UNIVERSITY OF CALIFORNIA, SAN DIEGO

Transcriptional Regulation of Murine B-1 and B-2 Cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Cody John Diehl

Committee in charge:

Professor Joseph L. Witztum, Chair
Professor Ronald M. Evans
Professor Christopher K. Glass
Professor Stephen M. Hedrick
Professor Cornelis Murre

2013

The Dissertation of Cody John Diehl is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____
Chair

University of California, San Diego

2013

DEDICATION

To my wife Emilee,

for her constant encouragement and loving support.

and

To my parents John and Jere,

for instilling in me a desire for learning.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I am sincerely grateful to my mentor Joseph Witztum, who has given me patient and insightful guidance at every stage during my studies. His kind encouragement has built my confidence and helped me to explore new ideas and goals. I am also very grateful for his thoughtfulness and kindness directed toward me at all times but especially at the time I became a father.

I'd also like to thank my committee members for their time and helpful discussions: Professor Christopher Glass who opened the doors of his lab to me and provided invaluable advice and resources that made many of my experiments possible, Professor Ron Evans who facilitated the nuclear receptor atlas experiment, and Professors Cornelis Murre and Stephen Hedrick for their insightful advice with regard to lymphocyte biology and experimentation.

Chapter 2, in full, is a reprint of the material with only slight formatting changes from how it appears in Research Resource: Comparative Nuclear Receptor Atlas: Basal and Activated Peritoneal B-1 and B-2 Cells in Molecular Endocrinology 2011. Diehl, Cody J; Barish, Grant D; Downes, Michael; Chou, Meng-Yun; Heinz, Sven; Glass, Christopher K; Evans, Ronald M; Witztum, Joseph L. Molecular Endocrinology 25 (3): 529. The dissertation author was the primary investigator and author of this paper.

Chapter 5, in part, is a reprint of results and materials to which the dissertation author contributed in the following publications:

S. von Vietinghoff, E. K. Koltsova, J. Mestas, C. J. Diehl, J. L. Witztum, K. Ley, Mycophenolate mofetil decreases atherosclerotic lesion size by depression of aortic T-lymphocyte and interleukin-17-mediated macrophage accumulation. *J Am Coll Cardiol* 57, 2194-2204 (2011).

Y. V. Mendez-Fernandez, B. G. Stevenson, C. J. Diehl, N. A. Braun, N. S. Wade, R. Covarrubias, S. van Leuven, J. L. Witztum, A. S. Major, The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. *Atherosclerosis* 214, 73-80 (2011),

The dissertation author was a co-author on these papers and only the results to which he contributed are presented herein.

Minna Kaikkonen and especially Nathan Spann from Dr. Christopher Glass' lab were extremely helpful and generous with their time as they taught me how to perform and analyze RNA- and ChIP-seq experiments. I am very grateful for their help and friendship. Dr. Grant Barish from Dr. Ron Evans' lab was also very helpful and instructive as I performed the B-cell nuclear receptor atlas. His help was very much needed and appreciated.

I'd also like to thank and recognize the help, advice, support, kindness, and training I received from my coworkers in the Wiztum lab. Dr. Sandy Chou helped me initiate my thesis project, taught me many of the techniques I still use on a daily basis, and offered timely and helpful mentorship during the initial stages of my dissertation. Fely Almazan has been my 'go-to-person' for technical advice in the lab and she has always been generous with her expertise, time, and even her homemade food. Drs. Ayelet Gonen and Soo-Ho Choi have provided helpful guidance and friendly discussions on a daily basis. I'd also like to acknowledge the help and/or guidance I received from Karsten Hartvigsen, Philipp Wiesner, Erica Montano, Xchu Que, Jennifer Pattison, Richard Elam, Maureen Hargrave, Andrew Li, Carole Banka, Yury Miller, and Sam Tsimikas.

Lastly I'd like to express deep gratitude to my family and especially my

wife Emilee Diehl for her constant love, support, and encouragement. Her confidence and belief in me helped me on a daily basis throughout graduate school. I would like to thank my parents, John and Jere Diehl for their support and love and for teaching me to love nature and embrace curiosity.

CURRICULUM VITAE

EDUCATION

2013 Ph.D., Biomedical Sciences, University of California San Diego

2007 B.S., Physiology, University of Arizona, Tucson, Arizona

HONORS AND AWARDS

American Heart Association Predoctoral Fellowship (2011)

Graduated Summa Cum Laude from University of Arizona (2007)

Academic Honors from University of Arizona: Honorable Mention (2001, 2002, 2004, 2007), Dean's List with Distinction (2002, 2006).

PUBLICATIONS

Sloniger JA, Saengsirisuwan V, **Diehl CJ**, Kim JS, Henriksen EJ. Selective angiotensin II receptor antagonism enhances whole-body insulin sensitivity and muscle glucose transport in hypertensive TG(mREN2)27 rats. *Metabolism-Clinical and Experimental.* 2005;54(12):1659-1668.

Sloniger JA, Saengsirisuwan V, **Diehl CJ**, Dokken BB, Lailerd N, Lemieux AM, Kim JS, Henriksen EJ. Defective insulin signaling in skeletal muscle of the hypertensive TG(mREN2)27 rat. *American Journal of Physiology-Endocrinology and Metabolism.* 2005;288(6):E1074-E1081.

Lemieux AM, **Diehl CJ**, Sloniger JA, Henriksen EJ. Voluntary exercise training enhances glucose transport but not insulin signaling capacity in muscle of hypertensive TG(mREN2)27 rats. *Journal of Applied Physiology.* 2005;99(1):357-362.

Ort T, Gerwien R, Lindborg KA, **Diehl CJ**, Lemieux AM, Eisen A, Henriksen EJ. Alterations in soleus muscle gene expression associated with a metabolic endpoint following exercise training by lean and obese Zucker rats. *Physiological genomics.* 2007;29(3):302-311.

Henriksen EJ, Teachey MK, Lindborg KA, **Diehl CJ**, Beneze AN. The high-fat-fed lean Zucker rat: a spontaneous isocaloric model

of fat-induced insulin resistance associated with muscle GSK-3 overactivity. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology.* 2008;294(6):R1813-R1821.

Muellenbach EA, **Diehl CJ**, Teachey MK, Lindborg KA, Archuleta TL, Harrell NB, Andersen G, Somoza V, Hasselwander O, Matuschek M. Interactions of the advanced glycation end product inhibitor pyridoxamine and the antioxidant -lipoic acid on insulin resistance in the obese Zucker rat. *Metabolism: clinical and experimental.* 2008;57(10):1465.

Muellenbach EM, **Diehl CJ**, Teachey MK, Lindborg KA, Hasselwander O, Matuschek M, Henriksen EJ. Metabolic interactions of AGE inhibitor pyridoxamine and antioxidant alpha-lipoic acid following 22 weeks of treatment in obese Zucker rats. *Life Sciences.* 2009;84(15-16):563-568.

Sun J, Hartvigsen K, Chou MY, Zhang Y, Sukhova GK, Zhang J, Lopez-Ilasaca M, **Diehl CJ**, Yakov N, Harats D, George J, Witztum JL, Libby P, Ploegh H, Shi GP. Deficiency of Antigen-Presenting Cell Invariant Chain Reduces Atherosclerosis in Mice. *Circulation.* 2010;122(8):808-820.

Wiesner P, Choi SH, Almazan F, Benner C, Huang W, **Diehl CJ**, Gonen A, Butler S, Witztum JL, Glass CK, Miller YI. Low Doses of Lipopolysaccharide and Minimally Oxidized Low-Density Lipoprotein Cooperatively Activate Macrophages via Nuclear Factor {kappa} B and Activator Protein-1: Possible Mechanism for Acceleration of Atherosclerosis by Subclinical Endotoxemia. *Circulation Research.* 2010;107(1):56-65.

Mendez-Fernandez YV, Stevenson BG, **Diehl CJ**, Braun NA, Wade NS, Covarrubias R, van Leuven S, Witztum JL, Major AS. The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. *Atherosclerosis.* 2011;214(1):73-80.

Miller YI, Choi SH, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, Boullier A, Gonen A, **Diehl CJ**, Que X, Montano E, Shaw PX, Tsimikas S, Binder CJ, Witztum JL. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res.* 2011;108(2):235-248.

Diehl CJ, Barish GD, Downes M, Chou MY, Heinz S, Glass CK, Evans RM, Witztum JL. Research Resource: Comparative Nuclear

Receptor Atlas: Basal and Activated Peritoneal B-1 and B-2 Cells. *Mol Endocrinol.* 2011.

von Vietinghoff S, Koltsova EK, Mestas J, **Diehl CJ**, Witztum JL, Ley K. Mycophenolate mofetil decreases atherosclerotic lesion size by depression of aortic T-lymphocyte and interleukin-17-mediated macrophage accumulation. *J Am Coll Cardiol.* 2011;57(21):2194-2204.

Barish GD, Yu RT, Karunasiri MS, Becerra D, Kim J, Tseng TW, Tai LJ, Leblanc M, **Diehl C**, Cerchietti L, Miller YI, Witztum JL, Melnick AM, Dent AL, Tangirala RK, Evans RM. The Bcl6-SMRT/NCoR cistrome represses inflammation to attenuate atherosclerosis. *Cell Metab.* 2012;15(4):554-562.

Tsiantoulas D, **Diehl C**, Witztum J, Binder CJ. B Cells and Humoral Immunity in Atherosclerosis. *Circulation Research.* 2014;in press.

ABSTRACT OF THE DISSERTATION

Transcriptional Regulation of Murine B-1 and B-2 Cells

by

Cody John Diehl

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor Joseph L. Witztum, Chair

Atherosclerosis is hallmarked by hypercholesterolemia but its progression is greatly modulated by the immune system. B cells, which produce antibodies, have been shown to positively or negatively affect atherogenesis (chapter 5 details various collaborative studies assessing the role of B-cells in various models of atherosclerosis). B-1 cells, which are innate-like B cells, have often been shown to inhibit atherosclerosis progression through secretion of natural antibodies, while adaptive B-2 cells have demonstrated both protective as well as injurious properties. B-1 and B-2 cells differ markedly in ontogenic, genetic, and phenotypic properties. While B-2 cell regulation has been extensively studied, the regulation of B-1

cells remains relatively mysterious. The overarching theme of this dissertation is to understand the transcriptional mechanisms that govern the unique properties of B-1 cells.

Members of the nuclear receptor superfamily of transcription factors regulate important processes in many immune cells. However the expression and function of nuclear receptors in B-1 cells was entirely unknown. I hypothesized that members of the nuclear receptor superfamily would be expressed in and regulate important innate immune functions of B-1 cells, such as survival, proliferation, and secretion of natural antibodies. Nuclear receptor expression was compared between B-1 and B-2 cells.

The function of the mineralocorticoid, glucocorticoid, and liver x receptors, which were expressed in B-1 cells, was analyzed *ex vivo.* No significant effect of mineralocorticoid receptor activation or antagonism was found in B-1 cells. LXR activation inhibited B-1 cell proliferation. Glucocorticoid receptor activation inhibited antibody production and potently induced apoptosis in B-1 cells.

