The Role and Regulation of CD1d in Normal and Pathological B Cells

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by

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Abstract

This work provides novel insights into the intersection between two critical areas of immunology, the CD1d-invariant NKT (iNKT) axis and B cells. CD1d is a non-polymorphic, MHC class I-like molecule, which presents phospho- and glycosphingo-lipid antigens to a subset of CD1d-restricted T cells called iNKT cells. CD1d is expressed on a variety of antigen presenting cells and the CD1d-iNKT cell axis regulates nearly all aspects of the innate and adaptive immune response. Expression of CD1d on B cells allows these cells to form cognate interactions with iNKT cells. Emerging evidence suggests, however, that expression of CD1d on B cells is variable, both on "normal" B cells during humoral immune responses, and also on "pathological" B cells in certain B cell disorders.

In this work, I investigate in detail the expression of CD1d on B cells across a range of conditions. Using both human and murine germinal centre (GC) B cells as a model for normal B cells, I show for the first time that CD1d expression changes dynamically, both at the surface protein and transcriptional level. CD1d falls to a nadir as a naïve B cell enters the GC, and subsequently rises again in post GC B cells. I then provide evidence that the loss of CD1d expression is paralleled in pathological B cells, specifically in Epstein-Barr Virus infection of B cells and the plasma cell disorder Multiple Myeloma. In these conditions, CD1d is again downregulated at both the surface and transcriptional level.

Having established that CD1d expression is lost in certain scenarios, I attempt to elucidate the biological significance of this downregulation. I approach this aim by attempting to constitutively express CD1d in murine GC B cells, and subsequently assessing the GC reaction. Constitutive expression of CD1d is achieved by two distinct methods, either by using adoptive transfer of *Cd1d1* transduced haematopoietic stem cells or by using a CD1d transgenic mouse model. I show that both of these approaches represent a feasible way to constitutively express CD1d in murine GC B cells, but cannot establish a definitive biological role for CD1d downregulation in murine GC B cells.

Finally, I investigate the transcriptional mechanisms governing the downregulation of CD1d, in either normal or pathological B cells. By analysing GC and MM B cells, I provide the first evidence that both retinoic acid signalling and bivalent chromatin domains act as a dual regulatory mechanism of CD1d. These findings demonstrate a new concept in the field of MM biology, specifically that MM B cells (like lymphomas) are able to "hijack" physiological pathways for disease propagation. Although previous work has highlighted the ability of all-trans retinoic acid (ATRA) alone to increase CD1d expression, this uncovering of a dual mechanism provides the rationale to use both ATRA and the polycomb-repressive complex inhibitor GSK343 to restore CD1d expression. I show that this is a more effective strategy than ATRA alone in increasing CD1d expression. Although this will need to be validated in further *in vivo* models, this ability to further increase CD1d expression may be of great therapeutic importance in the emerging field of tumour immunotherapy.

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Declarations

This work is my own, and all work is appropriately referenced.

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List of Abbreviations

ACTB	Beta-actin
ALDH	Retinaldehyde dehydrogenase
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine 7
APC	Antigen presenting cell
APML	Acute promyelocytic leukaemia
ApoE	Apolipoprotein E
ATRA	All-trans retinoic acid
Bcl	B cell leukaemia/lymphoma
B-CLPD	B-cell chronic lymphoproliferative disorder
BCR	B cell receptor
B-LCL	B-lymphoblastoid cell line
BM	Bone marrow
Breg	B regulatory cell
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukaemia
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
CSR	Class-switch recombination

CTD	C-terminal domain
DAPI	4',6-diamidino-2-phenylindole
DLBCL	Diffuse large B cell lymphoma
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ELF1	E74-like factor 1
ETS	E-twenty-six
EZH2	Enhancer Of Zeste 2
FASL	Fas Ligand
FB	Flow cytometry buffer
FBS	Foetal Bovine Serum
FDG	Fluorescein di-β-D-galactopyranoside
FL	Follicular lymphoma
GC	Germinal centre
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
H3K4me3	Trimethylation of histone H3 Lys 4

H3K27me3	Trimethylation of histone H3 Lys 27
HEL	Hen egg lysozyme
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFL	Human flt3-ligand
HLA	Human Leukocyte Antigen
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
ICOS	Inducible T-cell costimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IgV	Immunoglobulin variable region
iNKT	Invariant Natural Killer T
IRES	Internal ribosome entry site
lacZ	Beta-galactosidase
LB	Luria-Bertani
LEF-1	Lymphoid enhancer-binding factor-1
LDLR	Low-density lipoprotein receptor
LiCl	Lithium chloride
LMP	Latent membrane protein
mAB	Monoclonal antibody
mRNA	Messenger ribonucleic acid

MSCF	Murine stem cell factor
MSCV	Murine Stem Cell Virus
MHC	Major Histocompatibility Complex
$MgCl_2$	Magnesium Chloride
MGUS	Monoclonal gammopathy of uncertain significance
MLL	Mixed lineage leukaemia
MM	Multiple myeloma
MZ	Marginal zone
N-CoR	Nuclear receptor corepressor
Nef	Negative Regulatory Factor
NK	Natural Killer
NKT	Natural Killer T
NP-KLH	4-hydroxy-3-nitrophenyl-keyhole limpet hemocyanin
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PcG	Polycomb group
PD-1	Programmed cell death-1
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine 7
PerCP-Cy5.5	Peridinin chlorophyll-Cyanine 5.5
PETG	Phenyl-ethyl β-D-thiogalactopyranoside
PPARγ	Peroxisome proliferator-activated receptor

	gamma
PRC	Polycomb-repressive complex
QRT-PCR	Real time polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBC	Red blood cell
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RoDH	Retinol dehydrogenase
RPMI	Roswell Park Memorial Institute
RXR	Retinoid X receptor
SAM	S-(S′-adenosyl)-L-methionine
SAP	Signalling lymphocyte activation molecule associated protein
Sca-1	Stem cell antigen-1
SHM	Somatic hypermutation
shRNA	Short hairpin ribonucleic acid
SLE	Systemic lupus erythematosus
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SV40	Simian virus 40
TCR	T cell receptor

TD	T cell-dependent
TF	Transcription factor
TG	Transgenic
TGN	Trans-Golgi network
TI	T cell-independent
T_{FH}	T follicular helper
T_H	T helper
TrxG	Trithorax group
VSV-G	Vesicular stomatitis virus glycoprotein
XLP	X-linked lymphoproliferative disorder

Chapter 1

1 Introduction

1.1 CD1d

CD1d is a non-polymorphic, MHC class I-like, β2-microglobulin-associated molecule, which presents phospho- and glycosphingo-lipid antigens to a subset of immunoregulatory T cells called type I (or invariant) and type II NKT cells (Salio et al., 2014). While in rodents CD1d is the only lipid-presenting molecule, in humans there are in addition four other CD1 molecules (CD1a, b, c, and e), which interact with lipid-specific T cell subsets distinct to NKT cells.

1.1.1 The CD1 gene family

The 5 *CD1* genes in humans, termed *CD1A-E*, are grouped into two clusters on chromosome 1, group I (*CD1A-C* and *CD1E*) and group II (*CD1D*). Rodents lack *Cd1* group I genes, as a result of a chromosomal deletional event (Brigl and Brenner, 2004). There are two *Cd1d* genes in mice, *Cd1d1* and *Cd1d2* (sharing 95% homology), likely the result of a recent duplication event in evolution. The 2 genes are organised on chromosome 3 in a tail-to-tail fashion **(Figure 1.1)**. With the exception of the C57BL/6 (B6) strain in which *Cd1d2* bears a frameshift mutation (Park et al., 1998), mice express

both *Cd1d* genes although CD1d2 protein expression is generally lower and not functional (Chen et al., 1999).

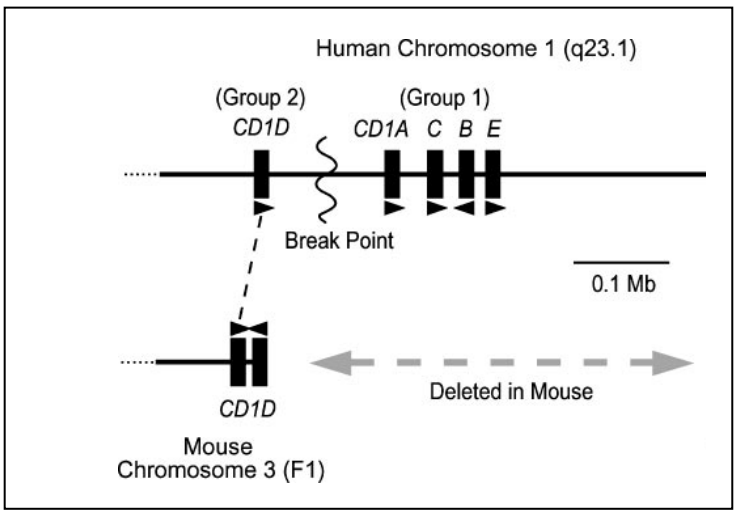


Figure 1.1. A genomic map of the human (chromosome 1) and mouse (chromosome 3) CD1 loci. *CD1 genes are shown as black boxes, and arrows indicate the direction of transcription. Dashed lines connect orthologous genes. (Adapted from Brigl and Brenner, 2004.)*

1.1.2 Assembly and expression of CD1d

CD1d molecules consist of a transmembrane heavy chain, which possesses a short C-terminal cytosolic tail, a hydrophobic transmembrane region, and an extracellular region that interacts non-covalently with β2-microglobulin. Following production, the CD1d heavy chains are transported into the endoplasmic reticulum (ER), where folding and association with β2-microglobulin occurs. Newly synthesized CD1d molecules are then rapidly delivered from the Golgi to the plasma membrane.

CD1d has a broad pattern of expression and is expressed on cells of both myeloid (monocytes, macrophages, dendritic cells) and lymphoid (B lymphocytes, thymocytes but not mature T cells) lineage (Brigl and Brenner, 2004; Exley et al., 2000). It is also expressed outside the haematopoietic system, for example on epithelial and vascular smooth muscle cells (Canchis et al., 1993). Expression of CD1d on B cells, the focus of this thesis, points to the potential of these cells to present lipid antigen to, and engage in cross-talk with, iNKT cells.

1.1.3 Lipid presentation by CD1d

The main known function of CD1d is to present phospholipid and glycolipid ligands to NKT cells. The crystal structure of CD1d has clearly shown that binding of glycolipids to CD1d is organised in such a way that the long lipid tails are accommodated in deep hydrophobic pockets defined by the α1 and α2 domains, whereas the sugar moieties within the polar head of the glycolipid protrude from the presentation groove and make contact with the NKT T cell receptor (TCR) (Koch et al., 2005; Zeng et al., 1997) **(Figure 1.2)**.

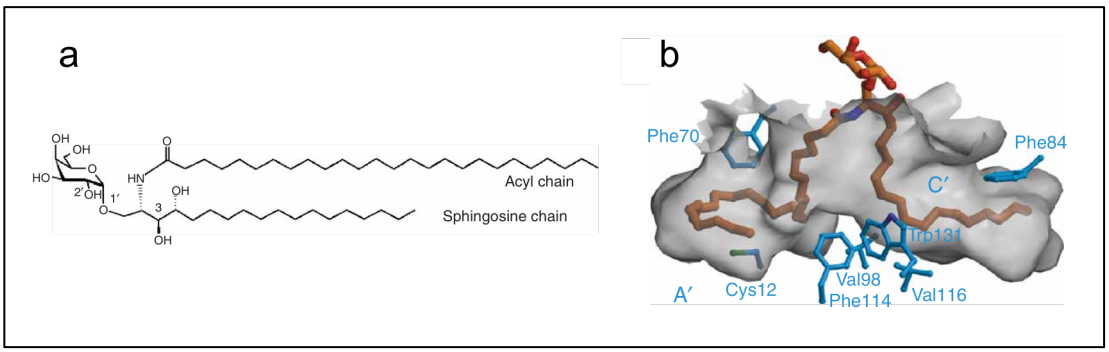


Figure 1.2. Glycolipid chemical structure and its binding to human CD1d

(a) *Structure of a typical CD1d-binding glycolipid (α-galactosylceramide) demonstrating the long lipid tails and a polar head comprised of sugar moieties.*

(b) *Crystallographic image of human CD1d, revealing the hydrophobic pockets A' and C', which accommodate the lipid tails of ligand. These channels communicate directly with the surface, allowing the polar head of the glycolipid to protrude from a presentation groove and interact with NKT cells. (From Koch et al., 2005.)*

Following synthesis in the ER, CD1d acquires endogenous lipid including glycosylphosphatidylinositol and phospholipids, before being transported to the plasma membrane (Gumperz, 2006). Central to its ability to function as an antigen presenting molecule, surface CD1d undergoes internalization and trafficking from the cell surface to endosomal and lysosomal compartments in the cytosol. In these compartments, CD1d exchanges its ligands with alternative lipids, before returning to the cell surface to present these (Brigl and Brenner, 2004) **(Figure 1.3)**.

This internalization and intracellular trafficking has been shown to depend on a tyrosine based sorting motif in the cytoplasmic tail of CD1d (YXXZ, where Y = tyrosine, X = any amino acid, and Z = bulky hydrophobic residue) (Chiu et al., 2002; Elewaut et al., 2003; Jayawardena-Wolf et al., 2001).

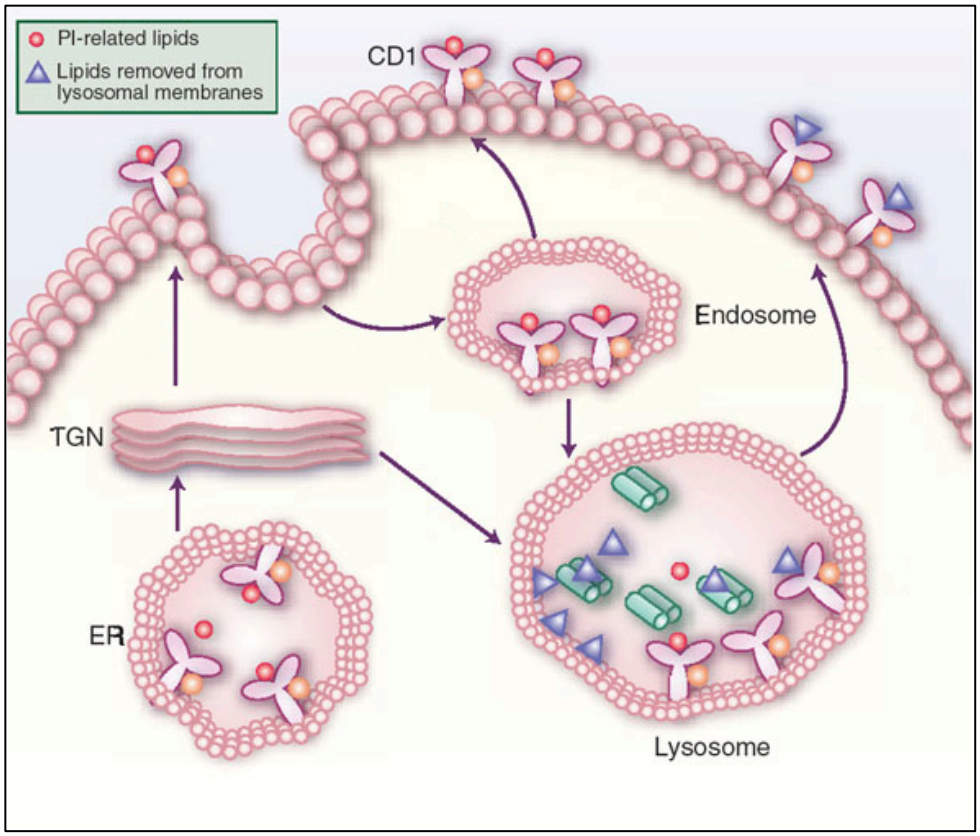


Figure 1.3. Trafficking and recycling of CD1d. *Following synthesis and assembly in the ER, CD1d loaded with endogenous lipid is transported to the plasma membrane. CD1d is, however, able to internalize and traffic back from the plasma membrane to endosomes and lysosomes. Newly synthesised CD1d may also directly move from the trans-Golgi network (TGN) to lysosomes. Within these lysosomal compartments, CD1d bound lipid may be exchanged for alternative lipid ligands, before CD1d is re-transported back to the plasma membrane to present antigen at the cell surface. This recycling of CD1d is key to the presentation of a diverse range of lipid antigens. (From Kronenberg, 2004.)*

The lipids presented by CD1d may be either endogenous to the cell or acquired by the cell from exogenous sources. Specifically, B cells may capture and internalize foreign lipid antigen directly through the B cell receptor (BCR), a concept that may be utilised in the design of novel lipid bound immunogens (Barral et al., 2008; Lang et al., 2005; Leadbetter et al., 2008). Alternatively, B cells may, like dendritic cells, be able to capture ApoE/lipid complexes via the low-density lipoprotein receptor (LDLR), in a BCR-independent manner (van den Elzen et al., 2005).

1.2 CD1d restricted T cells

Phospho- or glycolipid presented by CD1d is recognised by a set of immunoregulatory CD1d-restricted T cells, termed NKT cells as they were classically thought to express both a TCR and the NK receptor NK1.1. It is now known that the expression of such markers is not robust, and CD1d restricted T cells are now more stringently classified as either type I or type II NKT cells (Godfrey et al., 2004).

1.2.1 Type I NKT cells

These are also termed classical NKT cells or invariant NKT (iNKT) cells. A hallmark of iNKT cells is their use of a semi-invariant αβ TCR. In humans, it comprises an invariant TCRVα24–Jα18 chain paired nearly always with a non-invariant TCRVβ11 chain while in mice the homologous invariant TCRVα14–Jα18 chain pairs with a limited set of TCRVβ chains (TCRVβ2, 7 and 8).

iNKT cells are the best studied subset of CD1d-restricted T cells, and can be described as a type of innate-like lymphocyte which can bridge the innate and adaptive arms of the immune system (Brennan et al., 2013) **(Figure 1.4)**.

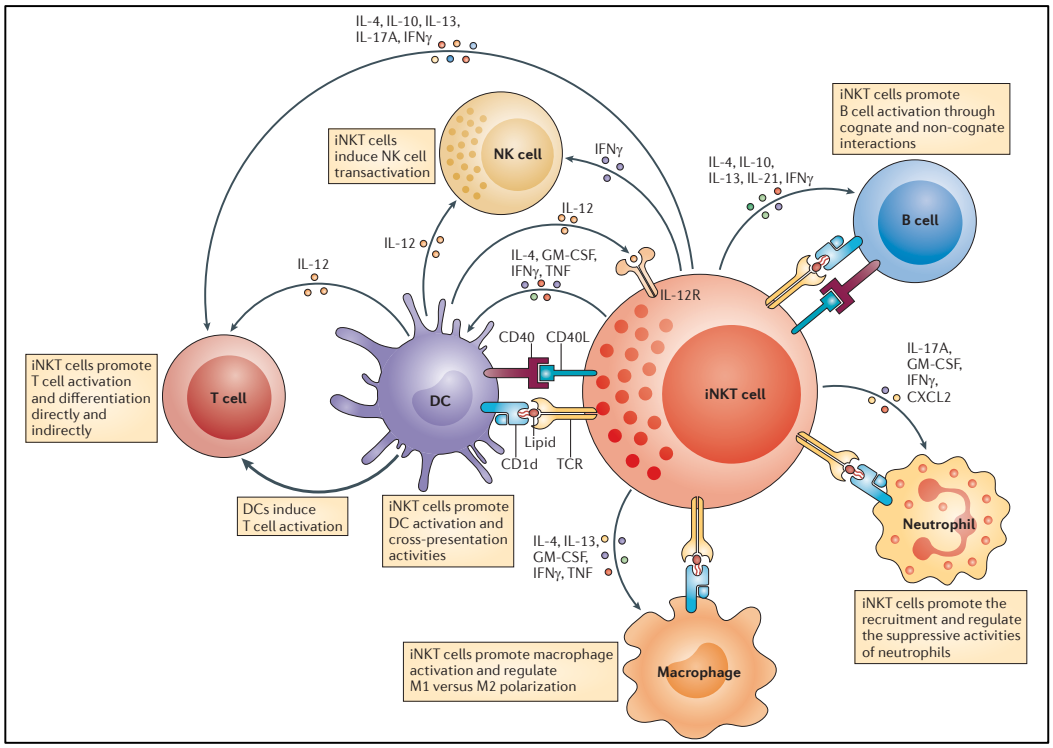


Figure 1.4. iNKT cells as a bridge between the innate and adaptive arms of the immune system. *iNKT cells are innate-like lymphocytes which can rapidly exert effector functions following activation. Through both cognate interactions with CD1d expressing cells, and non-cognate mechanisms including cytokine release, they are able to activate cells of both the innate and adaptive immune system. (From Brennan et al., 2013.)*

iNKT cells exist in a "poised effector state", and following activation, they can rapidly assume a T helper 1 (T_H1), T_H2 or T_H17 functional immune profile, and can also exhibit direct cytotoxicity using mediators such as perforin and Fas ligand (FASL) (Kronenberg, 2005). This diverse range of functions underpins the ability of the CD1d-iNKT axis to play a key role in anti-microbial, anti-tumour and autoimmune responses in mice (Berzins et al., 2011). More recently, the immunoregulatory importance of iNKT cells has also been demonstrated in the human setting in the context of acute graft vs host disease (Chaidos et al., 2012).

iNKT cells are activated in response to a range of endogenous and exogenous lipids, with the glycosphingolipid α-galactosylceramide (α-GalCer) being the prototypical and one of the most powerful, although not physiological (i.e. not synthesised in mammalian tissues), stimulating agonists (Kawano et al., 1997).

1.2.2 Type II NKT cells

In contrast to type I NKT cells, the CD1d-restricted type II NKT cells have a diverse repertoire of TCR alpha chains and are functionally less well characterised. Therefore, they will not be discussed further here.

1.3 CD1d in normal B cells

The CD1d molecule is expressed on mature, naïve and memory B cells, in plasma cells and also on B regulatory cells (Bregs) (Allan et al., 2011; Mauri and Bosma, 2012). The latter are a subset of immunoregulatory, IL-10-secreting B cells that immunophenotypically correspond to recent bone marrow emigrants called transitional B cells, and are implicated in the pathogenesis of autoimmune disorders (Mauri and Bosma, 2012).

1.3.1 The humoral immune response

The B cell immune response can be categorized as T cell-independent (TI) or T cell-dependent (TD). TI responses do not require direct interaction with T helper cells (T_H). They can be further classified as type 1, in which the B cell is stimulated by activating ligands (e.g. immunostimulatory DNA containing CPG motifs) that do not engage the BCR, or type 2, in which the BCR is engaged by multivalent epitopes such as polysaccharides. TI responses generally lead to extrafollicular antibody-producing cells rather than germinal centre (GC) formation, do not generate high affinity antibodies, and produce few plasma cells and atypical memory cells (MacLennan et al., 2003). The result is a rapid yet transient innate-like response that does not lead to enhanced recall responses.

TD responses occur through BCR activation by antigen in the presence of cognate help from a specialized subset of $CD4^+$ T_H cells, termed T follicular helper cells (T_{FH}) (Vinuesa et al., 2005). T_{FH} cells are regulated by the transcriptional repressor Bcl-6, use the chemokine receptor CXCR5 to home towards the B cell follicles, and express key molecules important for T/B cell interactions, including IL-21, PD-1, SAP, ICOS and CD40L (Ma et al., 2012). Activated B cells consequently proliferate and differentiate within the follicles, giving rise to GCs, the hallmark of the TD response.

The classic description of the GC is of a 'dark' zone consisting almost exclusively of densely packed proliferating B cells (termed centroblasts) and a 'light' zone consisting of smaller, non-dividing B cells (centrocytes) situated within a mesh of follicular dendritic cells (stromal antigen presenting cells), T cells and macrophages **(Figure 1.5)**. In reality, this compartmentalization of the GC is less well defined, and trafficking of cells in both directions between dark and light zones has been noted (Hauser et al., 2007; Schwickert et al., 2007). The GC is surrounded by non-activated naïve B cells, termed the mantle zone.

Within the GC environment, B cells undergo class-switch recombination (CSR), a DNA-level mechanism that brings about immunoglobulin heavy chain isotype switching. The vigorous proliferation of the centroblast also provides an opportunity for somatic hypermutation (SHM), a process by which the immunoglobulin variable region (IgV) of the rearranged antibody

genes of each B cell is modified, through introduction of non-random mutations. Both CSR and SHM are dependent on the enzyme activation-induced cytidine deaminase. Those B cells that express newly generated modified antibodies are selected for improved antigen binding in the light zone. This affinity maturation leads to generation of high affinity Ig-secreting, long-lived plasma cells and memory cells, thus ensuring a strong anamnestic response (Klein and Dalla-Favera, 2008).

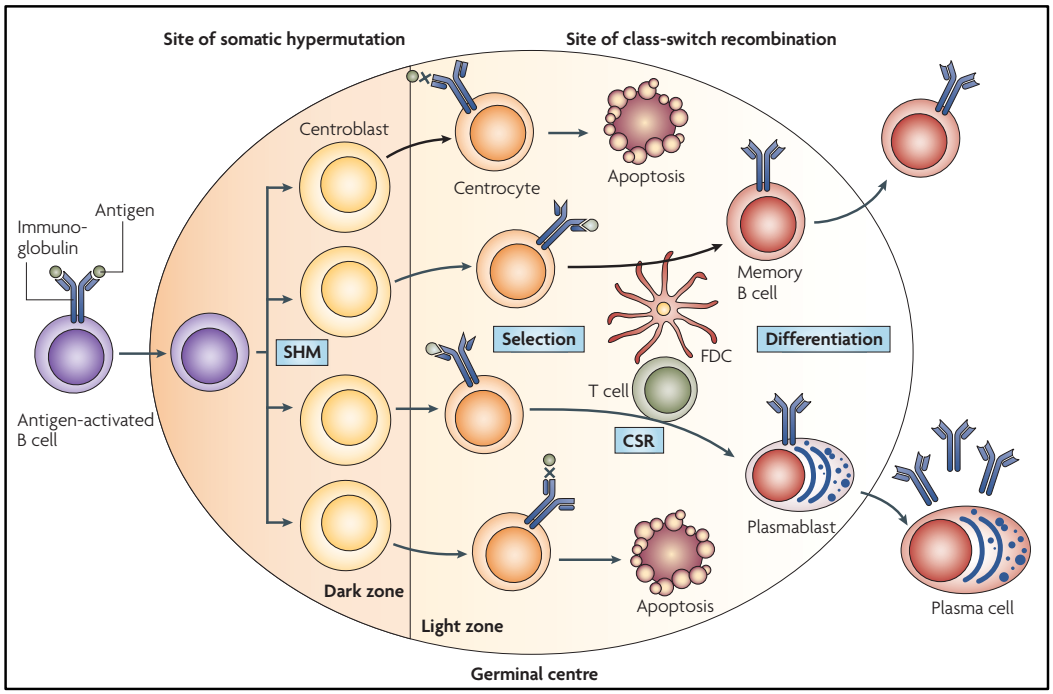


Figure 1.5. The germinal centre microenvironment. *Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) occurs, which may improve immunoglobulin receptor affinity for antigen (see main text). Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunising antigen. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR, see main text). Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells. (From Klein and Dalla-Favera, 2008.)*

1.3.2 Regulation of humoral immunity by CD1d and iNKT cells

Similar to conventional T cells, iNKT cells can also regulate, enhance and sustain humoral immune responses. Early studies revealed that administration of α-GalCer to mice induced iNKT cell activation, led to an IL-4-dependent activation of B cells, and in some cases an increase in total serum IgE levels (Singh et al., 1999). Using iNKT cell-deficient TCRJα18$^{-/-}$ mice, dependency of IgE responses on iNKT cells was subsequently shown in models of allergy, such as ovalbumin-induced asthma (Lisbonne et al., 2003). Similarly, the use of CD1d$^{-/-}$ mice has revealed impaired antibody responses to bacteria, including Borrelia species and Streptococcus pneumoniae polysaccharides (Belperron et al., 2005; Kobrynski et al., 2005). More recently, it was shown that iNKT cells play a critical role in the production of antibodies against the blood group A antigen (Tazawa et al., 2013). In comparison to wild type mice, iNKT cell-deficient mice did not develop increased anti-A levels (IgM and IgG) on immunisation with blood group A red blood cells. Furthermore, CD1d blockade by administration of a monoclonal antibody prior to immunisation also abrogated the anti-A response, in both wild type and humanised mice.

The precise nature of the interaction between iNKT and B cells can vary, and does not always depend on CD1d expression specifically by B cells. In order to further elucidate this interaction, investigators took advantage of the adjuvant function of lipid antigens, primarily α-GalCer.

1.3.3 Non-cognate interactions between iNKT and normal B cells

The help provided by iNKT to B cells may occur indirectly through non-cognate mechanisms. This principle was demonstrated by the generation of a series of murine bone marrow radiation chimeras, lacking CD1d or CD40 on B lymphocytes, or expressing CD1d or MHC II disjointly on antigen presenting cells (APCs) (Tonti et al., 2009). These experiments showed that B cell responses against nominal protein antigens could be enhanced by α-GalCer. This could occur in the absence of CD1d but not CD40 on B cells, and furthermore required co-expression of CD1d and MHC II on APCs. Taken together, the findings suggested a non-cognate mechanism for iNKT help to B cells, through licensing of APCs, increased T_{FH} cell activation and thus improved conventional T/B cell interactions **(Figure 1.6(a))**. In this role, α-GalCer is acting as a classical immunological adjuvant, stimulating early innate immune responses to aid the establishment of protective adaptive responses. This is underlined by pre-clinical studies in which vaccines containing α-GalCer mixed with protein antigens have been shown to induce more effective humoral and memory responses against several viral, parasitic and bacterial pathogens (Carreño et al., 2014).

1.3.4 Cognate interactions between iNKT and normal B cells

There is now firm evidence that B cells, through CD1d expression, are able to also form cognate interactions with iNKT cells, driving a novel form of humoral immune response. First, it was demonstrated that iNKT cells *in vitro* promoted proliferation of autologous B-lymphocytes and induced Ig production in a CD1d-dependent manner (Galli et al., 2003). iNKT cell help to B cells could occur both in the presence or absence of α-GalCer, suggesting that B cells expressing CD1d can present exogenous or endogenous lipid to iNKT cells. Further work in MHC class $II^{-/-}$ mice (which lack all class II-restricted T cells) provided support for this concept *in vivo*. Co-administration of α-GalCer with TD antigen was able to induce a limited antibody response in mice lacking T_h cells, implying that iNKT cells could act as an alternative, albeit less efficient, cognate partner for B cells (Galli et al., 2007; Lang et al., 2006). The CD1d-restricted nature of these cognate iNKT-B cell interactions was also shown to occur *in vivo,* using μMT mice. These mice lack mature B cells due to a targeted mutation in the μ immunoglobulin heavy chain. Following reconstitution of the mature B cell compartment in these mice with B cells from WT or $CD1d^{-/-}$ donors, α-GalCer enhanced antibody responses against NP-KLH, dependent on CD1d expression by B cells (Lang et al., 2008).

As a caveat, several of the studies up to this point had employed a similar immunisation strategy, using a combination of protein antigens mixed with

uncoupled α-GalCer. Such a combination is well suited to demonstrating the adjuvant properties of α-GalCer, but does not ensure efficient delivery to individual B cells of both the nominal antigen and the adjuvant lipid.

This was addressed by novel immunisation methodologies which aimed to target glycolipid to the BCR and generate lipid-specific B cell responses, either by using NP hapten directly conjugated to α-GalCer (Leadbetter et al., 2008) or protein antigen (hen egg lysozyme, HEL) and α-GalCer both linked to bead particles (Barral et al., 2008). In both cases, B cells activated by antigen can internalize both the antigen and the physically linked α-GalCer molecule through the BCR. Lipid can then bind to CD1d and subsequently be presented by the B cell to iNKT cells. Immunisation of mice in this way was shown to produce rapid elevation in the titres of hapten- or protein-specific IgM and IgG (Barral et al., 2008; Leadbetter et al., 2008). These immunogenic formulations are not able to generate MHC-II restricted epitopes, ruling out a role for a cognate T_h cell effect. Humoral responses were shown to be dependent, however, on the presence of iNKT cells, CD1d expression by B cells, CD40-CD40L signalling, CD80/CD86 co-stimulaton and interferon-γ (IFN-γ) production, strongly supporting a role for a cognate iNKT-B cell interaction.

This work was followed up by studies using the same immunisation techniques in order to establish the longer-term outcome for B cells that have received cognate help from iNKT cells. When NP-α-GalCer was used to

ensure lipid delivery specifically to B cells, the response was characterized by extrafollicular plasmablasts, GC formation, affinity maturation and a strong primary IgG response, dependent on IL-21 production by iNKT cells (King et al., 2012). There was however, impaired development of long-lived plasma cells and memory B cells, commensurate with a lack of an enhanced humoral memory response. Similar work involving the use of HEL-α-GalCer to induce stable iNKT-B cell cognate interactions also found evidence of IL-21 dependent GC reactions but a lack of long-lived plasma cells, memory B cells and enhanced recall responses (Chang et al., 2012). Finally, the outcomes of B cells in MHC class $II^{-/-}$ mice immunised with protein antigen and α-GalCer were explored (Tonti et al., 2012). In this different immunisation model, entailing use of mixed radiation chimeras and B cell transfer experiments, it was found that antigen-specific antibody responses occur in a CD1d- and CD40-dependent manner, suggestive of a cognate iNKT-B cell interaction. Furthermore, this was again accompanied by rapid but short-lived GC formation, and transient rather than long-lived antibody responses. Taken together, these studies suggest that the cognate interactions between CD1d-expressing B cells and iNKT cells thus generate a hybrid immune signature, termed the type 2 TD immune response, encompassing features of both the classic TD and TI responses **(Figure 1.6(b))**.

Intriguingly, these studies have revealed a subset of iNKT cells recapitulating features of T_{FH} cells, including expression of CXCR5, IL-21, PD-1 and CD28, all under control of the Bcl-6 transcriptional programme (Chang et al., 2012; Tonti et al., 2012). Using inducible knockout mice, it was further shown that signalling lymphocyte activation molecule associated protein (SAP), which is essential for iNKT cell development (see below), is also critical for this cognate B cell interaction (Detre et al., 2012). Termed $iNKT_{FH}$ cells, these are the specialised NKT cells, which, like their T cell counterparts, are responsible for interacting with the antigen-presenting B cell and driving the immune response. The non-cognate and cognate help offered by iNKT cells to B cells is highlighted in **Figure 1.6**.

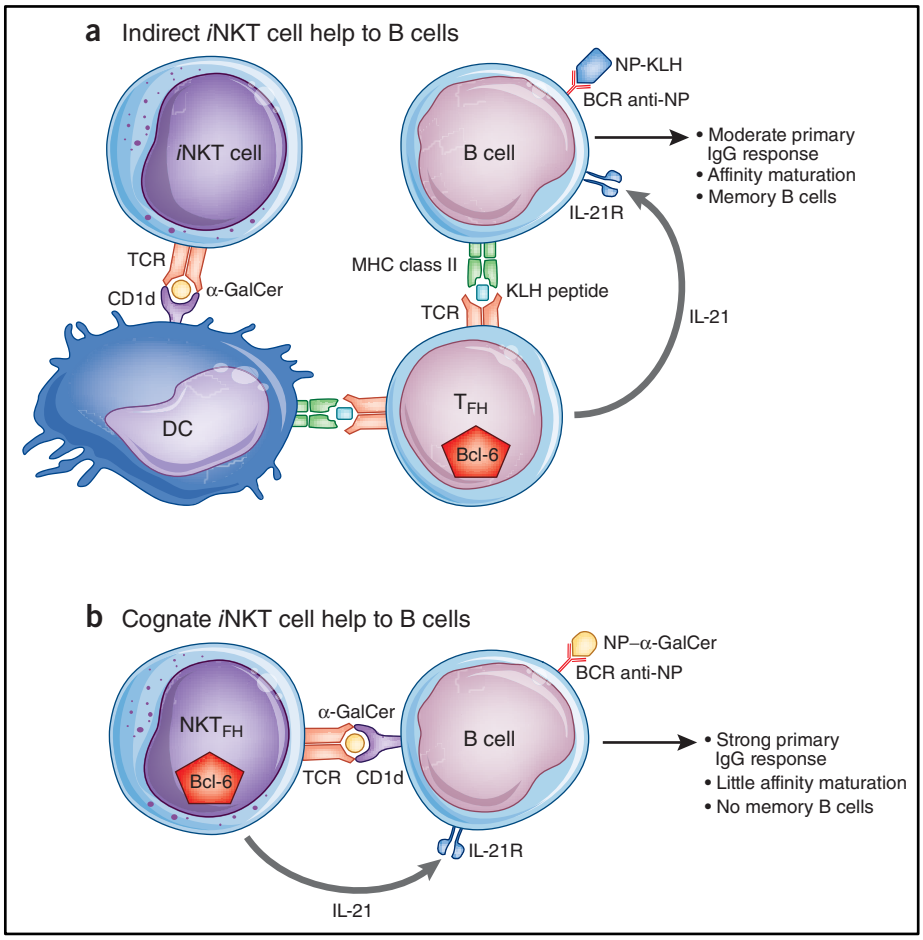


Figure 1.6. Non-cognate and cognate help from iNKT cells to B cells.

(a) *Non-cognate iNKT cell help. iNKT cells induce dendritic cell maturation, which then activate CD4⁺ T_H cells. These become T_{FH} cells, which provide help to B cells, leading to an immune response characterised by affinity maturation and memory development (termed the type 1 TD response).*

(b) *Cognate iNKT cell help. Presentation of lipid by B cells to iNKT cells can directly activate iNKT cells and lead to acquisition of the $iNKT_{FH}$ phenotype. These $iNKT_{FH}$ cells can then provide direct help to the B cell, which produces a rapid immune response lacking in affinity maturation and memory formation (termed the type 2 TD response). (From Lehuen and Fazilleau, 2012.)*

Recent *in situ* imaging studies have revealed that activated iNKT cells preferentially localize in the marginal zone (MZ) of the spleen, a specialized region in continuous contact with blood-borne antigens (Barral et al., 2012; King et al., 2013). The MZ contains several innate and innate-like lymphocytes, including MZ B cells, which may play a prominent role in responding rapidly to TI antigens (Cyster, 2000). It is of note that MZ B cells express high levels of CD1d protein and secrete mainly IgM and IgG3, the main antibody isotypes produced in response to lipid conjugated antigen (Lopes-Carvalho et al., 2005). Thus, it is plausible that these MZ B cells may be one of the predominant recipients of cognate $iNKT_{FH}$ help.

The physiological role of this type 2 TD response is not clear, as it may at first appear counterintuitive that there is rapid immune response with affinity maturation and yet this does not persist. It is possible that exposure in real life to pathogen lipid ligands may be more prolonged than that of soluble α-GalCer and thereby could sustain the presence of $iNKT_{FH}$ cell-dependent immune responses. Alternatively, pathogens may simultaneously stimulate both lipid and peptide B cell responses. The role of $iNKT_{FH}$-dependent GC formation may therefore be to provide a rapid response by lipid-specific B cells, which, although deliberately abrogated, can provide a platform of primed GCs that can be re-used by newly activated peptide-specific B cells. A more recent study has added further complexity to this issue (Bai et al., 2013). By immunising mice with liposomal nanoparticles containing both Streptococcus pneumoniae polysaccharides and α-GalCer,

the authors demonstrated extrafollicular B cell proliferation, leading to a prolonged, high affinity, class-switched antibody response and a strong anamnestic response. This was dependent on CD1d expression by dendritic cells and B cells, indicating a two-step cognate process. This outcome, incongruous with the previous studies, may be explained by the use of a different antigenic formulation, since polysaccharides are able to act as type 2 TI antigens; nevertheless, it demonstrates the potential to target cognate iNKT cell help to B cells in vaccination strategies.

1.3.5 Downregulation of CD1d in germinal centre B cells

In light of the concept that the help provided by $iNKT_{FH}$ cells may be purposely halted, it is notable that immunohistochemical staining of human lymph node tissue (Exley et al., 2000) reveals a clear lack of CD1d expression on GC B cells **(Figure 1.7)**. The *in vivo* biological mechanism and significance of this phenomenon, however, remain to be determined.

