigen-specific proliferation of lymph node cells with low levels of IL-4 but negligible IFNγ, suggesting that the immune response induced is of a predominantly T_{H2} cytokine profile that favours antibody-mediated immunity. Indeed, this was confirmed by finding high levels of serum IgG 1 and low levels of IgG2a.

This prompted me to investigate whether replication-deficient adenoviruses carrying the GFP gene could also be used to induce an immune response against GFP. I have found that a dose of 10^7 pfu of recombinant adenovirus was required for detectable antigen-specific responses after intradermal immunization of BALB/c mice, as lower doses were not effective. Thus, administration of Ad0(GFP) induced antigen-specific lymph node cells proliferation but only negligible antibody production. Incorporation of the NIK gene into the adenoviral vector, however, significantly increased both lymph node cell

proliferation and anti-GFP antibody production, suggesting that NIK has a potent adjuvant effect in the immunogenicity of the vector-encoded antigen. AdNIK(GFP)-induced antibody production consisted mainly of the IgG2a isotype with undetectable levels of IgG1, and lymph node cells produced IFNγ but not detectable IL-4. These findings suggest that although adenoviral immunization favours cell-mediated immune responses when compared to the humoral responses induced by the administration of recombinant protein and adjuvant, incorporation of NIK into the adenoviral vector significantly enhances that effect and skews the response towards a T_{h1} cytokine profile and cell-mediated immunity. That finding seems to echo the ability of NIK to induce IL-12 and IL-18 from immature dendritic cells (chapter 4). This effect is so strong that a single immunization is sufficient to overcome the genetic predisposition of BALB/c mice to generate T_{H2}-type responses (Heinzel F.P. et al. 1989).

I next examined whether the levels of antibody production induced by AdNIK(GFP) were long-lived and I found that 28 days and 56 days after immunization, high serum levels of total, IgG and IgG2a anti-GFP antibody were still present. These levels remained stable and did not increase or decrease significantly during that period. This is in contrast to the studies of others with naked DNA immunization, where IgG2a antibody levels against a vector-encoded model antigen, β-galactosidase, increased at later time-points (Raz E. et al. 1996).

Finally, I investigated whether a second administration of recombinant adenoviruses could boost the immune responses and thus provide superior immunity. For that purpose mice received a booster immunization of 10^6 pfu of recombinant adenovirus 56 days after the priming immunization, a dose which by itself is not capable of providing a useful primary response. I have found that administration of Ad0(GFP) to already Ad0(GFP) immunized mice induces high levels of antibody production that correlate with a mixed T_{H1}/T_{H2} response as both IgG2a and IgG1 levels could be measured. Similarly, administration of AdNIK(GFP) to AdNIK(GFP)-immunized mice further increases the total anti-GFP-specific antibody levels. However, although IgG2a levels are boosted, IgG1 levels decreased to almost undetectable levels, suggesting that the response remains

strongly skewed to the T_{H1} profile. In summary, these data show that a second administration of replication-deficient adenoviruses can boost the antibody levels against the vector-encoded antigen. But although this induces a mixed T_{h1}/T_{h2} response, incorporation of NIK into the adenoviral vector skews that response to the T_{h1} type. In addition, NIK increases both total and IgG2a antibody levels, suggesting that it has at the same time a potent adjuvant effect.

There are several questions that have not been addressed yet in this study. Thus, the efficacy of NIK in inducing cytotoxic T lymphocyte responses, immune responses against more relevant antigens and protective immunity against pathogenic organisms is not known. In addition, its efficacy in other vectors of genetic immunization such as naked DNA or other recombinant viruses, and in other strains of mice remains to be determined.

Nevertheless, this is the first study that makes use of an intracellular signalling molecule as an adjuvant to enhance the immunogenicity of genetic vaccines. It is based on the observation that the expression of NIK in immature DC coordinates the production of cytokines and chemokines, and the up-regulation of MHC antigen-presenting and costimulatory molecules. In vivo, incorporation of NIK into adenoviral DNA vectors leads to enhanced antigen-specific T cell and IgG2a antibody responses, that correlate with a T_{H1}-type of immunity (Mosmann T.R. and Coffman R.L. 1989).