The effect of dexamethasone, a synthetic glucocorticoid, upon the transcriptome B-1 and B-2 cells was compared. Surprisingly different sets of genes were regulated by dexamethasone in B-1 and B-2 cells. An analysis of the cistrome of GR was performed to test if the glucocorticoid receptor has unique genome-wide binding patterns between B-1 and B-2 cells. The GR cistrome was remarkably different between B-1 and B-2 cells. The unique binding of GR in B-1 and B-2 cells associated with cell-type specific enhancers. Future studies endeavor to identify cell-type specific lineage determining factors that control enhancer formation is B-1 versus B-2 cells

CHAPTER 1

INTRODUCTION

Atherosclerosis and inflammation

Atherosclerosis is defined as a hardening of the medium or large arteries characterized by the accumulation of fatty plaques in the artery wall. Sequelae of atherosclerosis include myocardial infarction, heart failure, peripheral vascular disease, and stroke (1). Together, these complications of atherosclerosis are the leading cause of death in the United States. Atherosclerosis develops over decades and is a condition principally characterized by elevated circulating cholesterol levels but is also marked by chronic inflammation of the arterial wall. The idea that atherosclerosis is a chronic inflammatory disease has become widely accepted and is supported by studies illustrating the capacity of the innate and adaptive immune system to alter its initiation and progression (2). These immune responses are targeted toward endogenously modified self-structures, exemplified by oxidized lipoproteins such as LDL. LDL enters the arterial intima where it is retained and oxidized. Oxidized LDL (OxLDL) induces vascular cells to release proinflammatory and chemotactic molecules, which attract lymphocytes and monocytes. As a result of OxLDL and its oxidized lipids, monocytes differentiate to macrophages, which now expresses enhanced content of so called “scavenger receptors.” Uptake of native LDL by LDL-receptors of macrophages is slow and does not lead to enhanced cholesterol accumulation as LDL receptor expression is down regulated by cholesterol accumulation. In contrast, OxLDL is specifically recognized by SRs, leading to enhanced uptake and as SR are not down-regulated by cholesterol content, such internalization of OxLDL ultimately leads to the formation of cholesterol-enriched “foam cells.”

Atherosclerosis is characterized by localized accumulation in the artery wall not only of modified LDL, but also of apoptotic cells, and cellular debris.

The involvement of the innate immune system in atherosclerosis functions to mediate the removal of oxidatively modified molecules, which are proinflammatory and proatherogenic. Wherease the involvement of the adaptive immune system can be categorized as an autoimmune-like response elicited by chronic tissue damage that alters self-molecules. These neo-self epitopes can be recognized by the antigen receptors of various lymphocytes. Antigen presenting cells (APCs) serve as a bridge between the innate and adaptive immune systems by recruiting antigen-specific adaptive immune T- and B-cells.

Lymphocytes and Atherosclerosis

Various immune cells have been observed within atherosclerotic lesions, including monoycytes/macrophages, dendritic cells, T cells, Natural Killer (NK) T cells, NK cells, and mast cells. Notably neutrophils and B cells have only rarely been described in atherosclerotic lesions (3,4). However, B cells have been found in the adventitia surrounding lesions and in draining lymph nodes (5). Lymphocytes as a whole are known to significantly modulate the initiation and progression of atherosclerosis (6). Immunodeficient mice with impaired B- and T- cell development, such as $Rag1^{-/-}$ or scid/scid mice, when crossed with mice that are genetically disposed to develop atherosclerosis, such as the $ApoE^{-/-}$ or $LDLR^{-/-}$ mice, show significantly reduced arterial plaque formation (7-10). These findings illustrate that lymphocytes are not necessary for the development of atherosclerosis, but importantly also shows that lymphocytes can play a profound effect in promoting lesion development. In some of these studies the atheroprotective effect of lymphocyte deficiency was lost as the mice aged or as plasma cholesterol levels became excessively high, indicating that lymphocytes may exert the most significant effect in the early stages of lesion development. When the atherogenic pressure of plasma

cholesterol is excessively high the profound modulatory role of lymphocytes appears to be lost. Among lymphyocyte subsets the majority of research in atherosclerosis has focused upon T cells. The current understanding is that T_H1 cells are strongly proatherogenic whereas T_{REG} cells are atheroprotective (11). The influence of T_H2 and T_H17 cells upon the pathophysiology of atherosclerosis remains unclear or ambiguous. Until relatively recently there was comparatively little focus upon the role of B cells and the antibodies that they secrete in atherosclerosis.

Initial research indicated that B cells in general were atheroprotective, but recent research indicates that various subsets of B cells appear to influence lesion development in different ways. The adaptive immune B-2 cell, which secretes predominantly IgG antibodies, appears to be proatherogenic whereas the innate-like B-1 cell, which secrete predominantly IgM isotype antibodies, may protect against plaque development. The role of other B-cell subsets such as regulatory, marginal zone, or the very recently described Innate Response Activator (IRA) B cells (12) in atherosclerosis have not yet been studied. Our laboratory first showed that immunization of rabbits and later mice with a model of OxLDL lead to atheroprotection, an observation soon confirmed by others (13-15). This lead us to suggest that a vaccine approach that enhanced B cell mediated IgG production to OxLDL could provide atheroprotection. Subsequently, Caligiuri et al. (16) demonstrated that splenectomy increased atherosclerosis in apoE mice, supporting an anti-atherogenic role of immune mechanisms. They went on to show that adoptive transfer of B cells from wild type mice rescued the increased atherosclerosis caused by splenectomy, while transfer of “educated” B cells from aged, atherosclerotic $ApoE^{-/-}$ mice significantly reduced lesion burden even compared to both sham op-

erated control mice. Interestingly, the adoptive transfer of whole splenocytes and T cells from atherosclerotic ApoE$^{-/-}$ mice also significantly reduced lesion burden in this study. However, the adoptive transfer of T cells reduced lesion burden only to the level of sham controls and had no additional protective function like the transfer of B cells from old *Apoe-/-* mice. Additionally, it was shown that the adoptive transfer of B cells resulted in a large increase in IgM and IgG titers to oxLDL in splenectomized mice. This strongly indicated that B cells and the antibodies they produce harbor protective immunity in the context of atherosclerosis. To directly examine the role of B cells in atherosclerosis Major et al. (17) transferred bone marrow from μMT mice, which have the gene for the μ-chain of the B cell receptor, disrupted and therefore lack B cells, into *Ldlr*$^{-/-}$ mice. As expected the lack of B cells led to a sharp decrease and eventual loss of total and specific anti-OxLDL antibodies. The absence of B cells and/or their secreted antibodies increased the lesion area at both early and late stages of atherosclerotic plaque development. In addition, a recent study from Doran et al. demonstrated that homing of B-2 cells to the aorta was also atheroprotective (18). These studies provide convincing evidence that B cells, including B-2 cells, protect from atherosclerosis.

However, other recent reports have provided evidence that B-2 cells are in fact proatherogenic. It was hypothesized that if B cells are atheroprotective their depletion would exacerbate the development of atherosclerosis. Using anti-CD20 monoclonal antibodies to deplete B cells from *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice (19,20) these investigators found unexpectedly that B cell depletion significantly decreased atherosclerosis. The authors of these studies explored possible explanations for the discrepancy between their data and the earlier reports for an atheroprotective role for B cells. They postulated that two major B cell sub-

sets, B-1 and B-2 cells have opposing activities with respect to atherogenesis. Ait-Oufella et al. reported that treatment with CD20 antibody caused a large reduction in total and specific IgG anti-OxLDL titers whereas IgM anti-OxLDL titers were only minimally perturbed (19). As B-1 cells are the major producers of IgM antibodies, it would appear that they were resistant to the depletion treatment protocol. In fact the authors found that peritoneal B-1 cells were retained to a greater extent than B-2 cells. Furthermore, others have shown in mice that the peritoneal cavity, the primary body location where B-1 cells are found, provides a protective niche for B cells during anti-CD20 immunotherapy (21). Kyaw et al. also hypothesized that the mechanism whereby anti-CD20 reduces atherosclerosis is by selective depletion of B-2 cells (20). They tested this by adoptively transferring B-2 cells into lymphocyte deficient ($RAG2^{-/-}$) or B cell deficient ($\mu MT^{-/-}$) *Apoe*$^{-/-}$ double knockout mice and found that B-2 cells in both cases significantly and profoundly increased the severity of atherosclerosis. Interestingly, they also adoptively transferred B-1 cells into $RAG2^{-/-}$ *Apoe*$^{-/-}$ mice and found that atherosclerosis was unchanged.

Thus, at present there is data to support both a protective as well as proatherogenic role of B-2 cells and further studies are needed to clarify the current contradictory data. On the other hand, most data supports an atheroprotective role of the B-1 cell population. For example, IgM secretory deficient mice (sIgM-/-), which have been genetically engineered to lack the capacity to secrete IgM but retain IgM surface expression on B cells, have been bred to the *Ldlr-/-* background . When fed a high cholesterol diet to induce atherosclerosis, the *sIgM-/-* x *Ldlr-/-* mice had dramatically more atherosclerosis than *Ldlr-/-* controls, demonstrating a profound anti-atherogenic role for plasma IgM (22). In yet another study, Kyaw et al. showed that transferring B-2 cells into

splenectomized mice did not rescue the enhanced atherosclerosis, but transfer of B-1 cells did (23). Furthermore, if B-1 cells from sIgM-/- mice were used, this protective effect was lost, again supporting a direct atheroprotective role of IgM. These studies strongly support an atheroprotective role for circulating IgM, which come predominantly from B-1 cells.

In summary, there is conflicting data on the role of B-2 cells in atherogenesis while the role of B-1 cells seems to be atheroprotective. It is possible and even likely that some of these discrepancies are due to varying subsets of B-2 cells and even B-1 cells. Increasingly subsets of B-2 cells are being defined, such as marginal zone B cells vs classical germinal center follicular B cells, as well as B regulatory cells, IgM+ Memory cells, naïve B cells and so forth. Similarly, various subsets of B-1 cells are beginning to be described, such as IRA B-1 cells (12), as well as B-1 cells that express plasma cell alloantigen 1 (PC1) differentially. PC1 high B-1 cells differ from B1 low B-1 cells in their origin, ability to secrete NAbs and IL-10 (24). Much work will be needed to clarify the roles of each of these B-2 and B-1 cell subsets on inflammation and atherogenesis to enable a more comprehensive understanding of the role of B cells in atherogenesis.

B-1 and B-2 Cells

Murine B lymphocytes are a heterogeneous population consisting of several defined subtypes, including among others, B-1 cells and B-2 cells (conventional B-cells). These B cell subpopulations can be distinguished on many levels, including: developmental patterns, extent of somatic recombination of immunoglobulin genes, antigen specificity, secreted antibody isotype profile, mode of selection and renewal, preferred anatomical location, surface phenotype, activation requirements, and function (see Table 1-1) (reviewed in

(25)). Despite these fundamental differences, relatively little is known of the basic cellular regulatory mechanisms of B-1 cells, and what distinguishes them from B-2 cells at this level. B-1 cells were so named because among B cells they appear first in development and are detectable in fetuses and neonates, whereas B-2 cells are not detectable until the end of the neonatal period (25). Until recently, it was thought that B-1 and B-2 cells derive from the same progenitor and that antigen specificity dictated their distinct phenotypes. Montecino-Rodriguez et. al (26) refuted this model by identifying a B-1 cell specific progenitor in fetal bone marrow, thereby demonstrating a distinct developmental pathway for B-1 cells. Furthermore, Wang and colleagues have further suggested distinct lineages even for B-1 cell subsets based on their expression of PC1 (24).