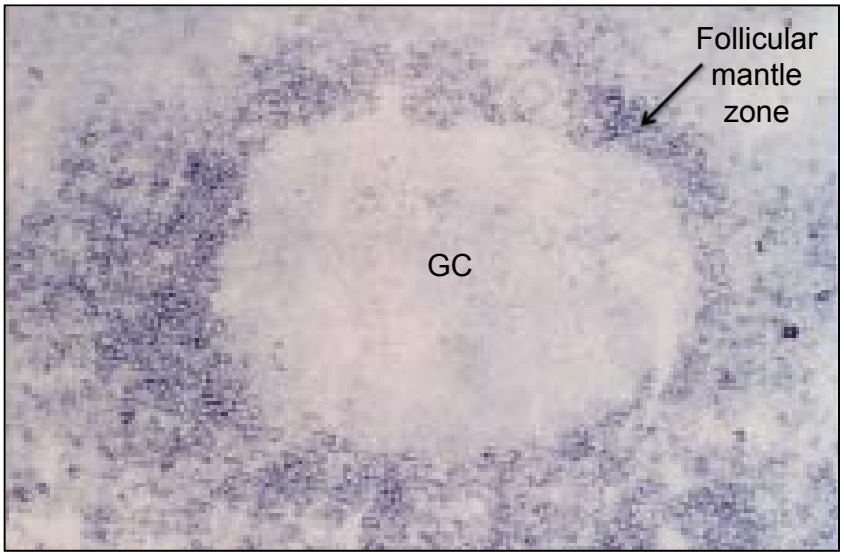


Figure 1.7. Immunohistochemical staining of a human lymph node for CD1d. *Lymph node stained with anti-CD1d monoclonal antibody (mAb), clone 51.1. Note the absence of CD1d staining within the GC in contrast to the strong CD1d staining in the surrounding mantle zone. Comparable results were obtained with the 27.1 and 42.1 anti-CD1d mAbs. (From Exley et al., 2000.)*

More recently, it was shown that transcriptional downregulation of CD1d can be induced in proliferating B cells activated *in vitro* (Allan et al., 2011). CD1d downregulation was found to occur at both the cell surface and the mRNA level in response to various activating ligands including anti-Ig antibodies, Staphylococcus aureus Cowan I bacteria (both of which stimulate the B cell receptor) and CD40 ligand (mimicking the T cell help observed *in vivo*). Mechanistically, CD1d downregulation was associated with a decrease in RARα signalling, and could be reversed by agonists of this pathway, including all-trans retinoic acid (ATRA) and the specific RARα agonist AM580. Following *in vitro* activation, CD1d downregulation occurred at 24 hours, reaching maximum effect at 5 days.

Upregulation of CD1d by AM580 predictably re-enabled lipid presentation. Following CD1d upregulation, lower doses of α-GalCer presented by B cells led to increased stimulation by iNKT cells and B cell proliferation. In contrast, higher doses of α-GalCer in fact induced an iNKT cell cytotoxic effect, leading to decreased numbers of B cells (Allan et al., 2011). If the *in vitro* activation of B cells can be considered analogous to the GC reaction, it can be speculated that these findings can be extended to the *in vivo* setting. A B cell CD1d-iNKT$_{FH}$ interaction in the GC, if too persistent, may similarly have the potential to induce iNKT cell cytotoxicity towards B cells. If appropriately timed, the downregulation of CD1d observed in the GC may therefore indeed exist to terminate such an interaction before it becomes disadvantageous, although this remains an area open to further investigation.

1.4 CD1d in pathological B cells

Mature B cells are implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE). In addition, they are targets for viral infection, especially by B cell-tropic viruses such as Epstein-Barr Virus (EBV), and for malignant transformation leading to a variety of B lineage malignancies including B-cell chronic lymphoproliferative disorders (B-CLPDs) and multiple myeloma (MM). Accumulating evidence, reviewed below, suggests an important role of the CD1d-iNKT cell axis in these processes with potential therapeutic applications.

1.4.1 CD1d and B cells in SLE

B cell dysfunction is central to the pathogenesis of SLE, a systemic autoimmune disorder. This is highlighted by the ability of the anti-CD20 mAb Rituximab to induce clinical remissions in a substantial fraction of patients (Ng et al., 2007). Recent work demonstrated a critical intersection of the CD1d-iNKT cell axis with B cells in health and in patients with SLE (Bosma et al., 2012). Specifically, presence of CD1d-expressing B cells and their direct interaction with peripheral blood (PB) iNKT cells appears to be indispensable for the α-GalCer-dependent *in vitro* expansion of iNKT cells. Much of this effect is mediated by transitional B cells (i.e. CD19+CD38^{hi}CD24^{hi}), which express the highest levels of CD1d amongst PB B cell subsets.

Bosma and colleagues have gone on to show that compared to healthy controls, patients with SLE have significantly lower frequency of PB iNKT cells, secreting abnormally low levels of IFNγ but high levels of IL-10. This finding correlates with considerably lower surface CD1d expression in patient than control B cells (but not monocytes), and in particular in Breg cells. Like GC B cells, loss of CD1d surface expression in SLE B cells is due, at least in part, to BCR signalling, which is increased in the disease in combination with other inflammatory stimuli such as IFN-α. Unlike the transcriptional loss observed in GC B cells, however, loss of CD1d expression in SLE is the result of an enhanced rate of CD1d internalisation in patient B cells. In patients who had a clinical response to Rituximab, restoration of the PB B cell pool following their initial depletion was associated with restoration of the frequency and function of iNKT cells and expression of CD1d on B cells.

While these findings provide important insights into the mechanisms that underpin the quantitative and qualitative iNKT cell defects in SLE, the role of dysfunctional iNKT cells in the pathogenesis of SLE and indeed of autoimmune disease in general remains unclear. In pre-clinical models, activation of the CD1d-iNKT cell axis can prevent or ameliorate established autoimmune disease (Simoni et al., 2013), providing impetus for development of clinical protocols that will aim to restore iNKT cell frequency and function in patients with autoimmune disorders including SLE.

1.4.2 CD1d and virally infected B cells

Immune evasion through downregulation of classical MHC molecules is a recognised strategy employed by viruses to withstand elimination by host defence mechanisms and help ensure their propagation (Finlay and McFadden, 2006). Highlighting the importance of CD1d in anti-viral immune responses, several viruses have been shown to downregulate expression of CD1d in APCs through post-transcriptional mechanisms. Herpes simplex virus type 1 primarily inhibits intracellular trafficking of CD1d, retaining newly synthesized molecule in the trans-golgi network and preventing recycling of endocytosed CD1d back to the cell surface (Rao et al., 2011; Yuan et al., 2006). In contrast, HIV (using Nef protein) (Chen et al., 2006) and human herpes virus 8 (using MIR-2 protein to ubiquitinate the cytoplasmic tail of CD1d) (Sanchez et al., 2005) act to enhance CD1d endocytosis. Other viruses, including lymphocytic choriomeningitic virus, vaccinia virus and vesicular stomatitis virus have also been shown to downregulate CD1d (Lin et al., 2005), although the mechanism(s) are unknown.

This raised the prospect that EBV, a ubiquitous human herpes virus that targets B cells (Thorley-Lawson and Gross, 2004), could employ a similar strategy. EBV infects more than 90% of the world's population (Amon and Farrell, 2005). The majority of primary infections by EBV occur during the first few years of life and are asymptomatic. Infection in adolescence or

adulthood leads to a transient large T cell response, causing the clinical syndrome of infectious mononucleosis (Hislop et al., 2007).

EBV has initial tropism for pharyngeal epithelial cells, but then spreads to local naïve B cells (via surface CD21 and HLA class II molecules) within tonsillar lymphoid tissue. Currently accepted models of chronic EBV infection propose that in order to persist in the host, EBV infects naïve B cells and drives B cell maturation through a GC-type reaction in order to produce memory B cells in which the virus can reside latently long term (Thorley-Lawson and Gross, 2004). EBV achieves this through expression of different genetic latency programmes (0-III) in different B cell subsets, associated with the transcription of different viral proteins **(Figure 1.8)**. The process is analogous to the formation of a GC environment following B cell activation by antigen stimulation of the BCR and stimulation of co-receptors by T_{FH} cells and dendritic cells. Specifically, LMP2a is a viral mimic of a constitutively active BCR (Mancao and Hammerschmidt, 2007), and likewise, LMP1 has been shown to functionally mimic an active CD40 receptor (Rastelli et al., 2007), thus replacing signals normally provided by antigen and T cell CD40 ligand respectively in the traditional GC reaction.

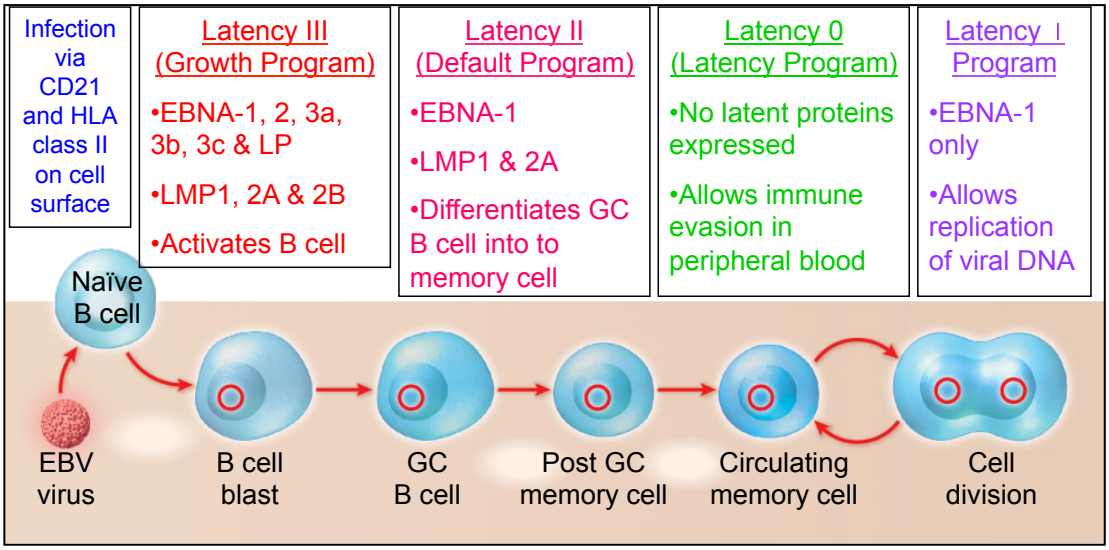


Figure 1.8. EBV directed B cell transformation through use of different transcriptional programmes. *Following initial infection of a naïve B cell via CD21 and HLA class II molecules on the B cell surface, EBV employs a range of transcriptional programmes, in order to transform the naïve B cell into a long lived memory B cell. The initial transcriptional programme, latency III (the growth programme) is associated with expression of 9 proteins, and leads to a rapidly proliferating activated B cell that enters a GC reaction (analogous to a centroblast). Signals within the GC cause the EBV transcription programme to change to latency II (the default programme). The expression of only 3 proteins in this programme, in conjunction with GC signals, leads to the differentiation of the proliferating GC B cell blast into a memory B cell that exits the GC environment. EBV resides long term within the circulating memory B cell, avoiding immune recognition by employing the latency 0 programme (the latency programme) in which no viral proteins are transcribed. Occasional division of the memory B cell requires replication of the viral DNA, associated with expression of EBNA-1 protein only (latency I programme). (Adapted from Thorley-Lawson and Gross, 2004.)*

It is possible that the imitation of the process of GC formation, and the consequent drastic downregulation of CD1d in EBV-infected cells, confers protection to the virus-propagating B cells from iNKT cell attack. Indeed, evidence from the study of patients with X-linked lymphoproliferative disorder (XLP) caused by mutations in *SH2D1A/SAP* or *XIAP,* further suggests that the CD1d-iNKT axis might be important in restraining EBV into a latent state. Individuals with XLP, in addition to other immune function defects, have profound defects in iNKT cell development associated with active EBV replication and EBV-associated lymphoproliferation (Pasquier et al., 2005; Rigaud et al., 2006).

Following on from these observations, the nature of the interaction between EBV infected B cells and iNKT cells was examined more recently *in vitro* (Chung et al., 2013). It was demonstrated that EBV infection of resting B cells can be mitigated by the presence of iNKT cells, indicating that EBV infected B cells are indeed a target for iNKT cells. However, in a parallel to the physiological activation of B cells, EBV infection of B cells rapidly leads to loss of expression of CD1d, abrogating further interaction with iNKT cells and thus providing a form of immune evasion. Similarly, like their physiologically activated counterparts, CD1d expression in EBV infected B cells *in vitro* could be restored by a RARα agonist, re-enabling the stimulation and cytotoxic effects of iNKT cells, even in the absence of exogenous antigen (Chung et al., 2013).

Downregulation of CD1d expression by EBV occurs at the transcriptional level, as opposed to post-transcriptional mechanisms employed by other viruses. Using chromatin immunoprecipitation, it was shown that transcriptional repression of *CD1D* in B cells occurs through binding of the LEF-1/β-catenin complex (members of the Wnt pathway) to the *CD1D* distal promoter upon EBV infection and that treatment with AM580 restores CD1d expression by inhibiting binding of LEF-1 in this region.

1.4.3 CD1d and malignant B cells

Numerous pre-clinical studies have demonstrated the power of the CD1d-iNKT cell axis to enhance (or in some cases inhibit) anti-tumour immunity (Terabe and Berzofsky, 2014). iNKT cells can directly target CD1d-expressing tumours (mostly of haematological, including lymphoid origin) (Li et al., 2014; Renukaradhya et al., 2008). Alternatively, by activating adaptive and innate immune responses, they can indirectly target CD1d-negative tumours (mostly of epithelial origin). Here I will focus the discussion on the evidence linking the CD1d-iNKT cell axis to the biology and clinical behaviour of mature B lineage malignancies, in particular MM and chronic lymphocytic leukaemia (CLL), two of the most common haematological malignancies.

Multiple Myeloma

MM is a malignant monoclonal plasma cell disorder characterized by end-organ damage including anaemia, hypercalcaemia, renal insufficiency and bone lesions (Mitsiades et al., 2004).

Survival and growth of the malignant plasma cell clone depends on accumulation of intrinsic oncogenic events and active cross-talk between the neoplastic plasma cells and tumour microenvironment (Anderson, 2007). T cell recognition of human leukocyte antigen (HLA) class I and II molecules on the surface of myeloma cells, leading to anti-myeloma immune responses, is an important component in this cross talk (Dhodapkar et al., 2003b; Yi et al., 1995), and it is well established that tumours, like viruses, downregulate HLA molecule expression as a means of immune escape and tumour progression (Algarra et al., 2000; Seliger et al., 2000).

Our group previously showed that CD1d is also dynamically downregulated on myeloma plasma cells during disease progression (Spanoudakis et al., 2009). Specifically, in primary myeloma samples, CD1d was highly expressed in pre-malignant and early stage disease, but was decreased, and subsequently lost, in advanced stage MM. Similarly, loss of CD1d expression was observed in most myeloma cell lines, both in terms of surface and mRNA expression, thus implicating this molecule as an important anti-survival factor in MM. These observations suggest that loss of CD1d is advantageous for the propagation of the malignant clone and permits

evasion of myeloma cells from iNKT cell-mediated immune surveillance, a process that would be further undermined by the defective (but reversible) production by iNKT cells of IFN-γ in patients with progressive disease (Dhodapkar et al., 2003a).

However, in addition to immune escape, CD1d may also act as a signalling conduit in myeloma cells. In myeloma cell lines with restored expression of CD1d, ligation of surface CD1d by mAb induced caspase-independent apoptosis (an effect dependent on the cytoplasmic tail of CD1d but not the Tyr residue required for endosomal trafficking of CD1d) (Jayawardena-Wolf et al., 2001; Spanoudakis et al., 2009). Thus, loss of CD1d may also be important in that it allows myeloma cells to avoid the triggering of CD1d-dependent, downstream anti-survival signalling pathways.

The therapeutic potential of the CD1d-iNKT cell axis was explored in a phase I trial in which autologous myeloid DCs loaded with α-GalCer were infused into patients with indolent myeloma (Richter et al., 2013). The procedure was safe and was associated with significant reduction of paraprotein level, indicating anti-myeloma activity. Notably, this small cohort of patients was also treated with lenalidomide, which had been previously found to enhance iNKT cell function in patients with myeloma (Chang et al., 2006).

B-cell chronic lymphoproliferative disorders

B-CLPDs are a heterogeneous group of diseases arising from the clonal expansion of mature B cells at different stages of differentiation (Catovsky, 1995). Immunophenotypic analysis of surface markers plays a key role in both the diagnosis and prognostication of B-CLPDs, especially CLL, and thus the potential of CD1d to be utilised in such a manner has been examined.

Several studies, each involving over 100 patients, found that surface expression of CD1d is significantly, although variably, downregulated in CLL compared with other B-CLPDs, and is lower than in normal B cells, such that CD1d expression levels below a set threshold are strongly predictive of a diagnosis of CLL. In addition, CD1d may also have a prognostic role, as higher CD1d expression levels are associated with reduced time to treatment and overall survival (Anastasiadis et al., 2014; Bojarska-Junak et al., 2014; Kotsianidis et al., 2011).

CD1d expression was also associated with several other known poor prognostic markers in CLL, including expression of ZAP-70, CD38 and CD49d, unmutated IgVH status and cytogenetic abnormalities (Anastasiadis et al., 2014; Bojarska-Junak et al., 2014; Fais et al., 2005). However, whether CD1d is a surrogate or an independent prognostic marker remains to be established. The latter would be more significant and would suggest that the CD1d molecule may actually play a direct role in the pathobiology of the disease. This concept is also strengthened by the observation that CD1d

expression in CLL, like myeloma, is dynamic, and changes during disease progression (Bojarska-Junak et al., 2014). However, while CD1d expression in myeloma decreases as disease progresses, CD1d expression in CLL cells tends to increase during disease progression. This may appear counter to the postulated role of CD1d as an anti-survival factor in myeloma. It is possible that the presence of CD1d provides downstream signals that are advantageous to the malignant CLL B cells. Alternatively, increased expression of CD1d may facilitate presentation of endogenous or exogenous lipids, possibly specific to the leukaemic clone, leading to cognate interactions with iNKT cells that would promote rather than inhibit survival of the leukaemic cell. This would chime well with the observation that the cognate B-iNKT cell interaction, in the presence of endogenous or low concentration of exogenous glycolipid ligand, promotes B cell proliferation *in vitro* (Galli et al., 2003), and with the finding that, unlike in MM, iNKT cells in CLL are fully functional (Weinkove et al., 2013).

In contrast to CLL, CD1d is highly expressed in other B-CLPDs such as splenic marginal zone lymphoma, lymphoplasmacytic lymphoma, mantle cell lymphoma and variant hairy cell leukaemia (Kotsianidis et al., 2011). With the exception of hairy cell leukaemia, these are largely incurable B cell malignancies and their high expression of surface CD1d would make them suitable targets for iNKT cell-based immunotherapeutic approaches.

1.5 Transcriptional regulation of CD1d expression

The changes in CD1d expression observed in normal and pathological B cells raises the question as to how such changes occur. In this section, I discuss our existing knowledge of the regulation of CD1d at the level of gene transcription, which as discussed above, has already been shown to play a role in CD1d expression in B cells activated *in vitro* or infected with EBV (Allan et al., 2011; Chung et al., 2013).

1.5.1 The interplay between transcription factors and chromatin

The transcriptional control of genes occurs at two connected levels. At the first, long established, level, DNA binding transcription factors (trans-factors or TFs) occupy specific DNA sequences at control elements (cis-elements), and recruit and regulate the transcription apparatus (Jacob and Monod, 1961). It is now recognized that this is supplemented by chromatin and its regulators, which permit accessibility of TFs and provide a second level of control (Allis and Jenuwein, 2016).

Transcription factors and the transcription apparatus

TFs regulate gene expression by initially forming a pre-initiation complex complex (Sikorski and Buratowski, 2009). TFs bind to specific DNA elements (enhancers) and to coactivators (protein complexes which do not

have DNA-binding properties of their own), which then recruit RNA polymerase II. Through physical looping of the DNA (stabilized by cofactors such as the protein cohesin), RNA polymerase II in turn binds to further TFs at the core promoter containing the transcription start site (Krivega and Dean, 2012).

RNA polymerase II begins transcription from the initiation site, but pause control factors cause it to stop tens of base pairs downstream (Adelman and Lis, 2012). In order for full transcription to proceed, binding of further TFs and coactivators are needed to recruit elongation factors such as P-TEFb. These phosphorylate the paused polymerase and the associated pause factors, allowing elongation to proceed (Luo et al., 2012). This sequence of events is summarised in **Figure 1.9**.

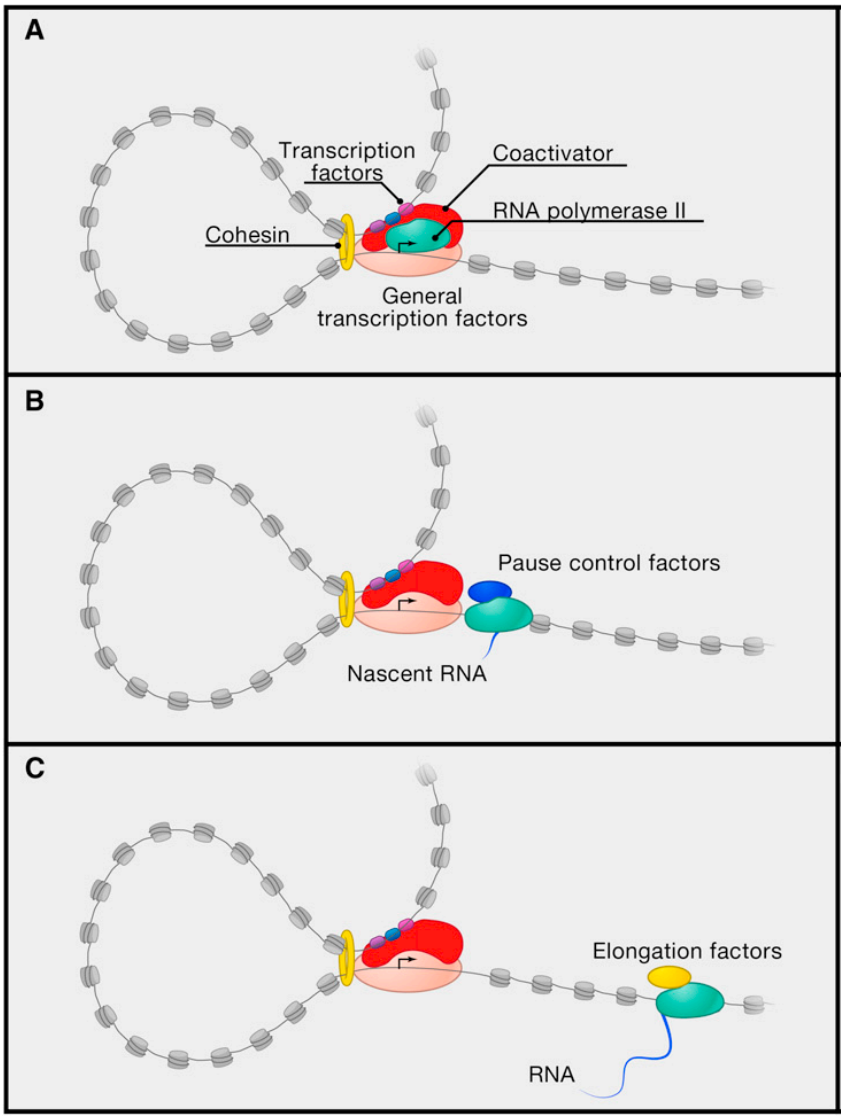


Figure 1.9. Transcriptional regulation by transcription factors and the transcription apparatus. *(A) The recruitment of transcription factors, coactivators and RNA polymerase II to enhancer and promoter regions allows initiation of transcription from the transcription start site. (B) Once initiated, transcription is subsequently paused in the early stages and completion of transcription is dependent on (C) further recruitment of transcription factors and elongation factors. (Adapted from Lee and Young, 2013.)*

Chromatin and its regulators

Within eukaryotic cell nuclei, DNA is condensed and structured into chromatin, by histone proteins (Campos and Reinberg, 2009). The functional unit of chromatin, termed the nucleosome consists of DNA wrapped around an octamer of four core histone proteins: H2A, H2B, H3 and H4. The core histones are arranged as a pair of H2A-H2B dimers, and an (H3)2(H4)2 tetramer in order to form the octamer. The conformation of chromatin can dictate gene expression by regulating the relative accessibility provided to TFs and the transcription apparatus. Tight packaging of nucleosomes into heterochromatin inhibits TF access, whereas euchromatin, with its relaxed chromatin, facilitates TF access and transcription (Allis and Jenuwein, 2016).

Thus, the modification of chromatin represents a powerful mechanism by which gene expression can be altered. Changes to chromatin can be imparted through chromatin remodeling, most notably by the ATP-dependent chromatin remodeling complexes of the SWI/SNF family, which mobilize nucleosomes to facilitate access of the transcription apparatus to DNA (Narlikar et al., 2013). At the level of the histones, nucleosome structure can be altered via the inclusion of histone variants. For example, histone variant H3.3 has been shown to be required for heterochromatin formation in the mouse embryo (Santenard et al., 2010).

Furthermore, histone proteins may be subject to post-translational modifications, which can act as powerful modulators of chromatin and gene expression (Bannister and Kouzarides, 2011). Transcription factors and apparatus recruit an array of histone-modifying enzymes that can either add (histone writers) or remove (histone erasers) acetylation, methylation, phosphorylation, sumoylation or ubiquitination marks. Such modifications may influence gene transcription in two main ways: firstly, by changing the physical interaction between histones and DNA to alter the chromatin structure, and secondly, by acting as a binding surface for the recruitment of other proteins (termed histone readers), which can then directly or indirectly influence transcription. The role of chromatin in transcription is highlighted in **Figure 1.10**.

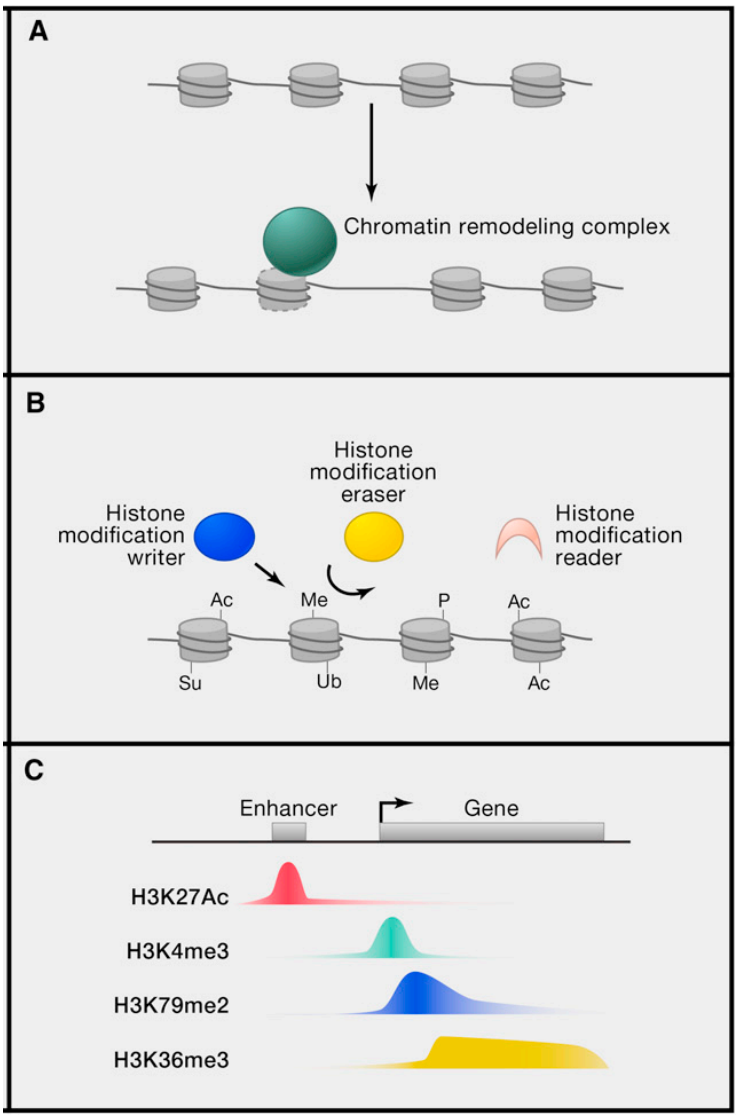


Figure 1.10. Transcriptional regulation by chromatin and its regulators. *(A) Chromatin structure can be regulated by ATP-dependent remodelling complexes that mobilize the nucleosome and allow access to TFs. (B) Transcriptional activity can also be influenced by proteins that add (writers), remove (erasers) or bind to (readers) histone post translational modifications. (C) Histone modifications can occur in characteristic patterns associated with gene transcription or repression. (Adapted from Lee and Young, 2013.)*

1.5.2 Transcription factor regulation of CD1d

Expression of CD1d is regulated by multiple transcription factors (TFs). In humans **(Figure 1.11)**, transcription of the human *CD1D* gene is regulated by a proximal promoter (from -106 to +24 in relation to ATG) and a distal promoter (from -665 to -202) (Chen and Jackson, 2004). The ubiquitous TF SP1 activates transcription by binding to the proximal promoter (Chen and Jackson, 2004; Yang and Chen, 2012), while LEF-1 represses *CD1D* transcription by binding to the distal promoter (Chen et al., 2010).

The upstream regulatory region of murine *Cd1d1* shows little conservation with that of the human *CD1D* gene. In mice, a minimal proximal promoter region has been identified, which is regulated by various members of the ETS family of TFs, including ELF1 in murine B cells and PU.1 in cells of myeloid lineage (Geng et al., 2005). It is of interest that single nucleotide polymorphisms in the proximal promoter of PWD inbred mice drastically reduce CD1d expression with consequent severe reduction in iNKT cell frequency (Borg et al., 2014).

Both human *CD1D* and murine *Cd1d1* genes share a retinoic acid response element (RARE) in a more distal promoter, located in the 5' untranslated region approximately 1.5 kb from ATG (Chen and Ross, 2007). The conservation of this promoter across species is suggestive of an important role in gene regulation, and indeed, RA signalling has been shown to play a

key role in CD1d expression. Thus, a further in depth discussion of this pathway is warranted in the following section.

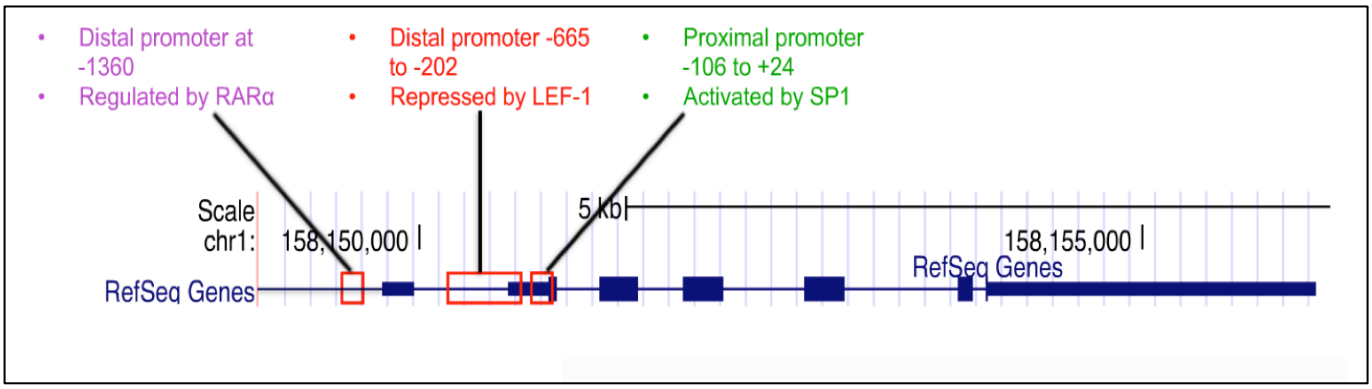


Figure 1.11. Summary of the transcriptional regulation of the human CD1D gene. *The human CD1D gene is reported to be regulated by multiple transcription factors, including SP1, LEF-1 and RARα. (Image adapted from the UCSC genome browser.)*

1.5.3 RA signalling pathway and role in CD1d regulation

RA synthesis

RA is the most biologically active of the retinoids, a family of molecules derived from vitamin A (retinol). Retinoids, obtained from the diet, are delivered to the cell as retinol bound to retinol-binding protein. Within the cell, retinol is enzymatically converted, first to retinal by retinol dehydrogenases (RoDHs) and then to RA by the retinaldehyde dehydrogenases (ALDHs) (Duester, 2000) **(Figure 1.12)**. There are several members of each of these enzyme classes, and in particular ALDH1A2 has been identified as a key rate-limiting enzyme in haematopoietic cells. The enzymatic process within cells is dependent upon retinoids binding to cytoplasmic retinoid-binding proteins (CRBP and CRABP). In addition to acting within cells where it is produced, RA may act in a paracrine manner and be taken up by neighbouring non-RA producing cells (Duester, 2008). During early development, this leads to a phenomenon of gradients of RA signalling activity that are distributed in a dynamic spatiotemporal manner.

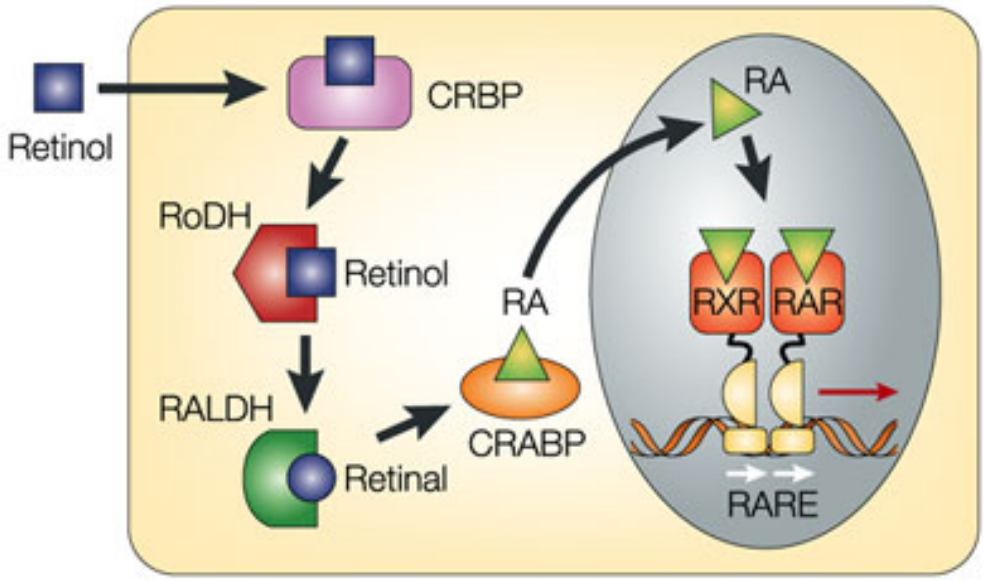


Figure 1.12. Pathway for the synthesis and mechanism of action of RA

A series of intracellular enzymatic steps converts retinol to RA. RA enters the nucleus and binds to RAR/RXR heterodimers, which activates transcription of the target gene. In addition, RA may leave the cell and act in a paracrine manner on neighbouring cells. (From Maden, 2002.)

Transcriptional regulation by RA

Following synthesis/uptake by neighbouring cells, RA enters the nucleus and influences gene activity by binding to receptors, which act as ligand activated nuclear transcription factors (Maden, 2002). There are two classes of these receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs). In humans and rodents, there are three types of RARs and RXRs (α, β and γ), and each has several isoforms. RARs and RXRs bind to form heterodimers, and these recognize consensus sequences in the regulatory elements of RA-responsive genes, known as RA response elements (RAREs). There is a marked functional redundancy between the RAR and RXR isotypes, with only RXRα-null mice exhibiting embryonic lethality. Thus, nuclear-receptor abundance is likely to be secondary to RA availability as the main determinant that drives RA signalling (Niederreither and Dollé, 2008).

The consensus is that in the absence of ligand, RAR/RXR heterodimers act as transcriptional repressors. Under these conditions, the RAR/RXR heterodimer binds a co-repressor complex that may include nuclear receptor co-repressor (N-CoR), and the histone modifiers histone deacetylase (HDAC) and Polycomb repressive complex 2 (PRC2, discussed in more details below). The binding of RA to the receptor leads to dissociation of the co-repressors and recruitment of co-activator proteins, including nuclear receptor co-activator (NCOA), histone acetyl-transferase (HAT) and Trithorax group proteins (also discussed in more detail below), leading to

transcription of the target gene (O'Malley and Kumar, 2009) **(Figure 1.13)**. During activation, co-activators bind to RAR, not RXR, which is consistent with RA-liganded RAR being the key regulatory component of RAR–RXR heterodimers (Rochel et al., 2011).

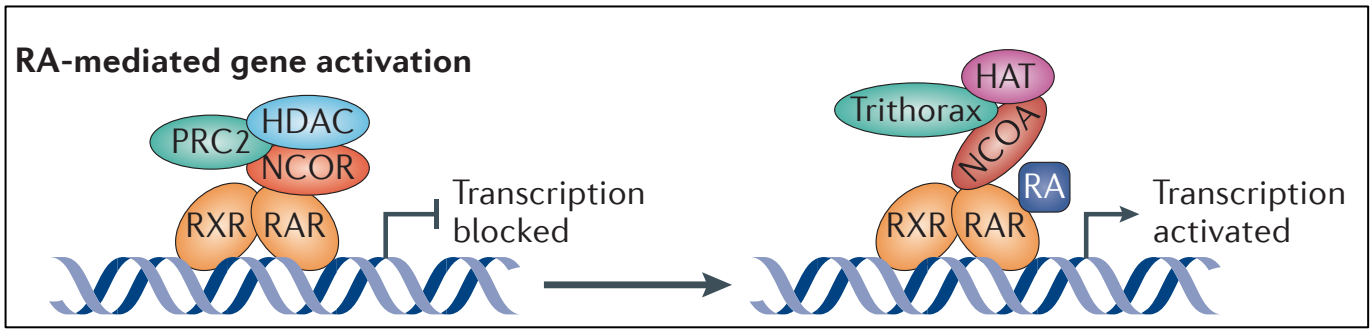


Figure 1.13. RA mediated transcriptional activation

RAR/RXR heterodimers act as recruitment hubs for transcriptional repressors, preventing gene transcription. Upon binding of RA ligand, there is displacement of repressor proteins and recruitment of activators, leading to active gene transcription.

Functions of RA signalling

RA signalling has a well established central role in vertebrate development (Cunningham and Duester, 2015). Beyond this developmental role, RA signalling has been shown to promote differentiation, cell cycle arrest and induce apoptosis in cells, and a diminution of RA signalling has been

implicated in the development and propagation of many tumours (Tang and Gudas, 2011). Utilisation of RA has been attempted in several malignancies, most notably acute promyelocytic leukaemia (APML) (Schenk et al., 2014), but also MM (Otsuki et al., 2003). More recently, increasing evidence has linked RA signalling directly to the regulation of CD1d.

As discussed above, a central role for RA signalling has been elucidated in B cells *in vitro*. To summarise, *in vitro* B cell activation, in response to various activating ligands including anti-Ig antibodies, Staphylococcus aureus Cowan I bacteria and CD40 ligand, was shown to downregulate CD1d in association with a decrease in RARα signalling (Allan et al., 2011). Similarly, EBV infection of B cells was also found to lead to loss of expression of CD1d (Chung et al., 2013). In both cases, CD1d expression could subsequently be restored through use of RARα agonists. Beyond B cells, Nagy and colleagues showed that activation of the peroxisome proliferator-activated receptor gamma (PPARγ) indirectly increased CD1d expression in human dendritic cells by increasing RA synthesis and subsequent activation of the RARα (Szatmari et al., 2006).

It is not known, however, if this role of RA signalling in CD1d regulation can be extended more broadly to other cells or even other B cell states, including *in vivo* GC B cells and MM B cells.

1.5.4 Potential role of bivalent chromatin in CD1d regulation

There are, to date, no reports examining a role for chromatin specifically in the regulation of CD1d. However, it can be speculated that certain chromatin modifications may have the potential to modulate CD1d expression, as discussed below.

Histone methylation has emerged as one of the most widely studied post-transcriptional modifications. Trithorax group (TrxG) proteins were discovered as activators of *Hox* genes in Drosophila (Schuettengruber et al., 2007). In mammals, the corresponding enzymes are SET1A, SET1B and mixed lineage leukaemia (MLL) proteins 1-4 (Shilatifard, 2012). These proteins catalyse the trimethylation of histone H3 Lys 4 (H3K4me3), a mark generally associated with active transcription.

Conversely, the Polycomb group (PcG) proteins were identified as silencers of *Hox* genes in Drosophila (Schuettengruber et al., 2007). In both mammals and flies, the PcG proteins form the multi-subunit Polycomb-repressive complexes (PRCs) 1 and 2. PRC2 catalyses the formation of H3K27me3 (trimethylation of histone H3 Lys 27), a pivotal mark in the establishment of repressive chromatin.

Genome-wide chromatin immunoprecipitation analyses have suggested that in undifferentiated embryonic stem cells, many developmental regulatory

gene loci are marked with a distinctive signature of both H3K4 and H3K27 trimethylation, termed bivalent marks (Bernstein et al., 2006). Such bivalent chromatin domains often mark lineage-regulatory genes, and might contribute to the precise unfolding of gene expression programmes during pluripotency and differentiation. In particular, it has been proposed that bivalent domains might repress lineage control genes (through H3K27me3 marking) during pluripotency while keeping them poised for activation upon differentiation (through H3K4me3 marking). The H3K27me3-mediated repression of developmental control genes might protect cells from the aberrant expression of lineage regulators and thus help maintain pluripotency. Conversely, it has been proposed that H3K4me3 poising of genes may lead to more efficient or more synchronous activation upon differentiation (Boettiger and Levine, 2009). H3K4me3 might also protect genes from permanent silencing, such as by repelling transcriptional repressors or blocking DNA methylation (Fouse et al., 2008). Thus, the combination of seemingly conflicting H3K4me3 and H3K27me3 marks in bivalent domains convey temporal and spatial precision to the expression of lineage control genes during pluripotency and differentiation **(Figure 1.14)**.