The implications of these findings are very important. First, NIK may be a very useful adjuvant for genetic immunization against viral and certain parasitic or bacterial infections, or even cancer vaccines that require strong cell-mediated immune responses. Second, the strong skewing effect induced by NIK towards T_{H1} immunity raises the possibility that it may be very useful for the treatment of allergy. In various studies, vaccination with allergen in the form of naked plasmid DNA has been shown to stimulate T_{H1}-type allergen-specific immune responses that confer long-lasting protection against allergy (Donnelly J.J. et al. 1997; Roman M. et al. 1997). But although this approach is successful in preventing allergic diseases, the therapy of ongoing conditions has been

limited, with the exception of a recent report showing that the incorporation of the IL-18 gene into the vector can successfully reverse airway hyperresponsiveness in mice (Maecker H.T. et al. 2001). The ability of NIK to induce strong T_{h1} immune responses make it an attractive way of reprogramming the responses against an allergen. Finally, these data define a novel family of vaccine adjuvants that consist of intracellular signalling molecules involved in the regulation of the immune response. Activation of the immune response in that way may provide a more physiological approach of enhancing immunogenicity by upregulating many functions involved in immunity, compared to the artificial expression of single cytokines or costimulatory molecules that may result in increased toxicity of vaccines and safety concerns.

CHAPTER 7

GENERAL DISCUSSION

Protection against infectous agents and cancer depends on the generation of an appropriate immune response. An immune response makes use of an enormous variety of cells and proteins that are capable of specifically recognizing and eliminating an apparently limitless number of foreign pathogens without in most cases damaging the host. Although usually beneficial, inappropriate or uncontrolled activation of the immune response can lead to extensive tissue damage that can be deleterious for the host. To intervene therapeutically to control the immune response when it is damaging for the host, or to induce it when it is beneficial, a good understanding of how the immune response is regulated is essential.

The immune response can be regulated at various levels including the recognition of the foreign agent, the presentation of antigen, the production of cytokines and chemokines and the up-regulation or down-regulation of cell surface molecules. These collectively result in the attraction and trafficking of immune cells towards the inflammed sites and their activation to exert appropriate effector functions to clear the invading organism. Central to these processes have been proposed to be the NF-κB transcription factors as they control the expression of cytokines, chemokines and adhesion molecules (reviewed by Baeuerle P.A. and Henkel T. 1994). The aim of this thesis was to validate the role of NF-κB in the regulation of the immune response in physiologically relevant cell types in humans and to dissect upsteam signalling pathways in that process.

One of the major obstacles of studying biochemical signalling pathways in primary cells is the difficulty of transfecting DNA, an essential tool in this process (Stacey K.J. et al. 1993; Zhong L. et al. 1999). To overcome this problem, I used an efficient adenoviral gene transfer technique recently developed by my colleagues (Foxwell B.J.M. et al. 1998) that consists of replication-deficient adenoviruses that have the E1 and E3 regions of their genome deleted. These deletions not only prevent adenoviral replication, but also remove the adenoviral genes known to subvert intracellular signalling (figure 7.1) and make the expression of the remaining endogenous adenoviral genes extremely inefficient (Hayder H. and Mullbacher A. 1996). This method has proved to be a potent tool for transfecting more than 95% of primary human macrophages, immature DC, HUVEC and HSF. Although one potential

criticism of this approach is the possibility that adenoviral vectors will themselves somehow alter intracellular signalling, no indication of such effects was observed in my experiments when uninfected cells were compared with cells infected with a number of control adenoviruses. Nevertheless, this possible problem is far outweighed by the limitations of transfection-permissive cell lines such as the 293 human embryonic kidney cells and HeLa epithelial cells that are most commonly used for signalling studies. These long-lived tumour cells not only have numerous chromosomal abnormalities and their intracellular pathways adapted for proliferation, but in addition, 293 cells are adenovirally transformed with the whole adenoviral genome that can more efficiently hijack the host signalling processes.