Table 1-1 Comparison of Murine B-1 and B-2 Cells

Class of Difference	Property	B-1 Cells	B-2cells
Genotypic	N-insertions in VDJ junctions	Few	Extensive
	V-gene usage	Non-random preference for D proximal V-genes	Apparently random
	V-region repertoire	Restricted	Diverse
	Somatic Hypermutation	Low-none	High
	Isotype secreted	IgM >> IgG	IgG >> IgM
Ontogenic	When first produced	Fetus	After birth
	Mode of Renewal	Self-renewing	Replaced from Bone marrow
	Mode of Selection	Positive selection (for endogenous antigens)	Negative Selection
Phenotypic/ Functional	Cell surface Phenotype (spleen)	$CD5^{+/-}$, $CD19^{hi}$, $CD1d^{mid}$, $CD23^{-}$, $CD43^{+}$, IgM^{hi}, IgD^{low},	$CD5^{-}$, $CD19^{mid}$, $CD1d^{hi}$, $CD21^{-}$, $CD23^{+}$, $CD43^{-}$, IgM^{low}, IgD^{hi}
	Primary location	Body Cavities (peritoneal, pleural)	Secondary lymphoid organs
	Spontaneous production of Immunoglobulin	High	Low
	Response to Carbohydrate antigen	Yes	Maybe
	Response to Protein Antigen	Maybe	Yes
	Requirement for T-cell help	No	Yes

Unlike B-2 cells, B-1 cells exhibit a limited repertoire of germline or near-germline encoded antigen receptors with little or no terminal deoxynucleotidyl transferase mediated nucleotide insertions (27,28). The antibodies secreted by B-1 cells are termed natural antibodies (NAbs) and by definition are secreted in the absence of external antigenic or immunogenic stimuli (reviewed in (25)). NAbs secreted by B-1 cells are predominantly but not exclusively IgM isotype (29,30) and they often have dual reactivity, binding to both self-antigens and antigens expressed on infectious pathogens. Thus, individual NAbs have broad but low-affinity to both self-antigens and antigens expressed on pathogens such as phosphocholine (31), phosphatidlylcholine (32), OxLDL (33), glycolipids (34), and carbohydrate epitopes (35). Our laboratory has shown that in some cases, the ability of a NAb to bind to both “self” and a pathogen is in fact due to the recognition of a common molecular motif present on a self-antigen and a foreign pathogen, such as the phosphocholine (PC) present on oxidized phospholipids of OxLDL and apoptotic cells, and the PC present on the cell wall of bacteria (36).

The mechanism of B-1 cell selection is incompletely understood, nevertheless it is appreciated that positive selection occurs during fetal and neonatal periods (37). This positive selection is thought to be made to endogenous ‘self-antigens’ because it occurs equally well in mice raised in germ free environments (38). The fact that NAbs bind to similar molecular motifs on exogenous antigens would serve as a further selecting agent later in life (39,40). In sharp contrast, developing self-reactive B-2 cells encountering cognate antigens undergo negative selection.

B-1 cells from adult mice adoptively transferred into irradiated recipient mice are able to reconstitute the B-1 cell population to normal levels, demon-

strating that B-1 cells are self-replenishing (41,42). In contrast, adoptively transferred adult B-2 cells are incapable of sustained reconstitution of recipient mice, only transfer of adult bone marrow can achieve this (41). Furthermore, B-1 cells are long-lived *in vitro* and constitute approximately 5% of all B cells (43). They are almost exclusively found in extra-lymphoid sites, predominantly the peritoneal cavity and pleural spaces (25,43). In the peritoneal cavity, B-1 cells are $CD19^{+}$, $CD23^{-}$, IgM^{hi}, IgD^{lo} and $Mac\text{-}1^{int.}$ (also known as CD11b) (36). When activated, B-1 cells migrate to the spleen and other secondary lymphoid tissues, lose Mac-1 expression and become $CD43^{+}$ (43). B-1 cells were first discovered and characterized according to their expression of the T-cell surface marker CD5 (44). Subsequently, a peritoneal B-1 cell subset was discovered lacking CD5 expression (45). $CD5^{+}$ B cells are referred to as B-1a cells and $CD5^{-}$ B-1 cells as B-1b cells. B-1a and B-1b cells differ in cellular origins (B-1b progenitors reside predominantly in adult and fetal bone marrow and B-1a progenitors in the fetal liver (46)), NAb specificity (46), and function (32). However, our laboratory has preliminary data and others have reported that B-1a cells can derive from adult bone marrow as well (47).

Conventionally, B-2 cells function as part of the adaptive immune response and produce high titers of high affinity antibodies specific for particular epitopes. B-1 cells as part of the innate immune system express and secrete “germline” encoded and purported broadly specific NAbs. NAbs and B-1 cell receptors are analogous to other receptors of the innate immune system in that they can bind to common pathogenic molecular motifs (so-called pathogen associated molecular patterns (PAMPs). Unlike B-2 cells, B-1 cells respond poorly to IgM cross-linking (48) and T-cell dependent antigens (43). Furthermore, B-1 cells have been shown to have superior capacity *in vitro* to

function as antigen presenting cells (49,50). B-1 cells also exhibit the macrophage-like ability of phagocytosis utilizing mannose and Fc receptors (50,51). Moreover, B-1 cells demonstrate a greater proliferative response when stimulated with lipopolysaccaride (52) (LPS, a TLR4 agonist) or TLR2 agonists (53) Furthermore, recent evidence has demonstrated that B-2 and B-1 cells differ quantitatively but not qualitatively in the expression of most of the murine TLRs and that stimulation of the TLRs in B-1 cells preferentially leads to plasma cell differentiation (53). Additionally, there is evidence that B-1 cells can fulfill an immunomodulatory role aside from their role in innate immunity. B-1 cells respond to various stimuli by secreting important regulatory cytokines such as Interleukin 10 (IL-10) (54). Additionally, recent reports have identified a so-called B Regulatory (Breg) cell that secretes IL-10 (55,56). The relationship of these cells, which are also referred to as B10 cells because of their production of IL-10, to B-1 cells is unknown, though they express similar cell surface markers including CD5 (25). IL-10 can suppress harmful immune responses by regulating the Th1/Th2 balance and directly down-regulating innate cell-mediated inflammatory responses (57). Furthermore, our lab has demonstrated that IL-10 can inhibit B-1 cells in an autocrine feedback loop, thereby decreasing B-1 cell proliferation and IgM secretion (unpublished results).

Besides their role in innate immune defense to common bacterial and viral pathogens, B-1 cells have been implicated with both positive and negative roles in various autoimmune conditions, inflammatory diseases (including atherosclerosis as discussed above), and human B-cell leukemias. Expanded B-1 cell-like populations have been observed in human autoimmune patients with Rheumatoid Arthritis and Sjogren's Syndrome (58). The NZB mouse, a model of Systemic Lupus Erythematosus (SLE) also possesses an

expanded B-1 cell population (59). Selective elimination of B-1 cells from the peritoneal cavity of NZB mice decreases the titers of anti-dsDNA IgM and IgG antibodies and the occurrence of lupus associated nephritis (60).

Our laboratory has shown that NAbs from B-1 cells that bind to OSE of OxLDL exert an anti-atherogenic effect through incompletely understood mechanisms (36,61). However, it is appreciated that many NAbs bind to epitopes on oxidized LDL and inhibit scavenger receptor mediated uptake of OxLDL by macrophages (36). Furthermore, it has been shown that NAbs can bind apoptotic cells, and by virtue of their ability to bind complement, can facilitate their clearance (42,62). Finally, there is extensive data implicating B-1–like cells in B-cell lymphomas and leukemias. A series of prototypic lymphomas isolated from mice possess a restricted set of B-cell receptors that are common to B-1 cells (63). Recent studies have shown that antibodies from human chronic lymphocytic leukemia cells target epitopes on OxLDL and apoptotic cells, which are commonly bound by antibodies from B-1 cells (64,65). Several transgenic mouse models expressing human genes implicated in B-cell neoplasms develop leukemic expansion of B cells that possess B-1 cell phenotypes (66). Indeed, we have recently extensively characterized a prevalent human CLL BCR and shown it binds prominently to MDA epitopes present on OxLDL, atherosclerotic tissue and apoptotic cells, similar to other NAbs to OSE we have cloned (67). Interestingly, a human equivalent to the mouse B-1 cell has only recently been identified based upon the criteria of spontaneous IgM secretion, efficient T cell stimulation, low rate of mutation in the BCR, and tonic intracellular signaling (68). Subsequent reports have refuted this claim based upon gene expression profiling and found that these ‘human B-1 cells’ more closely resemble pre-plasmablasts than mouse B-1

cells (69,70). Despite the obvious importance of B-1 cells in disease conditions and healthy immune function, little is known concerning basic signaling and regulatory mechanisms that govern their unique functions.

Nuclear Receptors

The studies undertaken in this doctoral dissertation have generally been to explore the unique regulation of B-1 cells. Specific focus was placed upon exploring the role of nuclear receptors (NRs) in regulating B-1 cells. NRs are a superfamily of structurally conserved ligand dependent transcription factors each possessing canonical DNA binding and ligand binding domains. There are 48 human and 49 mouse NRs. They endow cells with the capacity to directly control gene expression in response to developmental, physiological, and environmental signals (71). As a family they fulfill diverse and important roles in regulating developmental, reproductive, homeostatic, inflammatory, immune, and metabolic processes (72).

There are three broad classes of NRs segregated depending upon ligand- and DNA-binding properties (73). The first and most extensively studied class consists of the classic ligand-driven receptors typified by the estrogen and glucocorticoid receptors. They are generally cytoplasmic, and binding of ligand induces activation, dissociation from chaperone proteins, and translocation to the nucleus and subsequent transcription of target genes (74). The second class is comprised of the “orphan receptors,” which includes a diverse group of receptors for which ligands have not been identified or do not appear to be required. The third class consists of the metabolite-activated NRs or the so-called ‘adopted’ orphan receptors. They form obligatory heterodimers with the retinoid-X-receptor (RXR). Most RXR heterodimers in the absence of ligand are nuclear and bound to DNA and maintain active repression of tar-

get genes through association with co-repressors, histone deacetylases, and other chromatin-modifying factors (75). Upon ligand binding a conformational change of the receptor is initiated that causes the exchange of co-repressors for co-activators leading to the transcription of target genes. Examples include the peroxisome-proliferator-activated receptors (PPARs), the liver X receptors (LXRs), and the RXRs. Of particular interest in the context of innate immunity and B-1 cell biology are the LXRs and PPARs, which have been shown to be important regulators of both metabolic and inflammatory signaling (76).

Apart from regulating transcription through ligand-dependent activation and ligand-independent repression, some NRs can repress transcription in a ligand-dependent manner known as transrepression (73). Mechanisms responsible for transrepression are incompletely understood; even so, transrepression does not involve binding to receptor specific DNA response elements. Several transrepression mechanisms mediated by ligand activated NRs have been reported, including among others: direct interaction with other transcription factors (thereby inhibiting DNA binding of these transcription factors), competition for limiting pools of coactivators, and interactions with transcriptional co-repressors (thereby preventing their degradation and preserving a repressed transcriptional state)(74).