More recently, it has become apparent that this model may be extended, and establishment of bivalent histone modifications may occur in other undifferentiated states, including GC B cells and B cell malignancies (Béguelin et al., 2013; Caganova et al., 2013). This raises the question of whether the regulation of CD1d in particular may be brought about in part

by such bivalent domains. As discussed above, RA signalling, implicated in the regulation of CD1d, may involve recruitment of histone modifiers including PcG and TrxG proteins. The use of RARα agonists to restore CD1d expression in B cells activated *in vitro* or infected by EBV has highlighted that CD1d is not permanently silenced in these cells. This may be achieved by use of bivalent marking, which represses CD1d but keeps it poised for activation in response to RA.

However, the role of bivalent domains as a chromatin modification able to regulate CD1d remains hypothetical. Further work is needed to confirm if changes to the chromatin are associated with modulation of CD1d expression, and how such changes interact with the transcriptional apparatus, including that related to RA signalling.

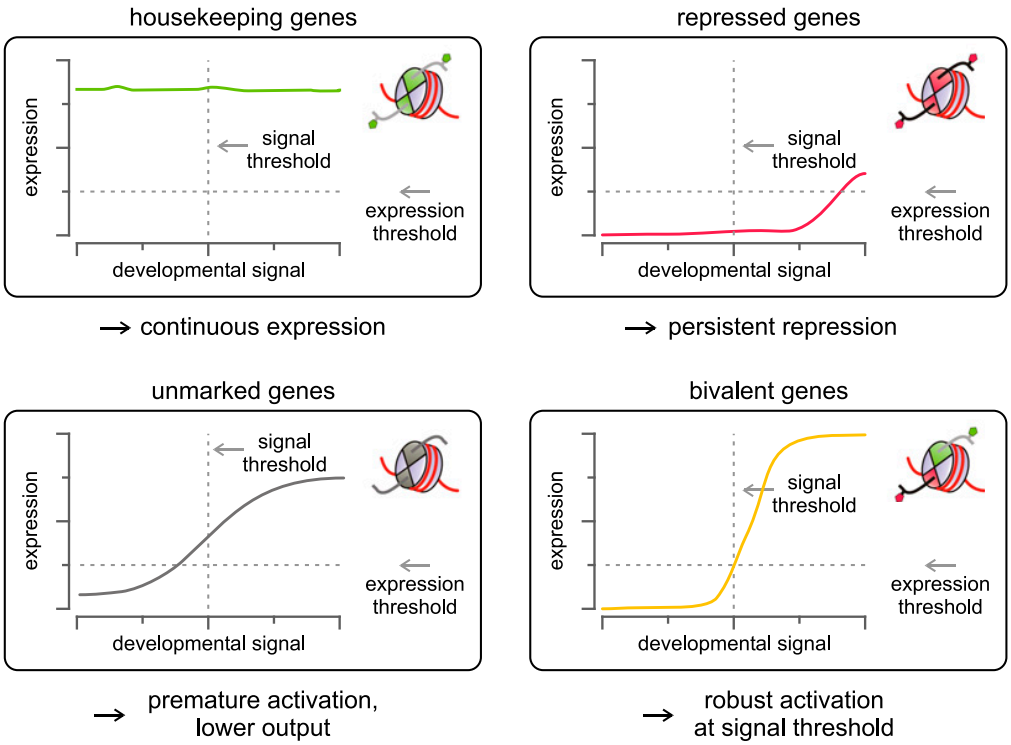


Figure 1.14. Model of gene expression in response to developmental signals

In this model, bivalent genes, marked by H3K4me3 and H3K27me3 histone modifications, are initially characterised by tightly repressed gene expression. Upon appropriate stimulation, gene expression is robust and synchronous. In this manner, bivalent domains achieve close temporal and spatial control of gene expression. (From Voigt et al., 2013.)

1.6 Aims of this work

The aim of this project is to explore the role and regulation of CD1d in normal and pathological B cells, building upon the existing information described above. Specifically, the project aims to:

1. Further characterise the expression of CD1d in normal and pathological B cells.

2. Investigate the biological significance of changes in CD1d expression in B cells.

3. Investigate the transcriptional mechanisms regulating expression of CD1d in normal and pathological B cells.

Chapter 2

2 Materials and methods

Details of the reagents used and their origin are shown in Appendix A and buffers, media and solutions in Appendix B. The primers used and the expected amplicon sizes are detailed in Appendix C.

2.1 Cells and media

All cells were cultured in a humidified, 5% CO_2, 37°C incubator. Cells were stored resuspended in Foetal Bovine Serum (FBS) with 10% dimethyl sulfoxide (DMSO) and frozen overnight at -80°C in a cell-freezing vessel containing isopropanol, after which they were transferred to liquid nitrogen.

2.1.1 Cell lines

Human EBV transformed B-lymphoblastoid cell lines (B-LCLs), human multiple myeloma (MM) cell lines and the murine myeloma cell line 5TGM1 were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with FBS, L-glutamine, penicillin/streptomycin, HEPES, sodium pyruvate and β2-mercaptoethanol ($RPMI^+$).

293T cells and the 293T derived Phoenix ecotropic cell line were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS, L-glutamine and penicillin/streptomycin ($DMEM^+$).

Selection of viral production competent Phoenix cells was done using Diptheria toxin (1μg/mL) and Hygromycin B (300μg/mL) in $DMEM^+$ for 1 week followed by recovery for 1 week in $DMEM^+$ alone.

2.1.2 Treatment of cell lines

To investigate the transcriptional control of CD1d, human MM cell lines were treated with different agents. All-trans retinoic acid (ATRA) was used at concentrations from $1x10^{-6}$M to $1x10^{-8}$M. The EZH2 inhibitor, GSK343A, was used at a concentration of $1x10^{-6}$M. Agents were provided in powder form. In order to achieve required concentrations of these agents, neat solutions were produced by reconstituting the powder in DMSO, followed by dilution in cell culture medium. Following treatment with agents, cells were cultured as described above and assessed at different time points, between 4 and 96 hours.

2.1.3 Primary cells

Primary human tonsil cells were kindly provided as single cell suspensions by Professor Ioannis Kotsianidis, Democritus University of Thrace, Greece. Collection and investigation of primary human tissue was approved by the local research ethics committee (National Research Ethics Service Committee East of England, reference number: 11/H0308/9).

Single cell suspensions of murine splenocytes were prepared by cell straining of pulped spleens using a syringe plunger, 40μm cell strainer and RPMI+ medium. Isolated splenocytes were subjected to red blood cell (RBC) lysis on ice by incubating with an ammonium chloride-based RBC Lysis Buffer for 5 minutes, prior to washing in Phosphate Buffered Saline (PBS) and use in downstream experiments.

Bone marrow (BM) cells were acquired by syringe flushing of dissected mice femora and tibiae (27G needle and RPMI medium).

2.1.4 Enrichment of murine BM cells for HSCs

Murine BM cells were enriched for HSCs by using the Miltenyi lineage cell depletion kit. In this process, lineage negative BM cells were isolated by depletion of BM cells expressing a panel of "lineage" antigens (negative selection). To achieve this, lineage positive BM cells were indirectly magnetically labelled using a cocktail of biotin-conjugated monoclonal antibodies (anti-CD5, B220, CD11b, Anti-Gr-1, 7-4, and Ter-119 antibodies), as the primary labelling reagent, and anti-biotin monoclonal antibodies, conjugated to magnetic MicroBeads, as the secondary labelling reagent. The magnetically labelled lineage cells were depleted by retaining them on a column in the magnetic field of a MACS® Separator. The unlabelled lineage negative cells passed through the column, and were collected as the HSC enriched BM cells.

HSC enriched BM cells were cultured in serum-free medium (Stemspan, SFEM) containing a cytokine cocktail of murine stem cell factor (mSCF), murine interleukin-3 (mIL-3), human flt3-ligand (hFL), and human interleukin-11 (hIL-11). Cells were cultured overnight, in a 12 well plate at a density of $1–1.5\times10^6$ cells per well, prior to retroviral transduction, as described below.

2.2 Mice

Wild type C57BL/6 mice (CD45.1 and CD45.2) were purchased from Charles River, UK.

CD1d transgenic (CD1d Tg) mice were provided by and used in conjunction with Professor Chyung-Ru Wang of Northwestern University, Chicago, USA.

RARE-hsp68LacZ transgenic mice, which express the beta-galactosidase (*lacZ*) gene under the control of a retinoic acid responsive element (RARE), were purchased from The Jackson Laboratory, USA, through Charles River, UK.

2.2.1 Maintenance

UK animal care and experimental procedures were approved by the Imperial College Local Ethical Review Committee and were conducted in accordance with the UK Home Office Animals Act of 1986.

Wild type C57BL/6 mice and RARE-hsp68LacZ transgenic mice were maintained at the animal facility of Central Biomedical Services, Imperial College London. CD1d Tg mice were housed at the animal facility of Northwestern University, Chicago, USA.

2.2.2 Immunisation of mice

In order to induce GC formation, mice were immunised on Day 0 by intraperitoneal injection of a single 100μg dose of the TD-antigen 4-hydroxy-3-nitrophenyl-keyhole limpet hemocyanin (NP-KLH), precipitated in Imject™ Alum Adjuvant. Spleens were harvested on day 10 for assessment of a GC reaction.

2.2.3 Adoptive transfer of HSCs

As detailed in other parts of this chapter, bone marrow cells, derived from WT C57BL/6 mice (congenic strain CD45.1), underwent enrichment for HSCs and subsequent viral transduction. These transduced cells were adoptively transferred to recipient WT C57BL/6 mice (congenic strain CD45.2).

Recipient mice were conditioned for adoptive transfer by sub-lethal irradiation, using 2 doses of 450rad, 6 hours apart. Following the second dose of irradiation, transduced donor cells were transferred by tail vein injection to recipient mice at a dose of $1X10^6$ cells/mouse.

At 8 weeks following transfer of cells, peripheral blood of recipient mice was obtained from the tail vein and analysed for evidence of donor cell engraftment (by GFP expression of total peripheral blood leucocytes).

2.3 Immunohistochemistry and histology

Immunohistochemical analysis of archived splenic paraffin sections obtained from patients undergoing splenectomy was performed by Professor Kikkeri Naresh of Imperial College London. (Collection and investigation of primary human tissue was approved by the local research ethics committee as described above.) In brief, human splenic tissue was sliced and fixed in 10% buffered formal saline for 18–24 hours and processed by paraffin embedding. 4-μm-thick sections were cut and stained with hematoxylin and eosin stain, and anti-CD1d antibody (clone 42.1).

2.4 Flow cytometry

2.4.1 Cell staining

Cell lines and primary cells were washed in PBS with 2% FBS (flow cytometry buffer, FB) and then resuspended in 100µL FB. Staining was performed by incubating cells at 4^{o}C for 20min in the presence of fluorophore-conjugated monocloncal antibodies (mAbs), as detailed below (approximately 10µg/ml of each antibody). Where staining was carried out with a biotinylated antibody, cells were subsequently washed and resuspended in FB, and a secondary staining step was performed using fluorophore-conjugated streptavidin. Details of antibodies used in flow cytometry, including the manufacturer, can be found in Appendix A.

2.4.2 Staining of human tonsillar B cells

The following anti-human mAbs were used: anti-CD19-PerCP-Cy5.5, anti-IgD-Alexa Fluor 647, anti-CD10-Brilliant Violet 421, anti-CD38-Pe-Cy7, and anti-CD1d-PE (clone 51.1) (or appropriate PE conjugated isotype control antibody).

2.4.3 Staining of murine splenic B cells

The following anti-mouse mAbs were used: anti-CD45.1-Brilliant Violet 605, anti-B220-Alexa Fluor 700, anti-CD19-PerCP-Cy5.5, anti-IgM-Brilliant Violet 421, anti-IgD-Brilliant Violet 421, anti-CD21-APC-Cy7, anti-CD23-Pe-Cy7, anti-GL7-Alexa Fluor 647, anti-CD95-biotin (followed by secondary staining with streptavidin-Brilliant Violet 650), and anti-CD1d-PE (clone 1B1) (or appropriate PE conjugated isotype control antibody).

2.4.4 Staining of murine BM cells

Murine BM cells, pre- and post- HSC enrichment, were stained using the following anti-mouse mAbs: anti-Cd117-APC, anti-lineage cocktail-eFluor 450, anti-Sca-1-PE, anti-CD150-PerCP-Cy5.5, and anti-CD48-Pe-Cy7. (The anti-lineage cocktail contained anti-CD3, B220, CD11b, Anti-Gr-1, and Ter-119 antibodies.)

2.4.5 Staining of B-LCLs and MM cell lines

Human B-LCLs and human MM cell lines were stained using the following anti-human mAbs: anti-CD1d-PE (clone 42.1), anti-CD1d-PE and anti-CD1d-APC (clone 51.1). The murine MM cell line 5TGM1 was stained using the following anti-mouse mAbs: anti-CD1d-PE and anti-CD1d-Alexa Fluor 647 (clone 1B1). In all cases, staining was also performed with the appropriate isotype control antibody.

2.4.6 Fluorometric assay to measure beta-galactosidase activity

In order to measure beta-galactosidase activity in murine splenic B cells from RARE-hsp68LacZ transgenic mice, the ImaGene Green™ lacZ Gene Expression Kit was used. This kit provides a fluorescent β-galactosidase substrate, fluorescein di-β-D-galactopyranoside, with an additional 12-carbon lipophilic moiety (termed C_{12}FDG). In cells exhibiting beta-galactosidase activity, C_{12}FDG is broken down to produce a fluorescent product with similar excitation and emission characteristics to FITC.

The experimental protocol followed the manufacturer's instructions. Spleens were harvested from non-immunised mice and a single cell suspension in culture medium was made as described above. In order to inhibit endogenous lysosomal beta-galactosidase activity, 300μM

chloroquine was added to the suspension, and incubated for 30 minutes at 37°C. Splenocytes were subsequently centrifuged and resuspended in culture medium containing C_{12}FDG at a concentration of 10μM. Following 20 minutes incubation at 37°C, the reaction was terminated by addition of 1mM phenyl-ethyl β-D-thiogalactopyranoside (PETG), a strong inhibitor of beta-galactosidase.

Splenocytes were then centrifuged and resuspended in FB. Staining of murine splenic B cell populations using mAbs was subsequently performed as described above.

2.4.7 Flow cytometric analysis

Stained samples were washed and resuspended in PBS. 4',6-diamidino-2-phenylindole (DAPI) was added at the time of acquisition for dead cell exclusion.

Analysis of samples was performed using a BD LSR Fortessa analyser, and fluorescence activated cell sorting was performed using a BD FACS Aria. Further data analysis was performed using FlowJo software (Tree Star Inc. Oregon, USA).

2.5 Chromatin immunoprecipitation

2.5.1 Chromatin preparation

$5x10^6$ cells were used per immunoprecipitation. Cells were collected by centrifugation at 1200rpm. Chromatin was prepared using the Shearing ChIP kit (Diagenode). A formaldehyde-based buffer was initially used to cross link proteins to DNA. Sequential washing in cell lysis buffers allowed isolation of chromatin. Chromatin was sonicated at 4°C for 20 minutes in a 0.5/0.5 on/off cycle using high intensity in a Bioruptor UCD-200 (Diagenode). Post-sonication fragments of average 500bp length were confirmed on a 1.5% agarose gel. Sheared chromatin was either used immediately in downstream experiments or was flash frozen in liquid nitrogen and stored at -80°C for later use.

2.5.2 Immunoprecipitaion and DNA extraction

Chromatin was diluted at least 10 times in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCL pH 8, 167mM NaCl) containing protease inhibitors. The diluted chromatin was pre-cleared by incubation with magnetic beads (Dynabeads Protein G from Thermo Fisher Scientific) for 1hr at 4°C on a rotating wheel. Pre-cleared chromatin was incubated with 3µg of antibodies overnight at 4°C on a rotating wheel.

Protein G magnetic beads were added and incubated for 2-4hrs at 4°C on a rotating wheel. The immunoprecipitates were washed for 5 min at 4°C in a rotating wheel with low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8, 500mM NaCl), LiCl buffer (0.25M LiCl, 1% IGEPAL, 1% sodium deoxicolate, 1mM EDTA, 10mM Tris-HCl pH 8) and 2 times with TE. Immunocomplexes were eluted by adding 150µl elution buffer (50mM Tris-HCl pH 8, 50mM NaCl, 1mM EDTA and freshly added 1% SDS and 20mg/ml of RNaseA) at 65°C for 4h and a second time for 30 min. Both elutions were pooled and treated with proteinase K. DNA was purified by phenol extraction and DNA precipitation.

2.5.3 Re-ChIP assays

Re-ChIP assays were performed as described (Truax and Greer, 2012) with some modifications. Specifically, the primary chromatin IP protocol was performed as described above except for the wash steps, which were performed with protease inhibitor-containing buffers, and the chromatin elution step, which was performed by incubating the magnetic beads in 10mM DTT/TE for 30 min at 37°C. The second IP was performed by diluting the eluate at least 20X in ChIP dilution buffer followed by the second IP as described above for the first IP.

2.5.4 ChIP antibodies

Antibodies used were: anti-H3K4me3, anti-H3K27me3, anti-RNA polymerase II CTD phospho S2, anti-RNA polymerase II CTD phospho S5, anti-EZH2, anti-RARα and control IgG. Details of antibodies used in ChIP, including the manufacturer, can be found in Appendix A.

2.6 Nucleic acid techniques

In order to investigate gene expression, mRNA was extracted and converted to cDNA, which was subsequently utilised by real time polymerase chain reaction (QRT-PCR). QRT-PCR was also used to quantify genomic DNA following chromatin immunoprecipitation (ChIP).

2.6.1 RNA extraction

The Qiagen RNeasy plus kit was used to extract RNA from cell lines and primary cells, as per the manufacturer's instructions. Following cell lysis and homogenisation in the presence of β2-mercaptoethanol and a guanidine isothiocyanate – containing buffer, cell lysate was passed through a gDNA Eliminator spin column to remove genomic DNA. Ethanol was applied to the flow through, and the sample was applied to an RNeasy spin column, allowing RNA to bind to a specialised silica-gel column membrane. Contaminants were washed way from the column using ethanol-based buffers, and RNA was subsequently eluted using nuclease free water. The RNA obtained by this method was specifically enriched for mRNA, since the columnar isolation method effectively excludes RNA less than 200 nucleotides long. Quantification of RNA extraction was made spectrophotometrically using the NanoDrop™ 2000. Approximately 2μg of RNA was obtained per 1 million cells using this method. Extracted RNA was stored at -80^{o}C for later use or was used immediately in cDNA synthesis.

2.6.2 cDNA synthesis

cDNA synthesis was performed by using SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific), as per the manufacturer's instructions. mRNA was annealed to oligo(dT) at 65^{o}C for 5 minutes followed by cooling on ice. cDNA was synthesized by incubating the sample with Superscript III reverse transcriptase enzyme, $MgCl_2$, and dNTPs at 50^{o}C for 50 minutes. Superscript III reverse transcriptase is a version of the Moloney Murine Leukemia Virus Reverse Transcriptase that has been engineered with the introduction of several point mutations to reduce RNase H activity and provide increased thermal stability. Termination of the reaction was achieved by incubating at 85^{o}C for 5 minutes. Synthesised cDNA was stored at -20^{o}C for later use or was used immediately in QRT-PCR.

2.6.3 Real time polymerase chain reaction

In order to measure gene expression, QRT-PCR of template cDNA was performed using Taqman Gene Expression Master Mix and the appropriate Taqman Gene Expression Assays (Applied Biosystems), as per the manufacturer's instructions. Reactions were run in a 96 well plate in the Applied Biosystems 7500 Real Time PCR System using the following programme:

1. 50°C for 2min

2. 95°C for 10min

3. 40 cycles of: 95°C for 15s, 60°C for 1min

Expression was calculated relative to the reference gene, beta-actin (ACTB), using the ΔCT method (Schmittgen and Livak, 2008). Specifically, gene expression was calculated by the formula:

$$R=2^{-(C_t\text{Target}- C_t\text{Reference})}$$

Where:

R is the relative gene expression of the target gene of interest

C_t^{Target} is the threshold cycle for the target gene of interest

$C_t^{\text{Reference}}$ is the threshold cycle for the reference gene

QRT-PCR was also used to quantify genomic DNA isolated using ChIP. In this case, QRT-PCR was performed using SYBR Green/ROX qPCR mastermix. Appropriate primers for relevant genomic loci (see below for details of design), template DNA and qPCR mastermix were combined, as per the manufacturer's instructions. Reactions were run in a 96 well plate in the Applied Biosystems 7500 Real Time PCR System using the following programme:

1. 95°C for 10min
2. 40 cycles of: 95°C for 15s, 60°C for 30s, 72°C for 30s

Expression of immunoprecipitated DNA was calculated using the ΔCT method, as above. In this case, expression was calculated relative to the reference of either input DNA or DNA immunoprecipitated by control IgG antibody.

2.6.4 Primer design for QRT-PCR

In order to use QRT-PCR to perform gene expression analysis, Taqman Gene Expression Assays (Applied Biosystems) containing validated primers were used as described above.

The use of QRT-PCR, using SYBR Green/ROX qPCR mastermix, to measure genomic DNA after ChIP required design of specific QRT-PCR primers for the appropriate genomic loci. Amplicons for QRT-PCR were designed to be 50-200bp long. Primers were designed using OligoPerfect™ Designer (https://tools.lifetechnologies.com), were 21-24mer with annealing temperatures between 55-65°C. Primers were designed after ensuring that there were no potential self- annealing sites, 3' complementarity or potential for hairpin formation. The primers used and the expected amplicon sizes are detailed in Appendix C.

2.6.5 Preparation of purified plasmids

In order to expand plasmid DNA stocks for preparation of retro- and lentivirus, plasmids were transformed using DH5α E.coli (42°C for 45 seconds followed by 2 hours incubation with SOCS media). After overnight colony formation on Luria-Bertani (LB) agar plates (containing 100μg/mL ampicillin), colonies were picked and grown overnight in LB broth (again with 100μg/mL ampicillin). Endotoxin-free plasmid DNA was then extracted using the Qiagen endotoxin-free maxi preparation kit, as per the manufactuer's instructions.

2.7 Viral transduction

Retroviral gene transduction was used to stably express CD1d in HSC enriched murine BM cells. Lentiviral transduction was used in preliminary experiments (described in Appendix D) to knockdown CD1d in the murine myeloma cell line 5TGM1, using shRNA technology. Both techniques are detailed below.

2.7.1 Production of retrovirus

The MigR1-cd1d1-eGFP and MigR1-eGFP retroviral vectors were kindly provided though previous work in the lab by Dr Ming Hu and Dr William Murray-Brown. MigR1-cd1d1-eGFP is an IRES-containing bicistronic retroviral vector, which expresses murine *Cd1d1* and *eGFP* under control of a constitutive MSCV promoter. MigR1-eGFP is the corresponding control vector, which only expresses *eGFP* **(Figure 2.1)**.

The 293T derived Phoenix ecotropic cell line was kindly donated by Dr Istvan Bartok of Imperial College London. This cell line is a second-generation retrovirus producer cell line for the generation of helper-free ecotropic retroviruses. Phoenix cells were plated in 10cm cell culture dishes at $3x10^6$ cells per dish and $3x10^5$ cells/mL using DMEM+ 24 hours prior to

transfection. Media was changed 1 hour before transfection. Lipofectamine 2000® reagent was used to transfect cells with 12µg of DNA vector (either MigR1-cd1d1-eGFP or MigR1-eGFP retroviral vectors) for each dish as per instructions. Fresh DMEM+ media was added to cells after 14-18 hours incubation, and after 48 hours viral supernatant was harvested and spin filtered at 900g for 15 minutes at 4°C.

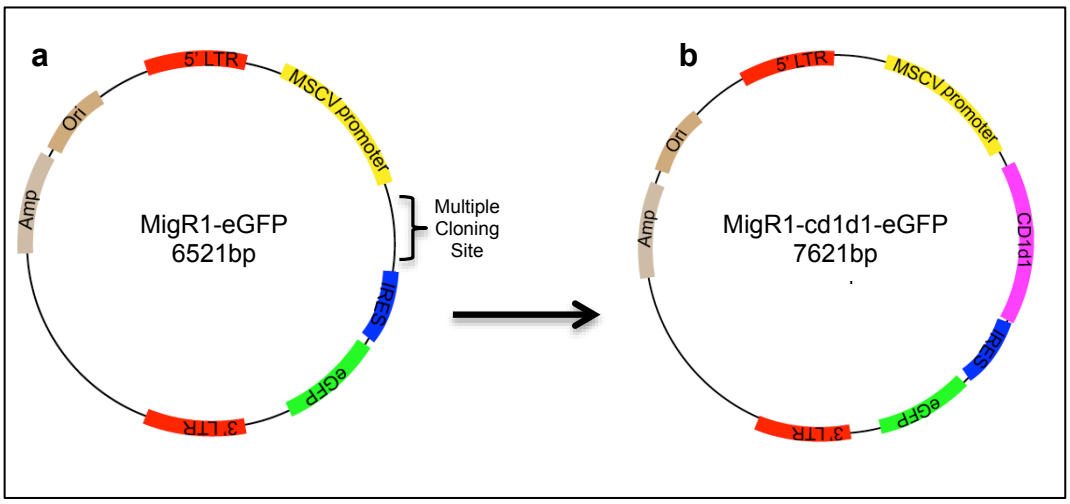


Figure 2.1. MigR1-eGFP and MigR1-cd1d1-eGFP retroviral vectors.

(a) *The MigR1-eGFP control vector contains a constitutively active MSCV promoter. A multiple cloning site allows for insertion of a transgene. Simultaneous expression of eGFP is achieved through use of an internal ribosome entry site (IRES). An ampicillin resistance gene (Amp) confers antibiotic resistance.*

(b) The MigR1-cd1d1-eGFP vector was produced through cloning the Cd1d1 transgene into the multiple cloning site of the MigR1-eGFP vector, and simultaneously expresses murine Cd1d1 and eGFP genes.

2.7.2 Retroviral transduction of HSC enriched BM cells

Transduction of HSC enriched BM cells was performed in 12 well plates (1–1.5 × 10^6 cells per well) coated with Retronectin (Takara Clontech) at a concentration of 10µg/cm^2. The Retronectin-coated wells were preloaded with retroviral supernatants by centrifugation for 20 to 60 minutes at 4 °C (900–1000g). The medium was aspirated, and HSC enriched BM cells were added in serum-free cytokine containing medium, as specified earlier, and incubated overnight at 37°C and 5% CO_2. The transduction was repeated after 24 hours by transferring non-adherent cells to new six-well plates that were coated with Retronectin and preloaded with retrovirus particles. The remaining non-adherent cells were recovered by washing the plate with PBS. After these cells were pelleted by centrifugation, they were suspended in new cytokine supplemented medium and combined with the other cells in the new plates. 24 hours after the second transduction, cells were transplanted by tail vein injection into recipient mice, as described above.

2.7.3 Production of shRNA expressing lentivirus

An shRNA clone set against murine *Cd1d1* was obtained from Genecopoeia. This set consisted of four lentiviral vectors expressing shRNAs against murine *Cd1d1* and one control lentiviral vector expressing a scrambled shRNA. The 3rd generation lentiviral vector psi-LVRU6MP was used, in which shRNA expression was driven by the constitutive U6 promoter. In addition each vector expressed mCherry under control of a SV40 promoter, to assess transfection and transduction efficiency **(Figure 2.2)**.

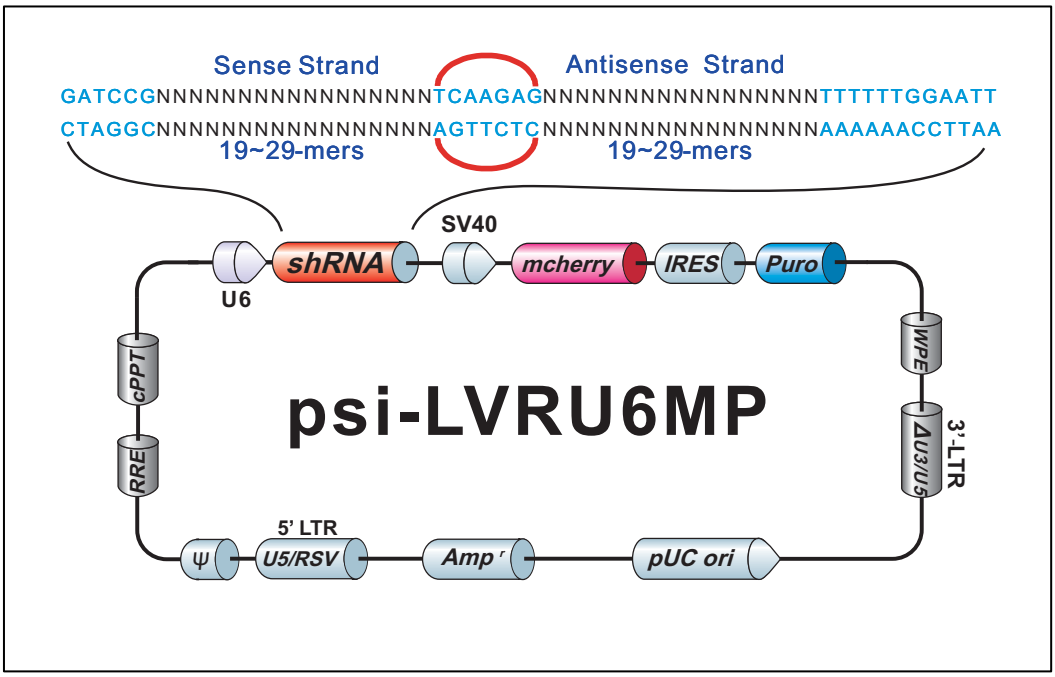


Figure 2.2. psi-LVRU6MP lentiviral vector. *This 3rd generation lentiviral vector contains an shRNA expression cassette driven by the U6 promoter, and a gene for the fluorescent protein mCherry under control of the SV40 promoter. An ampicillin resistance gene (Amp) confers antibiotic resistance.*

Production of shRNA expressing lentivirus was performed through transient transfection of 293T cells. 293T cells were plated in 10cm cell culture dishes at $3x10^6$ cells per dish and $3x10^5$ cells/mL using DMEM+ 24 hours prior to transfection. Media was changed 1 hour before transfection. Lipofectamine 2000® reagent was used to transfect cells with 5µg of psPAX2 (a 2nd generation helper plasmid containing Gag, Pol, Rev, and Tat), 5µg of psPAX2 (a VSV-G-expressing envelope vector) and 10µg of shRNA lentiviral vector as per instructions. Fresh DMEM+ media was given to cells after 14-18 hours incubation, and after 48 hours viral supernatant was harvested and spin filtered at 900xg for 15 minutes at 4°C.

Collected lentiviral supernatant was concentrated by using the Lenti-X concentrator (Takara Clontech) as per the manufacturer's instructions. Briefly, 30ml viral supernantant was mixed with the Lenti-X Concentrator and incubated for a short time at 4°C. The mixture was then centrifuged at low speed to obtain a high-titre virus-containing pellet, which was then resuspended in 3ml DMEM+. Concentrated virus was used immediately in transduction of target cells.

2.7.4 Lentiviral transduction of 5TGM1 cells

5TGM1 cells were transduced by concentrated lentiviral supernatant by spinocculation. 5TGM1 cells were plated in a 24-well plate, prior to viral infection, at a density of 0.5×10^5 cells per well. Cells were resuspended in 1ml of concentrated viral supernatant. Polybrene was added at a concentration of 8μg/ml. The plate was sealed and cells were centrifuged for 30 minutes at 800g at 32°C. Following centrifugation, virus containing medium was removed, and cells were resuspended in fresh complete culture media. Cells were cultured as described above and transduction efficiency was assessed at 48 hours by mCherry expression.

2.8 Statistical analysis

The statistical significance of normally distributed data was analysed using the Student's t-test, otherwise the non-parametric Mann-Whitney U test was used. Data was analysed and graphs generated using GraphPad Prism (GraphPad Software, Inc.).

Chapter 3

3 Expression of CD1d in normal and pathological B cells

3.1 Expression of CD1d in human GC B cells

The expression of CD1d on naïve and memory B cells has been well characterised. However, other than the aforementioned image of human lymph node staining with anti-CD1d (Exley et al., 2000), there is a paucity of data in the literature pertaining to its expression in GC B cells. In order to address this, the expression of CD1d in GC B cells was examined using both immunohistochemical staining of human spleen and flow cytometric analysis of human tonsillar B cells.

3.1.1 Immunohistochemical staining of human splenic B cells

Immunohistochemical staining of human splenic tissue with anti-CD1d antibody (clone 42.1) was kindly performed by Professor Kikkeri Naresh of Imperial College London (see Chapter 2, section 2.3, for further details). This revealed very weak staining for CD1d in GC B cells, in direct comparison to the strong CD1d staining observed in the B cells of the follicular mantle zone **(Figure 3.1)**.

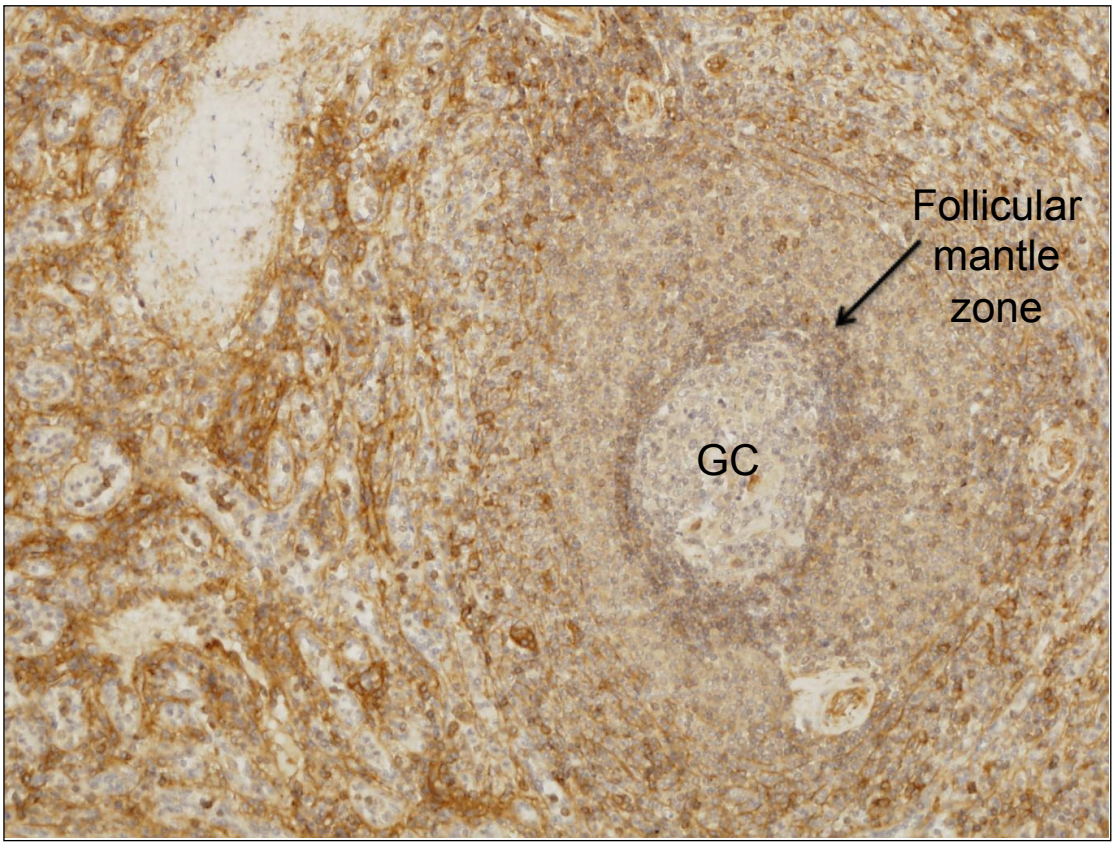


Figure 3.1. Immunohistochemical staining of normal human splenic tissue with anti-CD1d antibody. *Note the lack of CD1d staining in the GC, in comparison to the surrounding mantle zone which stains strongly for CD1d. Expression of CD1d can also be seen in scattered, inter-follicular plasma cells. (Staining with anti-CD1d antibody clone 42.1. Staining and acquisition of the image was kindly performed by Professor Kikkeri Naresh of Imperial College London) (n=1)*

3.1.2 Flow cytometric analysis of human tonsillar B cells

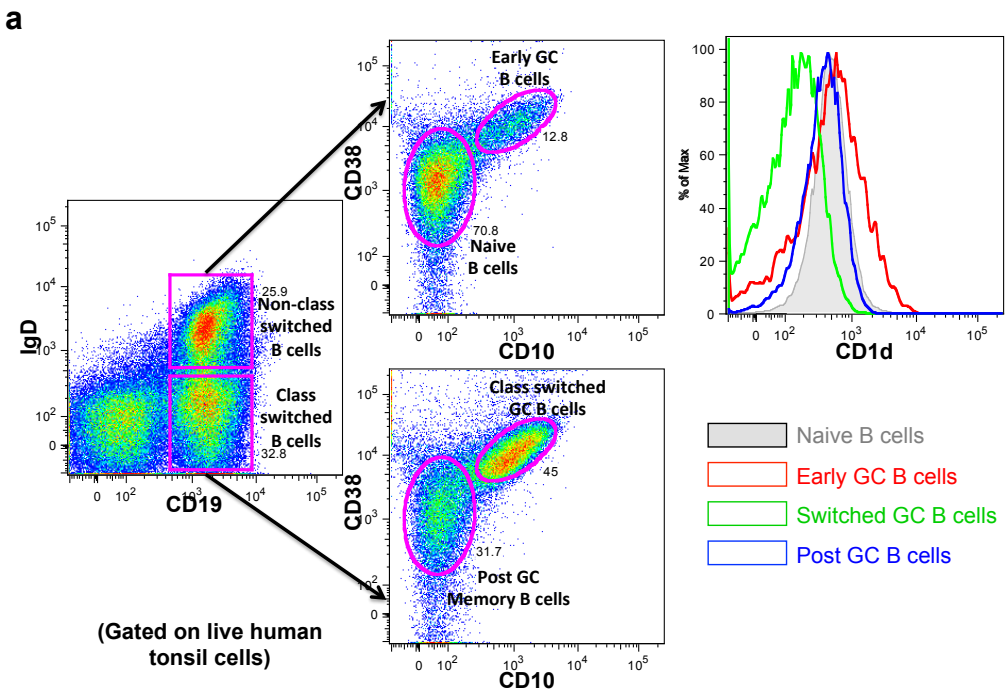
Gating strategy

Human tonsillar B cell populations were identified by flow cytometry according to known immunophenotypic markers previously described in the literature (Cook et al., 2003; Jackson et al., 2009). Full details of the antibodies used for analysis of tonsillar B cells can be found in Chapter 2, section 2.4.2. Non-class switched CD19+ B cells were identified as those carrying surface IgD. Within this population, use of the activation markers CD10 and CD38 could distinguish true naïve B cells (CD10-, CD38-) from early (non-class switched) GC BC cells (CD10+, CD38+).

Class switched B cells were defined as B cells without IgD expression (i.e., IgD-; this group encompassed B cells which had switched Ig class and also those B cells with on-going Ig receptor downregulation during the GC reaction which were imminently due to change Ig class). Again, use of the activation markers CD10 and CD38 distinguished established class switched GC B cells (CD10+, CD38+) from post GC memory B cells that were class switched but no longer activated (CD10-, CD38-).

Flow cytometric analysis of surface CD1d expression

By employing the gating strategy described above and staining with anti-CD1d antibody (clone 51.1), surface CD1d expression could be identified in different tonsillar B cell populations **(Figure 3.2 (a)).** Surface CD1d expression was found to be dynamic, decreasing as a naïve B cell entered the GC reaction, and subsequently rising again as the B cell left the GC **(Figure 3.2 (b))**.



b

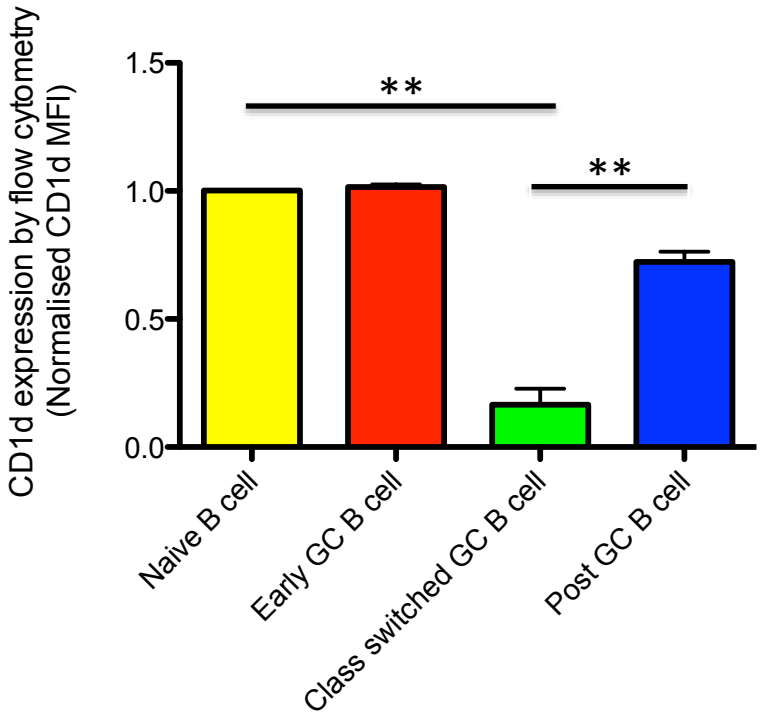


Figure 3.2. Dynamic surface expression of CD1d in the human germinal centre reaction.