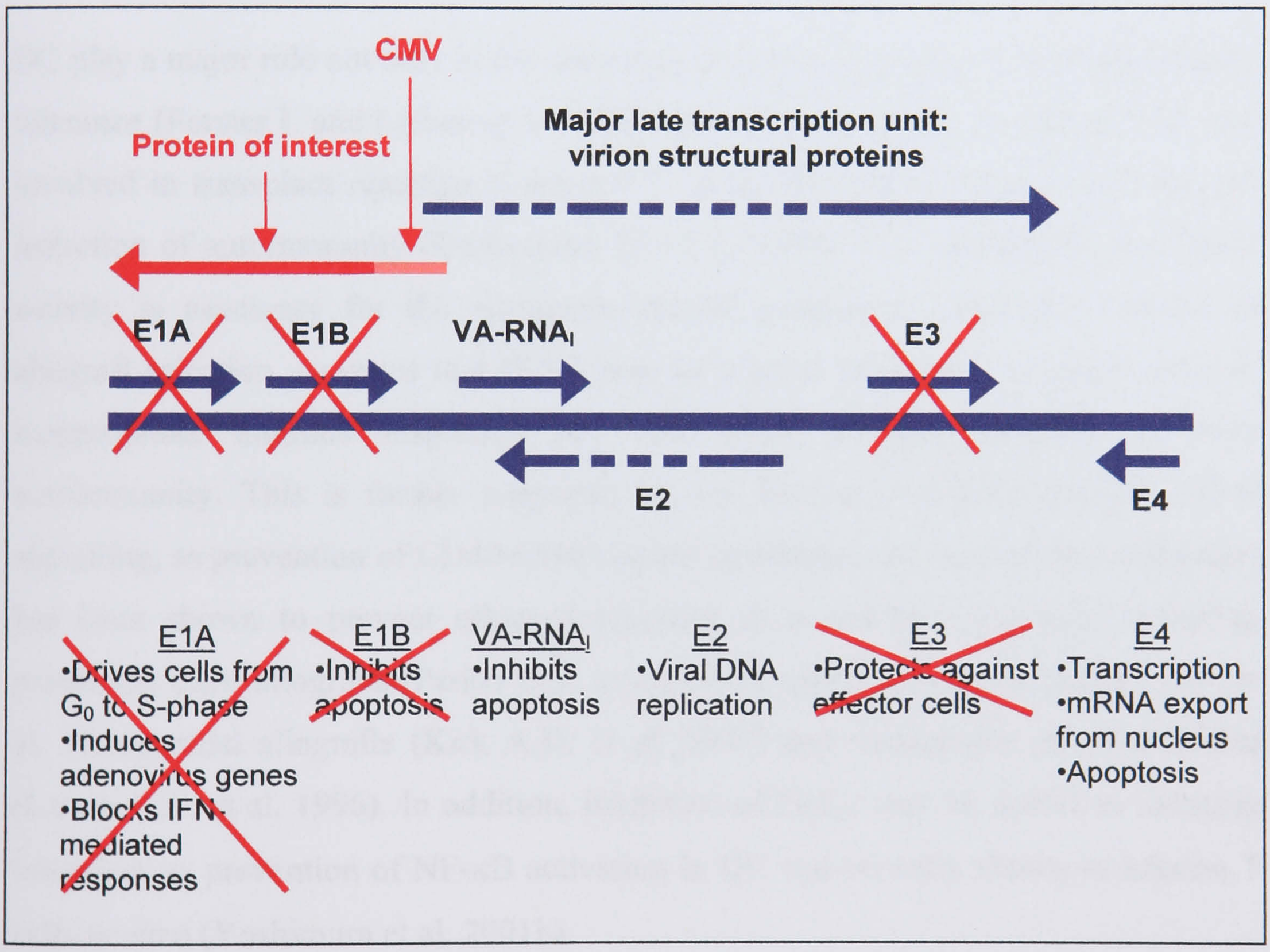


Figure 7.1: Replication-deficient adenoviruses as a tool to study intracellular signalling pathways. Deletion of the E1 region of the genome of adenoviruses of the Ad5 serotype renders them replication-deficient and prevents the expression of the remaining adenoviral genes. In addition, deletion of the E3 region removes almost all the adenoviral genes known to alter intracellular signalling pathways. These adenoviruses can then be engineered to express wild-type or mutant versions of intracellular signalling molecules and provide a powerful tool for biochemical studies into DNA transfection-resistant cell types.