Nuclear Receptors in Immune Cells

Extensive information exists regarding the expression and function of NRs in macrophages due to these cells' central role in antimicrobial defense, atherogenesis, autoimmunity, and other inflammatory diseases. Barish et al. (77) cataloged NR expression in murine macrophages and found that 28 of the 49 murine NRs are expressed with varying temporal patterns in association

with LPS and interferon γ stimulation of bone marrow derived macrophages. This study demonstrated the value of comprehensive cell-type specific expression profiling of the NR regulatory gene family to inform hypothesis-driven approaches for exploring a complex biological process.

On the other hand, few studies have explored the expression and function of NR in lymphocytes, even fewer exclusively in conventional B-cells, and none in B-1 cells. Schote and colleagues (78) analyzed the expression of 24 different NRs in CD4 and CD8 T cells, CD19 B cells, and CD14 monocytes collected from five human blood donors. They provided the first evidence for the expression of 12 NRs in these cell types. A more focused study explored the expression of the various retinoic acid NR isoforms in human B and T cells in the presence of a stimulus (all-trans-retinoic acid) (79). In conventional B cells and B lymphoma cell lines it has been demonstrated that PPARγ agonists can inhibit proliferation and induce apoptosis (80). Conversely, it was demonstrated that haploinsufficency for PPARγ enhances B cell proliferative responses (81). All of the above mentioned studies focused on NR expression in conventional B cells or B cell lymphoma cell lines and failed to specifically address the role of NR expression in B-1 cells. One line of research, however, has found a role for the Steroid and Xenobiotic nuclear orphan receptor (SXR) in negatively regulating B-1 cell development in the fetal liver (82).

Mineralocorticoid, Glucocorticoid, and Liver X Nuclear Receptors

As will be discussed in chapter 3, many of the studies performed as part of this thesis focused upon several of the NRs, namely the Mineralocorticoid Receptor (MR), Glucocorticoid Receptor (GR), and the Liver X receptors (LXR). MR and GR are highly related NRs, they share 94% and 57% amino

acid identity in their DNA binding and ligand binding domains, respectively (83,84). They bind the same DNA response element (84) and have similar binding affinities for several ligands (85). The mechanism of MR or GR specificity is poorly understood, but on a cellular level it is accomplished by the activity of 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), which inactivates glucocorticoids. Cells that strongly express 11βHSD2 selectively metabolize glucocorticoids so that they cannot effectively activate MR or GR, and are therefore selectively sensitive to mineralocorticoids through the MR (86). Traditionally, MR is thought to principally be expressed in the polarized epithelial cells of the distal nephron and colon, where when activated by aldosterone regulates ion homeostasis and water balance (87). MR expression has also been observed in non-epithelial cells such as hippocampal and hypothalamic neurons, cardiomyocytes, keratinocytes, and adipocytes (88). The role of MR in non-epithelial tissues and cells, including immune cells, is not yet well characterized. Nevertheless, several recent studies have explored the role of MR in specific immune cells, including: dendritic cells, macrophages and neutrophils (89-92). MR was found to orchestrate an important regulatory and often pro-inflammatory function in each of these innate immune cells.

The GR is the cellular sensor for glucocorticoids, which exert a wide range of anti-inflammatory and immunosuppressive activities in diverse cell types. GR's anti-inflammatory activity involves both repression of pro-inflammatory genes and the induction of anti-inflammatory molecules as well as the induction of apoptosis (93). The apoptotic effect of GR activation is especially prominent and well characterized in T lymphocytes. Additionally, GR is known to shift T helper cells toward a Th2 bias thereby stimulating humoral immunity (94). Although glucocorticoids are widely used to regulate B cell function in

patients, remarkably little is known of the mechanisms by which glucocorticoids regulate B cells, though there is evidence that they promote IgE production (95).

There are two LXR isoforms, LXRα and LXRβ, together these regulate the expression of genes involved in cholesterol metabolism (96). Naturally occurring cholesterol derivatives such as 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, and 24,25(*S*)-epoxycholesterol have been identified as LXR ligands (97). In a tissue specific manner, LXR agonists activate transcriptional programs that regulate cholesterol trafficking and whole body cholesterol content. LXR is highly expressed in the liver and regulates genes involved in cholesterol secretion into bile and bile-acid synthesis (96). In the intestine, LXR controls the expression of genes involved in cholesterol reabsorption (98). Finally, in peripheral tissues, LXR regulates genes that control reverse cholesterol transport (76). Importantly, LXRs have also been identified as regulators of inflammatory gene expression and innate immunity (74). Ligand activation of LXR antagonizes inflammatory gene expression downstream of TLR4, IL-1β, and tumor necrosis factor- α (TNF-α) signaling (99). Intriguingly, Bensinger et al. (100) recently reported that LXR signaling inhibits lymphocyte proliferation by inducing expression of cholesterol export transporters, thereby limiting cellular cholesterol levels that are needed for membrane synthesis. It is significant to note that this study examined LXR's effects on proliferation predominantly in T cells and to some extent in conventional B cells, but not in B-1 cells.

Specific Background and Research Goals

B-1 cells secrete NAbs, which in turn constitute the humoral branch of the innate immune system. B-1 cells, and especially the NAbs they secrete, have been implicated to play an important role in protection against atherosclerosis. Furthermore, they play important roles in host responses to bacterial and viral infections, as well as in various autoimmune conditions, inflammation, and in humans, B-1-like clones may be involved in B-cell leukemia. Despite their importance in health and disease, relatively little is known about what regulates the unique B-1 cell biology. Members of the nuclear receptor (NR) superfamily fulfill a central role in cellular homeostatic, metabolic, and inflammatory processes, and are likely important for immune cell regulation. Indeed, the functions of many NRs have been defined within select immune cells, particularly the macrophage. However, when I began this work little or nothing was known regarding the expression and function of any of the NRs in B-1 cells. I hypothesized that members of the NR superfamily were expressed in and regulate important innate immune functions of B-1 cells, such as survival, proliferation, and secretion of NAbs. Evidence supporting this hypothesis is presented in Chapters 2, 3, and 4. In Chapter 2, I analyzed the expression of all 49 mouse nuclear receptors in B-1 cells in the basal as well as TLR4 and TLR 2/1 stimulated conditions. Furthermore, I compared the expression of the NRs between B-1 cells, B-2 cells. In Chapter 3, I cultured primary B-1 cells in vitro and utilized readily available agonists and antagonists to explore the role of select nuclear receptors in regulating B-1 cell survival, proliferation, NAb production, differentiation, as well as various other parameters. The nuclear receptors studied in these experiments include the mineralocorticoid, glucocorticoid, and Liver X receptors. In Chapter 4, I focused my attention on the

role of the GR in B-1 and B-2 cells and explored on a genome-wide level the effect of GR activation on mRNA levels as well as GR DNA binding. As will be discussed in greater detail in Chapter 4, these studies utilized B-2 cells as a comparator, and found remarkable disparity between B-1 and B-2 cells in the binding patterns of GR throughout the genome as well as the mRNA levels of many genes. The studies in Chapter 4, which are still ongoing, are designed to establish the epigenetic and transcriptional mechanisms controlling the unique biology of B-1 cells.

Chapter 5 details various collaborations in which I participated, some of which have resulted in publications. Although these studies are not related to the role of NRs in B-1 cells they do explore in general terms the regulation of B-1 cells, the production of NAbs, and their roles in atherosclerosis.

References

1. Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell. 2001;104(4):503-516.

2. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res. 2002;91(4):281-291.

3. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. Circulation research. 2012;110(6):875-888.

4. Zhou X, Hansson GK. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. Scand J Immunol. 1999;50(1):25-30.

5. Watanabe M, Sangawa A, Sasaki Y, Yamashita M, Tanaka-Shintani M, Shintaku M, Ishikawa Y. Distribution of inflammatory cells in adventitia changed with advancing atherosclerosis of human coronary artery. Journal of atherosclerosis and thrombosis. 2007;14(6):325-331.

6. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol. 2008;8(10):802-815.

7. Reardon CA, Blachowicz L, White T, Cabana V, Wang Y, Lukens J, Bluestone J, Getz GS. Effect of immune deficiency on lipoproteins and atherosclerosis in male apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2001;21(6):1011-1016.

8. Song L, Leung C, Schindler C. Lymphocytes are important in early atherosclerosis. J Clin Invest. 2001;108(2):251-259.

9. Dansky HM, Charlton SA, Harper MM, Smith JD. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. Proc Natl Acad Sci U S A. 1997;94(9):4642-4646.

10. Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. Circulation. 2000;102(24):2919-2922.

11. Lichtman AH, Binder CJ, Tsimikas S, Witztum JL. Adaptive immunity in atherogenesis: new insights and therapeutic approaches. J Clin Invest. 2013;123(1):27-36.

12. Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, Tiglao E, Figueiredo JL, Iwamoto Y, Theurl I, Gorbatov R, Waring MT, Chicoine AT, Mouded M, Pittet MJ, Nahrendorf M, Weissleder R, Swirski FK. Innate response activator B cells protect against microbial sepsis. Science. 2012;335(6068):597-601.

13. Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. Proc Natl Acad Sci U S A. 1995;92(3):821-825.

14. Freigang S, Horkko S, Miller E, Witztum JL, Palinski W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. Arterioscler Thromb Vasc Biol. 1998;18(12):1972-1982.

15. George J, Afek A, Gilburd B, Levkovitz H, Shaish A, Goldberg I, Kopolovic Y, Wick G, Shoenfeld Y, Harats D. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. Atherosclerosis. 1998;138(1):147-152.

16. Caligiuri G, Nicoletti A, Poirier B, Hansson G. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. Journal of Clinical Investigation. 2002;109(6):745-753.

17. Major AS, Fazio S, Linton MF. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler Thromb Vasc Biol. 2002;22(11):1892-1898.

18. Doran AC, Lipinski MJ, Oldham SN, Garmey JC, Campbell KA, Skaflen MD, Cutchins A, Lee DJ, Glover DK, Kelly KA, Galkina EV, Ley K, Witztum JL, Tsimikas S, Bender TP, McNamara CA. B-cell aortic homing and atheroprotection depend on Id3. Circ Res. 2012;110(1):e1-12.

19. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vre E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF, Mallat Z. B cell depletion reduces the development of atherosclerosis in mice. J Exp Med. 2010;207(8):1579-1587.

20. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. J Immunol. 2010;185(7):4410-4419.

21. Hamaguchi Y, Uchida J, Cain DW, Venturi GM, Poe JC, Haas KM, Tedder TF. The peritoneal cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in mice. J Immunol. 2005;174(7):4389-4399.

22. Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO. Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation. 2009;120(5):417.

23. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, Bobik A, Toh BH. B1a B lymphocytes are atheroprotective by secreting

natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. Circ Res. 2011;109(8):830-840.

24. Wang H, Shin DM, Abbasi S, Jain S, Kovalchuk AL, Beaty N, Chen S, Gonzalez-Garcia I, Morse HC, 3rd. Expression of plasma cell alloantigen 1 defines layered development of B-1a B-cell subsets with distinct innate-like functions. Proc Natl Acad Sci U S A. 2012;109(49):20077-20082.

25. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat Rev Immunol. 2011;11(1):34-46.

26. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1B cell-specified progenitor. Nature Immunology. 2006;7(3):293-301.

27. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V-H-D-J(H) sequences from B-1a, B-1b, and conventional B cells. Journal of Immunology. 1997;158(3):1175-1186.

28. Li YS, Hayakawa K, Hardy RR. The Regulated Expression of B-Lineage Associated Genes During B-Cell Differentiation in Bone-Marrow and Fetal Liver. Journal of Experimental Medicine. 1993;178(3):951-960.