(a) Representative flow cytometric plot from a human tonsil sample displaying different human B cell populations and their corresponding CD1d expression. (Staining with anti-CD1d antibody clone 51.1.)

(b) Summary of surface CD1d expression in different B cell populations of human tonsil, as indicated by the MFI of CD1d antibody staining (normalised to the CD1d MFI of naïve B cells). (n=3 tonsils, mean and SEM shown)

3.2 Expression of CD1d in murine GC B cells

Although my data and previous work in the literature has highlighted the loss of CD1d in human GC B cells, the pattern of CD1d expression in murine GC B cells has not been previously reported. It would be important to establish if downregulation of CD1d is also a feature of the murine GC for two reasons: a) it would demonstrate that downregulation of CD1d expression is a process conserved across species and thus likely to be of functional significance and b) it would confirm the suitability of a murine model for determining the biological significance and mechanism of this phenomenon.

To address this need, I investigated CD1d expression in the murine spleen, using an established immunisation protocol to enrich for splenic GC B cells when required. Splenic B cells were analysed using flow cytometry to assess for surface CD1d expression and real time PCR to assess *Cd1d1* at the transcriptional level. As a further source of data, an online gene expression database was interrogated for murine B cell *Cd1d1* expression.

3.2.1 Generation of murine splenic GC B cells

In order to enrich the splenic GC B cell population and facilitate analysis, C57BL/6 mice were immunised with NP-KLH on day 0 according to published protocols (Vikstrom et al., 2010). NP-KLH consists of the BCR antigen NP conjugated to the peptide KLH and induces a TD immune response characterised by GC formation. Spleens were harvested on day 10 for assessment of GC formation and subsequent analysis (see Chapter 2, section 2.2.2, for further details of the immunisation process).

3.2.2 Flow cytometric analysis of murine splenic B cells

Gating strategy

Using a similar strategy to that used for human tonsillar B cells, murine splenic B cell populations were identified by flow cytometry according to known immunophenotypic markers previously described in the literature (Béguelin et al., 2013). Chapter 2, section 2.4.3, describes the antibodies used in this analysis. Non-class switched B220+ B cells were identified as those carrying surface IgM and/or IgD. Within this population, use of the activation markers GL7 and CD95 could distinguish true naïve B cells (GL7-, CD95-) from early (non-class switched) GC BC cells (GL7+, CD95+). True naïve B cells were further subdivided as follicular B cells (CD23+, CD21int) and marginal zone B cells (CD23-, CD21+).

Class switched B cells were defined as B cells with absent IgM and IgD expression (i.e., IgM-IgD-; this group encompassed B cells which had switched Ig class and also those B cells with on-going Ig receptor downregulation during the GC reaction which were imminently due to change Ig class). Again, use of the activation markers GL7 and CD95 distinguished established class switched GC B cells (GL7+, CD95+) from post GC B cells that were class switched but no longer activated (GL7-, CD95-).

Flow cytometric analysis of GC formation

Figure 3.3 (a) demonstrates the identification of class-switched B cells and GC B cells in both non-immunised and immunised mice. Although GC B cells (GL7+, CD95+) could be detected in the non-immunised spleen, it was found that the immunisation procedure led to a significant (approximately ten-fold) increase in GC B cell numbers **(Figure 3.3 (b))**. This facilitated their analysis, in particular with regards to isolating these cells for real time PCR (see below).

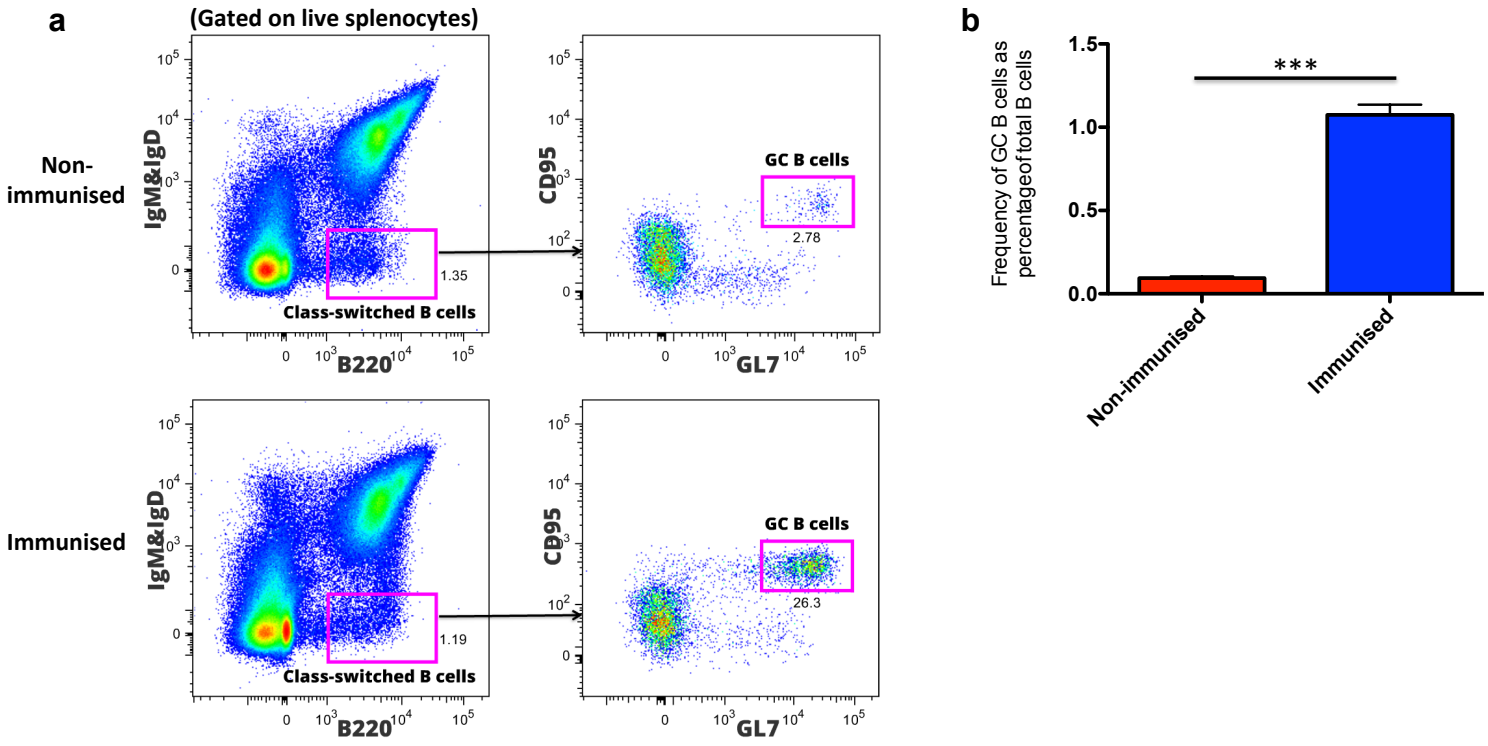


Figure 3.3. GC formation in non-immunised and immunised mice.

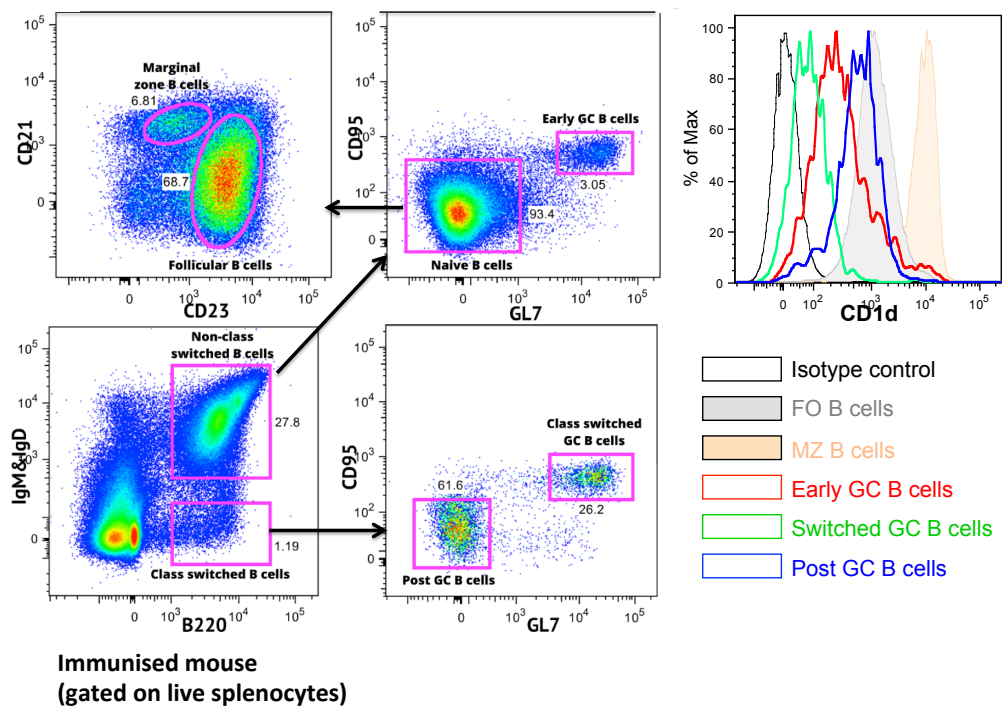
(a) *Representative flow cytometric plot showing enrichment of GC formation after immunisation with NP-KLH.*

(b) *GC B cell formation displayed as a percentage of total B cells. (n=3 mice per group, mean and SEM shown)*

Flow cytometric analysis of surface CD1d expression

By employing the gating strategy described above, surface CD1d expression could be identified in different splenic B cell populations by staining with anti-CD1d antibody (clone 1B1) **(Figure 3.4 (a))**. As observed previously in human tonsillar B cells, surface CD1d expression was found to be dynamic in the murine spleen, decreasing as a naïve B cell entered the GC reaction, and subsequently rising again as the B cell left the GC **(Figure 3.4 (b))**.

a



b

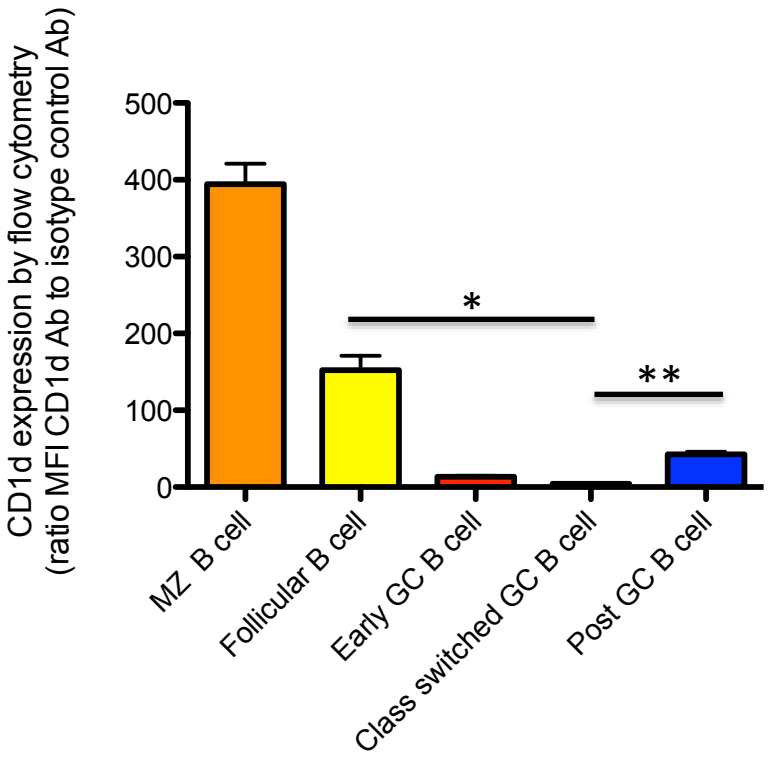


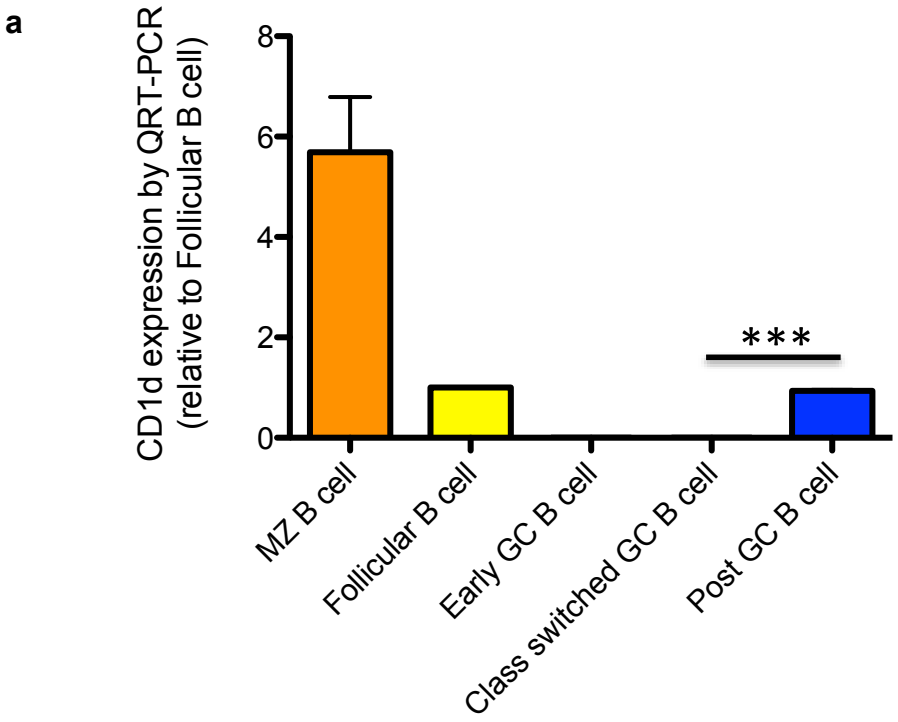
Figure 3.4. Dynamic surface expression of CD1d in the murine germinal centre reaction.

(a) Representative flow cytometric plot from an immunised C57BL/6 mouse displaying different splenic B cell populations and their corresponding CD1d expression. (Staining with anti-CD1d antibody clone 1B1.)

(b) Summary of surface CD1d expression in different splenic B cell populations of immunised mice, as indicated by the MFI ratio of CD1d antibody to isotype control antibody. (n=3 mice, mean and SEM shown)

3.2.3 Real time PCR analysis of murine splenic B cells

The murine splenic B cell populations described above were isolated using fluorescent activated cell sorting and subsequently real time PCR was performed to investigate *Cd1d1* expression at the transcriptional level (as described in Chapter 2, section 2.6.3). *Cd1d1* mRNA was again found to be dynamic, decreasing as a naïve B cell entered the GC reaction, and subsequently rising again as the B cell left the GC **(Figure 3.5 (a))**. This paralleled the changes seen in surface CD1d expression **(Figure 3.5 (b))**.



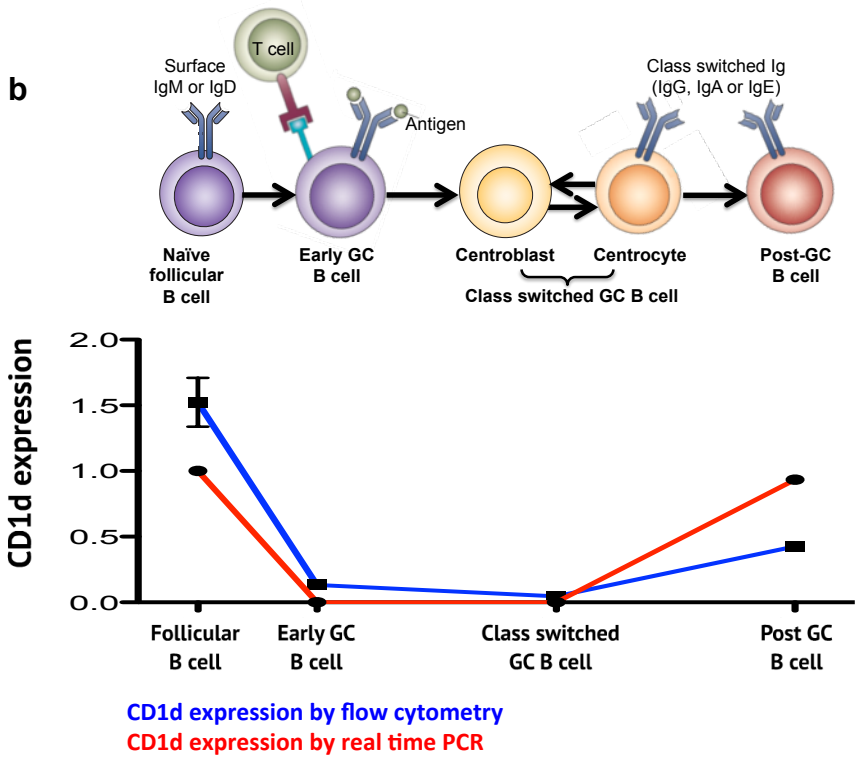


Figure 3.5. Dynamic Cd1d1 mRNA expression in the murine germinal centre reaction.

(a) Summary of Cd1d1 mRNA expression in different splenic B cell populations of immunised mice. Expression values are calculated relative to the reference gene beta actin, and are shown relative to the Cd1d1 expression value for follicular B cells. (n=3 mice, mean and SEM shown)

(b) Comparison of surface and transcriptional CD1d expression during the murine splenic GC reaction. CD1d expression values are calculated and expressed as described above in Figure 3.4 (b) and Figure 3.5 (a) (values calculated by flow cytometry are scaled down by a factor of 100 in order to use the same scale). (n=3 mice, mean and SEM shown)

3.2.4 In silico analysis of Cd1d1 expression using a public database

A public gene expression dataset (GSE11961) is available via the NCBI Gene Expression Omnibus data repository (Edgar et al., 2002) and provides unanalysed gene expression profiling data of murine splenic B cell populations by microarray (Affymetrix platform) (Kaji et al., 2012). Interrogation of this dataset was performed *in silico* using the NCBI GEO2R interactive web tool, which can be used to investigate the differential expression of different genes including murine *Cd1d1*. Analysis of this dataset confirms my previous experimental data, showing that although the *Cd1d1* gene is expressed in naïve marginal zone and follicular B cells, it is in the top 20 genes downregulated in the murine germinal centre. *Cd1d1* is then re-expressed in post GC memory B cells and plasma cells **(Fig 3.6)**.

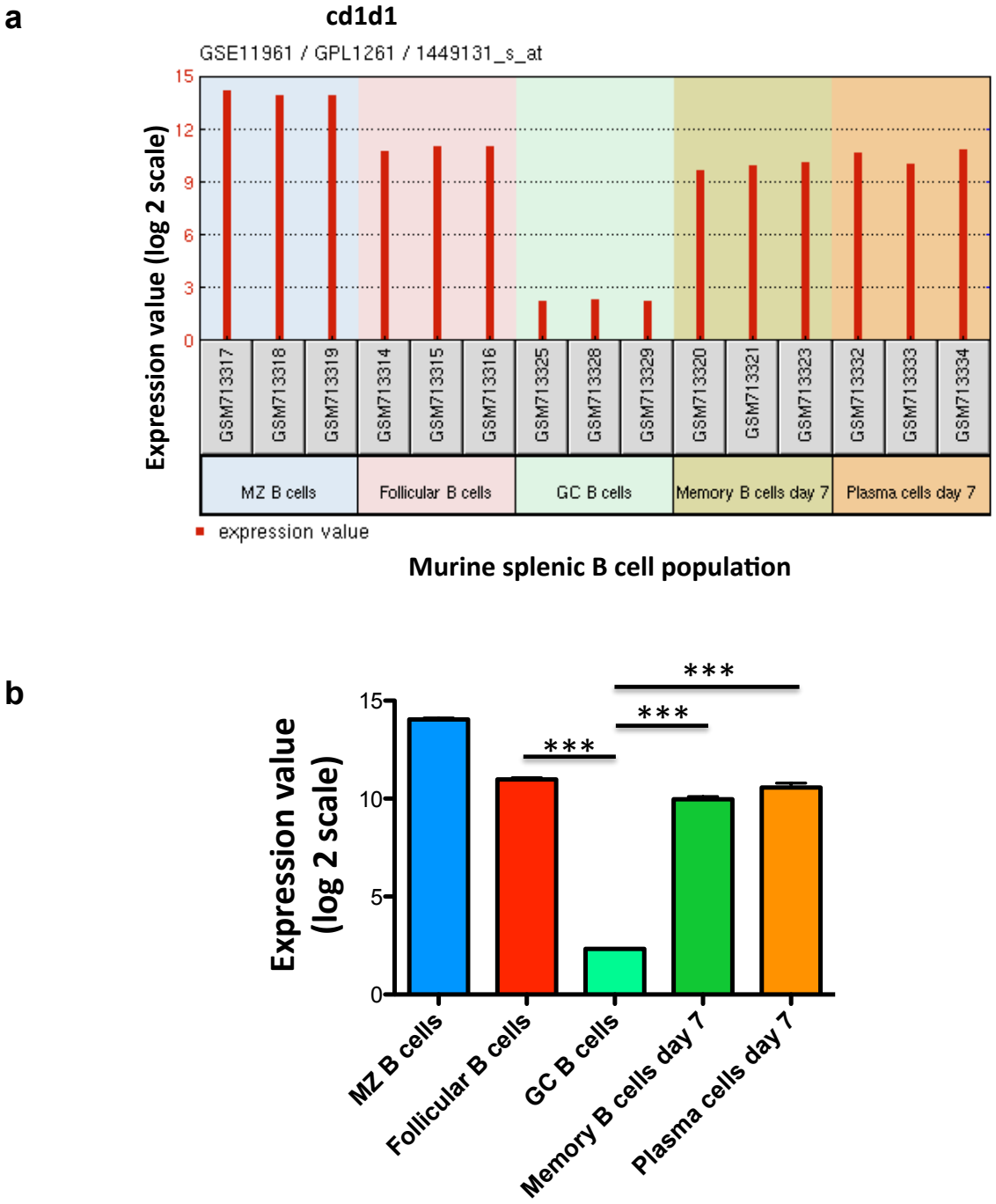


Figure 3.6. In silico analysis of Cd1d1 expression using a public database

(a) Affymetrix microarray data analysed by the NCBI GEO2R web tool, showing expression of murine Cd1d1 in different splenic B cell populations. (Note expression values are shown on a log2 scale.)

(b) Bar chart summary of expression data for Cd1d1 (mean and SEM shown). (Note expression values are shown on a log2 scale.)

3.3 Expression of CD1d in B-lymphoblastoid cell lines

Although appreciated that EBV can infect human B cells and bring about a GC-like reaction, as discussed in Chapter 1, section 1.4.2, the specific effect of EBV infection on B cell CD1d expression had not been reported at the start of this project. In order to initially address this issue, I studied B-lymphoblastoid cell lines (B-LCLs). These are immortalized cell lines produced by infection of naïve B cells by EBV, and represent an *in vitro* equivalent to newly infected *in vivo* B cells. In these cells, EBV employs the latency III transcriptional programme, and thus they represent a very useful cellular system for investigating the effect of EBV infection on CD1d expression in B cells. CD1d expression was determined at both the surface protein and transcriptional level, using flow cytometry and real time PCR respectively.

3.3.1 Flow cytometric analysis of B-LCLs

Five human B-LCLs were stained with anti-CD1d antibody (clone 42.1) or the corresponding isotype control antibody, and analysed by flow cytometry to assess surface CD1d expression (as further described in Chapter 2, section 2.4.5). The CD1d transduced cell line C1R-CD1d which expresses surface CD1d, was used as a positive control. Flow cytometric analysis revealed absent surface CD1d expression in all five B-LCLs, at levels comparable to expression of the isotype control antibody **(Figure 3.7)**.

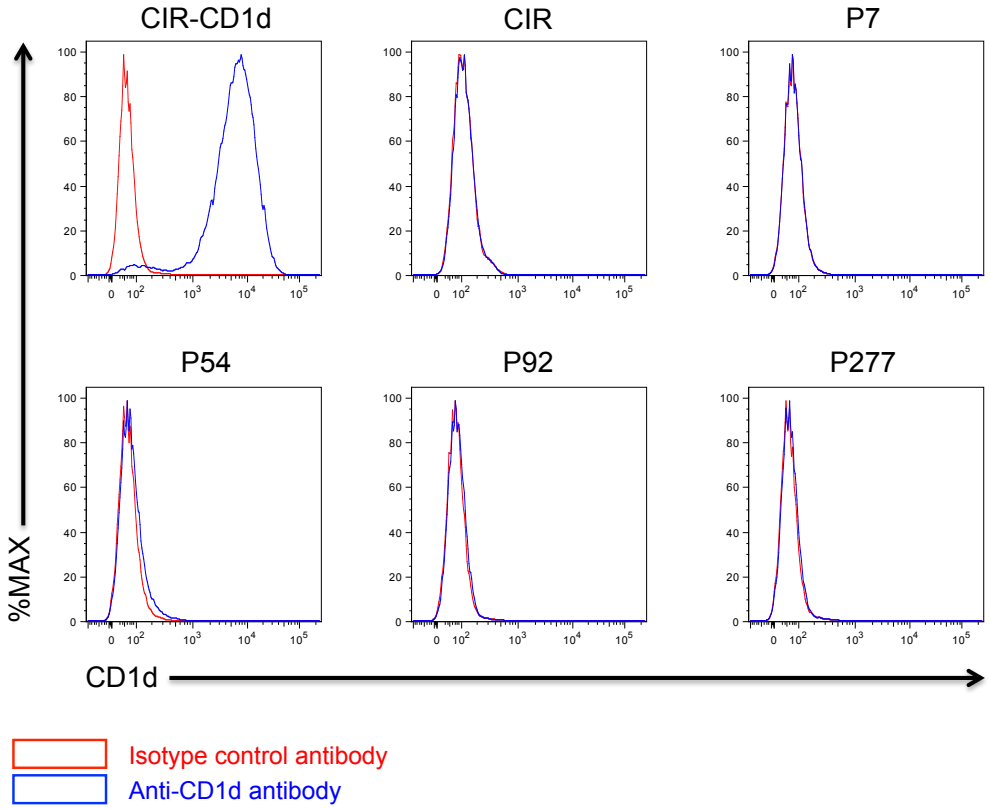


Figure 3.7. Flow cytometric analysis of surface CD1d expression in human B-LCLs. *In all 5 B-LCLs analysed, staining with anti CD1d antibody (clone 42.1) was found to be absent, at levels comparable to staining by isotype control antibody. Analysis of the cell line C1R-CD1d, as a positive control, revealed strong surface CD1d expression. (n=3 throughout)*

3.3.2 Real time PCR analysis of B-LCLs

Analysis of the same five B-LCLs by real time PCR (as described in Chapter 2, section 2.6.3) revealed an absence of *CD1D* mRNA in all cell lines, in comparison to detection of *CD1D* mRNA in peripheral blood B cells **(Figure 3.8)**. Thus the loss of CD1d expression in B-LCLs occurred at the transcriptional level.

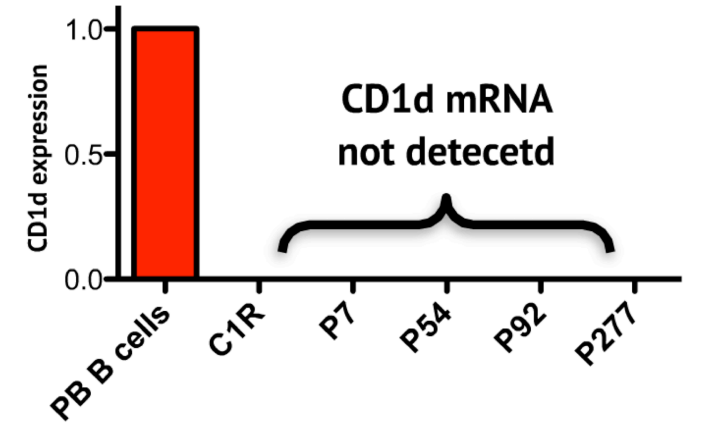


Figure 3.8. Real time PCR analysis of CD1D mRNA expression in human B-LCLs. *In all 5 B-LCLs analysed, no CD1D mRNA expression was detected, in comparison to the positive control of peripheral blood B cells. Expression values are calculated relative to the reference gene beta actin, and are shown relative to the CD1D expression value for peripheral blood B cells. (n=3)*

3.4 Expression of CD1d in human MM cell lines

The expression of CD1d in MM has previously been investigated by our group, as discussed in chapter 1, section 1.4.3, using both MM cell lines and primary cells. In order to corroborate and extend these findings, I undertook a more extensive analysis of MM cell lines. As for the analysis of B-LCLs, CD1d expression in MM cells lines was again determined at both the surface protein and transcriptional level, using flow cytometry and real time PCR respectively.

3.4.1 Flow cytometric analysis of MM cell lines

Seven human MM cell lines were stained with anti-CD1d antibody (clone 42.1) or the corresponding isotype control antibody, and analysed by flow cytometry to assess surface CD1d expression (as further described in Chapter 2, section 2.4.5). The CD1d transduced cell line C1R-CD1d and CD19+ PBMCs (i.e. circulating B cells), both of which express surface CD1d, were used as positive controls. Flow cytometric analysis revealed absent surface CD1d expression in all seven MM cell lines, at levels comparable to expression of the isotype control antibody **(Figure 3.9)**.

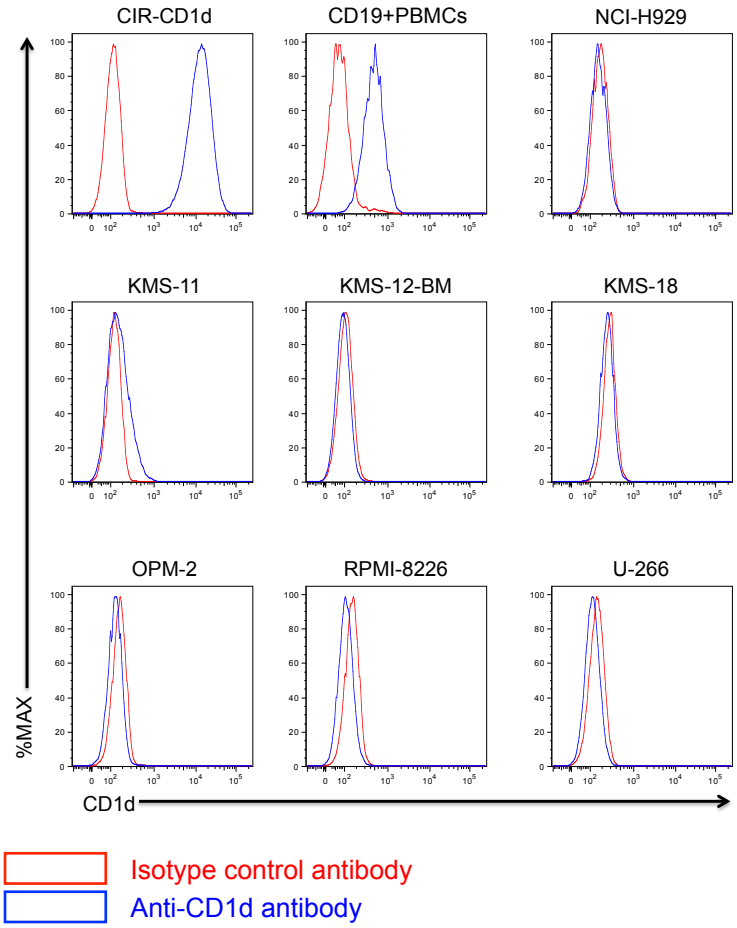


Figure 3.9. Flow cytometric analysis of surface CD1d expression in human MM cell lines. *In all 7 MM cell lines analysed, staining with anti CD1d antibody (clone 42.1) was found to be absent, at levels comparable to staining by isotype control antibody. Analysis of the cell line C1R-CD1d and CD19+PBMCs, as positive controls, revealed strong surface CD1d expression. (n=3 throughout)*

3.4.2 Real time PCR analysis of MM cell lines

Analysis of the same seven MM cell lines by real time PCR (see Chapter 2, section 2.6.3) revealed an absence of *CD1D* mRNA in all cell lines, in comparison to detection of *CD1D* mRNA in peripheral blood B cells **(Figure 3.10)**. Thus the loss of CD1d expression in MM cell lines occurred at the transcriptional level.

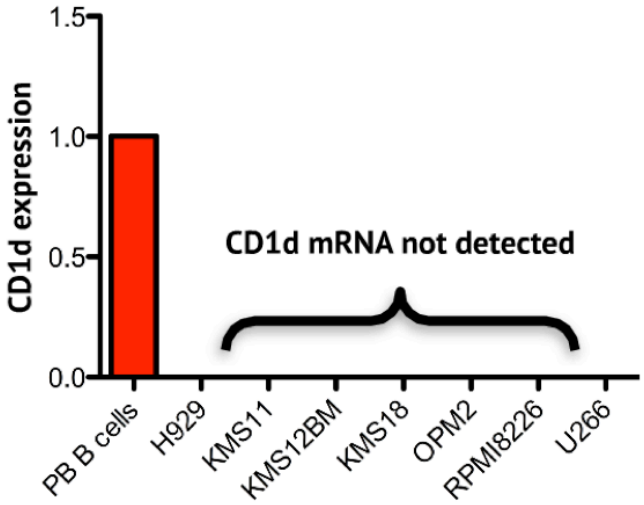


Figure 3.10 Real time PCR analysis of CD1D mRNA expression in human MM cell lines. *In all 7 MM cell lines analysed, no CD1D mRNA expression was detected, in comparison to the positive control of peripheral blood B cells. Expression values are calculated relative to the reference gene beta actin, and are shown relative to the CD1D expression value for peripheral blood B cells. (n=3)*

3.5 Discussion

3.5.1 Downregulation of CD1d in normal GC B cells

The studies detailed in this chapter have increased our understanding of the expression of CD1d in normal B cells during the GC reaction. The immunohistochemical staining of human spleen reinforces previous work by Exley et al. (2000) in demonstrating the *in situ* loss of CD1d expression in the GC of human lymphoid tissue.

Further important insights have been gained by the analysis of both human tonsillar and murine splenic tissue using flow cytomtery. By identifying specific cell populations within these tissues, I have shown for the first time that CD1d expression in normal B cells is dynamic, decreasing to a nadir as B cells enter the GC reaction, before increasing again as B cells leave the GC. The real time PCR and *in silico* approaches described above have shown that these dynamic changes occur at the transcriptional level.

The loss of CD1d expression in murine GCs is a hitherto undescribed phenomenon, and has important implications. The conservation across species of these dynamic changes in CD1d expression offers further support to the concept that this is a biologically significant phenomenon. Furthermore, it provides a platform from which murine models can be used to elucidate the biological significance and mechanisms of this

donwnregulation of CD1d, themes that will be explored in subsequent chapters.

3.5.2 Downregulation of CD1d in pathological B cells

The analysis of B-LCLs provides evidence that EBV infection of B cells leads to loss of CD1d expression at the transcriptional level. At the start of this project, this was a previously unreported finding. Similarly, in an extensive analysis of MM cell lines, CD1d was also found to be absent at the transcriptional level. MM cell lines are derived from cells of patients with advanced disease, and thus this builds upon previous work within our group that CD1d expression in MM is lost with disease progression.

The complete loss of CD1d at both the mRNA and protein level in these two different types of pathological B cells is striking, and provides a basis from which these cell lines can be used to further investigate the biological significance and mechanisms of this phenomenon. Furthermore, there are parallels that can be drawn with the dramatic transcriptional loss of CD1d in normal GC B cells. This raises the intriguing possibility that the downregulation of CD1d in normal and pathological B cells may share similar biological significance and/or biological mechanisms, and this will be explored in subsequent chapters.

3.5.3 Future work

Although my finding that EBV infection can abrogate CD1d expression in B-LCLs was previously unrecognized, a subsequent publication by another research group has now demonstrated and expanded upon these findings (Chung et al., 2013). This work is discussed further in Chapter one. At this point, I made the decision to focus my work, both in this project and in the future, on MM B cells as a model for pathological B cells, as the potential to uncover novel findings was greater in this area.

With regards to MM cells, there is potential to expand the characterisation of their CD1d expression, including use of further cell lines and use of primary patient samples. In the latter case, it would be useful to identify a cohort of patients, which could be prospectively followed in order to obtain samples at different stages of disease progression and correlate this with CD1d expression. Beyond this, I aim in the future to further expand this theme by addressing other B cell malignancies in a similar manner, including CLL and B cell lymphomas.

Chapter 4

4 Biological significance of changes in CD1d expression in B cells

As discussed in Chapter 1, section 1.3.4, there is clear evidence of a powerful cognate relationship between iNKT cells and CD1d expressing B cells, which can augment responses in certain B cell subsets. Yet it is also established, through both my work and previous work (Allan et al., 2011; Chung et al., 2013; Exley et al., 2000), that human CD1d is downregulated (a) in normal B cells following *in vitro* activation or as part of the GC reaction, and (b) in pathological B cells, including EBV infection and multiple myeloma, as part of the disease process. Furthermore, I have established that a similar loss of CD1d is also observed in murine GC B cells. However, the biological significance of this phenomenon *in vivo* remains poorly understood.

According to the *in silico* analysis performed in Chapter 3, *Cd1d1* is in the top 20 genes downregulated in murine GC B cells. This marked downregulation, in addition to the conservation of the loss of CD1d in GC B cells across different species, are both suggestive of an underlying purpose to this phenomenon. Although GC B cells, EBV transformed B-LCLs and advanced MM B cells are protean in their biological course, they share a phenotype characterised by a high degree of cellular proliferation (Klein and Dalla-Favera, 2008). I postulate that this high rate of proliferation makes these cells more vulnerable to iNKT mediated cellular cytotoxicity in comparison

to their non-proliferative counterparts. This thus necessitates downregulation of CD1d in order to obtund an iNKT-B cell interaction before it becomes detrimental to the B cell.

It is not clear why a highly proliferative B cell would be more susceptible to cellular cytotoxic attack from an iNKT cell. Such proliferative cells may be more effective than non-proliferative B cells in presenting lipid ligands to iNKT cells. An alternative explanation may involve metabolic reprogramming in proliferative cells (Marelli-Berg et al., 2012), leading to presentation of an altered lipid repertoire, consisting of lipid ligands with much higher affinity for iNKT cells in comparison to non-proliferative B cells. Indeed, the modulation of endogenous CD1d-presented lipids has previously been described in dendritic cells, where Toll-like receptor ligands were shown to effect change in the lipid repertoire and consequently lead to enhanced iNKT responses (Salio et al., 2007). Thus, it is plausible that such a phenomenon may exist in B cells in response to cellular proliferation.

In this chapter, I will detail two related approaches I have taken to address this hypothesis that downregulation of CD1d is essential to survival and propagation of highly proliferative B cells. These approaches share the principle of constitutively expressing CD1d in murine B cells *in vivo*, such that it is no longer downregulated in GC B cells. By subsequently assessing the output of the GC reaction in these mice, and comparing this to mice in

which CD1d is downregulated appropriately, the significance of the role played by this loss of CD1d expression can be investigated.

4.1 Constitutive expression of CD1d in GC B cells using adoptive transfer of *Cd1d1* transduced cells

4.1.1 Experimental design

An initial approach to constitutively express CD1d in murine GC B cells used the principle of retroviral transduction of a CD1d expression construct into isolated murine haematopoietic stem cells (HSCs), and adoptive transfer of these cells into mice. This was performed using the MigR1-cd1d1-eGFP expression construct, an IRES-containing bicistronic retroviral vector, which constitutively expresses murine *Cd1d1* and *eGFP* under control of a MSCV promoter.

An outline of this approach is described below and a general schematic representation of these procedures is illustrated in **Figure 4.1**. Further details of experimental procedures can be found in Chapter 2, sections 2.1.3 (harvest of bone marrow cells), 2.1.4 (enrichment for HSCs), 2.7.1 (production of retrovirus), 2.7.2 (retroviral transduction) and 2.2.3 (adoptive transfer of transduced cells).

Bone marrow cells were harvested from C57BL/6 wild type mice (congenic strain CD45.1). Using magnetic depletion of lineage positive cells, bone marrow cells were enriched for HSCs. By defining HSCs as Lin-, CD117+, Sca-

1+, CD150+, CD48- **(Figure 4.2 (a))**, magnetic depletion was found to lead to approximately a 10-fold enrichment in HSCs **(Figure 4.2 (b))**. HSC enriched bone marrow cells were cultured overnight in serum free medium containing the cytokines mSCF, mIL-3, hIL-11 and hFLT-3 ligand, and then were subsequently transduced for 48 hours using either MigR1-cd1d1-eGFP or MigR1-eGFP retroviral supernatants. On day 4, transduced cells were transferred intravenously by tail vein injection into sub-lethally irradiated (2X450rad) C57BL/6 recipients (congenic strain CD45.2). Four mice received MigR1-cd1d1-eGFP transduced cells (henceforth designated **MigR1-cd1d1 mice**) and four mice received MigR1-eGFP transduced cells (**MigR1 control mice**), each at a dose of $1X10^6$ cells/mouse.