With this powerful tool in my hands, I have made some important observations. First, the **requirement of IKK2 for NF-κB activation and NF-κB-dependent cytokine**

production is cell type- and stimulus-specific. Thus, in DC and macrophages IKK2 is necessary for CD40 ligand- but not LPS-induced NF-κB activation, whereas in endothelial cells IKK2 is required for NF-κB activation in response to LPS as well as IL-1 and TNFα. The reason for that is not known but it clearly demonstrates that there is selective utilization of IKK2 in certain cell types, and further emphasizes the need to study signalling pathways in the cell types that are most relevant to the aim of the study. Second, **IKK2 and NF-κB activation are required for efficient DC antigen-presenting function** as expression of a kinase defective form in DC inhibited the allogeneic MLR. This could be due, at least partly, to the prevention of T cell CD40 ligand-induced DC activation as IKK2 is able to block that effect.

DC play a major role not only in the induction of immunity but also of immunological tolerance (Forster I. and Lieberam I. 1996; Kurts C. et al. 1997). In addition, DC are involved in transplant rejection (Larsen C.P. et al. 1990; Fu F. et al. 1996) and the induction of autoimmunity (Drakesmith H. et al. 2000). The observation that IKK2 activity is necessary for the allogeneic mixed lymphocyte reaction, a model of allograft rejection, suggests that IKK2 may be a good target for the suppression of inappropriate immune responses that take place in transplantation or even autoimmunity. This is further supported by the finding that IKK2 inhibits CD40 signalling, as prevention of CD40-CD40 ligand interactions by monoclonal antibodies has been shown to prevent allograft rejection in a number of models including pancreatic islets allografts (Parker D.C. et al. 1995), cardiac allografts (Larsen C.P. et al. 1996), renal allografts (Kirk A.D. et al. 1997) and xenogeneic skin transplants (Larsen C.P. et al. 1996). In addition, inhibition of IKK2 may be useful in inducing tolerance, as prevention of NF-κB activation in DC was recently shown to tolerize T cells *in vitro* (Yoshimura et al. 2001b).

CD40-CD40L interactions are also important in the pathogenesis of a number of inflammatory diseases as shown by the administration of CD40 or CD40L neutralizing antibodies. Neutralizing antibodies not only prevent disease induction in murine models of lupus nephritis (Mohan C. et al. 1995), diabetes (Balasa B. et al. 1997), oophoritis (Griggs N.D. et al. 1996), inflammatory bowel disease (Stuber E. et al. 1996), atherosclerosis (Mach F. et al. 1998), collagen-induced arthritis (Durie F.H.

et al. 1993) and experimental autoimmune encephalomyelitis (EAE) (Gerritse K. et al. 1996), but they also improve established EAE and experimental autoimmune thyroiditis (Carayanniotis G. et al. 1997). Inhibition, thus, of IKK2 activity could be another way of successfully blocking CD40-CD40 ligand interactions in a number of disease states.

In contrast to IKK2, NIK is not required for efficient DC antigen-presenting function in the allogeneic MLR nor for CD40L- or LPS-induced DC activation. This suggests that despite the major role that NIK plays in NF-κB activation in 293 human embryonic kidney cells or HeLa epithelial cells, in DC NIK is redundant or involved in a currently unidentified pathway. During this work, two studies have suggested that the role of NIK in NF-κB activation may be restricted to certain cell types such as lymphocytes and fibroblasts, and certain signals such as lymphotoxin β (Garceau N. et al. 2000; Matsushima A. et al. 2001; Yin L. et al. 2001). Unless, however, the role that NIK has in various systems becomes clear, strategies aimed at blocking its activity for therapeutic purposes will not be feasible.

Although NIK seems to have a redundant role in dendritic cells, overexpression of the wild-type form of it potently enhances their antigen-presenting function in the allogeneic MLR and the antigen-specific T cell proliferation. This is due to the ability of **NIK to activate sequentially IKK2 and NF-κB, and to coordinate in that way the induction of cytokines, chemokines, MHC antigen-presenting and costimulatory molecules**, and suggests that NIK may be a useful immunostimulatory molecule for the enhancement of immune responses when needed.