29. Avrameas S. Natural Autoantibodies - From Horror Autotoxicus to Gnothi Seaunton. Immunology Today. 1991;12(5):154-159.

30. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(5):2250-2255.

31. Masmoudi H, Motasantos T, Huetz F, Coutinho A, Cazenave PA. All T15 ID-Positive Antibodies (but not the majority of VHT15+ Antibodies) are Produced by Peritoneal CD5+ Lymphocytes-B. International Immunology. 1990;2(6):515-520.

32. Hayakawa K, Hardy RR, Honda M, Herzenberg LA, Steinberg AD. LY-1 B-Cells - Functionally Distinct Lymphocytes that Secrete IgM Autoantibodies. Proceedings of the National Academy of Sciences of the United

States of America-Biological Sciences. 1984;81(8):2494-2498.

33. Horkko S, Binder CJ, Shaw PX, Chang MK, Silverman G, Palinski W, Witztum JL. Immunological responses to oxidized LDL. Free Radical Biology and Medicine. 2000;28(12):1771-1779.

34. Kearney JF. Innate-like B cells. Springer Seminars in Immunopathology. 2005;26(4):377-383.

35. Kearney JF, Vakil M, Dwyer DS. Idiotypes and Autoimmunity. Ciba Foundation Symposia. 1987;129:109-122.

36. Binder CJ, Shaw PX, Chang MK, Boullier A, Hartvigsen K, Horkko S, Miller YI, Woelkers DA, Corr M, Witztum JL. The role of natural antibodies in atherogenesis. Journal of Lipid Research. 2005;46(7):1353-1363.

37. Hardy RR, Wei CJ, Hayakawa K. Selection during development of V(H)11(+)B cells: a model for natural autoantibody-producing CD5(+) B cells. Immunological Reviews. 2004;197:60-74.

38. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. European Journal of Immunology. 1997;27(6):1557-1563.

39. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, Witztum JL. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. Journal of Clinical Investigation. 2000;105(12):1731-1740.

40. Vale AM, Kapoor P, Skibinski GA, Elgavish A, Mahmoud TI, Zemlin C, Zemlin M, Burrows PD, Nobrega A, Kearney JF, Briles DE, Schroeder HW, Jr. The link between antibodies to OxLDL and natural protection against pneumococci depends on D(H) gene conservation. J Exp Med. 2013;210(5):875-890.

41. Kantor AB, Herzenberg LA. Origin of Murine B-Cell Lineages. Annual Review of Immunology. 1993;11:501-538.

42. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw

PX, Choi J, Perkmann T, B„ckhed F, Miller YI. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. The Journal of Clinical Investigation. 2009;119(5):1335.

43. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. Annual Review of Immunology. 2002;20:253-300.

44. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR. The Ly-1 B-Cell Lineage. Immunological Reviews. 1986;93:81-102.

45. Stall AM, Adams S, Herzenberg LA, Kantor AB. Characteristics and Development of the Murine B-1B (Ly-1 B-Sister) Cell-Population. Annals of the New York Academy of Sciences. 1992;651:33-43.

46. Herzenberg LA, Tung JW. B cell lineages: documented at last! Nature Immunology. 2006;7(3):225-226.

47. Gu H, Forster I, Rajewsky K. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. The EMBO journal. 1990;9(7):2133-2140.

48. Rothstein TL, Kolber DL. Anti-Ig Antibody Inhibits the Phorbol Ester-Induced Stimulation of Peritoneal B-Cells. Journal of Immunology. 1988;141(12):4089-4093.

49. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. Current Opinion in Immunology. 2001;13(2):195-201.

50. Vigna AFG, Godoy LC, de Almeida SR, Mariano M, Lopes JD. Characterization of B-1b cells as antigen presenting cells in the immune response to gp43 from Paracoccidioides brasiliensis in vitro. Immunology Letters. 2002;83(1):61-66.

51. Almeida SR, Aroeira LS, Frymuller E, Dias MAA, Bogsan CSB, Lopes JD, Mariano M. Mouse B-1 cell-derived mononuclear phagocyte, a novel cellular component of acute non-specific inflammatory exudate. International Immunology. 2001;13(9):1193-1201.

52. Murakami M, Tsubata T, Shinkura R, Nisitani S, Okamoto M, Yoshioka

H, Usui T, Miyawaki S, Honjo T. Oral-Administration of Lipopolysaccharides Activates B-1 Cells in the Peritoneal-Cavity and Lamina Propria of the Gut and Induces Autoimmune Symptoms in an Autoantibody Transgenic Mouse. Journal of Experimental Medicine. 1994;180(1):111-121.

53. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. Journal of Immunology. 2007;178(12):7779-7786.

54. O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. European journal of immunology. 1992;22(3):711-717.

55. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. Immunological Reviews. 2008;224:201-214.

56. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. J Clin Invest. 2011;121(11):4268-4280.

57. Pestka S, Krause CD, Sarkar D, Walter MR, Shi YF, Fisher PB. Interleukin-10 and related cytokines and receptors. Annual Review of Immunology. 2004;22:929-979.

58. Viau M, Zouali M. B-lymphocytes, innate immunity, and autoimmunity. Clinical Immunology. 2005;114(1):17-26.

59. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The Ly-1 B-Cell Subpopulation in Normal, Immunodefective, and Autoimmune Mice. Journal of Experimental Medicine. 1983;157(1):202-218.

60. Murakami M, Honjo T. B-1 Cells and Autoimmunity. Annals of the New York Academy of Sciences. 1995;764(1):402-409.

61. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nature Medi-

cine. 2003;9(6):736-743.

62. Ogden CA, Kowalewski R, Peng YF, Montenegro V, Elkon KB. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. 2005.

63. Pennell CA, Arnold LW, Locascio N, Lutz PM, Willoughby PB, Haughton G. The CH Series of Murine B-Cell Lymphomas - Identification of Cross-Reactive Idiotypes and Restricted Antigen Specificities. Current Topics in Microbiology and Immunology. 1984;113:251-257.

64. Catera R, Silverman GJ, Hatzi K, Seiler T, Didier S, Zhang L, Herv, M, Meffre E, Oscier DG, Vlassara H, Scofield RH, Chen Y, Allen SL, Kolitz J, Rai KR, Chu CC, Chiorazzi N. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol Med.14(11-12):665-674.

65. Myhrinder AL, Hellqvist E, Sidorova E, Soderberg A, Baxendale H, Dahle C, Willander K, Tobin G, Backman E, Soderberg O, Rosenquist R, Horkko S, Rosen A. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood. 2008;111(7):3838-3848.

66. Hardy RR. B-1B cells: development, selection, natural autoantibody and leukemia. Current Opinion in Immunology. 2006;18(5):547-555.

67. Que X, Widhopf GF, 2nd, Amir S, Hartvigsen K, Hansen LF, Woelkers D, Tsimikas S, Binder CJ, Kipps TJ, Witztum JL. IGHV1-69-encoded antibodies expressed in chronic lymphocytic leukemia react with malondialdehyde-acetaldehyde adduct, an immunodominant oxidation-specific epitope. PLoS One. 2013;8(6):e65203.

68. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. J Exp Med. 2011;208(1):67-80.

69. Covens K, Verbinnen B, Geukens N, Meyts I, Schuit F, Van Lommel L, Jacquemin M, Bossuyt X. Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype. Blood. 2013;121(26):5176-5183.

70. Mabbott NA, Gray D. Identification of Co-Expressed Gene Signatures in Mouse B1, Marginal Zone and B2 B-cell Populations. Immunology. 2013.

71. Giguere V. Orphan nuclear receptors: From gene to function. Endocrine Reviews. 1999;20(5):689-725.

72. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The Nuclear Receptor Superfamily - The 2nd Decade. Cell. 1995;83(6):835-839.

73. Glass CK, Ogawa S. Combinatorial roles of nuclear receptors in inflammation and immunity. Nature Reviews Immunology. 2006;6(1):44-55.

74. Bensinger SJ, Tontonoz P. Integration of metabolism and inflammation by lipid-activated nuclear receptors. Nature. 2008;454(7203):470-477.

75. Glass CK, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. Genes & Development. 2000;14(2):121-141.

76. Castrillo A, Tontonoz P. Nuclear receptors in macrophage biology: At the crossroads of lipid metabolism and inflammation. Annual Review of Cell and Developmental Biology. 2004;20:455-480.

77. Barish GD, Downes M, Alaynick WA, Yu RT, Ocampo CB, Bookout AL, Mangelsdorf DJ, Evans RM. A nuclear receptor atlas: Macrophage activation. Molecular Endocrinology. 2005;19(10):2466-2477.

78. Schote AB, Turner JD, Schiltz J, Muller CP. Nuclear receptors in human immune cells: expression and correlations. Mol Immunol. 2007;44(6):1436-1445.

79. Ballow M, Wang XC, Xiang SN, Allen C. Expression and regulation of nuclear retinoic acid receptors in human lymphoid cells. Journal of Clinical Immunology. 2003;23(1):46-54.

80. Padilla J, Kaur K, Cao HJ, Smith TJ, Phipps RP. Peroxisome proliferator activator receptor-gamma agonists and 15-deoxy-Delta(12,14)-PGJ(2) induce apoptosis in normal and malignant B-lineage cells. Journal of

Immunology. 2000;165(12):6941-6948.

81. Setoguchi K, Misaki Y, Terauchi Y, Yamauchi T, Kawahata K, Takashi K, Yamamoto K. Haploinsufficiency of peroxisome proliferator-activated receptor-gamma affects the B cell proliferative response and the severity of experimentally-induced arthritis. Arthritis and Rheumatism. 2001;44(9):S88-S88.

82. Casey SC, Nelson EL, Turco GM, Janes MR, Fruman DA, Blumberg B. B-1 Cell Lymphoma in Mice Lacking the Steroid and Xenobiotic Receptor, SXR. Mol Endocrinol. 2011.

83. Funder JW. Mineralocorticoid receptors: distribution and activation. Heart failure reviews. 2005;10(1):15-22.

84. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. Cloning of Human Mineralocorticoid Receptor Complementary-Dna - Structural and Functional Kinship with the Glucocorticoid Receptor. Science. 1987;237(4812):268-275.

85. Rupprecht R, Reul JMHM, van Steensel B, Spengler D, Söder M, Berning B, Holsboer F, Damm K. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. European Journal of Pharmacology: Molecular Pharmacology. 1993;247(2):145-154.

86. Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid Action - Target Tissue-Specificity Is Enzyme, Not Receptor, Mediated. Science. 1988;242(4878):583-585.

87. Stockand JD. New ideas about aldosterone signaling in epithelia. American Journal of Physiology- Renal Physiology. 2002;282(4):F559.

88. Pascual-Le Tallec L, Lombes M. The mineralocorticoid receptor: A journey exploring its diversity and specificity of action. Molecular Endocrinology. 2005;19(9):2211-2221.

89. Bergmann A, Eulenberg C, Wellner M, Rolle S, Luft F, Kettritz R. Aldosterone Abrogates Nuclear Factor {kappa} B-Mediated Tumor Necrosis Factor {alpha} Production in Human Neutrophils via the Mineralocorti-

coid Receptor. Hypertension. 2010;55(2):370.