At 8 weeks following transfer of cells, peripheral blood of recipient mice was analysed for evidence of donor cell engraftment (by GFP expression of total peripheral blood leucocytes). Engrafted mice were subsequently immunised, and spleens were harvested at day 10 for analysis of GC formation, all as previously described in Chapter 3, sections 3.2.1 and 3.2.2.

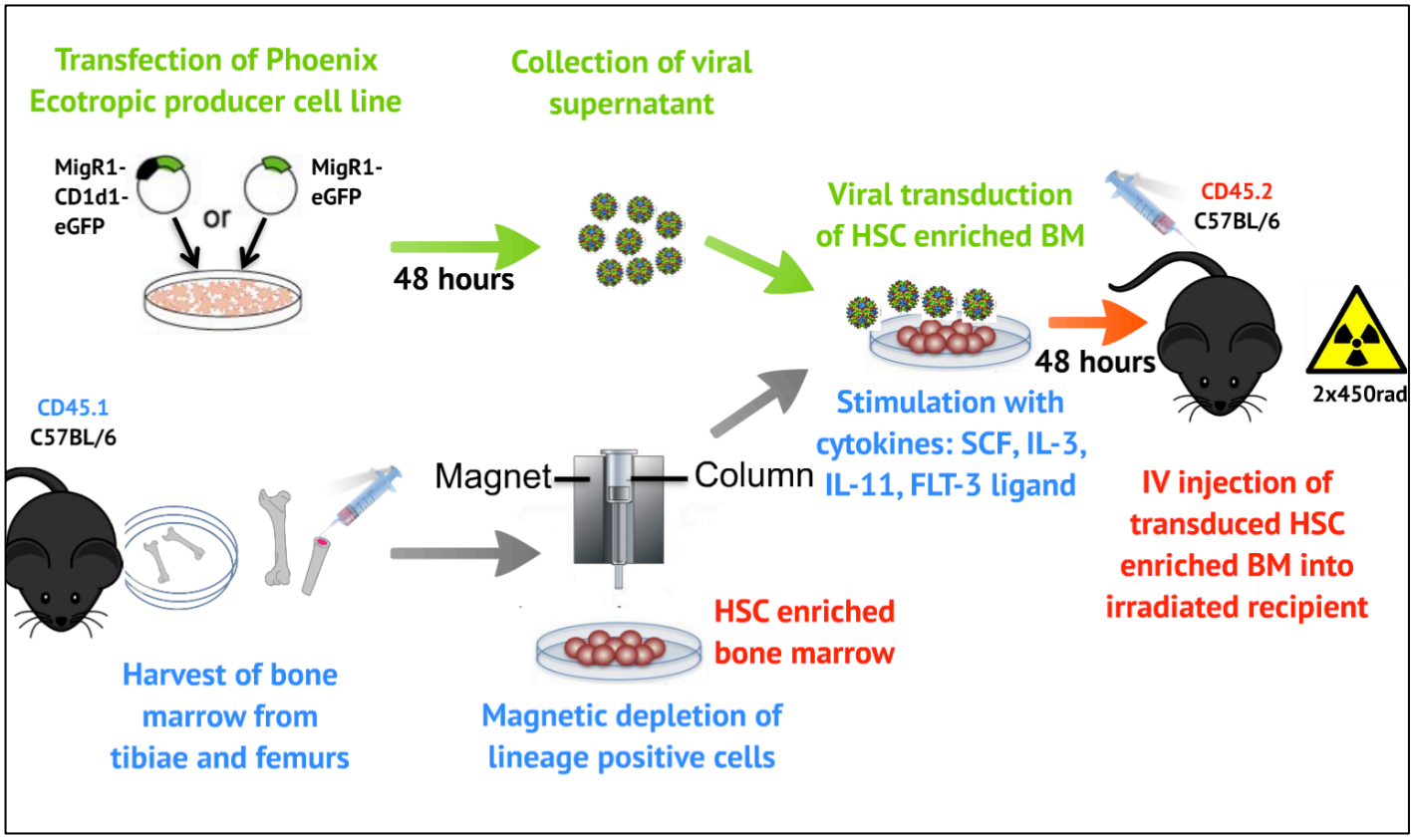


Figure 4.1. Schematic outline of an experimental approach to constitutively express CD1d in murine B cells using adoptive transfer of Cd1d1 transduced HSCs. *Bone marrow cells were harvested from C57BL/6 wild type mice (congenic strain CD45.1) and then enriched for HSCs using magnetic columnar depletion of lineage positive cells on day 1. Following overnight culture in serum free medium containing the indicated cytokines, HSC enriched marrow cells were transduced on day 2 for 48 hours by pre-prepared retroviral supernatants of either the MigR1-cd1d1-eGFP or MigR1-eGFP vectors. On day 4, transduced cells were then tail vein injected into sub-lethally irradiated (2X450rad) C57BL/6 recipients (congenic strain CD45.2). Using this approach, donor engraftment could be expected at 8 weeks post transplant. (n=4 mice for each vector)*

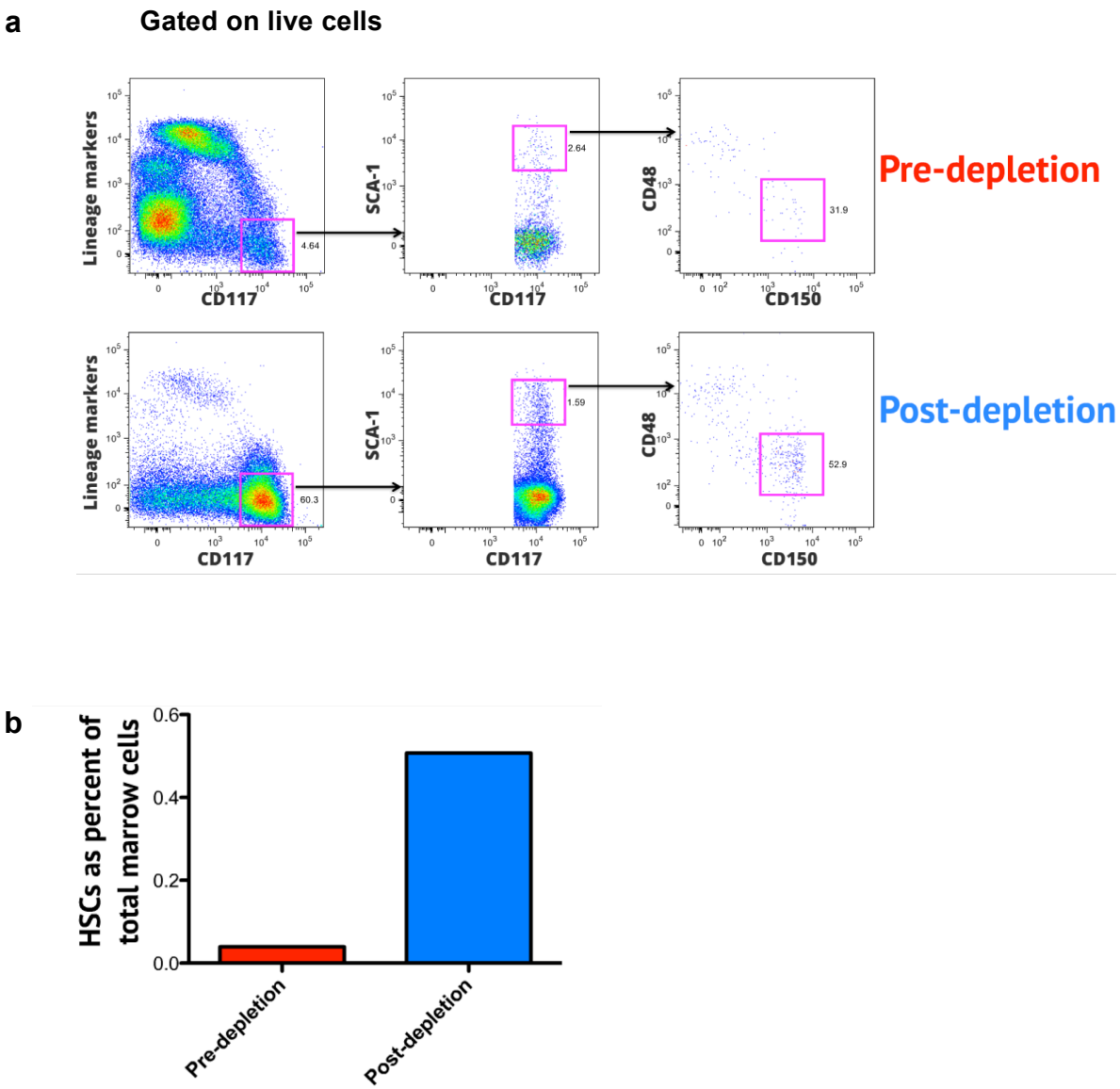


Figure 4.2. Enrichment of bone marrow cells for HSCs using magnetic columnar depletion of lineage positive cells.

(a) *Flow cytometric analysis of the HSC population in bone marrow cells. By defining HSCs as Lin-CD117+Sca-1+CD150+CD48-, their relative frequency in murine bone marrow could be assessed pre and post magnetic columnar depletion of lineage positive cells.*

(b) *Frequency of HSCs in murine bone marrow (as a percentage of total bone marrow cells) pre and post depletion of lineage positive cells. (n=1)*

4.1.2 Flow cytometric analysis of splenic B cells in recipient mice

In the initial experiment, three out of four MigR1-cd1d1 mice had evidence of donor engraftment at 8 weeks, but only one MigR1 control mouse displayed evidence of donor engraftment at this same time point. This unfortunately precluded a comprehensive comparison of the two sets of mice. However, immunisation and splenic analysis of engrafted mice still provided an opportunity to (a) demonstrate as a proof of principle that this experimental approach can lead to constitutive expression of CD1d in splenic B cells and (b) provide preliminary data for GC formation, with a view to further experiments.

Gating strategy

Murine splenic B cells were analysed for GC formation using a similar strategy to that employed in Chapter 3, section 3.2.2, with the use of additional markers to take into account the transplant procedure and to more stringently identify B cells. Engrafted transplanted donor cells were identified by expression of the congenic marker CD45.1, and of these, transduced donor cells could be distinguished from non-transduced donor cells by GFP expression. Within each of these populations, B cells were identified by combined expression of the B cell markers B220 and CD19.

As described in section 3.2.2, expression of IgM and/or IgD was used to differentiate non-class switched B cells (IgM+ and/or IgD+) from class switched B cells (IgM-IgD-). In each of these populations, expression of the activation markers GL7 and CD95 again identified early non-class switched and class switched GC B cells respectively. In the non-class switched B cell population, use of the markers CD21 and CD23 could again be used to identify naïve follicular and marginal zone B cells. A representative flow cytometry plot is shown in **Figure 4.3**, highlighting the comparative analysis that could be made between non-transduced and transduced B cell populations.

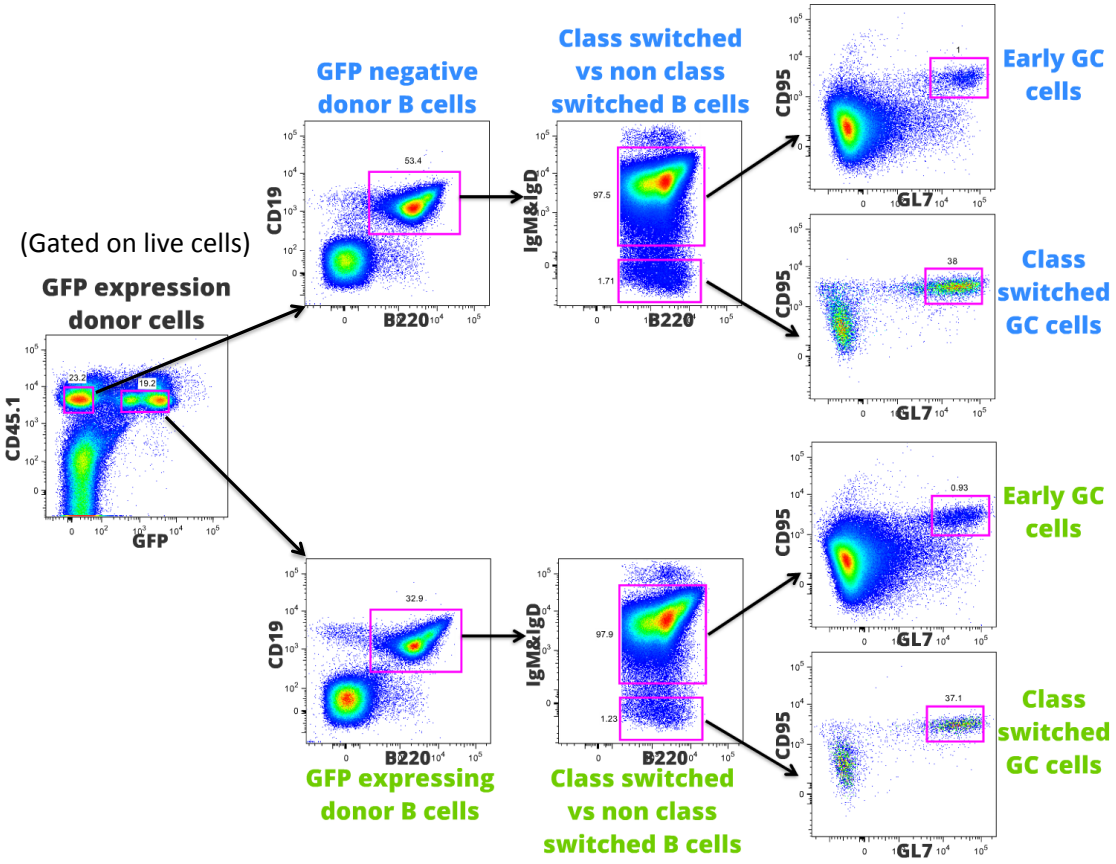


Figure 4.3. Flow cytometric analysis of murine splenocytes following adoptive transfer of donor cells.

Murine splenocytes were analysed following immunisation at 8 weeks post adoptive transfer of HSC enriched BM cells transduced with the MigR1-cd1d1-eGFP retroviral vector. The congenic marker CD45.1 identified engrafted donor cells, and GFP was used to identify those donor cells that had been transduced by the vector. In both transduced and non-transduced donor cells, an identical analysis could be performed, using a similar strategy to that previously employed, allowing a direct comparative analysis. Specifically, splenic B cells were identified by dual expression of B220 and CD19. Again, expression of IgM and/or IgD was used to differentiate non-class switched B cells (IgM+ and/or IgD+) from class switched B cells (IgM-IgD-). In each of these populations, expression of the activation markers GL7 and CD95 again identified early non-class switched and class switched GC B cells respectively. (Plots representative of 3 MigR1-cd1d1 mice and 1 MigR1 control mouse.)

Flow cytometric analysis of donor cell engraftment, transduction and CD1d expression

By assessing expression of CD45.1, the percentage of donor, engrafted splenic cells could be determined in the three MigR1-cd1d1 mice, and the one mouse MigR1 control mouse. It was observed that adoptive transfer of cells according to this model led in all cases to the generation of mixed chimeras, as would be expected by the use of a low non-myeloablative dose of radiation in this conditioning protocol **(Figure 4.4 (a))**. There was a higher level of engraftment observed in MigR1-cd1d1 mice. The reason for this is not clear, and this finding of course carries the caveat that a limited number of mice were analysed. In all cases, the engraftment of donor cells was sufficient to allow a subsequent meaningful analysis of donor B cell populations.

The proportion of these donor cells exhibiting stable transduction *in vivo* could subsequently be assessed by GFP expression **(Figure 4.4 (b))**. Transduction efficiency was found to be highest in the MigR1 control mouse (54% versus 18-44.5% for MigR1-cd1d1 mice). Although the limited numbers prevent firm conclusions to be drawn, a higher level of transduction would indeed be expected in cells transduced by the smaller MigR1-eGFP vector (which does not contain the *Cd1d1* transgene). Again, in all cases, the transduction of donor cells was sufficient to allow subsequent analysis of transduced donor B cell populations.

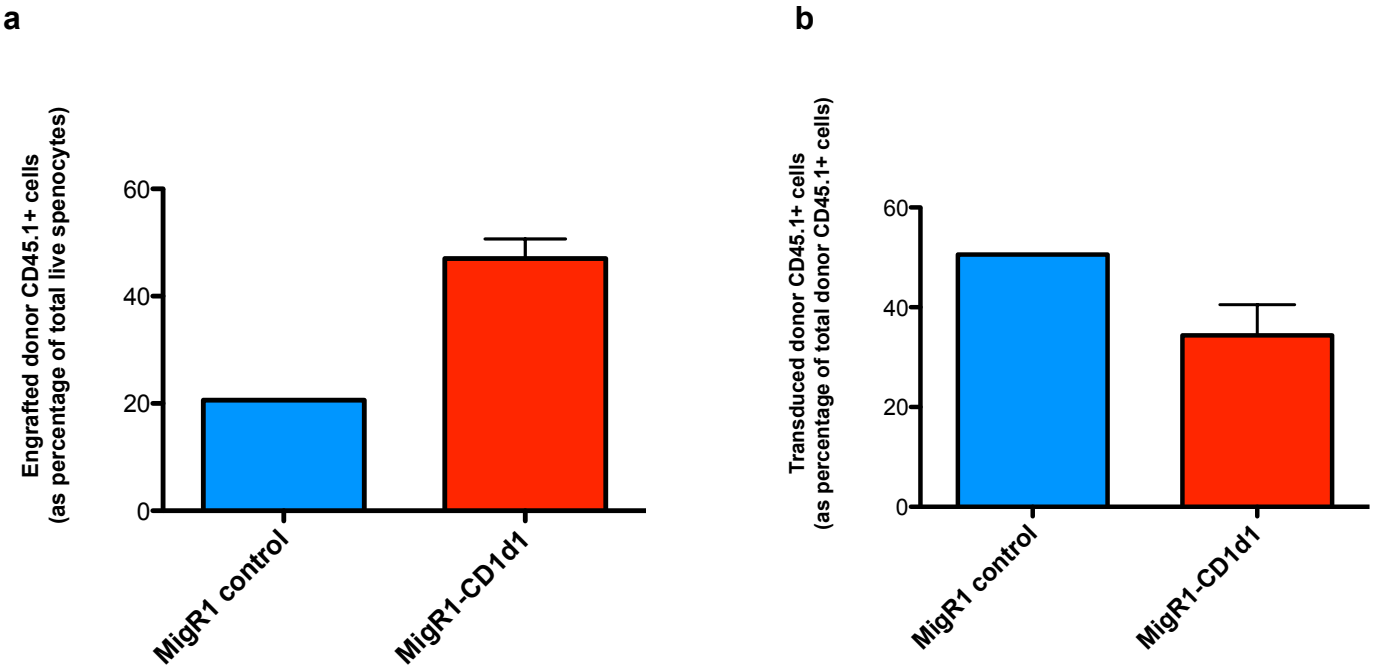


Figure 4.4. Donor cell engraftment and transduction following adoptive transfer of MigR1-eGFP (MigR1 control mouse) or MigR1-cd1d1-eGFP transduced cells (MigR1-cd1d1 mice).

(a) *Frequency of donor, engrafted cells (expressed a percentage of total live splenocytes).*

(b) *Frequency of transduced donor engrafted cells (expressed a percentage of total donor engrafted cells).*

(n=1 mouse for MigR1 control group and n=3 mice for MigR1-cd1d1 group, mean and SEM shown)

By staining with anti-CD1d antibody (clone 1B1), expression of CD1d in transduced donor cells was compared to that in non-transduced donor cells. As expected, transfer of MigR1-cd1d1-eGFP transduced cells led to constitutive expression of CD1d in all transduced donor cells, in comparison to non-transduced donor cells, where CD1d was expressed at physiological levels (including loss of CD1d expression in GC B cells). In contrast, transplantation of MigR1-eGFP transduced cells led to identical, non-constitutive, CD1d expression in transduced and non-transduced donor cells, both at physiological levels. **(Figure 4.5)**

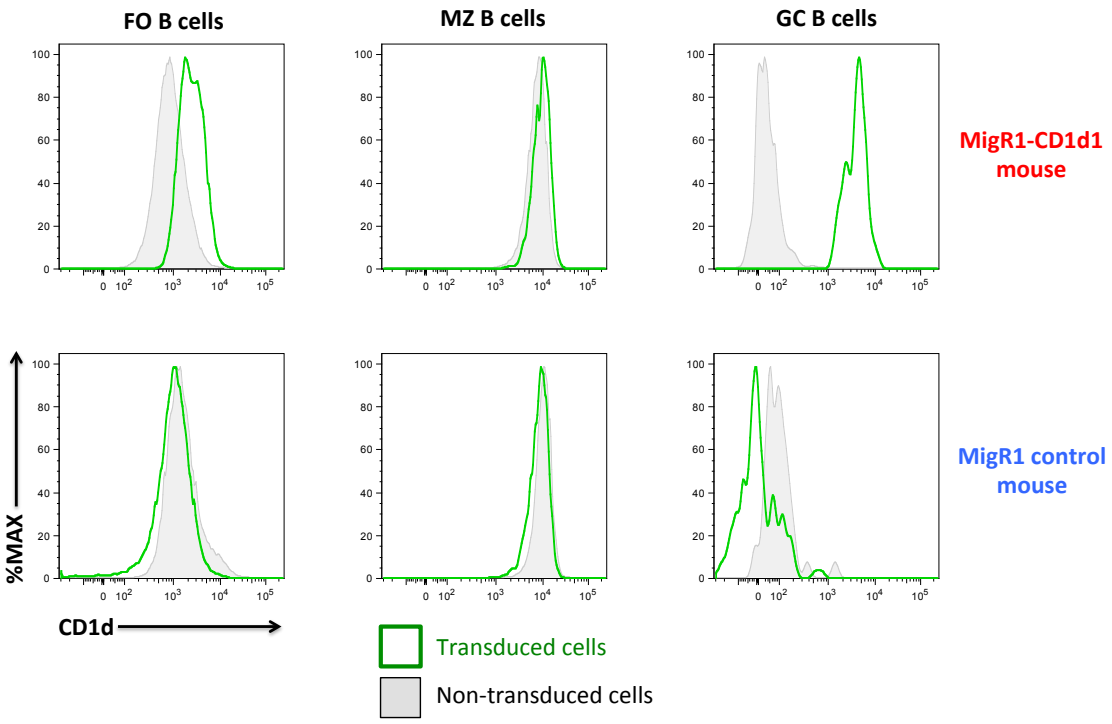


Figure 4.5 CD1d expression in transduced and non-transduced cells in vivo following adoptive transfer of MigR1-eGFP (MigR1 control mouse) or MigR1-cd1d1-eGFP transduced cells (MigR1-cd1d1 mice).

Transduced cells in the MigR1-cd1d1 mouse express constitutively high CD1d in comparison to non-transduced cells, which express CD1d at physiological levels (with highest levels observed in MZ B cells and lowest levels observed in GC B cells, as previously noted). In contrast, both transduced and non-transduced cells in the MigR1 control mouse express CD1d at physiological levels. (FO=follicular B cells, MZ=marginal zone B cells, GC=germinal centre B cells) (n=1 mouse for MigR1 control group and n=3 mice for MigR1-cd1d1 group)

In summary, these results provide proof of principle that adoptive transfer of MigR1-cd1d1-eGFP transduced murine HSCs can lead to stable engraftment of transduced donor cells able to generate splenic B cells, including GC B cells, which constitutively express CD1d.

Flow cytometric analysis of GC formation

In order to assess the impact of constitutive CD1d expression on GC formation, the frequency of donor class switched GC B cells was calculated as a percentage of total donor B cells.

In the initial analysis, the frequency of GC B cells was assessed in the transduced (GFP+) population only, and direct comparison made between the MigR1-cd1d1 mice and MigR1 control mouse **(Figure 4.6 (a))**. In opposition to the hypothesis, this revealed an approximately 2-fold increase in GC B cells in the MigR1-cd1d1 group.

This direct comparison makes the assumption that the two sets of mice can generate comparable immune responses, other than the difference caused by donor reconstitution of cells transduced by different vectors (i.e. constitutive CD1d expression).

In order to validate this, I used the non-transduced (GFP-) donor cells as an internal control. If the immune responses of the MigR1-cd1d1 and MigR1 control mice are otherwise comparable, then one would expect that a direct

comparison of the frequency of GC B cells in the non-transduced population would yield similar results between the two sets of mice. However, as shown in **Figure 4.6 (b)**, a clear difference was also noted in the GC B cell frequency between the MigR1-cd1d1 and MigR1 control mice in the non-transduced population, with approximately a 3 fold increase in GC B cells in the MigR1-cd1d1 group.

The reason for this discrepancy is not clear. All mice were age matched and treated according to standardized transplant and immunisation protocols. However, these results suggest that immune reconstitution and/or responses to immunisation differ between the two sets, independent of CD1d expression on B cells.

As an attempt to account for this variability and standardize results between the two sets of mice, percentages of transduced GC B cells were calculated relative to the corresponding percentage in the donor non-transduced GC B cell population, to give a normalised frequency. Using this approach, a reduction could be observed in the frequency of class switched GC B cells following constitutive expression of CD1d, but this did not reach statistical significance **(Figure 4.6 (c))**.

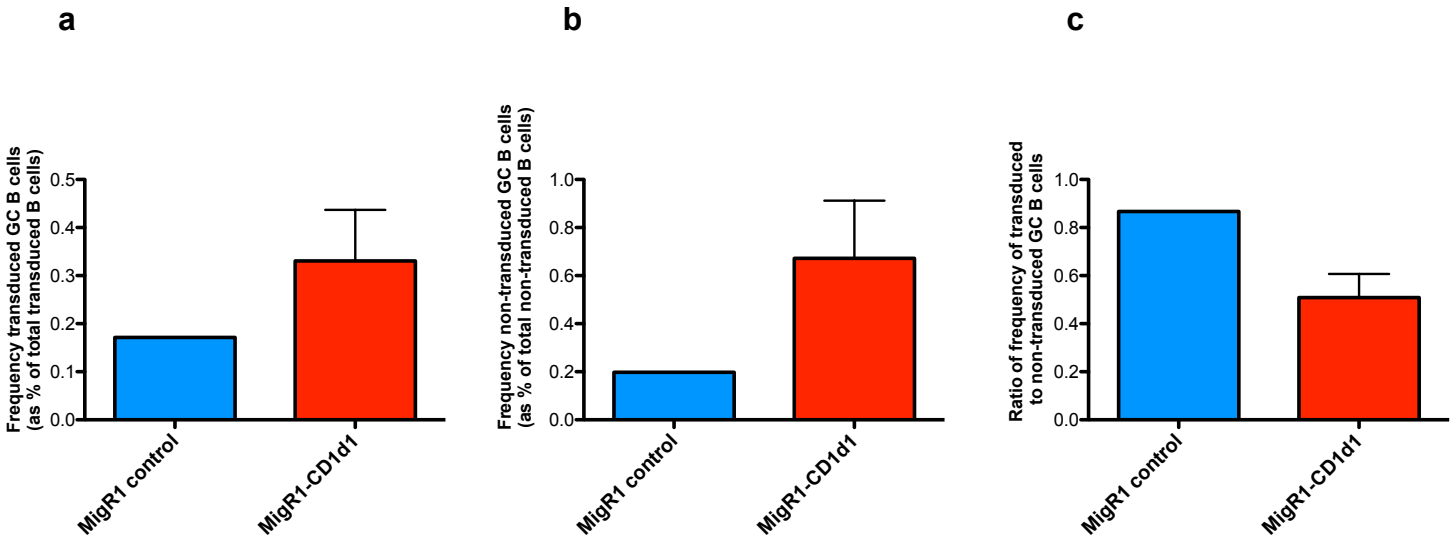


Figure 4.6. Frequency of donor class switched GC B cell populations in MigR1 control and MigR1-cd1d1 mice.

(a) *Frequency of transduced GC B cells in MigR1 control and MigR1-cd1d1 mice (expressed a percentage of total transduced B cells).*

(b) *Frequency of non-transduced GC B cells in MigR1 control and MigR1-cd1d1 mice (expressed a percentage of total non-transduced B cells).*

(c) *Ratio of frequency of transduced GC B cells to non-transduced GC B cells in MigR1 control and MigR1-cd1d1 mice. By calculating a ratio, the data can be normalised to take into account variations in the immune response between individual mice.*

(n=1 mouse for MigR1 control group and n=3 mice for MigR1-cd1d1 group, mean and SEM shown.)

In summary, this experiment demonstrated that it is possible to constitutively express CD1d in murine splenic B cells, including GC B cells, using the adoptive transfer of CD1d transduced HSCs. However, there are limitations associated with this transplant approach, specifically lack of engraftment in all mice, and a variable immune response between engrafted mice. Thus, although this system could generate preliminary results, the interpretation of these was not clear. I therefore decided to explore an alternative approach, in which CD1d is constitutively expressed in a transgenic mouse model, thereby avoiding the possible limitations of a transplant experiment.

4.2 Constitutive expression of CD1d in GC B cells using a transgenic mouse model

4.2.1 Experimental design

The generation and analysis of a CD1d transgenic (CD1d Tg) mouse was first described by Chun and colleagues (Chun et al., 2003). In this mouse model, established in a line of C57BL/6 mice, the murine *Cd1d1* gene is in theory under constitutive expression in all tissues, driven by the promoter of a classical MHC class I gene H-2K^b. In the initial analysis, it was shown that CD1d is indeed expressed at a high level (approximately 1-2 log fold higher than wild type controls) in a variety of cell types, including splenocytes **(Figure 4.7)**. Thus, the CD1d Tg mouse offers a model in which CD1d is potentially constitutively expressed in GC B cells, allowing an analysis of the significance of CD1d downregulation.

All experiments in this section were performed in collaboration with Professor Chyung-Ru Wang of Northwestern University, Chicago, USA, who has developed the CD1d Tg mouse. I planned, designed protocols and analysed results for all experiments. Experiments were performed by Professor Wang, in her lab in Chicago.

In this experiment, age matched (10 week old) C57BL/6 wild type and CD1d Tg mice were immunised by intraperitoneal injection of NP-KLH in alum adjuvant, as per the protocol used in Chapter 3, section 3.2.1. On day 10, spleens were harvested for analysis of GC formation by flow cytometry (as detailed in Chapter 3, section 3.2.2) and peripheral blood was analysed by ELISA for serum NP-specific antibody formation.

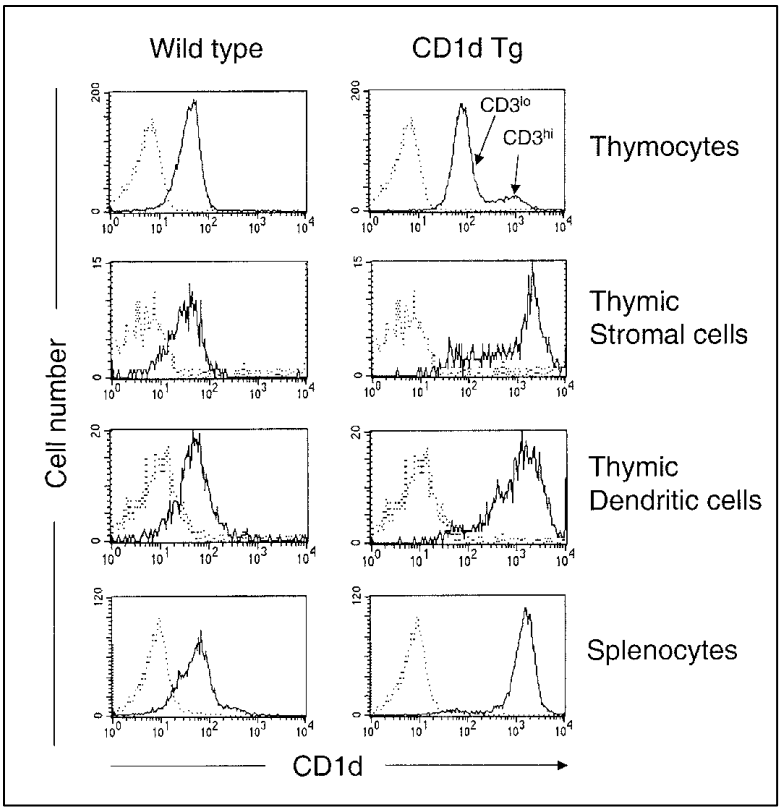


Figure 4.7. CD1d expression in CD1d Tg mice. *Flow cytometric analysis of cell surface expression of CD1d in WT control and Tg mice. Cells were stained with FITC-anti-CD1d mAb (5C6) (solid line) or appropriate isotype control (dotted line). (From Chun et al., 2003)*

4.2.2 Flow cytometric analysis of splenic B cells in CD1d Tg mice

As discussed above, previous work had shown the high expression of CD1d in splenocytes of the CD1d Tg mouse. Flow cytometry was used to assess if this expression was constitutive within GC B cells, or if CD1d was still subject to downregulation, and furthermore the potential impact of constitutive CD1d expression on GC B cell formation.

Gating strategy

Splenic B cell populations were identified and analysed for CD1d expression and GC formation using a similar gating strategy to that employed in Chapter 3, section 3.2.2 **(Figure 4.8)**.

Flow cytometric analysis of CD1d expression in splenic B cell populations

Figure 4.8 shows the expression of CD1d in follicular, marginal zone and GC B cells in CD1d Tg and wild type mice, both before (**Figure 4.8 (a)**) and 10 days following immunisation (**Figure 4.8(b)**). It was observed that irrespective of immunisation, all B cell populations in the CD1d Tg mouse, including GC B cells, express CD1d at similar constitutively high level. This is in contrast to wild type mice where, as previously observed, CD1d is expressed at physiological levels, with highest levels in the marginal zone B cells and loss of CD1d expression GC B cells.

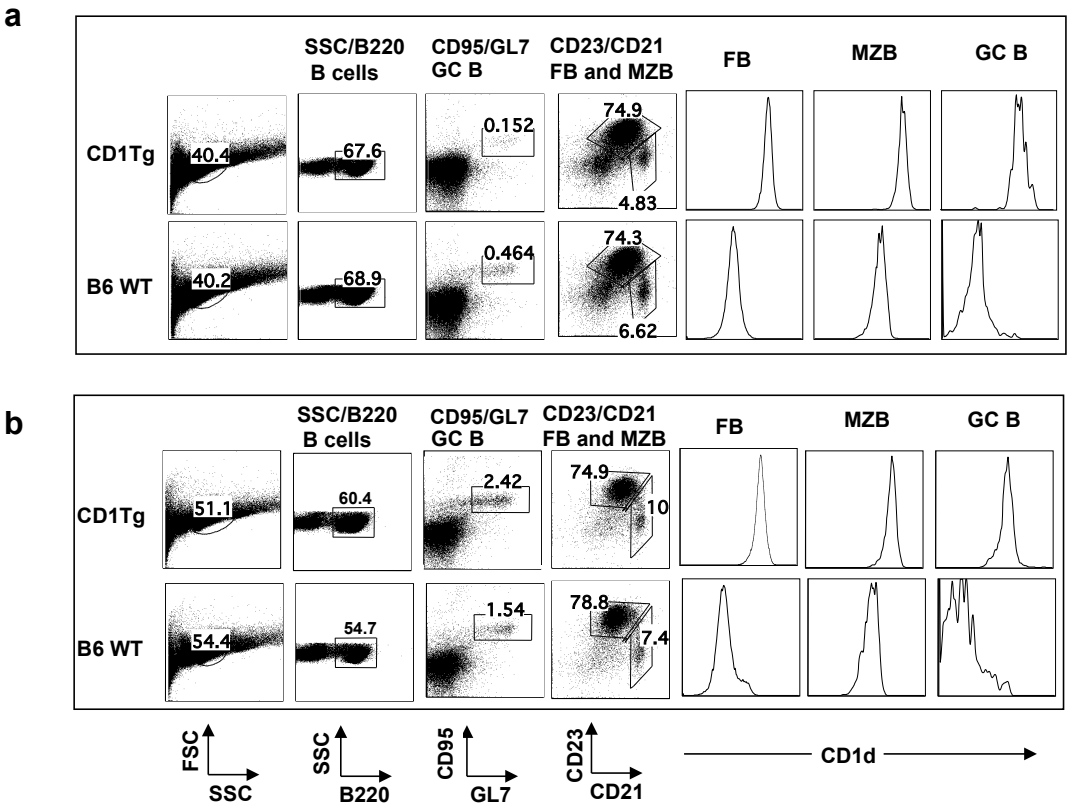


Figure 4.8. CD1d expression in splenic B cell populations in CD1d Tg mice. *Representative flow cytometric analysis of CD1d in CD1d Tg and C57BL/6 (B6) WT control mice, in both* ***(a)*** *non-immunised mice and* ***(b)*** *immunised mice. Using a similar gating strategy to that previously employed, the B cell marker B220 is used to label murine splenic B cells. Within this population, follicular B cells (CD23+, CD21int), marginal zone B cells (CD23-, CD21+) and total GC B cells (GL7+, CD95+) can be identified and stained for CD1d. Irrespective of immunisation, all B cell populations in the CD1d Tg mouse, including GC B cells, express CD1d at similar constitutively high levels. This is in contrast to wild type mice, where as previously observed, CD1d is expressed at physiological levels, and is subject to downregulation in GC B cells. (n=3 mice in each group)*

Flow cytometric analysis of GC formation

Following immunisation, the frequency of GC B cells (as a percentage of total B cells) was compared in CD1d Tg and wild type mice (**Figure 4.9 (a)**). No significant difference was observed in the frequency of GC B cells between the two sets of mice following immunisation (**Figure 4.9 (b)**).

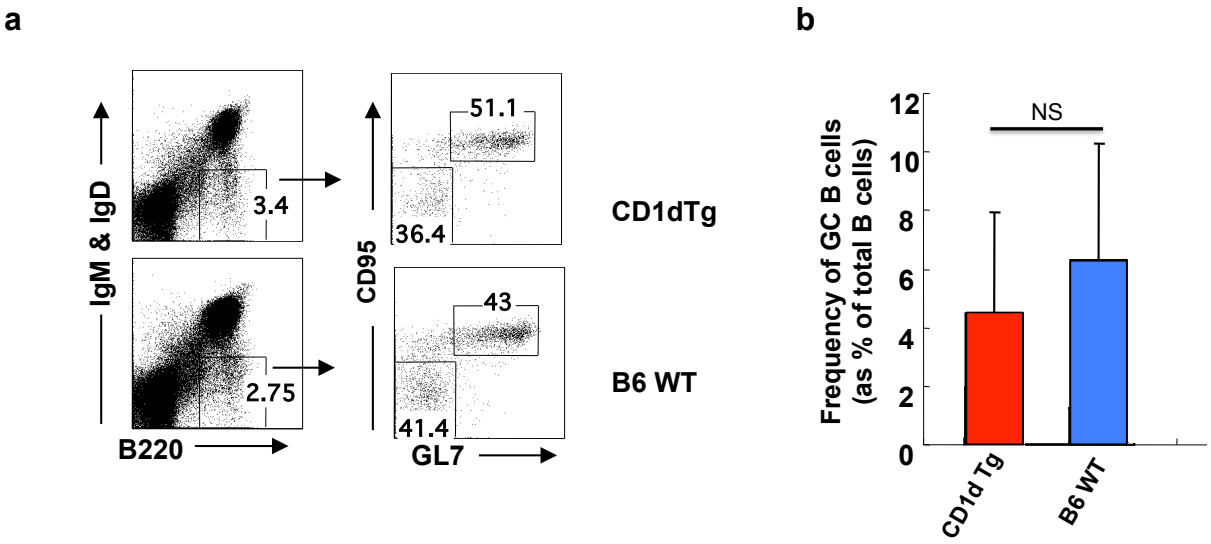


Figure 4.9. GC formation in CD1d Tg mice.

(a) *Representative flow cytometric analysis of class switched GC B cell formation in CD1d Tg and C57BL/6 WT control mice following immunisation.*

(b) *Summary of frequency of GC B cells (as a percentage of total B cells) following immunisation in CD1d Tg and C57BL/6 WT control mice.*

(n=3 mice in each group, mean and SEM shown)

4.2.3 Analysis of peripheral blood for anti-NP antibody formation

The use of a transgenic mouse model in which all GCs expressed CD1d, as opposed to a mixed chimera transplant model in which only a proportion of GCs constitutively expressed CD1d, allowed a meaningful analysis of total GC output in the peripheral blood, by measurement of anti-NP antibodies, using ELISA. Consistent with their similar frequency for GC formation, both CD1d Tg and wild mice exhibited similar levels of anti-NP serum antibodies **(Figure 4.10)**.