The main clinical intervention that requires the enhancement of the immune response rather than its inhibition is immunization. The observation that **NIK enhances the immune response against a vector-encoded antigen *in vivo*** confirmed the physiological significance of the *in vitro* studies and suggested that NIK may be a useful adjuvant for genetic vaccines. The strong skewing effect that NIK has towards a T_{H1} type of immune responses implies that the best use of NIK as an adjuvant would be against viral and certain parasitic or bacterial infections, or even cancer vaccines that require strong cell-mediated immune responses. In addition, it supports another

potential use of NIK that is in the treatment of allergy. As various studies have shown that the generation of T_{H1}-type allergen-specific immune responses not only confers long-lasting protection against allergy (Donnelly J.J. et al. 1997; Roman M. et al. 1997) but can also reverse it (Maecker H.T. et al. 2001), NIK may constitute an attractive new approach to reprogramming the responses against allergen.

The most unexpected findings described in this thesis, however, came from the study of MyD88, the adaptor molecule involved in IL-1R/TLR signalling (reviewed by O'Neill L.A. and Dinarello C.A. 2000). The finding that inhibition of MyD88 activity in M-CSF-differentiated macrophages and immature DC activates them in the absence of further stimulation, is in complete contrast to the current litterature. Although this observation can be an artifact of the overexpression of a MyD88 form with a deletion it its death domain, a factor that requires the construction of regulated expression vectors to be controlled, strong evidence supports a possible inhibitory role for MyD88. First, co-expression of MyD88wt can abrogate the stimulatory effect that the inhibitory form of MyD88 has, showing that the effect is specific for MyD88. Second, the identification of Tollip, a new component of the IL-1 pathway, that plays an inhibitory role in IRAK phosphorylation and p54 MAPK or NF-κB activation suggests that there is an inhibitory mechanism that prevents IRAK phosphorylation in the absence of ligand binding. This is further supported by findings in Drosophila, where the toll receptor has an inhibitory domain in its C-terminus that blocks ligand-independent Toll signalling by impeding the catalytic activity of Pelle, an IRAK homologue (Shen B. and Manley J.L. 1998). As this region is not conserved in IL-1R/TLR family members in mammals suggests that they may have evolved other mechanisms of a similar inhibitory function. Third, the fact that MyD88 possesses a death domain leaves open the possibility that MyD88 may be employing a inhibitory mechanism that is similar to that employed by the TNF receptor family members for the prevention of ligand-independent signalling (figure 7.2). My studies together with these of others in MyD88-deficient mice (Kawai T. et al. 1999) indicate that our current understanding on how TLRs signal is very poor and a lot needs to be done before making use of these pathways therapeutically. The early assumption that IL-1R/TLR signal by using a similar framework of downstream mediators is now being

questioned as there is evidence that this is not the case between distinct TLRs (Kawai T. et al. 1999; Ozinsky A. et al. 2000)