90. Herrada AA, Contreras FJ, Marini NP, Amador CA, Gonzalez PA, Cortes CM, Riedel CA, Carvajal CA, Figueroa F, Michea LF, Fardella CE, Kalergis AM. Aldosterone Promotes Autoimmune Damage by Enhancing Th17-Mediated Immunity. Journal of Immunology. 2010;184(1):191-202.

91. Rickard AJ, Morgan J, Tesch G, Funder JW, Fuller PJ, Young MJ. Deletion of mineralocorticoid receptors from macrophages protects against deoxycorticosterone/salt-induced cardiac fibrosis and increased blood pressure. Hypertension. 2009;54(3):537.

92. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Sch++tz G, Lumeng CN, Mortensen RM. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. The Journal of Clinical Investigation. 2010;120(9):3350-3364.

93. Tuckermann JP, Kleiman A, McPherson KG, Reichardt HM. Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis. Critical Reviews in Clinical Laboratory Sciences. 2005;42(1):71-104.

94. Franchimont D. Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. Annals of the New York Academy of Sciences. 2004;1024:124-137.

95. Zen M, Canova M, Campana C, Bettio S, Nalotto L, Rampudda M, Ramonda R, Iaccarino L, Doria A. The kaleidoscope of glucorticoid effects on immune system. Autoimmun Rev. 2011;10(6):305-310.

96. Peet DJ, Turley SD, Ma WZ, Janowski BA, Lobaccaro JMA, Hammer RE, Mangelsdorf DJ. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell. 1998;93(5):693-704.

97. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature. 1996;383(6602):728-731.

98. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. Journal of Biological Chemistry. 2002;277(21):18793-18800.

99. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nature Medicine. 2003;9(2):213-219.

100. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell. 2008;134(1):97-111.

CHAPTER 2

Comparative Nuclear Receptor Atlas:
Basal and Activated Peritoneal B-1 and B-2 cells

Abstract

Naïve murine B cells are typically divided into three subsets based upon functional and phenotypic characteristics: innate-like B-1 and marginal zone (MZ) B cells versus adaptive B-2 cells, also known as follicular or conventional B cells. B-1 cells, the innate-immune like component of the B cell lineage are the primary source of natural antibodies, and have been shown to contribute to autoimmune diseases, human B-cell leukemias, and inflammatory disorders such as atherosclerosis. On the other hand, B-2 cells are the principal mediators of the adaptive humoral immune response and represent an important pharmacological target for various conditions including Rheumatoid Arthritis, Lupus erythematosus, and lymphomas. Using the resources of the Nuclear Receptor Signaling Atlas (NURSA) program, we used quantitative real-time PCR (qPCR) to assess the complement of the 49 murine nuclear receptor superfamily expressed in quiescent and toll-like receptor (TLR) stimulated peritoneal B-1 and B-2 cells. We report the expression of 24 nuclear receptors in basal B-1 cells and 25 nuclear receptors in basal B-2 cells, with in some cases dramatic changes in response to TLR 4 or TLR 2/1 stimulation. Comparative nuclear receptor profiling between B-1 and peritoneal B-2 cells reveals a highly concordant expression pattern, albeit at quantitatively dissimilar levels. We also found that splenic B cells express 23 nuclear receptors. This catalog of nuclear receptor expression in B-1 and B-2 cells provides data to be utilized to better understand the specific roles of nuclear receptors in B cell function, chronic inflammation and autoimmune disease.

Introduction

Murine B cells are heterogeneous and comprised of various subsets that can be distinguished by surface phenotype, anatomical localization, requirement for activation, mode of replenishment, diversity of immunoglobulin gene segment usage, and immunological function. Functionally B cells are typically divided into the innate-like B-1 cells and marginal zone (MZ) B cells versus adaptive B-2 cells, also known as follicular or conventional B cells (reviewed in (1)). B-1 cells are a primordial subset of B cells that secrete natural antibodies that are not part of the adaptive immune system as they have no memory, but rather harbor a strong component of the TLR dependent innate immune response. Natural antibodies provide a rapid and preformed defense against invading pathogens (2) and are hypothesized to fulfill a homeostatic role by binding to apoptotic cells, mediating their rapid clearance (3). B-1 cells themselves can be divided into at least two subsets based upon their expression of CD5 (B-1a are CD5+ and B-1b are CD5-) and are found predominately in the peritoneal cavity and pleural space. Additionally, B-1 cells have been suggested to fulfill a regulatory role, through the secretion of select cytokines in inflammatory and immune diseases (4). Besides their role in innate immune defense to common bacterial and viral pathogens, B-1 cells have been implicated with both positive and negative roles in various autoimmune conditions, inflammatory diseases (including atherosclerosis), and human B-cell leukemias (2;5-7). B-2 cells re-circulate and are continually replenished from bone marrow precursors cells. They are abundant in the spleen, lymph nodes,

peripheral blood, and are also found in smaller numbers in the peritoneal and pleural spaces. Through cooperation with T cells, they are stimulated to produce high-affinity antibodies, which constitute the adaptive humoral immune response and are therefore critically important in host immune defense. As both B-1 and B-2 cells fulfill vital roles in immunity, as well as pathological roles in certain diseases, it is important to gain insight into the regulation of these cells and possible strategies for pharmacologic manipulation. Because nuclear receptors are a prototypic regulatory family that controls and integrates the basic functions of many immune cells, the aim of this study is to define the repertoire of expressed nuclear receptors in B-1 and B-2 cells.

Nuclear receptors are important regulators of gene transcription and represent a significant class of pharmacological targets. Various studies have documented their expression and roles in inflammation and immunity, particularly in macrophages or dendritic cells (8;9). Numerous reports have also explored the role of individual nuclear receptors in lymphoid cells, but comparatively few studies have investigated the expression and role of the nuclear receptor superfamily as a whole in these cells. It has been reported that at least twelve of the human nuclear receptors are expressed in various immune cells including T and B lymphocytes (10). There is no report detailing the expression or function of nuclear receptors in B-1 cells. Given the established importance of nuclear receptors in other immune cells, such as the macrophage, we sought to identify the full complement of nuclear receptors expressed within unstimulated and stimulated B-1 and B-2 cells.

Compared to conventional B-cells (B-2 cells), little is known regarding the activation requirements and regulation of B-1 cells. Similar to B-2 cells, they express Toll-like receptors (TLRs), but in response to TLR activation,

they preferentially differentiate into immunoglobulin secreting plasma cells (11). Toll-like receptors are “pattern recognition receptors” that sense both exogenous and endogenous pathogens, and induce innate and adaptive immunity (12;13). This study will focus on activation of B cells by two distinct TLR agonists: Kdo2-Lipid A, which is a highly pure lipopolysaccharide (LPS) sub-structure that activates TLR-4 (14), and Pam_3CSK4, which is a synthetic triacylated lipopeptide that activates the TLR-2 and TLR-1 complex (15). Intracellular signaling through TLR-4 and TLR-2/1 involves the use of the MyD88 and Trif adaptor proteins. TLR4 uses both MyD88 and Trif cascades, whereas TLR-2/1 signals exclusively via a MyD88 dependent pathway (12). B-1 cells have been shown to proliferate and differentiate in response to TLR-4 and TLR-2 specific ligands (11;16;17). TLR-4 activation has been shown to induce the transcriptional repressor Blimp-1 mRNA in B-1 cells, which is necessary for immunoglobulin secretion (18). In B-2 cells, TLR-4 and TLR-2 stimulation have been shown to induce proliferation, cytokine secretion, and class switch recombination (11;19-22). Comparison of the effects of TLR-2 and TLR-4 activation on nuclear receptor expression will enable future hypothesis driven approaches to better understand B-1 and B-2 cellular responses to innate immune signals.

Quantitative expression profiling in basal and TLR stimulated B-1 and B-2 cells identified the expression of 24 and 25 nuclear receptors, respectively. With a few notable exceptions, the same cohort of nuclear receptors was expressed in both B-1 and B-2 cells, albeit in many cases at drastically different levels. The robust changes observed in the temporal pattern of expression of these receptors in response to either TLR-2/1 or TLR-4 stimulation suggests that inflammatory signaling modulates the expression of many of the nuclear

receptors in B cells. In the supplemental material we also provide comparative data for nuclear receptor expression in B cells isolated from the spleen. This report provides the first comprehensive study of nuclear receptors in B-1 and B-2 cells and provides new data to better understand the biology and regulation of these cells.

Results

Primary B-1 and B-2 cells were isolated from the peritoneal exudate cells of 16 to 20-week old female C57BL/6 mice by fluorescent activated cell sorting. B-1 cells were purified to greater than 98% purity as the $CD3^-$, $CD19^+$, and $CD23^-$ population, whereas B-2 cells were purified to greater than 97% purity as the $CD3^-$, $CD19^+$, and $CD23^+$ population (Fig. 2-1). The purified B-1 or B-2 cells were then exposed to media containing Kdo2-Lipid A or Pam_3CSK4 and the expression of the 49 murine nuclear receptors was assessed over a 72h time course using a high-throughput qPCR platform. RNA was isolated from cells harvested at 0, 6, 24, and 72h after stimulation (Fig. 2-1) along with a parallel set of control samples from non-stimulated B-1 cells. We attempted to maintain a parallel set of B-2 cells in culture in the absence of stimulation, but the cells did not survive the duration of the experiment, consistent with the known requirement for stimulation of B-2 cells in culture.

The Atlas of B-1 and B-2 cell Nuclear Receptors

Gene expression analysis of the nuclear receptor superfamily in B-1 cells revealed the presence of mRNA transcripts for 24 of the 49 known receptors. These include eight members of the endocrine receptor family, which are activated by high affinity lipophilic hormones, six adopted orphan receptors,

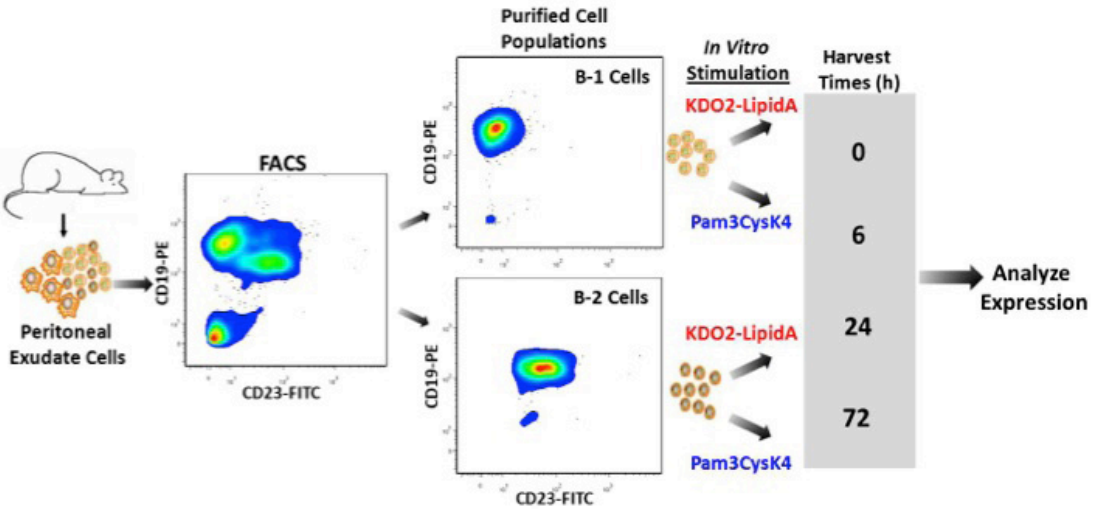
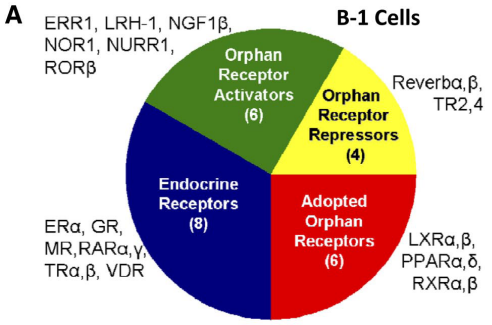
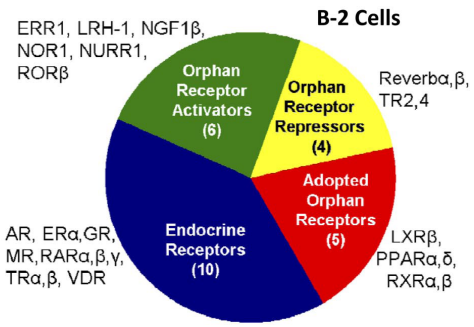