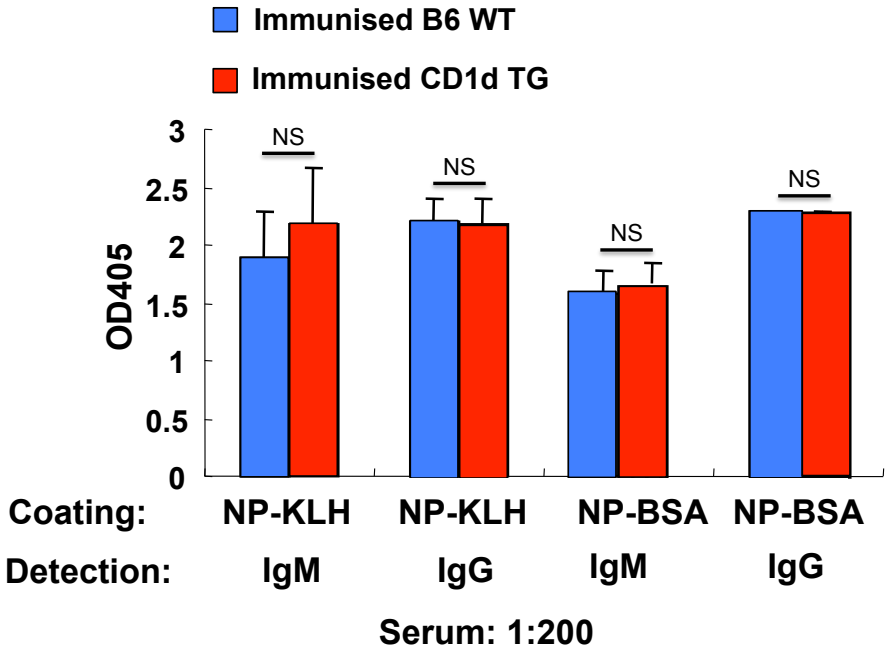


Figure 4.10. Anti-NP antibody formation in CD1d Tg mice. *Following immunisation with NP-KLH, peripheral blood from CD1d Tg and control C57BL/6 mice was analysed at day 10 using ELISA, for anti-NP IgM and IgG. (n=3 in each group)*

4.3 Discussion

4.3.1 Biological significance of CD1d downregulation in GC B cells

The two experimental approaches detailed in this chapter have attempted to assess the biological significance of CD1d downregulation in the GC, through forced constitutive expression of CD1d in these B cells.

In the initial approach, use of adoptive transfer of *Cd1d1* transduced HSCs was shown to be an effective method for establishing constitutive expression of CD1d in GC B cells. Following immunisation, it was shown that there was no significant difference in GC formation between mice receiving *Cd1d1* transduced HSCs or control vector transduced HSCs. As discussed above, this transplant experiment was limited by inconsistent engraftment, which reduced analysis to three mice receiving *Cd1d1* transduced cells and only one mouse receiving cells transduced by the control vector. Furthermore, variable immune responses were observed in transplanted mice, which further limited direct comparative analysis directly between transduced GFP+ populations.

In view of these limitations, an alternative secondary approach was undertaken, using a transgenic mouse model, the CD1d Tg mouse, in which CD1d was constitutively expressed in many different cell populations, including GC B cells. Use of this mouse model again demonstrated no

significant difference in GC formation or antibody production, in direct comparison to age matched wild type controls in which CD1d is downregulated in the GC.

Thus, from these experiments, the biological significance of CD1d downregulation in GC B cells remains unclear. CD1d downregulation does not appear essential for the formation of GCs or subsequent antibody production.

Beyond the limitations of these experiments discussed above, there are further caveats to be appreciated. In the original paper describing the CD1d Tg mouse, Chun and colleagues demonstrate an increase in thymic negative selection of NKT cells (Chun et al., 2003). Consequently these mice exhibit reduced numbers of NKT cells, and the residual NKT cells escape negative selection by exhibiting both an altered Vβ usage and a reduced sensitivity to antigen. The authors show that this increase in negative selection is mediated by high expression of CD1d on bone marrow derived thymic stromal cells, and in particular dendritic cells. Thus, although it was not assessed, this effect may also be present in the transplantation experiments described earlier in this chapter, where the adoptive transfer of HSCs transduced with the MigR1-cd1d1-eGFP vector also leads to high expression of CD1d in all BM cells. In both cases, a reduction in number and function of NKT cells would have important consequences with regards to the assessment of GC formation. If loss of CD1d in GC B cells is a mechanism to

avoid extrinsic NKT cell-mediated cytotoxicity towards these cells, then constitutive expression of CD1d in GC B cells may not be sufficient to demonstrate this if the effect of NKT cells is negated by increased negative selection.

In addition, the approach to immunisation used in these experiments warrants further consideration. In all experiments, immunisation was performed using the T cell-dependent immunogen NP-KLH. Although this is effective in generating GC B cell formation, it does not provide an exogenous source of lipid, which B cells can present directly to iNKT cells. It is possible that the effect of constitutional CD1d expression on GC B cells may only be unmasked if GC B cells are provided with an exogenous source of lipid to present (i.e. endogenous GC lipids may not be high affinity ligands for iNKT cells), and thus NP-KLH alone would be insufficient for this purpose.

4.3.2 Future work

To take this work further in the future, I would continue with the approach of using of a transgenic mouse model to constitutively express CD1d in GC B cells. However, I would refine this model, such that constitutive CD1d expression is limited to GC B cells only. This could be achieved in theory by expressing the *Cd1d1* transgene under a GC specific promoter, such as for

example Bcl-6 (the master regulator of the GC) (Basso and Dalla-Favera, 2010) or activation-induced cytidine deaminase (AICDA, which regulates the antibody diversification processes of SHM and CSR in the GC) (Xu et al., 2007). By constitutively expressing CD1d only in GC B cells, this would limit the potential effects of persistent high CD1d expression in other cells (either haematopoietic or non-haematopoietic), such as the potential increased negative selection of NKT cells documented by Chun et al.

The immunisation approach of a novel mouse model can also be refined. The use of NP-KLH can be augmented by the addition of α-GalCer, with the aim of providing an exogenous source of lipid for presentation by GC B cells. This may be needed to precipitate iNKT-mediated cytotoxicity if GC B cell endogenous lipids lack sufficient affinity towards iNKT cells. Alternatively, the novel immunisation methodologies discussed in Chapter 1, which aim to target glycolipid to the BCR and generate lipid-specific B cell responses, either by using NP hapten directly conjugated to α-GalCer (Leadbetter et al., 2008) or protein antigen (hen egg lysozyme, HEL) and α-GalCer both linked to bead particles (Barral et al., 2008), may prove even more effective in this context.

The approaches documented in this chapter have focussed on using the GC reaction as a model to assess the *in vivo* biological significance of CD1d downregulation. As discussed in previous chapters, CD1d is also downregulated in pathological B cells, including EBV infection and multiple

myeloma. The *in vivo* biological significance of these phenomena is, however, more difficult to ascertain. EBV is a human B cell disease and thus would require a humanised animal model to investigate further. *In vivo* multiple myeloma studies are traditionally performed using xenograft models. These models make use of an immunosuppressed host environment to enable multiple myeloma engraftment, but this immunosuppressive environment clearly precludes their use in investigating the CD1d-iNKT axis.

A murine multiple myeloma cell line, the 5TGM1 cell line, is able to recapitulate features of multiple myeloma when injected in non-immunosuppessed C57BL/KaLwRij mice. By altering CD1d expression in this cell line, this therefore offers an opportunity to assess the *in vivo* biological significance of the CD1d-iNKT axis in the pathobiology of multiple myeloma. Preliminary work in characterising and altering CD1d expression in this cell line has commenced, and is detailed further in Appendix D.

Chapter 5

5 Transcriptional mechanisms regulating changes in CD1d expression in B cells

The mechanisms regulating the changes in CD1d expression in both normal and pathological B cells remain only explained in part. In this chapter, I will attempt to expand our understanding of this area, in particular by focussing on MM B cells. Specifically, I will look at two distinct mechanisms that may exert transcriptional control on CD1d, namely retinoic acid (RA) signalling and bivalent histone modification-mediated transcriptional repression. As previously detailed in Chapter 1, section 1.5, there is already some evidence for the role of these pathways in B cells and/or CD1d regulation, but this is limited and does not extend to B cell malignancies such as MM.

It is recognised that neoplastic transformation of cells may involve aberrations of normal cellular processes. Specific to this field, it is increasingly appreciated that GC pathways are 'hijacked' during lymphomagenesis (Basso and Dalla-Favera, 2015). MM is a malignancy of plasma cells, the terminally differentiated B cell. Normal plasma cells are relatively quiescent in comparison to proliferative GC B cells, and there is little evidence connecting GC pathways to MM. However, as discussed in Chapter 1, the loss of CD1d in both the GC and advanced MM is marked, both at the surface protein and transcriptional level, and this may provide evidence as to sharing of certain biological pathways. I therefore

hypothesise that as MM develops and progresses, utilisation of physiological GC mechanisms by the MM cells may result in a greater proliferative capacity **(Figure 5.1)**. In order to provide evidence for this, I will attempt to demonstrate parallels between the use of RA and histone modification pathways in GC and MM B cells.

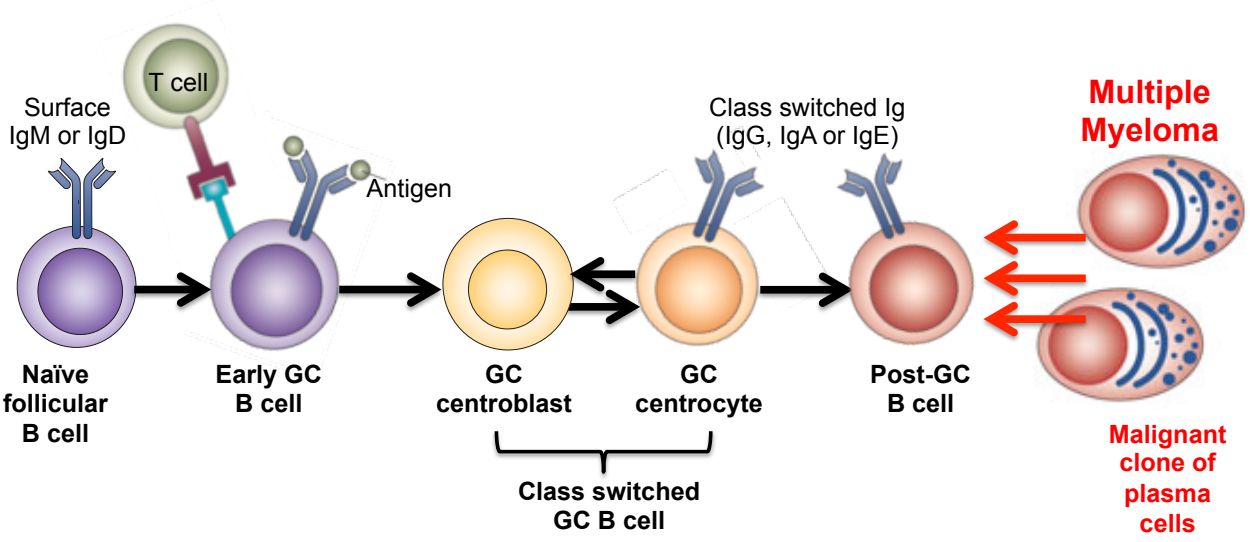


Figure 5.1. Proposed utilisation of GC B cell pathways by MM B cells. *As MM progresses, acquisition of a more proliferative phenotype may be achieved by MM B cells 'hijacking' physiological pathways, normally utilised by GC B cells.*

5.1 Regulation of CD1d expression by retinoic acid signalling

As discussed in Chapter 1, specifically section 1.5.3, there is accumulating evidence for the role of RA signalling in the regulation of CD1d. Nagy and colleagues showed that activation of the peroxisome proliferator-activated receptor gamma (PPARγ) indirectly increased CD1d expression in human dendritic cells by increasing RA synthesis and subsequent activation of the RARα (Szatmari et al., 2006). Specific to B cells, *in vitro* B cell activation, in response to various activating ligands including anti-Ig antibodies, Staphylococcus aureus Cowan I bacteria and CD40 ligand, was shown to downregulate CD1d in association with a decrease in RARα signalling (Allan et al., 2011). Similarly, EBV infection of B cells was also found to lead to loss of expression of CD1d (Chung et al., 2013). In both cases, CD1d expression could subsequently be restored through use of RARα agonists.

It is not known if this role of RA signalling in CD1d regulation can be extended more broadly to other B cell states. Herein, I have investigated the relationship between RA signalling and CD1d expression in MM B cells, aiming to upregulate Cd1d through use of RA. Furthermore, I have attempted to draw parallels to RA signalling in GC B cells *in vivo*, where CD1d expression is also downregulated.

5.1.1 Regulation of CD1d in MM B cells by RA signalling

In silico analysis of RA signalling in MM using a public database

As discussed in Chapter 1, section 1.4.3, the disease course of MM spans a wide trajectory. MM begins as the pre-malignant plasma cell dyscrasia monoclonal gammopathy of uncertain significance (MGUS), which may progress to classical MM. Further disease progression then leads to advanced MM, including the aggressive form of the disease called plasma cell leukaemia.

In order to assess RA signalling across the disease spectrum of MM, I took advantage of a public gene expression dataset (GSE2113), available via the NCBI Gene Expression Omnibus data repository (original data from Mattioli et al., 2005). By interrogation of this dataset, gene expression profiling data can be analysed for plasma cells at each of the aforementioned disease stages in MM.

Specifically, I used this dataset to investigate gene expression of RA target genes as a measure of RA signalling. Consistent with its large range of pleiotropic effects in development and adult tissue homeostasis, RA has been established to influence transcription of hundreds of target genes (Balmer and Blomhoff, 2002; Delacroix et al., 2010). Many of these genes are regulated indirectly by RA, and thus their gene expression may be influenced by other pathways. Thus, I investigated two genes that are

known to be directly regulated by RA based on previous studies (Balmer and Blomhoff, 2002), as these are most likely to to be indicative of changes in RA signalling. These genes were *EGR1* (Edwards et al., 1991; Larsen et al., 1994) and *TGM2* (Allan et al., 2011).

The expression of these two RA target genes can be shown to decrease as the plasma cell disorder progresses from MGUS, through MM, to plasma cell leukaemia (**Figure 5.2).** In both cases, there is a significant decrease in target gene expression in the advanced plasma cell leukaemia state in comparison to the early MGUS disease state. Although limited to only a few genes, this *in silico* data analysis suggests that disease progression in MM is indeed associated with a general decrease in RA signalling.

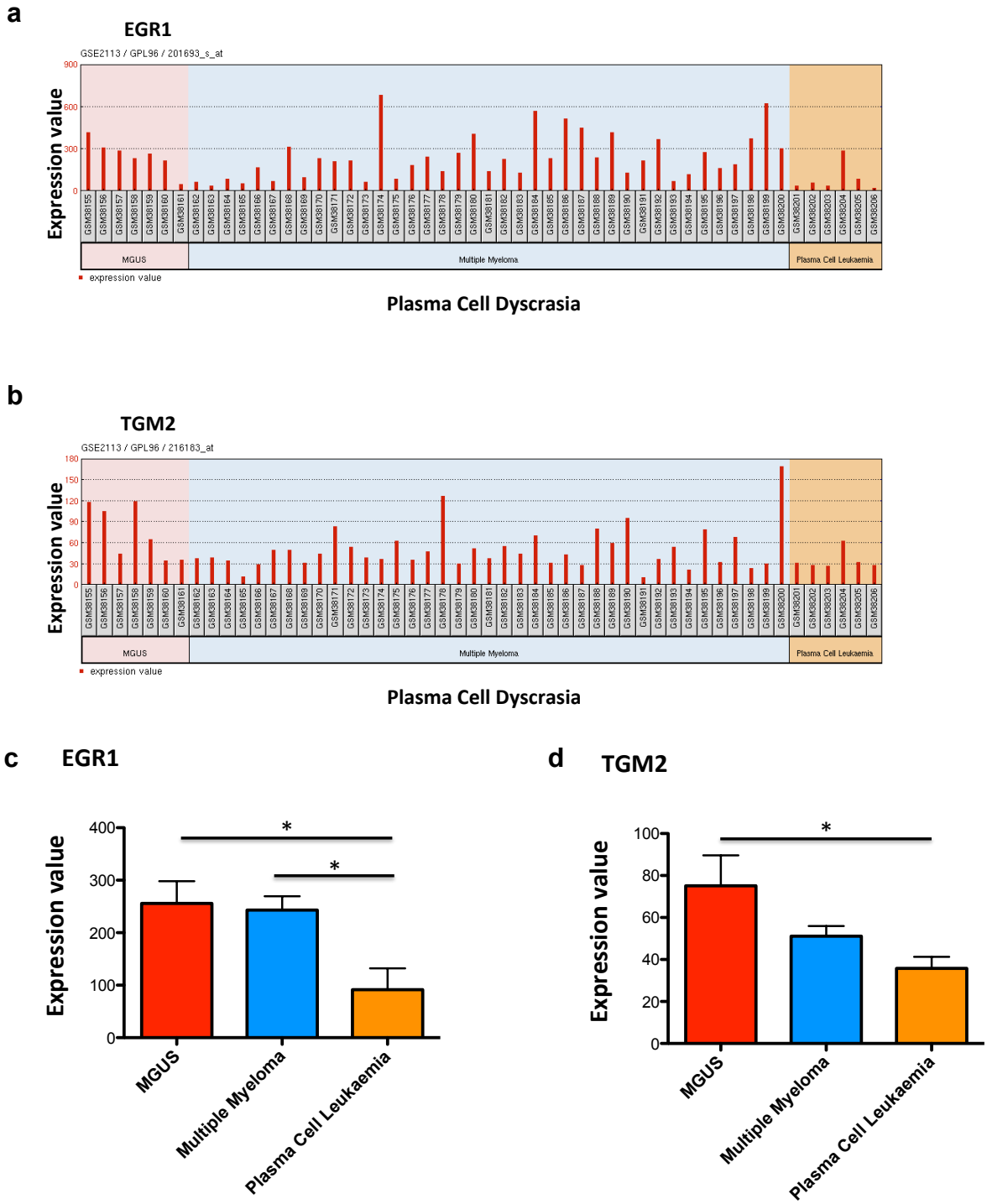


Figure 5.2. In silico analysis of RA target gene expression using a public database.

(a and b) *Affymetrix microarray data analysed by the NCBI GEO2R web tool, showing expression of RA target genes (a) EGR1 and (b) TGM2 in plasma cells at different stages of disease progression in MM.*

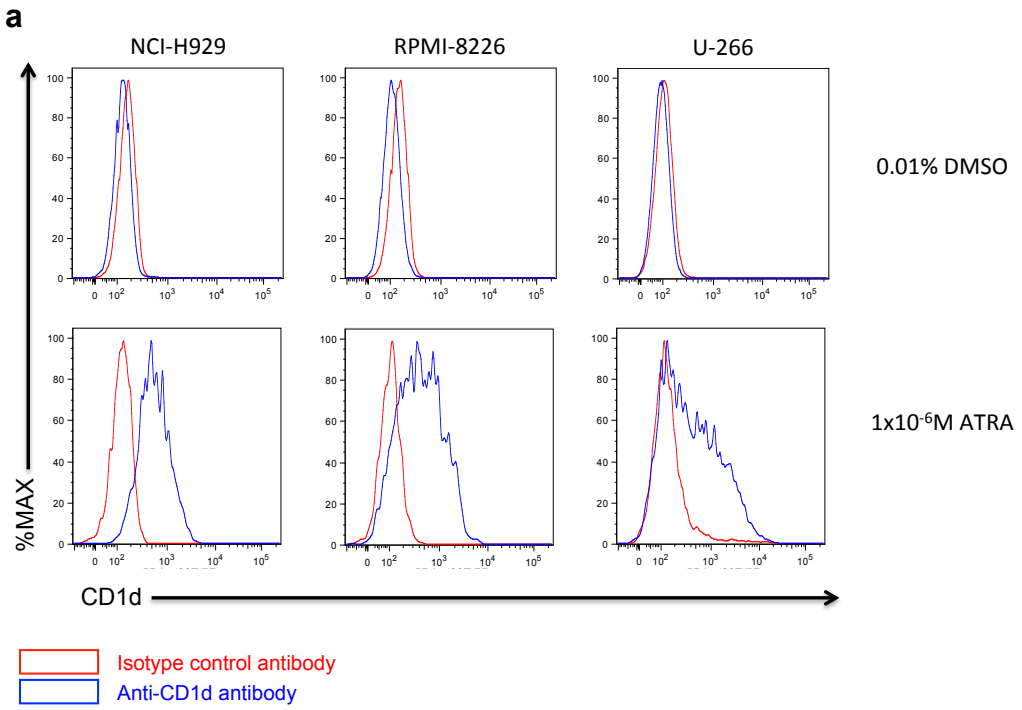
(c and d) *Bar chart summary of expression data for genes (c) EGR1 and (d) TGM2 (mean and SEM shown).*

Restoration of CD1d expression in MM cell lines using RA

The concept that RA signalling is downregulated in MM with disease progression provides the rationale that RA may, as in B cells activated *in vitro* and EBV infected B-LCLs, be able to restore CD1d expression at the transcriptional and subsequently protein level.

This hypothesis was tested *in vitro* using MM cells lines, which as discussed in Chapter 3, section 3.4, represent an advanced form of MM and do not express CD1d mRNA and surface protein. A full description of methods used to treat cell lines can be found in Chapter 2, section 2.1.2. Three different MM cell lines, NCI-H929, RPMI-8226 and U266, were initially treated with all-trans retinoic acid (ATRA). The endogenous "physiological" concentration of RA is approximately $1x10^{-9}$M (Collins, 2002). In order to attempt to invoke a response in these MM cell lines, a higher "pharmacological" dose of $1x10^{-6}$M of ATRA was chosen as the initial dose. This value is consistent with the concentration of ATRA used in therapy for other human conditions such as APML, and is supported by other reports in the literature (Bidad et al., 2011; Herault et al., 2004).

Surface CD1d expression was assessed at 48 hours by flow cytometry. At this time point, treatment with $1x10^{-6}$M ATRA led to restoration of surface CD1d expression in all three cell lines **(Figure 5.3)**.


a
NCI-H929
RPMI-8226
U-266
0.01% DMSO
1x10-6M ATRA
%MAX
CD1d
Isotype control antibody
Anti-CD1d antibody

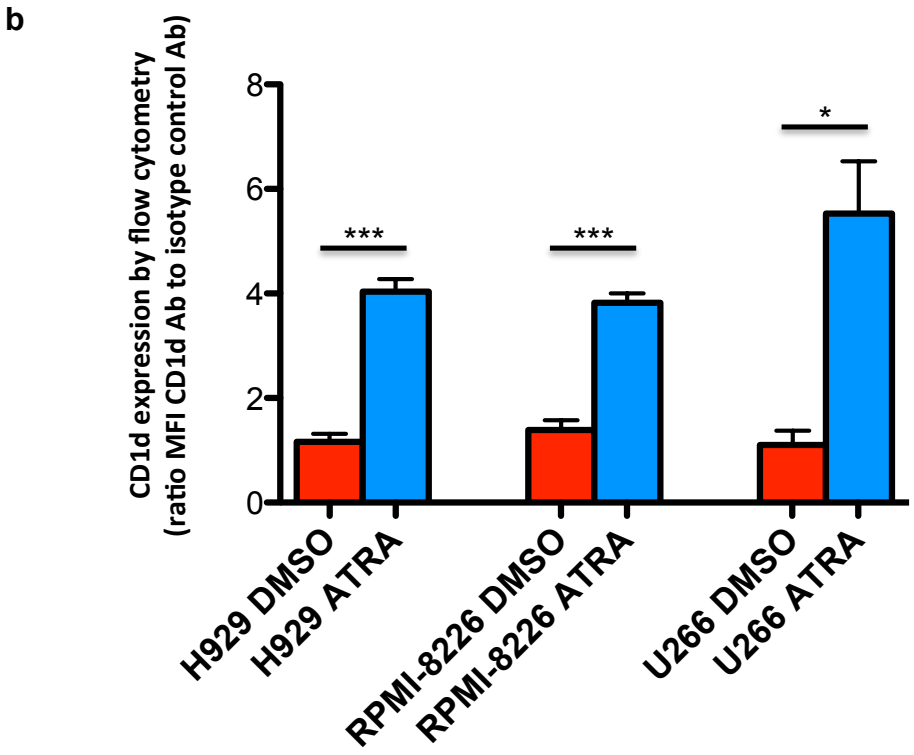

b
CD1d expression by flow cytometry
(ratio MFI CD1d Ab to isotype control Ab)
0
2
4
6
8
***
***
*
H929 DMSO
H929 ATRA
RPMI-8226 DMSO
RPMI-8226 ATRA
U266 DMSO
U266 ATRA

Figure 5.3. Flow cytometric analysis of surface CD1d expression in MM cell lines at 48 hours post treatment with ATRA.

(a) *Representative flow cytometric plots showing restoration of CD1d expression at 48 hours following treatment with 1X10^{-6}M ATRA, in comparison to 0.01% DMSO control. CD1d surface expression was assessed by staining with anti-CD1d antibody (clone 42.1) and comparing to staining by the appropriate isotype control antibody.*

(b) *Summary of surface CD1d expression in MM cell lines following treatment with 1X10^{-6}M ATRA or 0.01% DMSO, as indicated by the MFI ratio of CD1d antibody to isotype control antibody. (n=3 for each cell line, mean and SEM shown)*

By further studying the U266 cell line, it could be determined that restoration of surface expression of CD1d by ATRA is both time and dose dependent **(Figure 5.4)**.

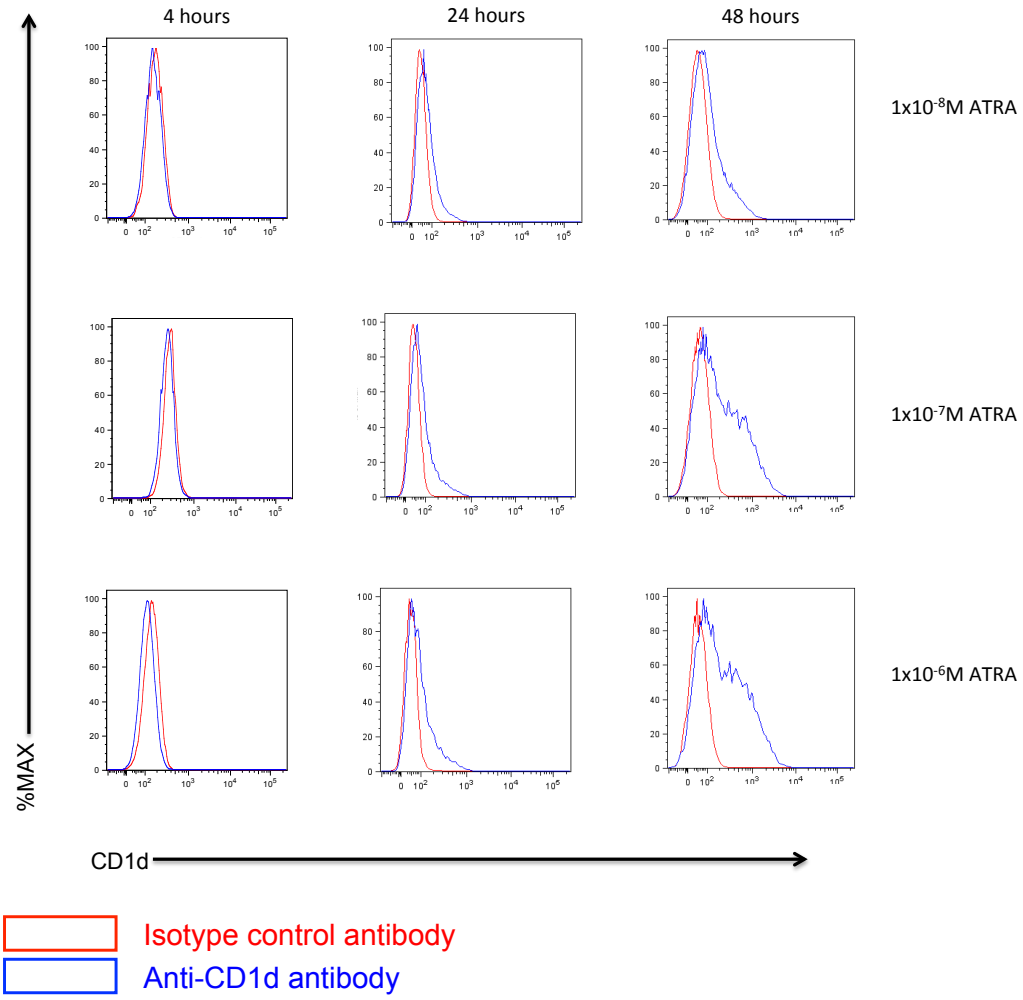


Figure 5.4. Flow cytometric analysis of surface CD1d expression in MM cell line U266 at different time points and different concentrations of ATRA*. Representative flow cytometric plots shown. Response to ATRA was found to be both time and dose dependent. (n=1)*

In addition to flow cytometry, the effect of ATRA on *CD1D* expression at the transcriptional level was assessed using real time PCR. Treatment of myeloma cell lines NCI-H929, RPMI-8226 and U266 with $1x10^{-6}$M ATRA led to a significant increase in *CD1D* mRNA at 48 hours, in comparison to treatment with 0.01% DMSO. Of note, although a greater increase in *CD1D* mRNA was observed at 48 hours, a significant increase in *CD1D* transcription was observed as early as 4 hours post treatment, indicating that ATRA is exerting a direct transcriptional effect on *CD1D* **(Figure 5.5)**.

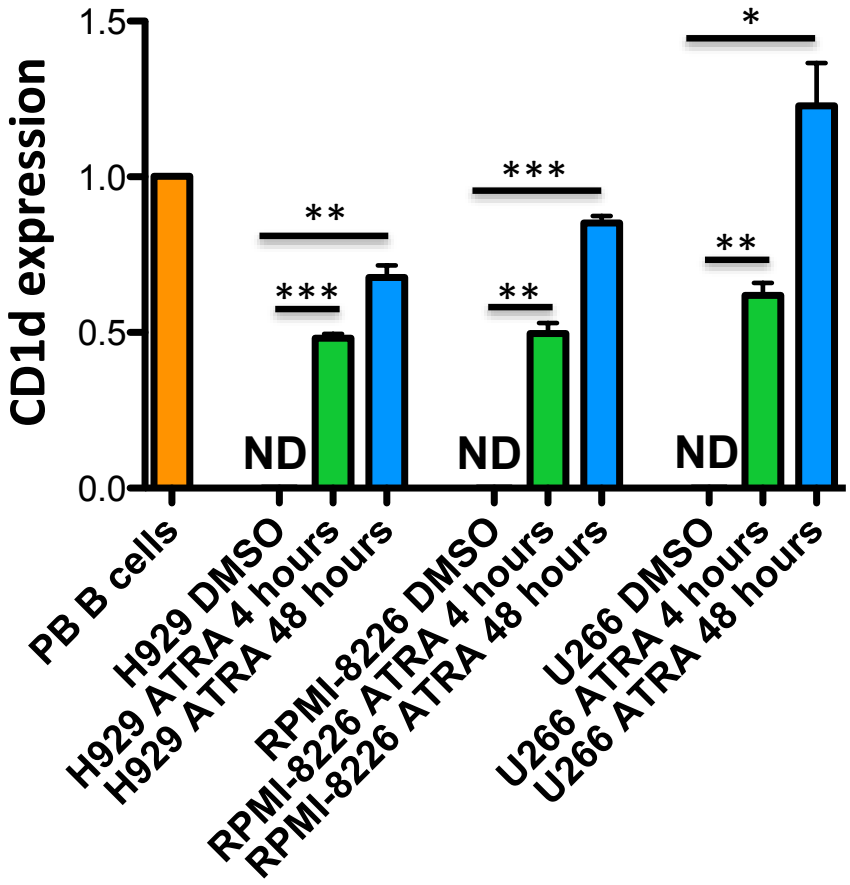


Figure 5.5 Real time PCR analysis of CD1D mRNA expression in human MM cell lines treated with ATRA.

In all 3 MM cell lines analysed, CD1D mRNA expression was significantly increased following treatment with 1x10^{-6}M ATRA, both at 4 hours and 48 hours, in comparison to treatment with 0.01% DMSO alone. Expression values are calculated relative to the reference gene beta actin, and are shown relative to the CD1D expression value for untreated peripheral blood B cells. (ND means CD1D mRNA Not Detected.) (n=3 for each cell line, mean and SEM shown)

5.1.2 Parallels to RA signalling in GC B cells

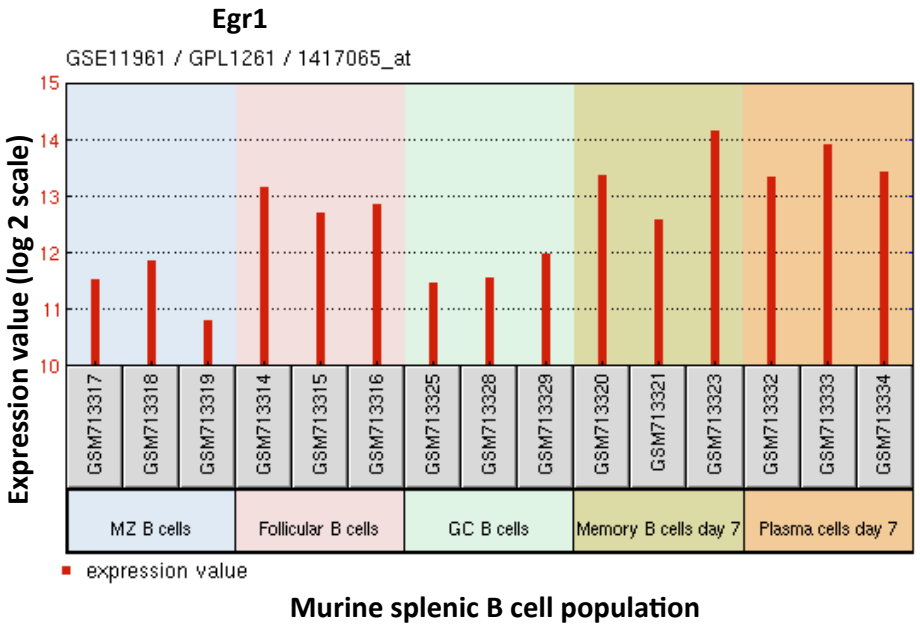
The decrease in RA signalling associated with CD1d downregulation (and subsequent restoration of CD1d with ATRA) in advanced myeloma, highlights the importance of this pathway for CD1d expression. The clear loss of CD1d expression in GC B cells raises the question of whether advanced MM B cells, in downregulating RA signalling, are mimicking a physiological pathway observed in GC cells. The downregulation of RA signalling and CD1d in B cells activated *in vitro* and EBV infected B-LCLs, both of which share similarities to *in vivo* GC B cells (as discussed in Chapter 1) is suggestive of this phenomenon. However, as of yet, there is no evidence to demonstrate a decrease in RA signalling in GC B cells. I aimed to address this through several approaches including *in silico* analysis of a current gene expression database, real time PCR and an *in vivo* model.

In silico analysis of RA signalling in GC B cells using a public database

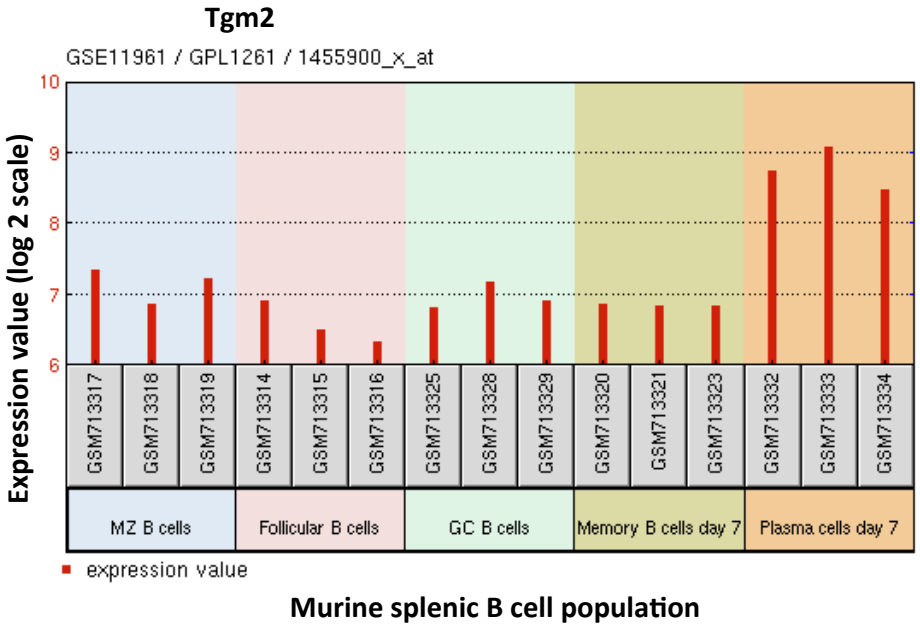
Using the public gene dataset (GSE11961) initially described in Chapter 3, section 3.2.4, which provides unanalysed microarray gene expression profiling data of murine splenic B cell populations (Kaji et al., 2012), the differential expression of RA target genes can be assessed during the GC reaction. As described above in section 5.1.1 for the analysis of malignant plasma cells, *Egr1* and *Tgm2* were chosen as known RA target genes for this analysis **(Figure 5.6)**.

In the case of *Egr1*, the dataset shows significantly higher expression of this gene in follicular B cells and post GC B cells (memory B cells and plasma cells) in comparison to GC B cells. The expression of *Tgm2* in naïve and GC B cells is not as clearly demarcated as *Egr1* in this dataset, but it is notable that there is a significantly higher level of expression of this gene in plasma cells compared to GC B cells. Similar to the analysis of advanced myeloma, this *in silico* data analysis is suggestive that GC B cells are indeed associated with a lower level of RA signalling, especially in comparison to normal plasma cells.

a


Egr1
GSE11961 / GPL1261 / 1417065_at
Expression value (log 2 scale)
15
14
13
12
11
10
GSM713317
GSM713318
GSM713319
GSM713314
GSM713315
GSM713316
GSM713325
GSM713328
GSM713329
GSM713320
GSM713321
GSM713323
GSM713332
GSM713333
GSM713334
MZ B cells
Follicular B cells
GC B cells
Memory B cells day 7
Plasma cells day 7
expression value
Murine splenic B cell population

b


Tgm2
GSE11961 / GPL1261 / 1455900_x_at
Expression value (log 2 scale)
10
9
8
7
6
GSM713317
GSM713318
GSM713319
GSM713314
GSM713315
GSM713316
GSM713325
GSM713328
GSM713329
GSM713320
GSM713321
GSM713323
GSM713332
GSM713333
GSM713334
MZ B cells
Follicular B cells
GC B cells
Memory B cells day 7
Plasma cells day 7
expression value
Murine splenic B cell population

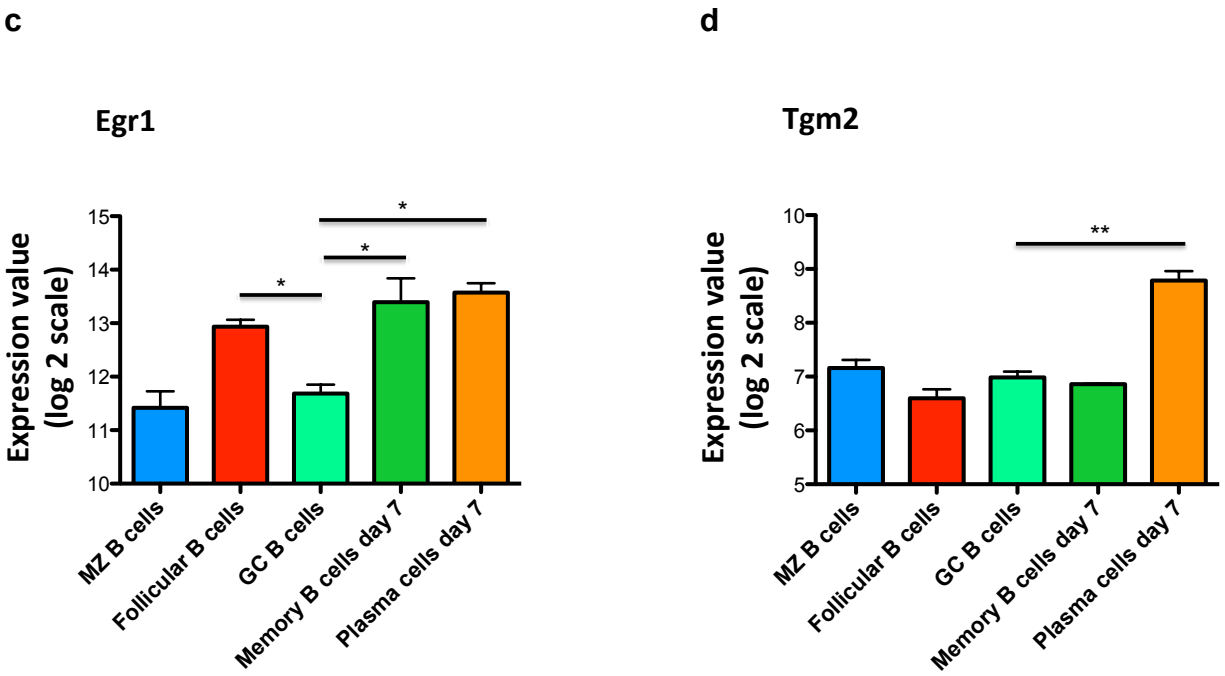


Figure 5.6. In silico analysis of RA target gene expression using a public database.