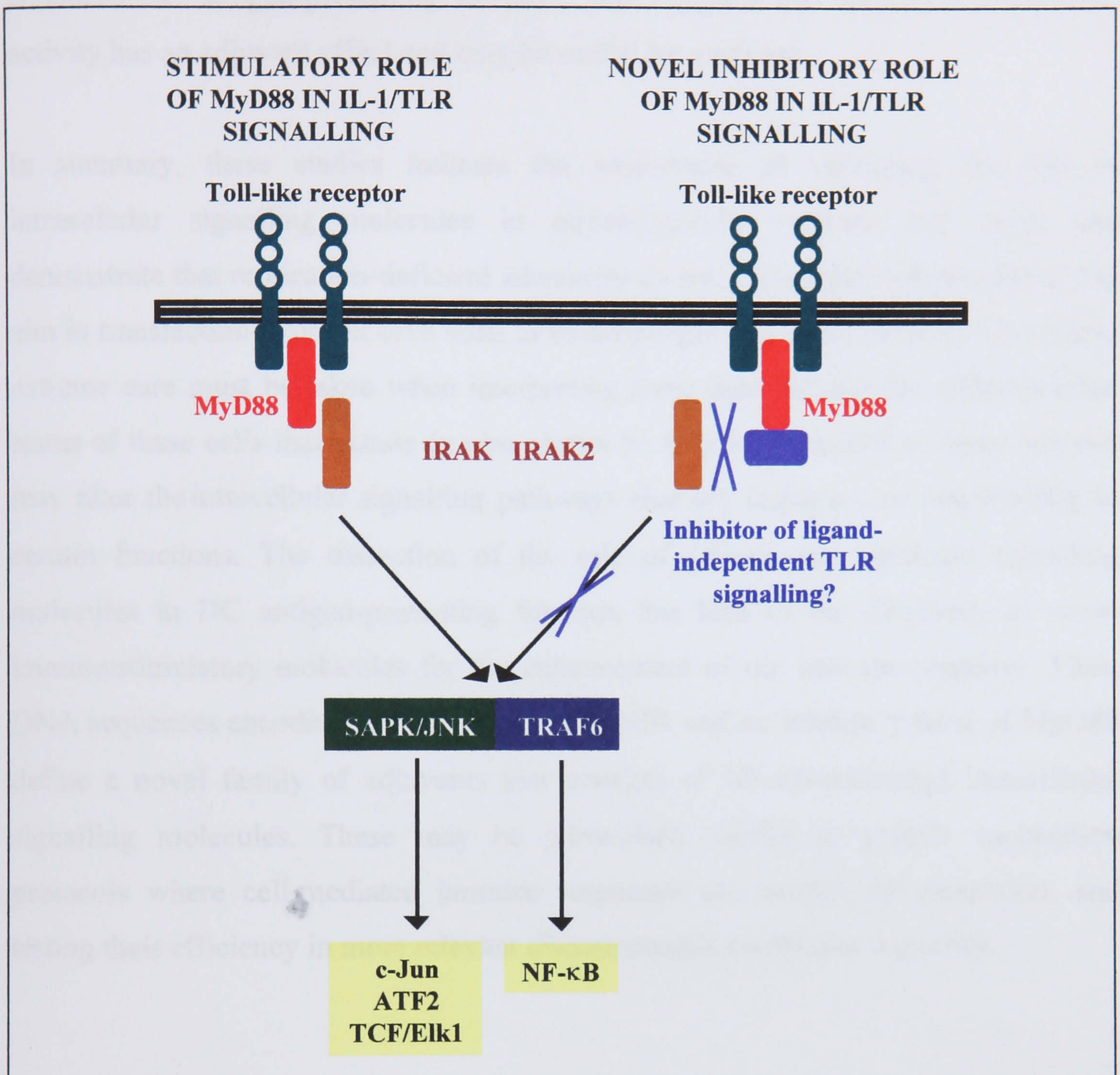


Figure 7.2: Hypothetical model for the inhibitory role of MyD88 in DC and macrophages. MyD88 may prevent ligand-independent TLR signalling in DC and macrophages by recruiting an inhibitory molecule to its death domain, thus preventing its interaction with IRAK. Deletion of its death domain allows the activation of IRAK to take place and a TLR-like pathway to be initiated.

Whichever the mechanism by which **MyD88 prevents ligand-independent TLR signalling in macrophages and DC**, inhibition of MyD88 activity allows the activation of a typical TLR pathway to take place, with the phosphorylation of IRAK and the activation of IKK2 and NF-κB. The mechanism of MyD88dn activation is clearly cell-type specific, and must involve interactions with members of the TLR family restricted to macrophages and DC such as TLR3 (Muzio M. et al. 2000), but

others cannot be excluded. In DC, the effect of Myd88dn bears similarities with that of wild-type NIK overexpression as it up-regulates costimulatory molecules and enhances DC antigen-presenting function, and suggests that inhibition of MyD88 activity has an adjuvant effect and may be useful for vaccines.

In summary, these studies indicate the importance of validating the role of intracellular signalling molecules in physiologically relevant cell types and demonstrate that replication-deficient adenoviruses are a powerful tool to achieve this aim in transfection-resistant cells such as macrophages and dendritic cells. Even then, extreme care must be taken when interpreting these data because the differentiation status of these cells that occurs *in vivo* cannot be exactly mimicked *in vitro*, and this may alter the intracellular signalling pathways that are important or rate-limiting in certain functions. The dissection of the role of NF-κB and upstream signalling molecules in DC antigen-presenting function has lead to the discovery of novel immunostimulatory molecules for the enhancement of the immune response. Thus, DNA sequences encoding a wild-type form of NIK and an inhibitory form of MyD88 define a novel family of adjuvants that consists of NF-κB-activating intracellular signalling molecules. These may be particularly useful in genetic vaccination protocols where cell-mediated immune responses are needed for protection, and testing their efficiency in more relevant disease models constitutes a priority.

"One never notices what has been done; one can only see what remains to be done..."

Marie Curie

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