Fig.2-1 Experimental Design Peritoneal B-1 or B-2 cells were isolated from CB57BL6/J mice using Fluorescence Activated Cell Sorting (FACS). B-1 cells were selected as CD3- (gate not shown), CD19+ and CD23-, and B-2 cells as CD3- (gate not shown), CD19+ and CD23+. Primary B-1 or B-2 cells were exposed to Kdo2-Lipid A or Pam3CysK4 and harvested at the indicated time points. Samples were processed and subjected to quantitative PCR analysis.

which are activated by low-affinity dietary lipids, and ten orphan receptors, including six constitutive activators and four constitutive repressors (Fig. 2-2A) (23). Figure 2-2B tabulates the expressed and non-expressed receptors in B-1 cells according to their classification along with their nomenclature. (The mean cycle threshold (Ct) values from qPCR reactions for these measurements are given in a Supplemental Table 2-1).

Qualitatively, peritoneal B-2 cells express a similar assortment of nuclear receptors in comparison to peritoneal B-1 cells, with a few notable exceptions (Fig. 2-2C-D). B-2 cells were found to express 25 of the 49 murine nuclear receptors, including ten endocrine receptors, five adopted orphan receptors, and ten orphan receptors, comprised of six constitutive activators and four constitutive repressors. Notably, mRNA transcripts for the androgen receptor (AR) were expressed more abundantly and consistently in B-2 cells, whereas they were only detected sporadically in B-1 cells. Additionally, RARβ,





B

Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Expressed	**Expressed**	**Expressed**
ERα (NR3A1) **GR** (NR3C1) **MR** (NR3C2) **RARα,γ** (NR1B1,1B3) **TRα,β** (NR1A1, 1A2) **VDR** (NR1I1)	**LXRα,β** (NR1H2, 1H3) **PPARα,δ** (NR1C1,1C2) **RXRα,β** (NR2B1,2B2)	**ERR1** (NR3B1) **LRH-1** (NR5A2) **NGF1B** (NR4A1) **NOR1** (NR4A3) **NURR1** (NR4A2) **Reverbα,β** (NR1D1,1D2) **RORβ** (NR1F2) **TR2,4** (NR2C1,2C2)
Unexpressed	**Unexpressed**	**Unexpressed**
AR* (NR3C4) **ERβ** (NR3A2) **PR** (NR3C3) **RARβ*** (NR1B2)	**CAR** (NR1I4) **FXRα, β** (NR1H4,1H5) **PPARγ*** (NR1C3) **PXR** (NR1I2) **RXRγ** (NR2B3)	**COUP TF 1,2,3** (NR2F1,2F2,2F4) **DAX1** (NR0B1) **ERR2,3** (NR3B2,3B3) **GCNF** (NR6A1) **HNF4α,γ** (NR2A1, 2A2) **PNR** (NR2E3) **RORα,γ** (NR1F1,1F3) **SF-1** (NR5A1) **SHP** (NR0B2) **TLX** (NR2E1)

D

Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Expressed	**Expressed**	**Expressed**
AR (NR3C4) **ERα** (NR3A1) **GR** (NR3C1) **MR** (NR3C2) **RARα,β,γ** (NR1B1,1B2,1B3) **TRα,β** (NR1A1, 1A2) **VDR** (NR1I1)	**LXRβ** (NR1H2) **PPARα,δ** (NR1C1,1C2) **RXRα,β** (NR2B1,2B2)	**ERR1** (NR3B1) **LRH-1** (NR5A2) **NGF1B** (NR4A1) **NOR1** (NR4A3) **NURR1** (NR4A2) **Reverbα,β** (NR1D1,1D2) **RORβ** (NR1F2) **TR2,4** (NR2C1,2C2)
Unexpressed	**Unexpressed**	**Unexpressed**
ERβ (NR3A2) **PR*** (NR3C3)	**CAR** (NR1I4) **FXRα,β** (NR1H4,1H5) **LXRα*** (NR1H3) **PPARγ*** (NR1C3) **PXR** (NR1I2) **RXRγ** (NR2B3)	**COUP TF 1,2,3** (NR2F1,2F2,2F4) **DAX1** (NR0B1) **ERR2,3** (NR3B2,3B3) **GCNF** (NR6A1) **HNF4α,γ** (NR2A1, 2A2) **PNR** (NR2E3) **RORα,γ** (NR1F1,1F3) **SF-1** (NR5A1) **SHP** (NR0B2) **TLX** (NR2E1)

Fig.2-2 The Composition of Nuclear Receptors Expressed in Peritoneal B-1 and B-2 cells. A, Twenty-four of 49 known nuclear receptors are expressed in the peritoneal B-1 cell. These include eight endocrine receptors, six adopted orphan receptors that bind low-affinity dietary lipids, and ten orphan receptors, including six constitutive activators and four constitutive repressors. **B,D**, Tabular listing of nuclear receptors expressed or non-expressed in (B) peritoneal B-1 cells or (D) peritoneal B-2 cells with their unified nomenclature system names listed in parentheses (94). **C**, Twenty-five of 49 known nuclear receptors are expressed in peritoneal B-2 cells. These include ten endocrine receptors, five adopted orphan receptors that bind low-affinity dietary lipids, and ten orphan receptors, including six constitutive activators and four constitutive repressors. Receptors were deemed unexpressed if cycle threshold (Ct) values exceeded 35. The actual Ct values are given in the Supplemental Table 2-1 in comparison to simultaneously determined Ct values for the same receptors in splenic B cells and bone marrow derived macrophages. *Trace or inconsistent expression detectable.

which was detected intermittently at trace levels in B-1 cells, was expressed more consistently and abundantly in B-2 cells. Finally, whereas LXRα was expressed at low levels in B-1 cells (average Ct value 34.8), its expression in B-2 cells (average Ct value 35.2) was still lower and below the cut off to be classified as expressed. Stimulation with Kdo2-Lipid A or Pam_3CSK4 dramatically and uniquely altered the quantitative pattern of receptor expression in both peritoneal B-1 and B-2 cells over time.

Basal expression of nuclear receptors in B-1 and B-2 cells in the absence of stimulation.

A quantitative comparison of the basal expression levels at time 0 of mutually expressed nuclear receptors between basal peritoneal B-1 and B-2 cells reveals markedly disparate expression patterns for most receptors (Fig. 2-3). Notably, it was observed that thyroid receptor beta (TRβ) and TRα were expressed at over 20-fold and 4-fold higher levels respectively in peritoneal B-2 than in B-1 cells. Interestingly, TRα knock-out mice or mice that have impaired thyroid hormone production display impaired B cell development (24;25). Nearly all of the NRs were expressed more abundantly in B-2 cells than in B-1 cells, with peroxisome-proliferator activated receptor alpha

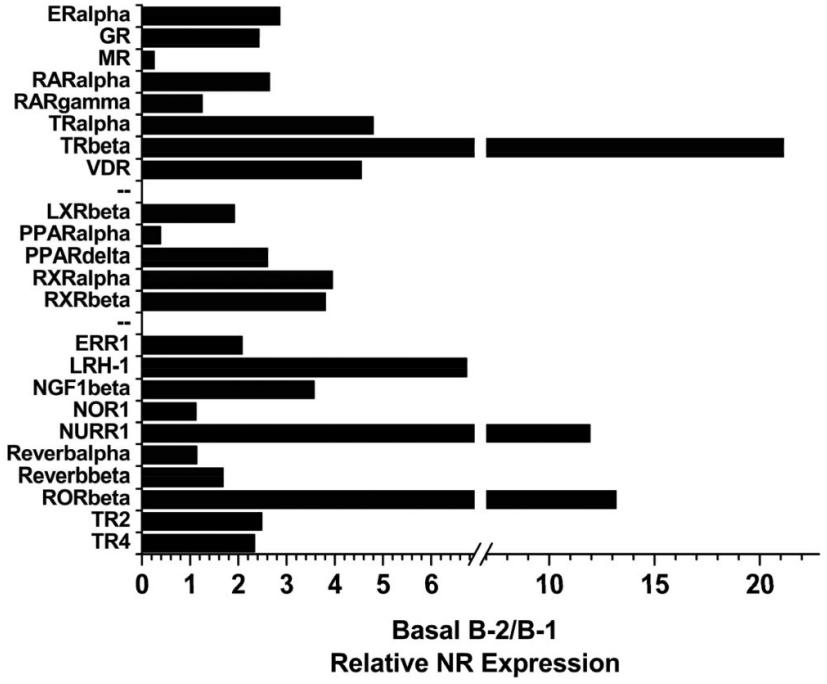


Fig.2-3 Relative Nuclear Receptor Expression in Basal B-2 cells versus Basal B-1 cells. Basal expression at time zero (normalized to 36B4) in peritoneal B-2 cells relative to peritoneal B-1 cells. B-2 cells displayed moderately higher expression of many of the nuclear receptors and marked enhanced expression of RARβ, TRβ, LRH-1 and RORβ. In contrast, MR and PPARα expression levels were dramatically higher in B-1 cells.

(PPARα) and the mineralocorticoid receptor (MR) being the exceptions. MR expression levels in B-1 cells were about 4-fold higher than in B-2 cells. This finding suggests that the MR may be playing a prominent role specifically in B-1 cell biology. In contrast, it is interesting to also note that B-2 cells express twice the levels of the glucocorticoid receptor (GR). Since MR and GR both bind glucocorticoids, it will be of interest to investigate whether the dissimilar expression levels of these receptors results in unique gene expression patterns when stimulated. The analysis of the quantitative pattern of nuclear receptor gene expression in B-1 versus B-2 cells has great potential for uncovering the roles of these receptors in these related but distinct immune cell types.

As noted above, we were unable to maintain B-2 cells in culture in the absence of TLR stimulation over the 72h time course of study. However, B-1 cells could be maintained in culture in the absence of stimulation (Fig 2-4A), and the expression patterns of nuclear receptors in these cells maintained in fresh media in the absence of either Kdo2-Lipid A or Pam_3CSK4 were found to be quite dynamic over the entire 72h time period. This dynamic expression is perhaps due to the reduction in serum concentration (from 20% to 10%) at the time the cells were placed in the media for the beginning of the time course experiment, or a response to the accumulation of metabolites during the experiment, or could be due to natural circadian patterns of expression.