(a and b) *Affymetrix microarray data analysed by the NCBI GEO2R web tool, showing expression of RA target genes (a) Egr1 and (b) Tgm2 in different murine splenic B cell populations. (Note expression values are shown on a log2 scale.)*

(c and d) *Bar chart summary of expression data for genes (c) Egr1 and (d) Tgm2 (mean and SEM shown). (Note expression values are shown on a log2 scale.)*

Real time PCR analysis of murine splenic B cells

As described in Chapter 3, section 3.2, different murine splenic B cell populations can be identified by flow cytometry from the harvested spleens of immunised mice, and furthermore can be isolated using fluorescent activated cell sorting. In order to complement the *in silico* analysis above, real time PCR was performed to investigate *Tgm2* expression at the transcriptional level. *Tgm2* mRNA was found to decrease as a naïve B cell entered the GC reaction, subsequently rising again as the B cell left the GC **(Figure 5.7)**. This data differs from the above *in silico* analysis by showing a higher level of *Tgm2* expression in naïve B cells compared to GC B cells, but again highlights that *Tgm2* expression is significantly higher in post GC cells than in GC B cells. This offers further support to the concept of diminution of RA signalling in GC B cells.

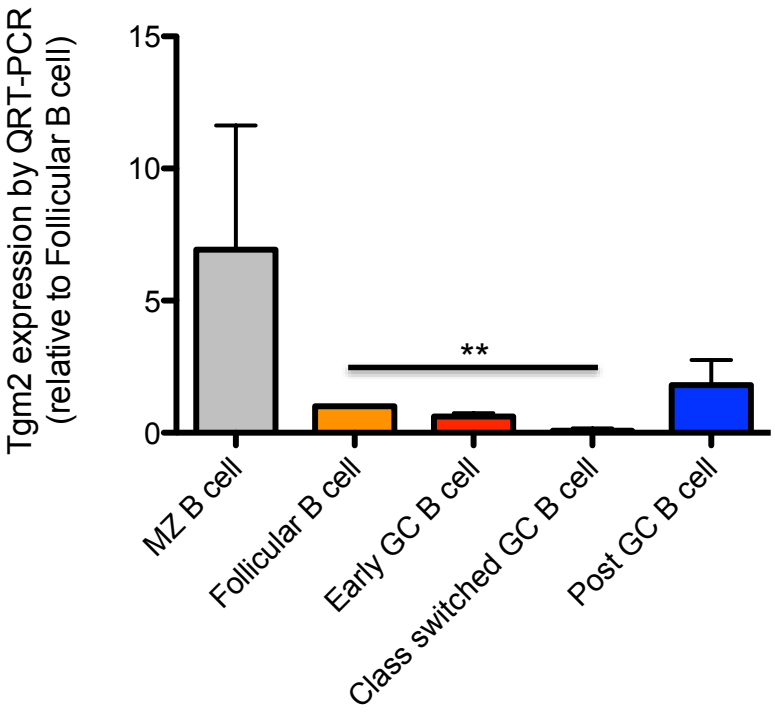


Figure 5.7. Gene expression of RA target gene Tgm2 in the murine germinal centre reaction.

Summary of Tgm2 mRNA expression in different splenic B cell populations of immunised mice. Expression values are calculated relative to the reference gene beta actin, and are shown relative to the Tgm2 expression value for follicular B cells. (n=3 mice, mean and SEM shown)

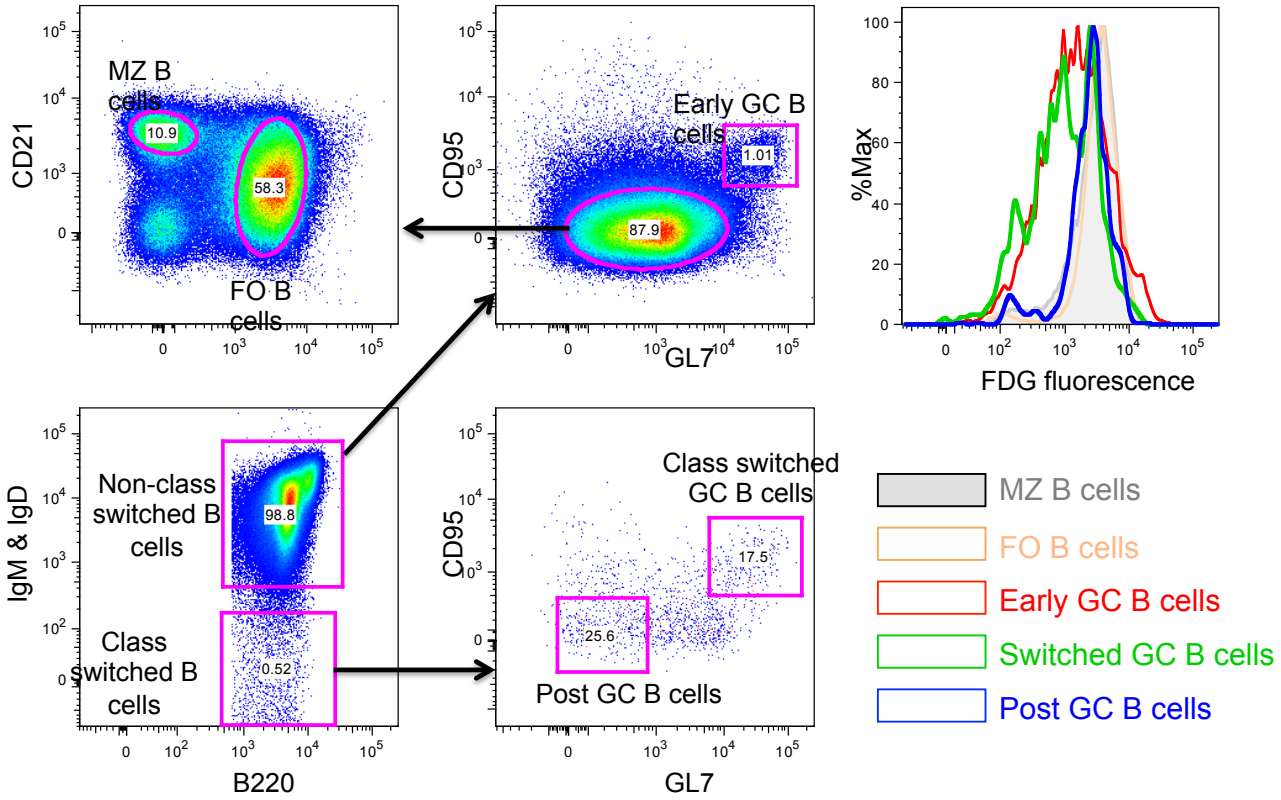
Use of an in vivo model to assess RA signalling in GC B cells

The Tg(RARE-Hspa1b/lacZ)12Jrt/J transgenic mouse strain (common name RARE-hsp68LacZ) expresses the beta-galactosidase (*lacZ*) gene under the control of a RARE (Rossant et al., 1991). RA signalling is indicated by beta-galactosidase activity, to allow tracking of the RA signalling pattern.

I utilised this mouse model to investigate RA signalling in murine splenic B cell populations. Murine spleens (from non-immunised mice) were harvested and analysed by flow cytometry as previously described in Chapter 3, section 3.2.2, in order to identify individual splenic B cell populations. In addition, a fluorometric assay was employed using a fluorescein-based galactosidase substrate, C_{12}FDG. This is cleaved only by cells expressing beta galactosidase (i.e. cells in which RA signaling is present), to produce a fluorescent substrate. The degree of fluorescence is thus a surrogate marker of RA signalling. A further description of this fluorometric assay can be found in Chapter 2, section 2.4.6.

By combining this assay with flow cytometric identification of individual splenic B cell populations, RA signalling could be assessed in these different populations, including GC B cells. It was observed that RA signalling, as measured by substrate fluorescence, falls to a nadir in GC B cells, before rising again in post GC B cells. This is again consistent with a loss of RA signalling in GC B cells **(Figure 5.8)**.

a


MZ B cells
10.9
58.3
FO B cells
CD21
Early GC B cells
1.01
87.9
CD95
GL7
%Max
FDG fluorescence
Non-class switched B cells
98.8
Class switched B cells
0.52
IgM & IgD
B220
Class switched GC B cells
17.5
25.6
Post GC B cells
CD95
GL7
MZ B cells
FO B cells
Early GC B cells
Switched GC B cells
Post GC B cells

b

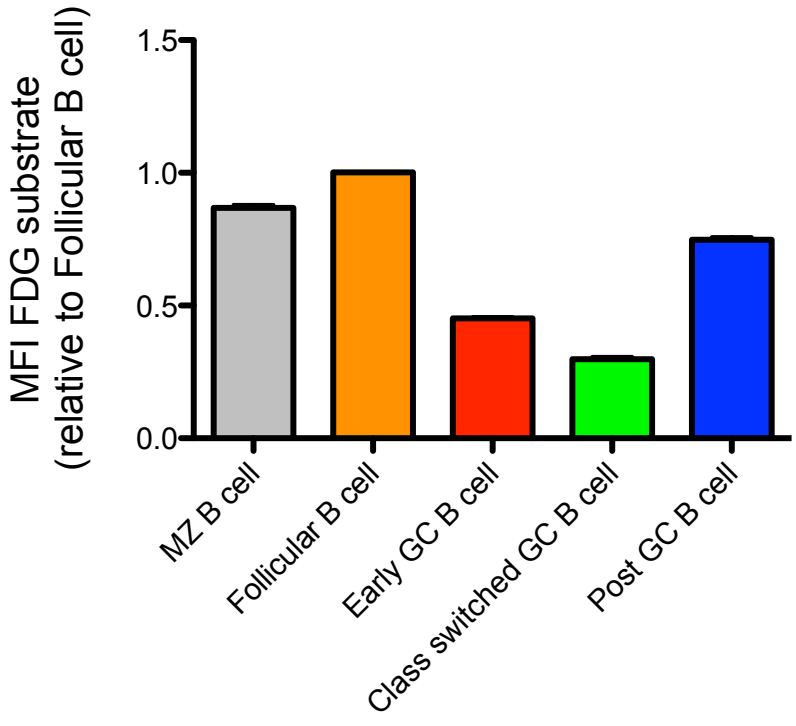

MFI FDG substrate
(relative to Follicular B cell)
1.5
1.0
0.5
0.0
MZ B cell
Follicular B cell
Early GC B cell
Class switched GC B cell
Post GC B cell

Figure 5.8. RA signalling in murine splenic B cell populations

(a) Representative flow cytometric plot from a non-immunised RARE-hsp68LacZ *mouse displaying different splenic B cell populations and the corresponding fluorescence of the FDG substrate (a surrogate marker for beta-galactosidase activity and hence RA signalling).*

(b) Summary of FDG substrate fluorescence in different splenic B cell populations of non-immunised RARE-hsp68LacZ *mice. Fluorescence for each B cell population is shown as the MFI value, relative to the MFI value for follicular B cells (n=2 mice, mean and SEM shown).*

Taken together, this data indicates that the loss of CD1d in GC B cells is correlated with a decrease in RA signalling. This is suggestive that advanced MM plasma cells, in also losing RA signalling and CD1d expression, may therefore be 'hijacking' a physiological mechanistic pathway for disease propagation.

5.2 Regulation of CD1d expression by bivalent chromatin

The role of chromatin in regulating CD1d remains a poorly defined area, but it is plausible that this may represent a mechanism of controlling gene expression.

As detailed in Chapter 1, section 1.5.4, many developmental regulatory gene loci are marked with a distinctive signature of both H3K4 and H3K27 trimethylation, termed bivalent marks (Bernstein et al., 2006). This bivalent chromatin is suggested to convey temporal and spatial precision to the expression of lineage control genes during pluripotency and differentiation.

Furthermore, it has become apparent that this model may be extended, and establishment of bivalent histone modifications may occur in other undifferentiated states, including GC B cells and B cell malignancies (Béguelin et al., 2013; Caganova et al., 2013). Thus, bivalent chromatin may be a potential mechanism by which CD1d is regulated in these B cell states.

As discussed in Chapter 3, sections 3.1.2, 3.2.2 and 3.2.3, the expression of CD1d in the GC is dynamic, with CD1d expression rapidly falling to undetectable levels in GC B cells, before rising again rapidly as post GC B cells emerge. It would therefore make sense for the regulation of CD1d to employ such bivalent marks. The use of H3K27me3 would ensure CD1d expression is efficiently shut down in GC B cells, whereas H3K4me3 marking

would ensure CD1d is poised for rapid reactivation following the GC reaction. Similarly, as discussed above, the use of RA to restore CD1d expression in MM cells has highlighted that CD1d is not permanently silenced in these cells. Again, this may be achieved by use of bivalent marking, which represses CD1d but keeps it poised for activation in response to RA.

There are, however, currently no reports in the literature to specifically link bivalent histone modifications to CD1d regulation. I therefore explored this concept in MM cell lines through experimental approaches, and then attempted to extend these findings to GC B cells using in silico approaches.

5.2.1 Regulation of CD1d in MM cells by bivalent chromatin

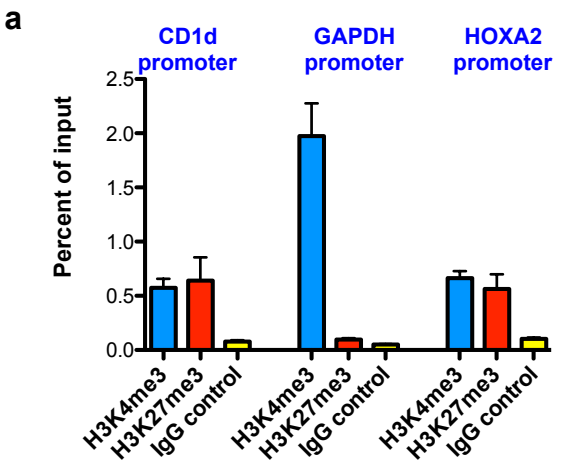
Investigation of the epigenetic regulation of MM cells using ChIP

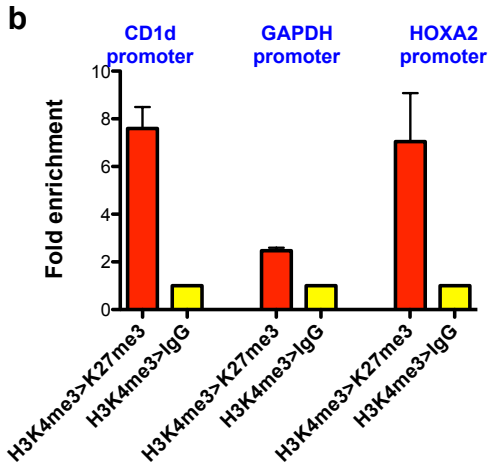
The multiple myeloma cell line U266 was further investigated by chromatin immunoprecipitation (ChIP), in order to study the epigenetic landscape of *CD1D*. A full description of this technique can be found in Chapter 2, section 2.5. Specifically, ChIP primers were designed to analyse the upstream regulatory element of the *CD1D* gene, approximately 1.5kb from the ATG translational start site, consistent with the reported location of the RARE. Analysis of control genomic loci was also undertaken, namely the upstream regulatory regions of *HOXA2* (a putative target of polycomb mediated repression, known to be marked by bivalent histone modifications) and *GAPDH* (a transcriptionally active housekeeping gene). The process of primer design is further described in Chapter 2, section 2.6.4.

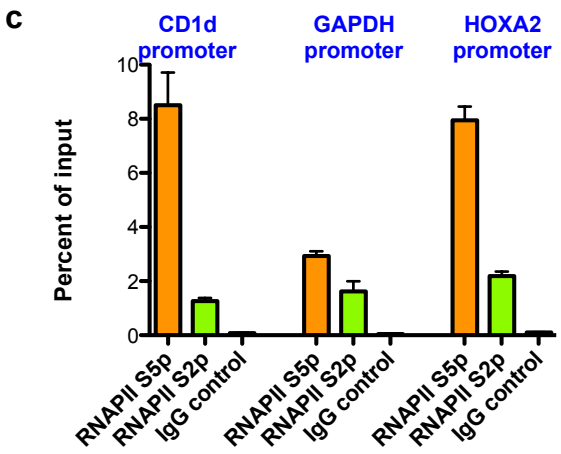
ChIP data for U266 is shown in **Figure 5.9**. It can be seen that the upstream *CD1D* promoter is marked by both H3K4me3 and H3K27me3, suggestive of a bivalent domain **(Figure 5.9 (a))**. Although such ChIP assays point towards bivalency, they cannot unequivocally establish the coexistence of both marks on the same allele in a given cell, and rather may be due to mixed cell populations. To further establish bivalency at the *CD1D* promoter, sequential Re-ChIP was used to enrich first for H3K4me3 and then subsequently for H3K27me3 **(Figure 5.9 (b))**, again demonstrating the bivalent nature of the *CD1D* promoter. Bivalent domains are also

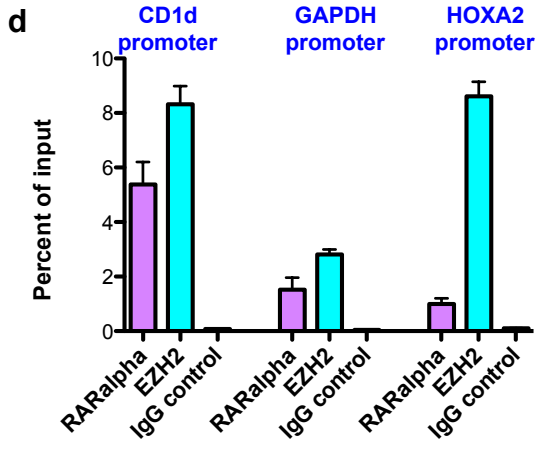
characterised by the serine phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNAPII) (Brookes et al., 2012). It is suggested that the RNAPII of poised genes is associated with higher levels of serine 5 phosphorlylation (S5p) than serine 2 phosphorylation (S2p), whereas actively transcribed genes are associated with both forms of RNAPII. **Figure 5.9 (c)** demonstrates that the *CD1D* promoter region is associated with a skewing towards RNAPIIS5p rather than RNAPIIS2p. Taken together, this data is suggestive of a poised, bivalent regulatory region of *CD1D* in U266 cells.

Having previously established that *CD1D* expression is regulated by retinoic acid, ChIP was used to establish the presence of RARα at the *CD1D* promoter **(Figure 5.9 (d))**. Similarly, the imposition of the repressive H3K4me27 mark at the *CD1D* promoter by PRC2 was established by using ChIP to confirm the presence of EZH2 (a key component of PRC2) at the *CD1D* promoter **(Figure 5.9 (d))**. Again, sequential Re-ChIP was used to confirm that RARα and EZH2 are not disparate, but rather interact on the same allele **(Figure 5.9 (e))**.


a
CD1d promoter
GAPDH promoter
HOXA2 promoter
Percent of input
2.5
2.0
1.5
1.0
0.5
0.0
H3K4me3
H3K27me3
IgG control


b
CD1d promoter
GAPDH promoter
HOXA2 promoter
Fold enrichment
10
8
6
4
2
0
H3K4me3>K27me3
H3K4me3>IgG


c
CD1d promoter
GAPDH promoter
HOXA2 promoter
Percent of input
10
8
6
4
2
0
RNAPII S5p
RNAPII S2p
IgG control


d
CD1d promoter
GAPDH promoter
HOXA2 promoter
Percent of input
10
8
6
4
2
0
RARalpha
EZH2
IgG control

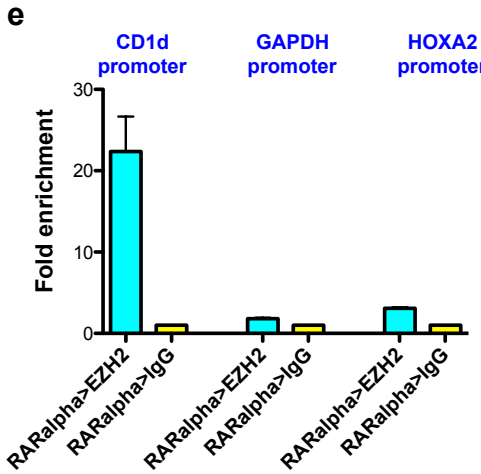

e
CD1d promoter
GAPDH promoter
HOXA2 promoter
Fold enrichment
30
20
10
0
RARalpha>EZH2
RARalpha>IgG

Figure 5.9. ChIP analysis of the CD1D promoter in U266 MM cells

(a) ChIP analysis of H3K4me3 and H3K27me3 mark recruitment at the CD1D promoter. ChIP data is shown as a percentage of the input chromatin. The promoter regions of GAPDH, a transcriptionally active housekeeping gene and HOXA2, a known bivalent repressed gene, are shown as controls. Immunoprecipitation by an IgG control antibody is shown as an additional negative control.

(b) Sequential Re-ChIP analysis of H3K4me3 followed by H3K4me27 at the CD1D promoter. ChIP data is shown as fold enrichment in comparison to a second immunoprecipitation with an IgG control antibody. The promoter regions of GAPDH and HOXA2 are shown as controls.

(c) ChIP analysis of RNAPIIS5p and RNAPIIS2p recruitment at the CD1D promoter. ChIP data is shown as a percentage of the input chromatin. The promoter regions of GAPDH and HOXA2 are shown as controls. Immunoprecipitation by an IgG control antibody is shown as an additional negative control.

(d) ChIP analysis of RARα and EZH2 recruitment at the CD1D promoter. ChIP data is shown as a percentage of the input chromatin. The promoter regions of GAPDH (not reported to be regulated by RARα or EZH2) and HOXA2 (known to be regulated by EZH2 but not RARα) are shown as controls. Immunoprecipitation by an IgG control antibody is shown as an additional negative control.

(e) Sequential Re-ChIP analysis of RARα followed by EZH2 at the CD1D promoter. ChIP data is shown as fold enrichment in comparison to a second immunoprecipitation with an IgG control antibody. The promoter regions of GAPDH and HOXA2 are shown as controls. .
(n=3, mean and SEM shown)

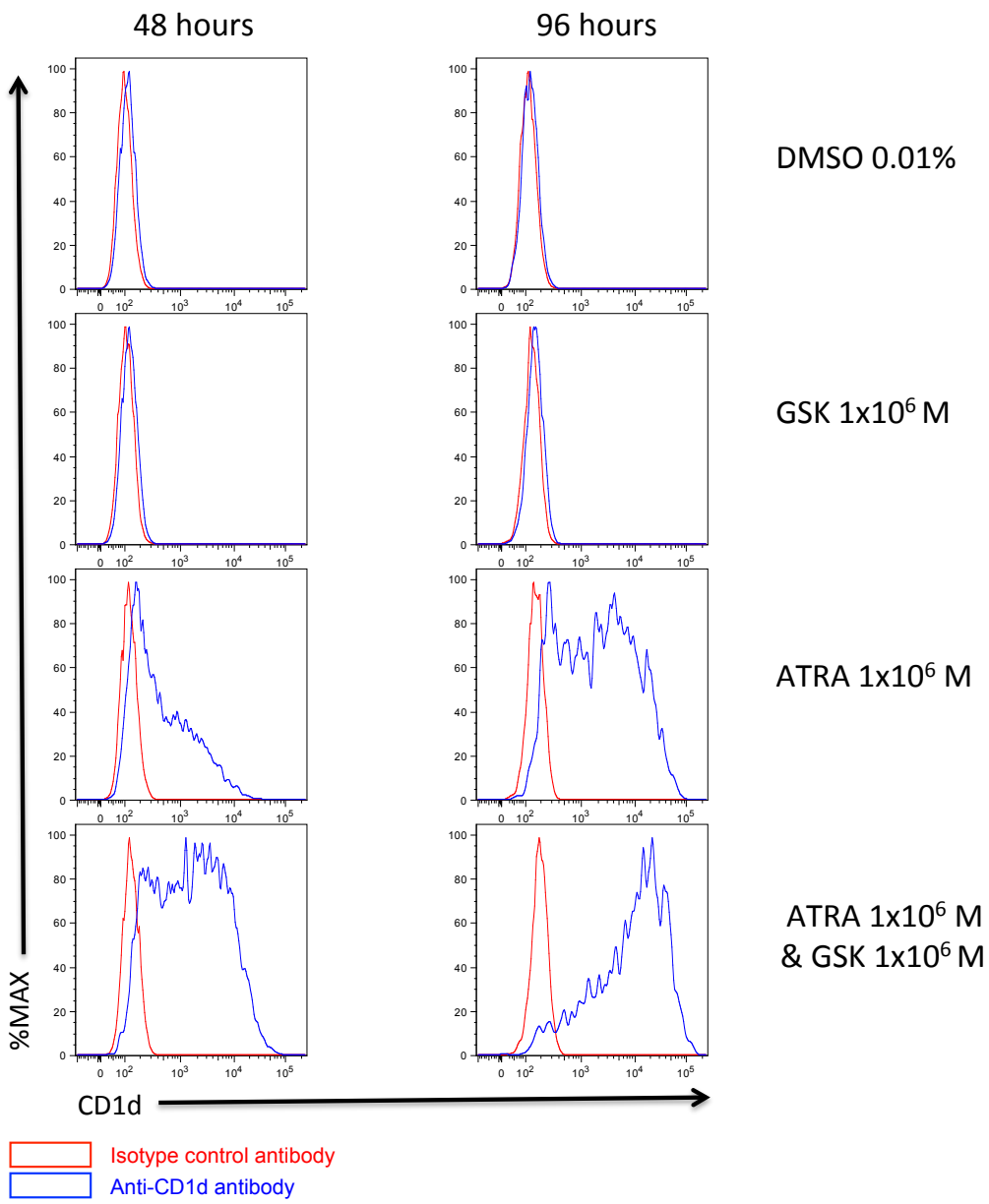
Synergistic restoration of CD1d expression in U266 cells by RA and a PRC inhibitor

The repression of CD1d in U266 through imposition of bivalent marking raises the possibility that inhibition of PRC may represent a novel strategy to re-express CD1d. This concept was investigated through a pharmacological approach, using the EZH2 inhibitor GSK343. EZH2 catalyses the trimethylation of H3K27 via transfer of a methyl group from the cofactor S-(S′-adenosyl)-L-methionine (SAM). GSK343 docks to the SAM binding site of EZH2 and subsequently acts through competitive inhibition against SAM. Through this mechanism, it is reported that GSK343 inhibits EZH2 with an IC50 of $4x10^{-9}$M and is over 1000-fold selective for other histone methyltransferases except EZH1 (60-fold selectivity).

A full description of methods used to treat cell lines can be found in Chapter 2, section 2.1.2. U266 multiple myeloma cells were treated with either ATRA, GSK343 or a combination of both agents. As in earlier experiments, ATRA was used at a ”pharmacological” dose of $1x10^{-6}$M. In terms of dosing GSK343, it was noted that although the compound has an IC50 of $4x10^{-9}$M, higher doses of at least $1x10^{-6}$M were required to achieve more than 90% inhibition of EZH2 (Verma et al., 2012). Thus, a dose of $1x10^{-6}$M for GSK343 was used, consistent with other reports of using this compound on B cells (Béguelin et al., 2013).

In order to allow sufficient time for inhibition of EZH2, surface CD1d expression was assessed at 48 hours and 96 hours by flow cytometry, as detailed in Chapter 2, section 2.4.5 **(Figure 5.10 (a))**. Consistent with previous results described above, ATRA treatment led to a time dependent increase in surface CD1d expression. GSK343 treatment alone did not lead to restoration of CD1d expression. However, a combination of ATRA and GSK343 restored CD1d surface expression to approximately a 5-fold higher level than treatment with ATRA alone **(Figure 5.10 (b))**.

a


48 hours
96 hours
DMSO 0.01%
GSK 1x10⁶ M
ATRA 1x10⁶ M
ATRA 1x10⁶ M & GSK 1x10⁶ M
%MAX
CD1d
Isotype control antibody
Anti-CD1d antibody

b

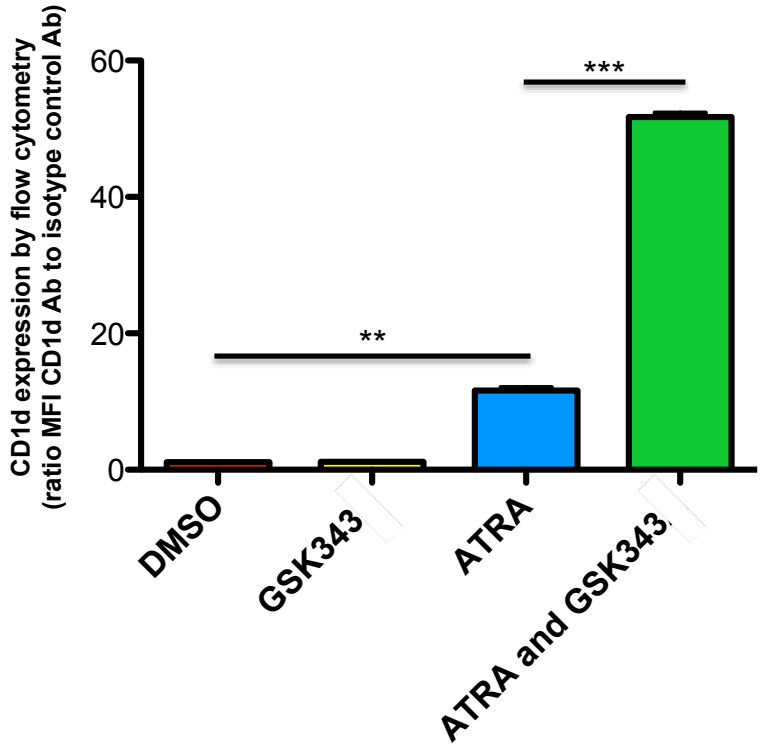


Figure 5.10. Flow cytometric analysis of surface CD1d expression in MM cell line U266 post treatment with ATRA and/or GSK343

(a) *Representative flow cytometric plots showing restoration of CD1d expression at 48 and 96 hours in the U266 cell line following treatment with ATRA 1X10^{-6}M, GSK343 1X10^{-6}M, or a combination of both. Treatment with 0.01% DMSO was used as a control. CD1d surface expression was assessed by staining with anti CD1d antibody (clone 42.1) and comparing to staining by the appropriate isotype control antibody.*

(b) *Summary of surface CD1d expression at 96 hours in U266 following treatment with 0.01% DMSO, ATRA 1X10^{-6}M, GSK343 1X10^{-6}M, or a combination of treatments, as indicated by the MFI ratio of CD1d antibody to isotype control antibody. (n=3 for each cell line, mean and SEM shown)*

In addition to flow cytometry, the effect of treatment with ATRA and/or GSK343 on *CD1D* expression at the transcriptional level was assessed using real time PCR (described in Chapter 2, section 2.6.3). Consistent with the flow cytometry results, treatment of U266 with control DMSO or $1x10^{-6}$M GSK343 alone did not increase *CD1D* mRNA. Treatment with $1x10^{-6}$M ATRA led to a significant increase in *CD1D* mRNA at 96 hours, an effect which was again increased by approximately 5-fold by treatment with a combination of both ATRA and GSK343 **(Figure 5.11)**.

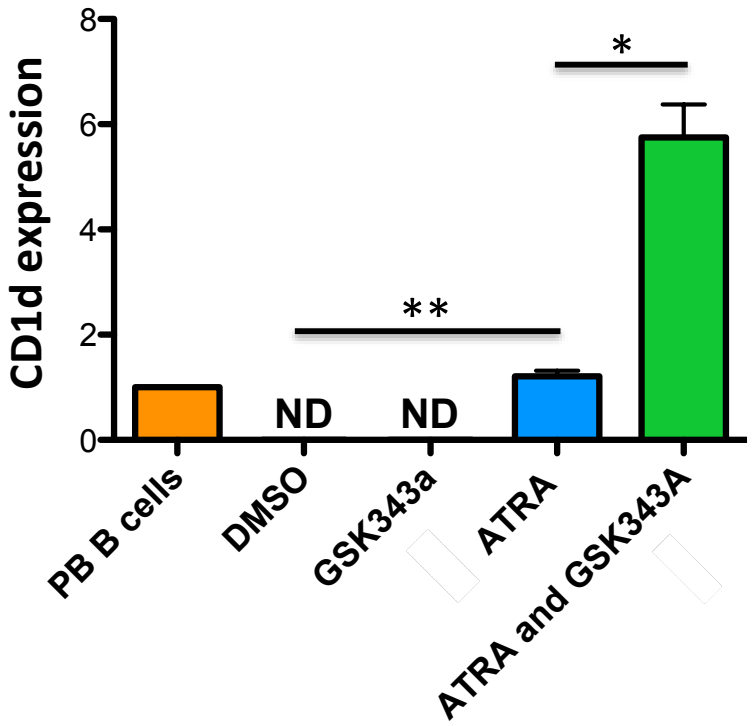


Figure 5.11 Real time PCR analysis of CD1D mRNA expression in MM cell line U266 at 96 hours post treatment with ATRA and/or GSK343

No effect on CD1D mRNA was observed by treatment with 0.01% DMSO control or 1x10^{-6}M GSK343 alone. CD1D mRNA expression was significantly increased following treatment with 1x10^{-6}M ATRA at 96 hours. Combined treatment with 1x10^{-6}M ATRA and 1x10^{-6}M GSK343 further increased CD1D mRNA by approximately 5-fold in comparison to treatment with ATRA alone. Expression values are calculated relative to the reference gene beta actin, and are shown relative to the CD1D expression value for untreated peripheral blood B cells. (ND means CD1D mRNA Not Detected.) (n=3 for each cell line, mean and SEM shown)

5.2.2 Parallels to the use of bivalent chromatin in GC B cells

Although there are no reports in the literature detailing the regulation of CD1d by bivalent domains, the use of online databases allows the findings described above in MM cells to be extended to other B cells, using *in silico* approaches.

In silico analysis of B-LCLs

The UCSC genome browser (an online application provided by the Genome Bioinformatics Group of UC Santa Cruz) provides Chromatin Immunoprecipitation sequencing (ChIP-seq) data (via the Broad Institute) for the B-LCL GM12878. As shown in **Figure 5.12**, the upstream region of the *CD1d* gene in this cell line is marked by both H3K4me3 and H3K27me3, consistent with bivalent poising of the *CD1d* promoter.

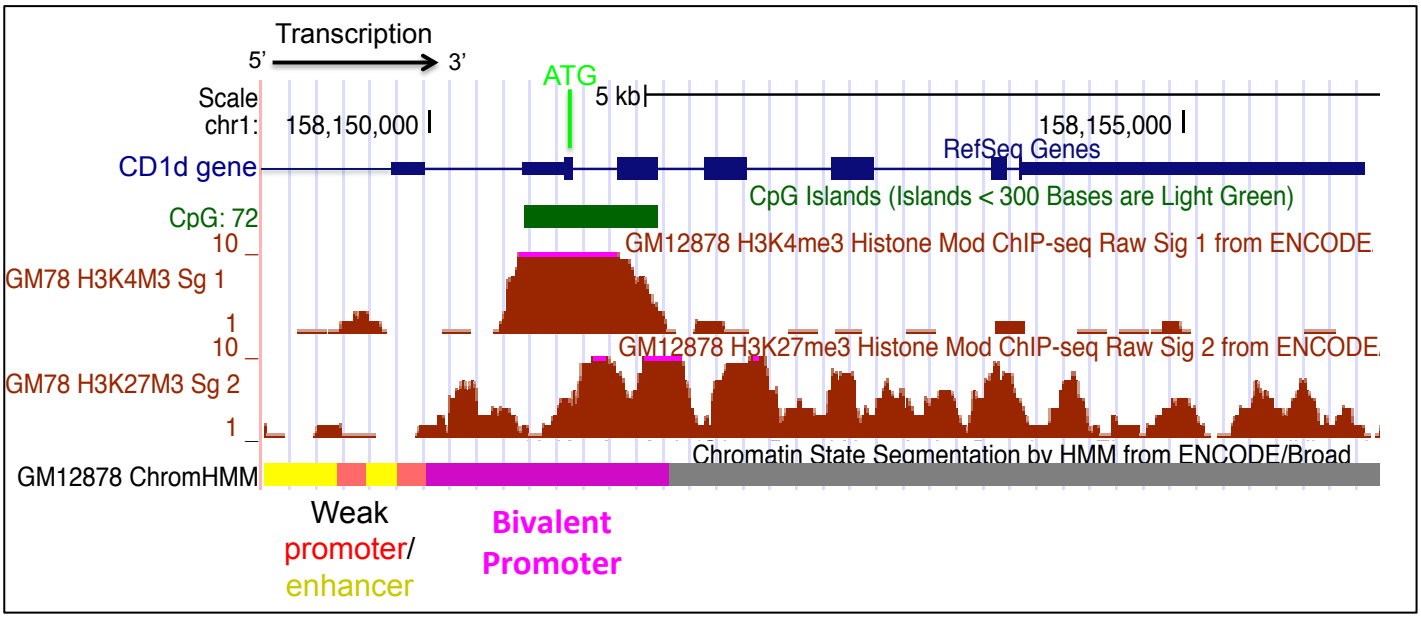


Figure 5.12. In silico analysis of CD1D histone methylation using ChIP-seq data for B-LCL GM12878.

ChIP-seq data, provided by the Broad Institute, is shown analysed in the UCSC genome browser. The proximal upstream regulatory region of the CD1d gene is marked by both H3K4me3 and H3K27me3, consistent with a bivalent poised promoter.

As discussed earlier, the development of EBV-infected B-LCLs can be considered as closely related to that of GC B cells. This thus offers a first line of evidence that CD1d regulation in GC B cells may also be in part due to bivalent histone modifications.

In silico analysis of murine GC B cells

Specific to GC B cells, Caganova and colleagues have provided an online dataset (Gene Expression Omnibus database, GSE50912) containing ChIP-seq data for murine GC B cells, using antibodies against H3K4me3 and H3K27me3. This data thus allows an analysis of bivalent marking in the *Cd1d1* gene in murine GC B cells.

By downloading WIG files for this data and incorporating the data into the UCSC genome browser, it can be seen that the upstream regulatory region of the murine *Cd1d1* gene is marked by both H3K4me3 and H3K27me3 **(Figure 5.13)**. The location of this bivalent histone methylation signature is approximately 1500bp from the ATG translational start site (i.e. the location of the RARE in the distal *Cd1d1* promoter region). This is again highly suggestive of a poised bivalent domain correlating with CD1d downregulation, and parallels the observations described above in MM B cells and B-LCLs.

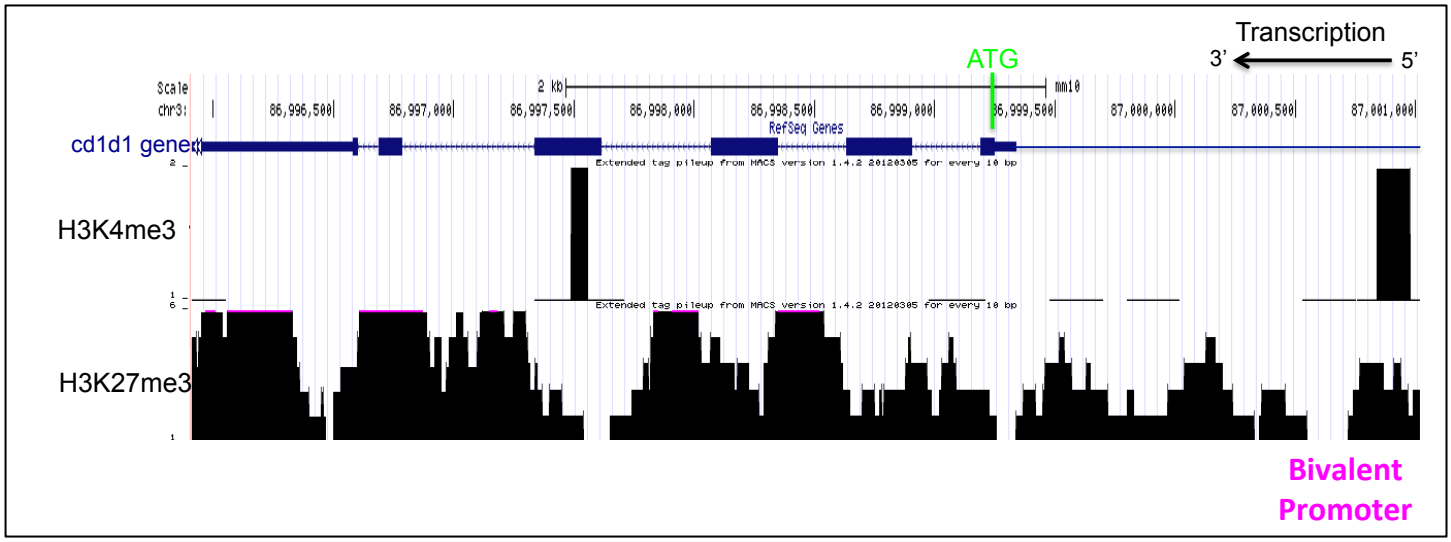


Figure 5.13. In silico analysis of Cd1d1 histone methylation using ChIP-seq data for murine GC B cells.

ChIP-seq data (from Gene Expression Omnibus database GSE50912) is shown analysed in the UCSC genome browser. The distal upstream regulatory region of the Cd1d1 gene, approximately 1500bp upstream from the ATG translational start site, is marked by both H3K4me3 and H3K27me3, consistent with a bivalent poising of the promoter.

5.3 Discussion

5.3.1 Role of RA signalling

The findings detailed in this chapter have revealed important insights into the transcriptional control of CD1d in B cells. Like activated B cells and EBV infected B cells, an initial level of control is provided in MM B cells by RA signalling. Consistent with the loss of CD1d in advanced MM, *in silico* analysis is suggestive of a decrease in RA signalling in MM B cells during disease progression. The corollary of this is the restoration of CD1d expression in MM B cells using ATRA, a hitherto unreported phenomenon, demonstrating the influence of RA on CD1d expression in these cells. Similarly, several approaches have been utilised in this chapter to show that GC B cells are also marked by a decrease in RA signalling, coincident with the fall in CD1d expression in these cells.

It is not clear what is driving the decrease in RA signalling in these B cells. As discussed earlier, the B cells in which CD1d downregulation has been reported share a highly proliferative phenotype. Does this proliferative phenotype lead to a decrease in RA signalling, or is the fall in RA signalling the cause of cellular proliferation? In addition, one can consider that there may be a third factor, which causes cellular proliferation and a decrease in RA signalling independently. Activated, GC and EBV infected B cells share,

for example, activation of B cell receptor pathways, and these may play a key role in modulating RA signalling and cellular proliferation.

The ability of ATRA to restore CD1d expression allows B cells to present lipid ligand to iNKT cells. It would be of interest to investigate if ATRA plays a more global role in promoting B-iNKT cell interactions. As discussed in Chapter 4, toll-like receptor ligands were shown to effect change in the lipid repertoire in dendritic cells and consequently lead to enhanced iNKT responses (Salio et al., 2007). It is possible that ATRA may also have an effect on the endogenous lipid repertoire, promoting not only CD1d expression, but also presentation of high affinity lipid ligands to iNKT cells. This modulation of endogenous lipids by ATRA has not previously been reported in the literature and would be of interest to investigate in the future. For example, a B cell line such as C1R-CD1d (which constitutively expresses CD1d) could be used in an iNKT cellular cytotoxicity assay, in the presence and absence of ATRA. If ATRA increased iNKT cytotoxicity towards B cells, then this would be an effect independent of CD1d and would warrant further investigation, such as lipid profiling of ATRA treated B cells in comparison to non-treated B cells.

The role of RA signalling in controlling CD1d expression in B cells needs to be put into the context of our current knowledge of RA in B cell biology. It has previously been reported that ATRA can induce the growth inhibition of MM B cells through several mechanisms, including up-regulation of

p21/Cip1, down-regulation of the IL-6/IL-6R auto/paracrine loop and modulation of Fas apoptotic antigen (Otsuki et al., 2003). It is thus intuitive that re-expression of CD1d may be another mechanism by which ATRA can impede the propagation of myeloma. It is, however, more difficult to reconcile the role of ATRA in mature B cell development. It is reported that ATRA augments primary and secondary humoral B cell responses in response to antigenic stimuli, through many actions including enhancing B cell proliferation, class switch recombination, and the generation of antibody-secreting plasma cells. This is manifest in ATRA offering a protective effect against infection and enhancing vaccination responses (Ertesvåg et al., 2009; Ross et al., 2011). If there is a biological advantage provided by the downregulation of CD1d in the GC, then the use of ATRA, an agent which restores CD1d expression in B cells, is counterintuitive to this, and thus further investigation is needed to analyse why ATRA is therefore not detrimental to humoral immunity.

5.3.2 Role of bivalent chromatin

The ChIP analysis of the U266 MM cell demonstrates for the first time a further level of control of CD1d expression, through the use of bivalent marking. Such bivalent domains ensure CD1d is effectively repressed, but not permanently silenced, and instead remains poised for re-expression (consistent with the immediate response of *CD1D* gene transcription to

treatment with ATRA). The use of sequential Re-ChIP not only further confirms the bivalency of the *CD1D* promoter, but also permits the demonstration of the interaction between RARα and the PRC2 component EZH2 at the *CD1D* promoter. The importance of PRC2-mediated repression in regulating CD1d expression is further demonstrated by the dramatic increase in CD1d expression achieved by treating U266 cells with ATRA and the EZH2 inhibitor GSK343 compared to ATRA alone.

Several studies show that elevated levels of EZH2 correlate with poor prognosis in solid tumors, including those in prostate, breast and kidney (Kleer et al., 2003; Varambally et al., 2002; Wagener et al., 2010). Specific to B cells, somatic activating mutations in EZH2 have been identified in follicular lymphoma (FL), and GCB diffuse large B cell lymphoma (DLBCL), leading to increased H3K27me3 (Morin et al., 2010; 2011). Similarly, there is increasing evidence for the role of EZH2 in MM propagation (Kalushkova et al., 2010; Popovic et al., 2014). These findings suggest that dysregulation of H3K27me3, through EZH2 over-expression or point mutations, silences target genes important in tumour growth and survival. Indeed, pharmacological targeting of EZH2 may represent an effective treatment strategy for B cell disorders such as lymphomas (Béguelin et al., 2013). The work in this project again highlights the active role of PRC2 in advanced MM. By demonstrating that the *CD1D* gene is a target of PRC2, it introduces the novel concept that PRC2 may act to enhance immune evasion as one mechanism of propagating tumour growth and survival.

The exact hierarchical nature of the interaction between RA signalling and bivalent histone marking warrants further investigation. It is intriguing that whereas ATRA alone can induce *CD1D* transcription, and treatment with both GSK343 and ATRA greatly augments this response, treatment with GSK343 alone has no effect on *CD1D* transcription. This is suggestive that RARα is the main transcriptional hub responsible for regulation of *CD1D* and RA is thus the main driver. PRC proteins are recruited to this hub as a further layer of transcriptional control, but their inhibition alone is still not sufficient to overcome the other co-repressors associated with RARα in the absence of RA. To investigate this concept in future work, further detailed ChIP analysis will be required to examine RARα, its associated co-repressors and associated bivalent marks following treatment with ATRA alone, GSK343 alone and the combination of both agents.

5.3.3 Utilization of GC pathways by MM cells

The concept that GC pathways can be 'hijacked' by B cell lymphomas for disease propagation (Basso and Dalla-Favera, 2015) has yet to be extended to other B cell malignancies including MM. The work in this chapter has provided evidence that both the loss of RA signalling and imposition of bivalent marks to repress CD1d expression are shared phenomena, utilised by both MM B cells and GC B cells. Thus, MM B cells may also 'hijack' physiological GC pathways.

The work in this chapter to model the epigenetic landscape in GC B cells has predominantly utilised an *in silico* approach. In order to confirm these findings, further work in the future is needed to investigate GC B cells experimentally in greater depth. Specifically, naïve, GC and post GC B cells (either human or murine) could be isolated by FACS as previously described and analysed by ChIP analysis. If such parallels can be validated, then the sharing of these transcriptional programmes may have implications beyond CD1d expression. Specifically, it would provide a rationale to investigate MM more closely for use of other GC programmes, some of which may provide novel therapeutic targets.

5.3.4 Therapeutic restoration of CD1d

Not only does the transcriptional regulation of CD1d provide an interesting theoretical framework, but knowledge of these pathways also allows an opportunity for more effective therapeutic restoration of CD1d. Consistent with previous reports in the literature for other B cell states, pharmacological doses of ATRA can restore CD1d expression in MM B cells. For the first time, I have shown that combined use of ATRA with PRC inhibition leads to a greater (approximately 5-fold) increase in CD1d expression compared to ATRA alone. This may be of importance, for example, in therapeutic interventions, where restoration of CD1d on MM B cells is a prerequisite for use of iNKT immunotherapy.

Further work in the future is required to take this potential therapeutic avenue forward. It would need to be shown, through *in vitro* cellular cytotoxicity assays, that the CD1d produced in response to ATRA and GSK343 is fully functional and capable of presenting lipid to iNKT cells. Furthermore, such assays would allow an assessment of the effect of the level of CD1d restoration (i.e. is a greater effect achieved with both ATRA and PRC inhibition compared to ATRA alone in terms of iNKT lysis). In addition, *in vivo* models would then need to be used, to assess if CD1d can be restored pharmacologically in MM cells *in vivo*. Through adoptive transfer of iNKT cells (as a model of iNKT immunotherapy), the advantage of *in vivo* restoration of CD1d could again be tested. Exploration of such avenues is important and warrants priority. In the emerging field of cancer immunotherapy, where iNKT immunotherapy may indeed have a role, effective restoration of CD1d may indeed prove to be useful addition to the armamentarium of MM treatment.

Conclusions

6 Conclusions

The CD1d-iNKT axis has emerged over the last two decades as an exciting area of T cell immunobiology. A role for this axis has been implicated in a wide range of conditions, including infections, malignancies and autoimmune disorders, and exploitation of the interaction between iNKT cells and other cells may herald therapeutic benefit (Berzins et al., 2011).

This project has focussed on the interaction between iNKT cells and B cells, by investigating the regulation of CD1d expression on these cells. Specifically, a detailed study has been undertaken of the evidence of loss of CD1d expression in B cells, the biological significance of this downregulation, and the transcriptional mechanisms underpinning these changes in expression. Furthermore, this study has in parallel addressed both physiological "normal" B cells and "pathological" B cells from certain disease states, including EBV infection and MM.

In Chapter 3, both human tonsillar and murine splenic GC B cells were used as a model for normal B cells. It was shown that the pattern of CD1d expression is consistent across species. CD1d falls to a nadir as a naïve B cell enters the GC, and subsequently rises again in post GC B cells. Although previous evidence exists for the *in situ* loss of CD1d expression in the GC (Exley et al., 2000), this is the first data to show how the expression of CD1d changes dynamically, both at the surface protein and transcriptional level.

The loss of CD1d was shown to be paralleled in pathological EBV infected and MM B cells, again at the surface and transcriptional level. A natural extension of this work would be to investigate if this loss of CD1d expression can occur in other disease states. There is already some evidence that CD1d expression may change on B cells in the autoimmune disorder SLE (Bosma et al., 2012) and B cell malignancies such as CLL (Bojarska-Junak et al., 2014). The change in CD1d expression in these conditions does not, however, completely follow the transcriptional loss observed in GC, EBV and MM B cells. In SLE, loss of CD1d on B cells has been suggested to occur as a result of increased CD1d internalisation. Conversely, in CLL, CD1d expression is reported to increase with disease progression. These findings warrant further investigation, to confirm the nature of CD1d changes in these conditions. But the current work in Chapter 3 stands by itself to provide a convincing picture of the downregulation of CD1d in normal and pathological B cells.

The conservation of CD1d downregulation in GC B cells across species, and the *in silico* demonstration in Chapter 3 that CD1d is in the top 20 most downregulated genes in the GC, is suggestive of the biological significance of this phenomenon. In Chapter 4, it was hypothesised that presentation of lipid antigen by GC B cells to iNKT cells, while initially advantageous to B cells, may prove detrimental to these B cells if prolonged. Thus, the purpose of CD1d downregulation in GC B cells may be to obtund presentation of exogenous lipid antigen to iNKT cells. Alternatively a model can be put

forward that unites the loss of CD1d in GC, EBV infected and MM B cells. Specifically, it was postulated that the high proliferative nature of these cells may make them more vulnerable to iNKT mediated cellular cytotoxicity, possibly through presentation of an altered endogenous lipid repertoire with higher affinity for iNKT cells. Although only speculative at this stage, support for this model is provided by previous evidence for the metabolic reprogramming of proliferative cells (Marelli-Berg et al., 2012) and also the report that APCs are capable of changing their lipid repertoire, leading to enhanced iNKT responses (Salio et al., 2007).

In order or investigate this theory, two separate *in vivo* approaches were employed, both sharing the theme of constitutively expressing CD1d in GC B cells, in order to assess if this led to iNKT mediated cytotoxicity. In the initial approach, a chimeric mouse model was produced by adoptive transfer of *Cd1d1* transduced HSCs to irradiated recipients. These experiments provided proof of principle that such a mouse chimera is capable of constitutively expressing CD1d in GC B cells in the proportion of B cells transduced by the *Cd1d1* expressing vector. These experiments were limited, however, by inconsistent engraftment and variable immune responses between different transplanted mice, and as such a definitive conclusion of as to the role of CD1d could not be reached.

This approach was superseded by a second approach, in which a previously generated CD1d transgenic mouse model was utilised. Again, it was shown

that these mice constitutively expressed CD1d in GC B cells. By comparing the production of GC B cells in these mice to their wild type counterparts, no significant difference in GC B cell frequency of antibody production was noted.

Such experiments would thus appear to refute an essential role of CD1d downregulation in GC B cells, but limitations remain. In particular, constitutive expression of high levels of CD1d on BM derived cells may lead to increased negative selection of iNKT cells in the thymus, and subsequent impairment of iNKT numbers and function. Thus, further work is warranted before firm conclusions can be drawn. This would include, for example, the use of a more refined mouse model in which CD1d could be constitutively expressed much more specifically only in B cells at the time of the GC reaction. Furthermore, these experiments would need to be carried out in the presence of exogenous lipid antigen (ideally a novel antigen which is targeted to the B cell). Until such future experiments are conducted, speculation will remain as to the purpose of this dramatic downregulation of CD1d in GC B cells.

The biological role of the downregulation of CD1d can also be addressed by investigating the pathological counterparts to normal B cells. Such approaches have traditionally been difficult to carry out *in vivo*. EBV is a human disease and thus would require a humanised murine model to investigate further. Furthermore, EBV B-LCL or MM B cell tumour models

typically utilise immunosuppressed murine strains, such as NOD scid gamma, in order to facilitate tumour engraftment, but this of course negates an investigation of the CD1d-iNKT axis. In contrast, a murine specific tumour cell line, 5TGM1, recapitulates features of multiple myeloma when injected into normal host mice, and thus may allow investigation of the role of CD1d on such cells. Appendix D details preliminary work with this cell line. 5TGM1 cells express CD1d and this can be reduced approximately 10 fold by shRNA knockdown of murine *Cd1d1*. This preliminary work thus forms the basis by from which further *in vitro* and *in vivo* studies can be conducted using this cell line, and offers an alternative approach to assess the biological role of CD1d.

The transcriptional mechanisms governing the downregulation of CD1d, in either normal or pathological B cells, is a further source of biological intrigue, and this was addressed in chapter 5. By focussing on MM cell lines, it was shown that both RA signalling and bivalent chromatin domains act as a dual regulatory mechanism of CD1d. This is of potential therapeutic importance. In the emerging field of tumour immunotherapy, restoration of CD1d in tumours may be of vital importance. Previous work in B cells activated *in vitro* and EBV infected B cells has revealed the ability of ATRA to restore CD1d in these B cells. The uncovering of a dual mechanism for downregulation of CD1d, encompassing not only RA signalling but also PRC mediated repression, has provided the rationale to restore CD1d in a MM cell line using a dual pharmacological approach of both ATRA and GSK343,

an inhibitor of the PRC component EZH2. Restoration of CD1d using this dual approach has not previously been reported in the literature, but is shown in this work to be a more effective strategy for restoration of CD1d. It is of interest that whereas ATRA is able to act alone to partially increase CD1d expression, GSK343 alone has no effect on CD1d surface levels. This provides evidence for a hierarchical model, where the RA receptor acts as a central transcriptional hub, and PRC components act as secondary components to fine-tune transcriptional control.

Although the experimental work in Chapter 5 has focussed on MM B cells, further work in this chapter, including *in silico* analysis, has attempted to uncover similar mechanisms in GC B cells. These findings are suggestive that loss of RA signalling and use of bivalent domains are both associated with loss of CD1d in GC B cells. To confirm these findings, primary ChIP of pre GC, GC and post GC B cells will need to be undertaken, to again elucidate the epigenetic landscape. However, these initial findings offer the first evidence that like lymphomas, MM B cells are able to hijack physiological pathways for disease propagation. This is an important concept, which if demonstrated for other pathways, may have therapeutic ramifications beyond CD1d.

Of course, additional transcriptional mechanisms may also be at work. Previous work in EBV infected B cells has highlighted that RA signalling may also modulate LEF-1 mediated transcriptional repression of CD1d (Chung et

al., 2013), and this remains a further area for investigation in MM and GC B cells. The question also remains as to what is controlling RA signalling in B cells. GC, EBV infected and MM B cells share, as discussed, a highly proliferative phenotype, but it is not clear if this is cause or consequence of a decrease in RA signalling. Perhaps the master regulator is BCR activation. BCR signalling pathways are by definition activated in GC B cells, but are also activated in EBV infected, SLE and some MM B cells, and this may be the initial trigger to modulate cellular proliferation, RA signalling and CD1d expression.

In summary, this body of work has answered many questions, but has opened up many new ones. **Figure 6.1** highlights the main points of our current understanding and areas for future investigation. CD1d expression on B cells represents a fascinating area of immunobiology in itself, but the close link between normal and pathological B cells means that research in this area may yield real therapeutic benefit in the future.

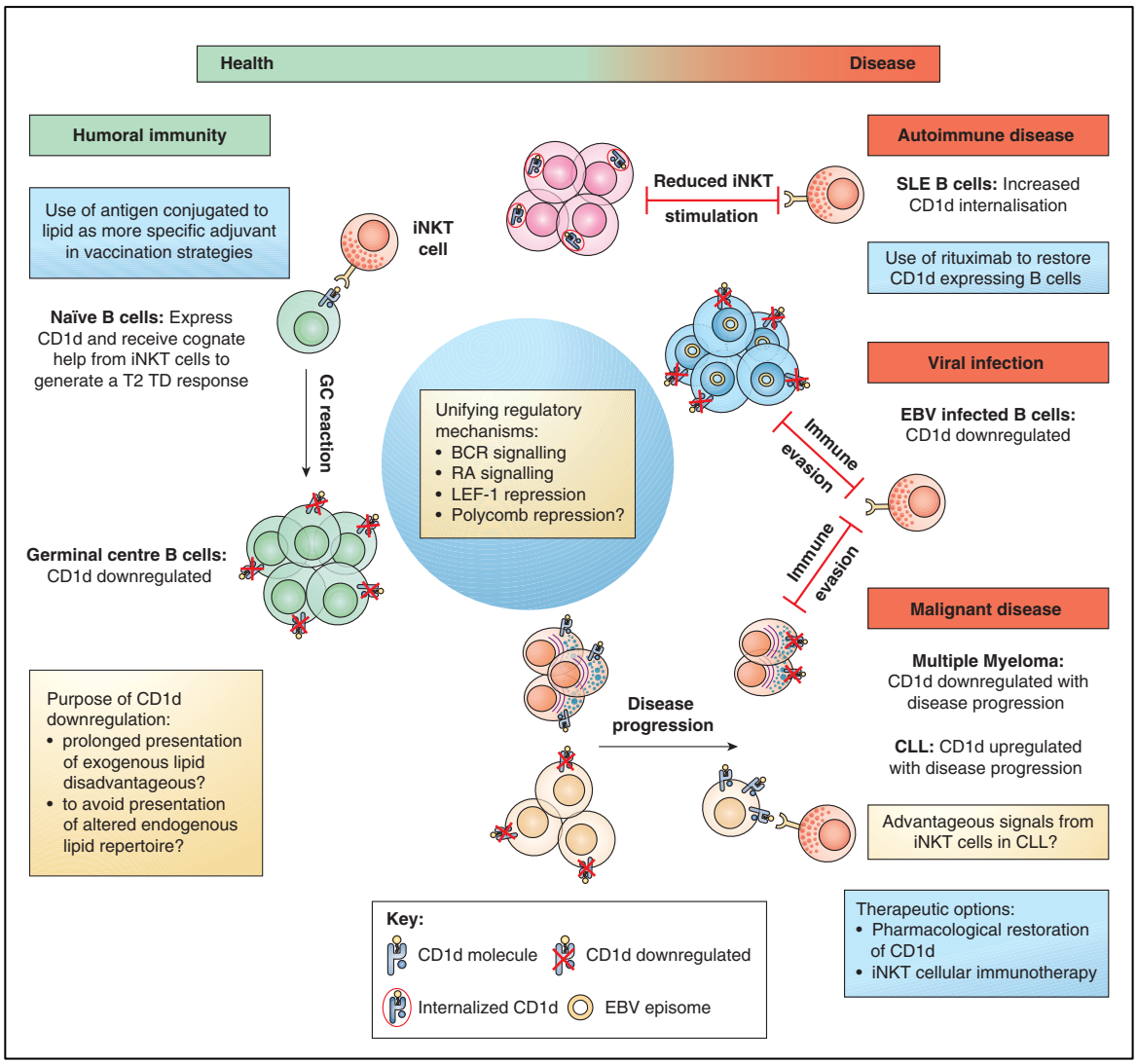


Figure 6.1. Dynamic expression and role of CD1d on B cells in health and disease.

Expression of CD1d on normal naïve B cells permits their cognate interaction with iNKT cells. This leads to the development of the type 2 TD immune response, followed by CD1d downregulation in the germinal centre. Pathological, SLE, EBV-infected or malignant B cells display abnormal CD1d expression, and regulation of CD1d in these cells may also involve utilisation of physiological pathways. Yellow highlighted boxes describe potential areas that warrant further research or may be exploited in the future for therapeutic benefit. (From Chaudhry and Karadimitris, 2014.)

Appendices

7 Appendices

Appendix A

Reagents used and their origin are detailed in the table below.

Product	Company	Catalogue Number
Anti-H3K4me3	Abcam	ab8580
Anti-H3K27me3	Abcam	ab6002
Anti-RNA polymerase II CTD phospho S2	Abcam	ab5095
Anti-RNA polymerase II CTD phospho S5	Abcam	ab5131
Anti-EZH2	Abcam	ab3748
Control IgG	Abcam	ab27472
Taqman Gene Expression Master Mix	Applied Biosystems	4369016
SYBR Green/ROX qPCR mastermix.	Applied Biosystems	4309155
Anti-human CD1d-PE (clone 42.1)	BD Biosciences	550255
Isotype control antibody-PE	BD Biosciences	555749
Anti-human CD19-PerCP-Cy5.5	Biolegend	302229
Anti-human IgD-Alexa Fluor 647	Biolegend	348227
Anti-human CD10-Brilliant Violet 421	Biolegend	108731
Anti-human CD38-Pe-Cy7	Biolegend	303515
Anti-human CD1d-PE (clone 51.1)	Biolegend	350305
Isotype control antibody-PE	Biolegend	400311
Anti-mouse CD45.1-Brilliant Violet 605	Biolegend	110737

Anti-mouse B220-Alexa Fluor 700	Biolegend	103231
Anti-mouse CD19-PerCP-Cy5.5	Biolegend	115533
Anti-mouse IgM-Brilliant Violet 421	Biolegend	406517
Anti-mouse IgD-Brilliant Violet 421	Biolegend	405725
Anti-mouse CD21-APC-Cy7	Biolegend	123417
Anti-mouse CD23-Pe-Cy7	Biolegend	101613
Anti-mouse GL7-Alexa Fluor 647	Biolegend	144605
Streptavidin-Brilliant Violet 650	Biolegend	405231
Anti-mouse CD1d-PE	Biolegend	123509
Isotype antibody-PE	Biolegend	400607
Anti-mouse CD150-PerCP-Cy5.5	Biolegend	115921
Anti-mouse CD48-Pe-Cy7	Biolegend	103423
Anti-human CD1d-APC (clone 51.1)	Biolegend	350307
Isotype control antibody-APC	Biolegend	400311
Anti-mouse CD1d-PE (clone 1B1)	Biolegend	123509
Isotype control antibody-PE	Biolegend	400607
Anti-mouse CD1d- Alexa Fluor 647 (clone 1B1)	Biolegend	123511
Isotype control antibody- Alexa Fluor 647	Biolegend	400626
NP-KLH	Biosearch Technologies	N-5060
Shearing ChIP Kit	Diagenode	kch-redmod-100
Anti-mouse CD95-biotin	eBioscience	13-0951
Anti-mouse Cd117-APC	eBioscience	17-1178
Anti-lineage cocktail-eFluor 450	eBioscience	88-7772-72
Anti-mouse Sca-1-PE	eBioscience	12-5981-81
Lineage Cell Depletion Kit, mouse	Miltenyi Biotech	130-090-858

mSCF	Peprotech	250-03
mIL-3	Peprotech	213-13
hFL	Peprotech	300-19
hIL-11	Peprotech	200-11
RNeasy plus kit	Qiagen	74134
Endotoxin-free maxi preparation kit	Qiagen	12362
FBS	Sigma-Aldrich	F7524
DMSO	Sigma-Aldrich	D5879
L-glutamine	Sigma-Aldrich	G7513
Penicillin-Streptomycin	Sigma-Aldrich	P4333
Diptheria toxin	Sigma-Aldrich	D0564
Hygromycin B	Sigma-Aldrich	H3274
ATRA	Sigma-Aldrich	R2625
GSK343	Sigma-Aldrich	SML0766
DAPI	Sigma-Aldrich	32670
Agarose	Sigma-Aldrich	A9539
Anti-RARα	Sigma-Aldrich	SAB4800047
shRNA set against murine *Cd1d1*	Source BioScience	MSH026730-LVRU6MP
40 µm Cell Strainer	Stem Cell Technologies	27305
RPMI 1640 Medium	Thermo Fisher Scientific	11875-093
HEPES	Thermo Fisher Scientific	15630-080
Sodium Pyruvate	Thermo Fisher Scientific	11360-070
β2-mercaptoethanol	Thermo Fisher Scientific	31350-010
DMEM	Thermo Fisher Scientific	11965-092

Imject™ Alum Adjuvant	Thermo Fisher Scientific	77161
PBS	Thermo Fisher Scientific	10010023
ImaGene GreenTM lacZ Gene Expression Kit	Thermo Fisher Scientific	I-2904
Dynabeads Protein G	Thermo Fisher Scientific	10003D
Proteinase K	Thermo Fisher Scientific	25530-049
DTT	Thermo Fisher Scientific	15508-013
SuperScript III First-Strand Synthesis Supermix	Thermo Fisher Scientific	18080-400
DH5α E.coli	Thermo Fisher Scientific	18265-017
Lipofectamine 2000	Thermo Fisher Scientific	11668030
Retronectin	Takara Clontech	T100B
Lenti-X Concentrator	Takara Clontech	631231

Appendix B

Shown below are buffer, media and solution recipes used throughout this project.

RPMI+

RPMI
10% FBS
2mM L-Glutamine
Penicillin (1000U/mL)
Streptomycin (1000mg/mL)
10mM HEPES
1mM Sodium Pyruvate
50uM β2-mercaptoethanol

DMEM+

DMEM
10% FBS
2mM L-Glutamine
Penicillin (1000U/mL)
Streptomycin (1000mg/mL)

Phosphate Buffered Saline (PBS, in 1L)

8g NaCl
0.2g KCl
1.44g Na_2HPO_4
0.24g KH_2PO_4
pH 7.4

Flow cytometry buffer (FB)

PBS

2% FBS

ChIP dilution buffer

0.01% SDS

1.1% Triton X-100

1.2mM EDTA

16.7mM Tris-HCL pH 8

167mM NaCl

Low salt buffer

0.1% SDS

1% Triton X-100

2mM EDTA

20mM Tris-HCl pH 8

150mM NaCl

High salt buffer

0.1% SDS

1% Triton X-100

2mM EDTA

20mM Tris-HCl pH 8

500mM NaCl

LiCl buffer

0.25M LiCl

1% IGEPAL

1% sodium deoxicolate

1mM EDTA

10mM Tris-HCl pH 8

LB (Luria broth, 1L)

5g NaCl

5g Yeast extract

10g Peptone

H_2O to 1L, pH 7.5

LB agar (1L)

15g agar in 1L LB

SOCS media (1L)

2% tryptone

0.5% yeast extract

10 mM sodium chloride

2.5 mM potassium chloride

10 mM magnesium chloride

10 mM magnesium sulfate

20 mM glucose

Appendix C

Primer sequences used for ChIP (in the 5’ to 3’ direction) and the expected amplicon sizes are detailed below.

Gene	Forward primer	Reverse primer	Expected amplicon size
CD1D	CCCTGAGAAAGTGACCTTGG	TGGCTGTTAGCTTTCAGTTCC	166
GAPDH	CCGGGAGAAGCTGAGTCATG	TTTGCGGTGGAAATGTCCTT	51
HOXA2	AGGAAAGATTTTGGTTGGGAAG	AAAAAGAGGGAAAGGGACAGAC	138

Appendix D

This section will describe preliminary work performed in using the 5TGM1 cell line as a means to investigate the biological significance of the loss of CD1d in the disease multiple myeloma.

5TGM1 cell line

5T33 myeloma cells arose spontaneously in aged C57BL/KaLwRij mice. (approximately 1 in 200 mice) and produced characteristic features of the human disease, including monoclonal gammopathy, bone disease and organ infiltration in late disease (Radl et al., 1988). A 5T33 cell line was derived from these cells (Manning et al., 1992), and injection of these cells into normal C57BL/KaLwRij mice can recapitulate features of multiple myeloma. The 5TGM1 cell line is a GFP-expressing myeloma cell line derived from 5T33, which in particular produces extensive myeloma bone disease (Garrett et al., 1997).

The biological significance of CD1d expression or loss on these murine myeloma cell lines has not previously been investigated. It is of note that, in contrast to traditional myeloma xenograft models which have utilised immunosuppressed murine strains such as NOD scid gamma, the ability of

5T33 and 5TGM1 myeloma cells to propagate in an imunocompetent C57BL/KaLwRij host allows the CD1d-iNKT axis to be studied *in vivo*.

Figure 7.1 shows GFP expression and surface CD1d expression of the 5TGM1 cell line by flow cytometry (see Chapter 2, section 2.4.5). It can be seen that these cells exhibit both high GFP and surface CD1d expression.

The expression of CD1d by these myeloma cells thus offers an opportunity to investigate the role of CD1d in disease propagation. Specifically, by knockdown of murine *Cd1d1*, the propagation of $CD1d^{hi}$ and $CD1d^{lo}$ cells, both *in vitro* and *in vivo* can be compared.

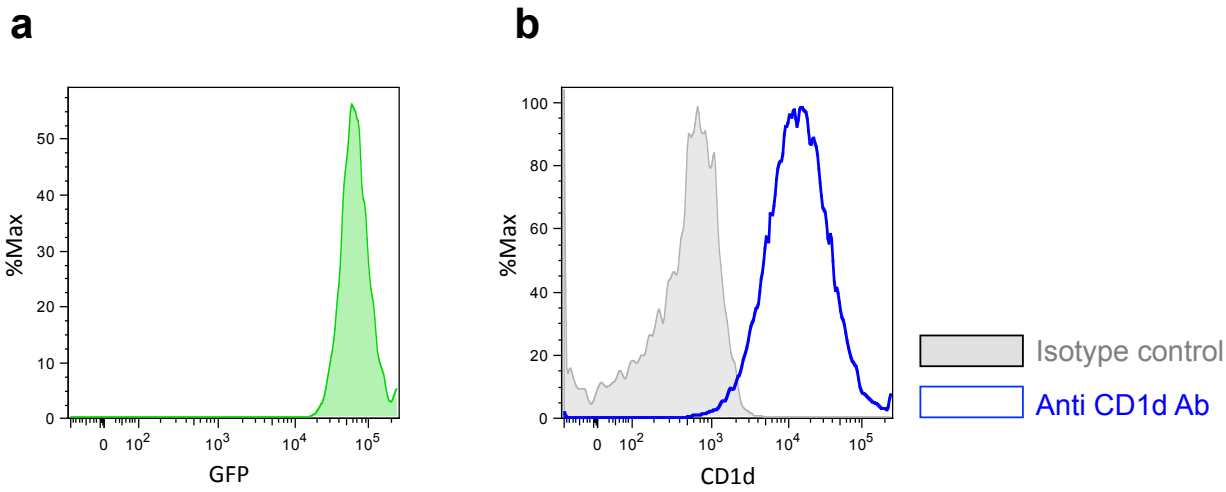


Figure 7.1. Baseline characteristics of 5TGM1 cell line

(a) FACS histogram showing strong GFP expression in 5TGM1 cells

(b) FACS histogram showing strong CD1d expression in 5TGM1 cells (staining with anti-CD1d antibody shown in comparison to isotype control antibody)

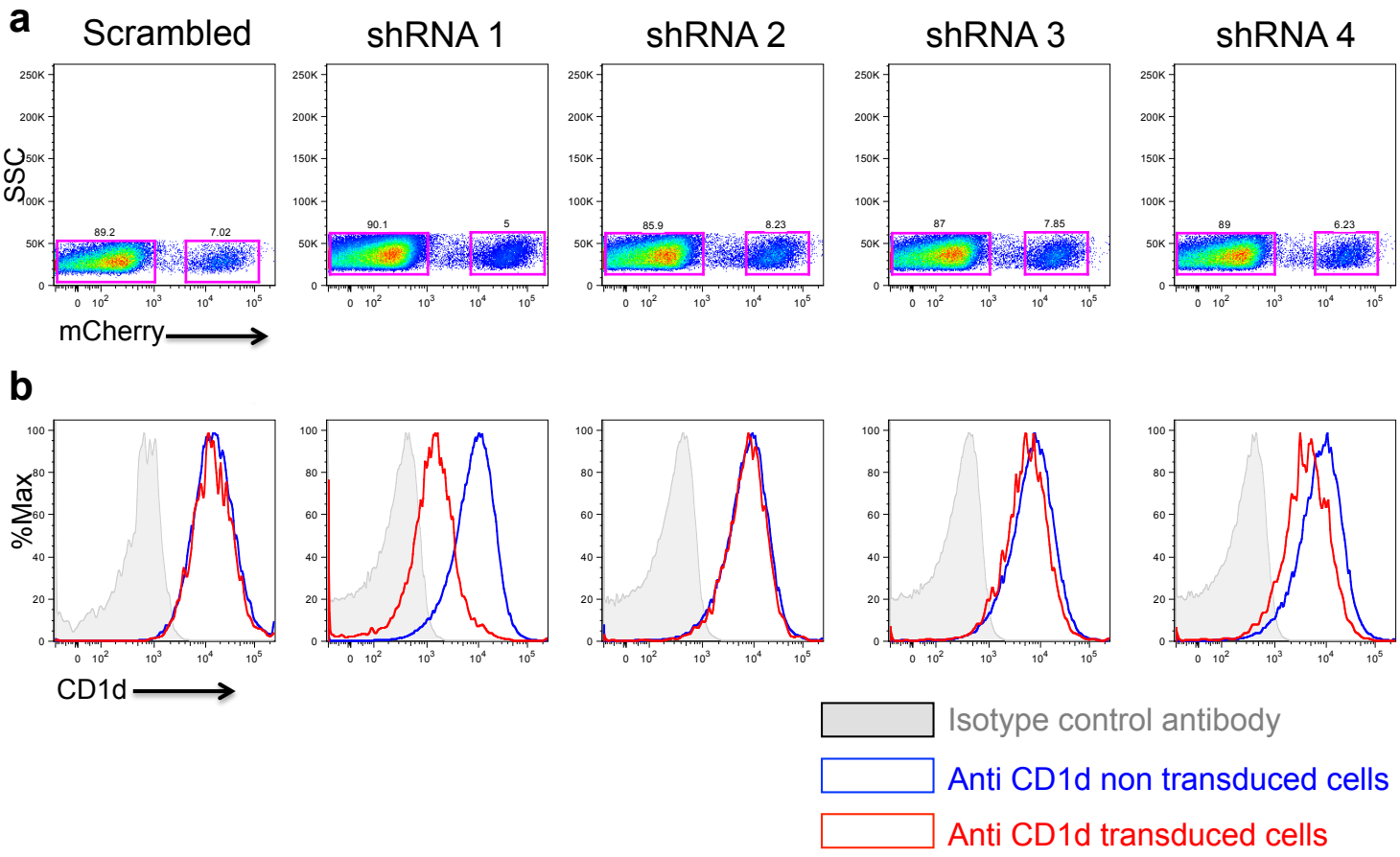
(n=3)

Knockdown of Cd1d1 in the 5TGM1 cell line

In order to knockdown murine *Cd1d1*, shRNA technology was employed, by using a commercially available shRNA set against *Cd1d1*. A full description of this method can be found in Chapter 2, sections 2.7.3 and 2.7.4. **Figure 7.2** shows surface CD1d expression (as assessed by flow cytometry) of the 5TGM1 cell line following transduction with either scrambled shRNA or shRNA directed against *Cd1d1* (shRNAs 1-4). In particular, shRNA1 is shown to produce the highest degree of *Cd1d1* knockdown, with approximately a 10 fold decrease in surface CD1d expression. This is consistent with evaluation of the transcriptional activity of *Cd1d1* by real time PCR (**Figure 7.3**). Although all shRNAs lead to a decrease in *Cd1d1* mRNA levels, the greatest effect is produced by shRNA1. It is notable that although shRNAs 2-4 produce more than a 50% reduction in *Cd1d1* mRNA, the effect on CD1d surface expression is minimal, indicating the high degree of transcriptional knockdown required to bring about a phenotypic effect.

The ability of shRNA to produce a 10-fold reduction in surface CD1d expression thus allows the propagation of both $CD1d^{hi}$ and $CD1d^{lo}$ 5TGM1 cell lines *in vitro*. These cell lines can be used in future experiments, both *in vitro* and *in vivo*, to investigate the biological significance of CD1d expression in multiple myeloma.

Gated on live cells:



c

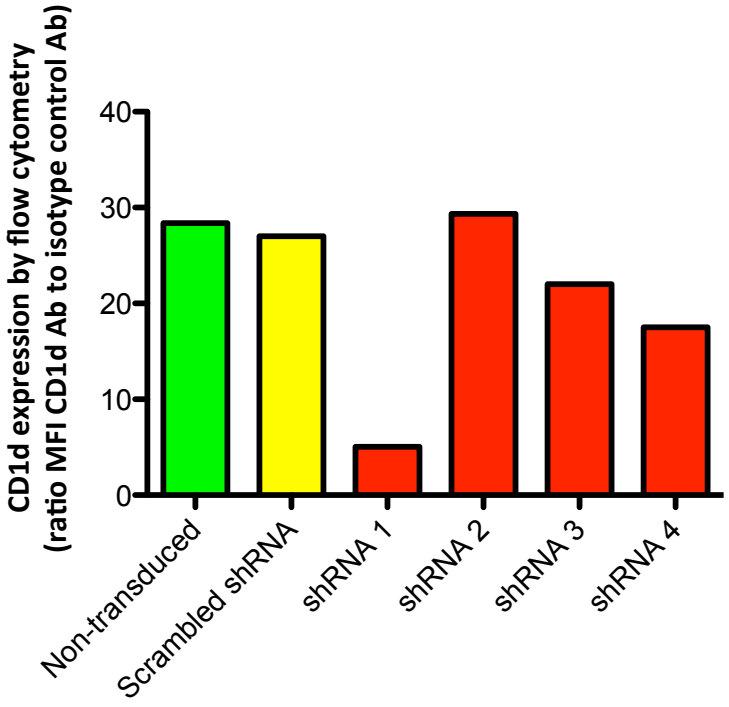


Figure 7.2. Surface CD1d expression of 5TGM1 cell line following shRNA transduction.

(a) FACS plots showing gating strategy to identify transduced cells. Since the 5TGM1 cell line expresses GFP, an shRNA vector expressing mCherry was used. (b) and (c) shRNA1 is shown to produce approximately a 10 fold reduction in surface CD1d expression, whereas shRNAs 2-4 produce minimal effect. (n=1)

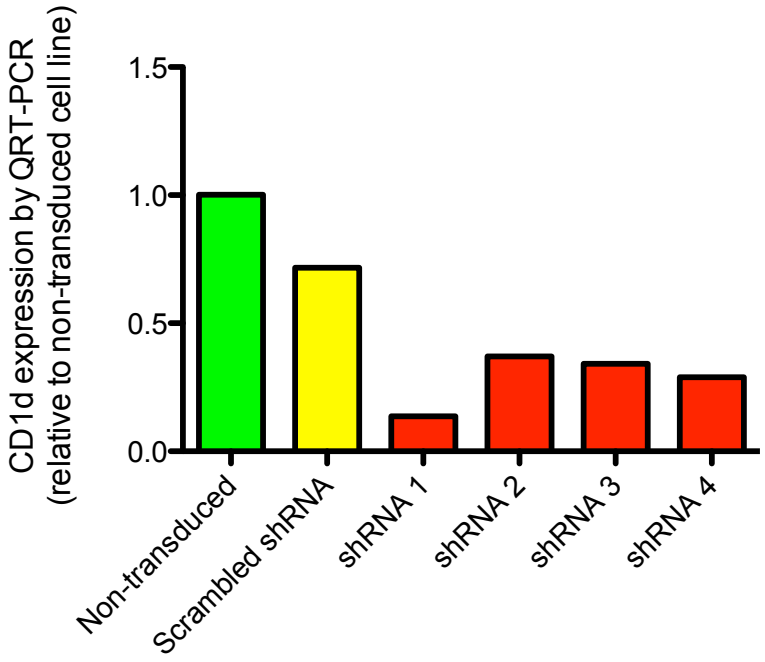


Figure 7.3. Cd1d1 mRNA expression of 5TGM1 cell line following shRNA transduction.

Consistent with the surface expression of CD1d, shRNA1 is shown to produce the highest knockdown of Cd1d1 mRNA. (n=1)

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