In the text below, we discuss nuclear receptor expression in B-1 and B-2 cells according to the “Receptor Family” classification scheme noted in Fig. 2-2 and comment on basal expression and responses to TLR stimulation of some but not all of the expressed receptors. Finally, we provide a profile of basal levels of nuclear receptor expression in a population of splenic B cells, and their responses to stimulation with TLR4 and TLR2 agonists.

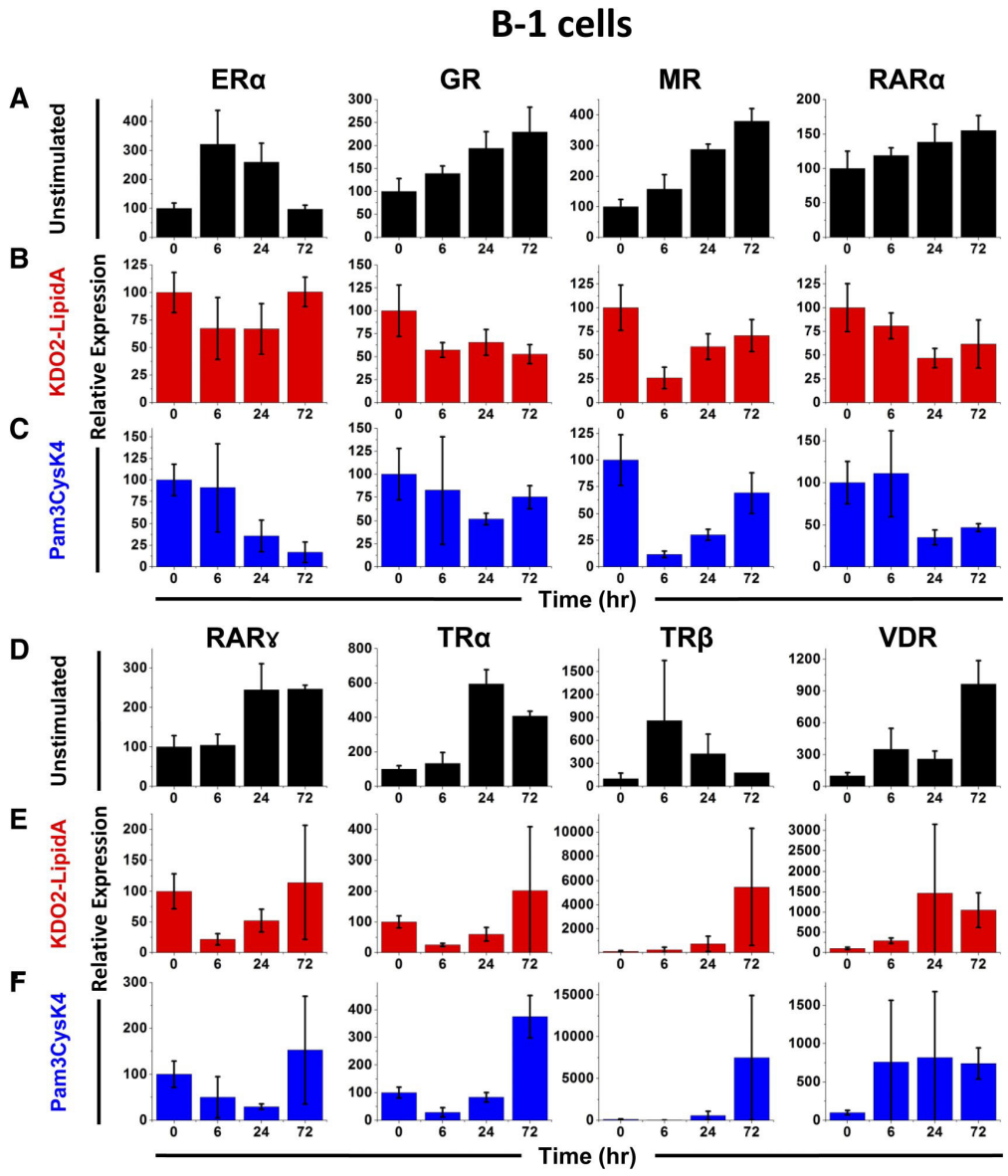


Fig.2-4 Basal and Stimulated Expression Profiles of B-1 Cell Endocrine Nuclear Receptors. A,D, Endocrine receptors in basal peritoneal B-1 cells generally display dynamic expression. ERα and TRβ display an initial peak in expression followed by down regulation. GR, MR, RARγ, TRα and VDR generally upregulate expression over time. RARα expression is not altered. **B,E,** Kdo2-Lipid A appears to induce the expression of TRβ and VDR. It has no effect on ERα or TRα expression levels. MR and RARγ were initially down regulated but then had increasing expression at subsequent time points. GR and RARα were down regulated. **C,F,** Pam3CSK4 had the same general effect as Kdo2-Lipid A on GR, MR, RARα, RARγ, TRβ, and VDR expression. TRα was upregulated and ERα was down regulated. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD.

The Endocrine Receptor Family

Eight of the 12 members of the endocrine receptor family are expressed in B-1 cells (Fig. 2-2A-B), including the estrogen receptor α (ERα), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), retinoic acid receptors α and ɣ (RARα,ɣ), thyroid receptors α and β (TRα,β), and the vitamin D receptor (VDR). In B-2 cells, two additional receptors were expressed, (Fig. 2-2C-D), the androgen receptor (AR) and the retinoic acid receptor β (RARβ). Progesterone receptor (PR) expression was also minimally detectable in 50% of the samples with an average Ct value of 33.8.

The RAR receptors are activated by acid derivatives of vitamin A and form heterodimers with the adopted orphan retinoid X receptors (RXRs) and bind to retinoic acid response elements. It is estimated that retinoic acid mediated activation of RAR/RXRs directly regulates more than 100 genes (26). In our B-1 cell culture system, in the absence of stimulation, RARα expression increased slightly over time whereas RARɣ expression was increased 2.5 times above basal at the 24h and 72h time points (Fig. 2-4A,D). Treatment with Kdo2-Lipid A and Pam_3CSK4 caused a reduction in RARα expression at later time points, whereas RARɣ expression was down regulated early but later recovered (Fig. 2-4B,C,E,F). Very similar expression patterns were observed in B-2 cells (Fig 2-5). RARβ expression was only slightly detectable in B-1 cells and was expressed inconsistently among replicates (data not shown). In B-2 cells on the other hand, RARβ was consistently detectable but only in unstimulated cells (Fig. 2-5C-D). It is generally believed that vitamin A and its derivatives acting predominantly through RARs are important for maintaining and stimulating the immune system and protecting against infectious diseases (27). It has been reported that RARα and RARɣ are expressed in human

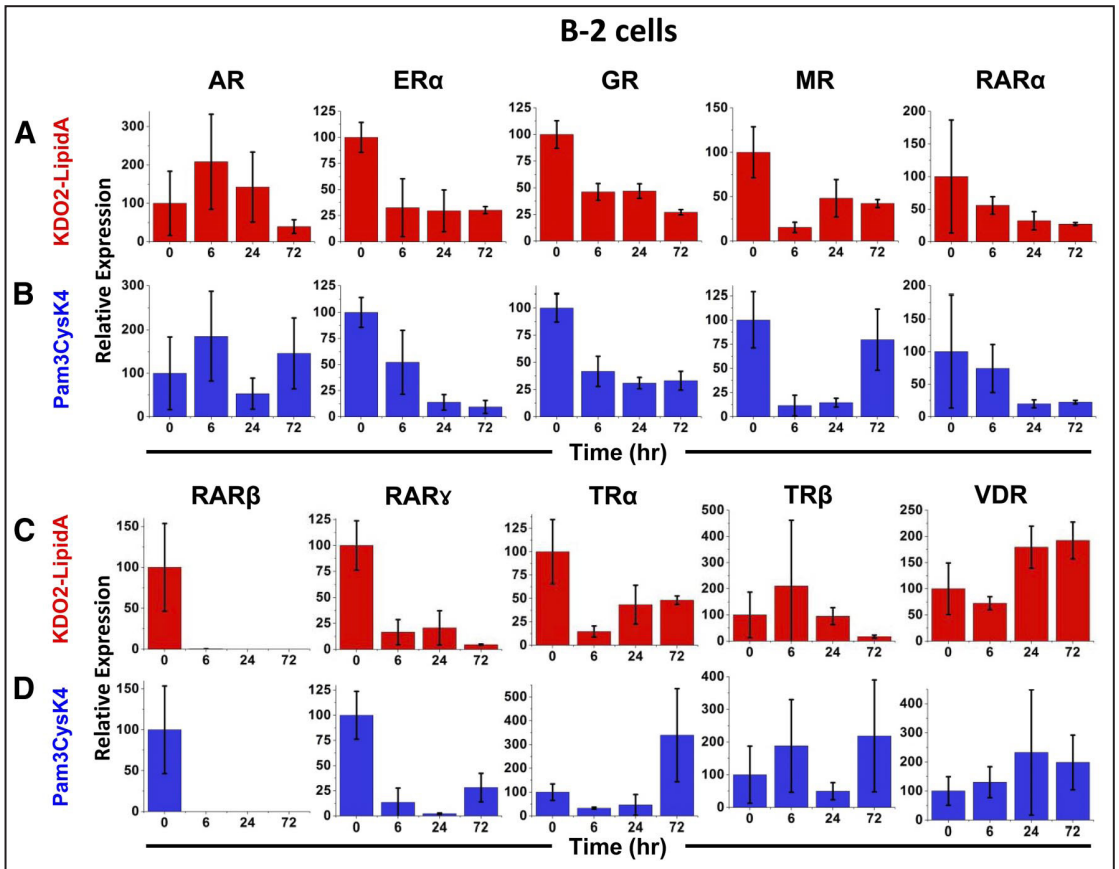


Fig.2-5 Stimulated Expression Profiles of B-2 Cell Endocrine Nuclear Receptors. A,C, Treatment of peritoneal B-2 cells with KDO2-LipidA, a TLR4 ligand. This led to a general down-regulation of ERα, GR, MR, RARα, β, γ, and TRα mRNA levels. AR and TRβ mRNA levels were initially up-regulated followed by subsequent down regulation. VDR expression levels slightly decreased initially but increased at later time points. **B,D**, Treatment with Pam3CSK4, a TLR2/1 ligand. It had the same general effect as KDO2-LipidA on ERα, GR, RARα, β, γ and VDR expression. AR, MR,TRα and β displayed a peak in expression at the 72 h time point. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD.

lymphocytes, including B and T cells (28). Various human and animal studies have demonstrated a regulatory capacity of retinoids on antibody responses (29). *In vitro* studies have demonstrated that retinoic acid can induce differentiation and increase antibody production of human B cell derived hybridomas and mouse splenic B cells (30;31). Interestingly, it has been shown that retinoic acid is able to induce a marked increase in IgM secretion from human umbilical cord blood mononuclear cells, a hypothesized human equivalent to

mouse B-1 cells (32). This leads to the interesting hypothesis that RARα and RARɣ may play an important role in regulating the spontaneous secretion of natural antibodies from B-1 cells.

The GR and MR nuclear receptors were expressed in both basal and stimulated B-1 cells. Unstimulated B-1 cells displayed increasing levels of expression of both GR and MR over time. By the 72h time point, GR expression levels had more than doubled whereas MR expression had increased by almost 4-fold (Fig. 2-4A). When B-1 cells were stimulated with Kdo2-Lipid A, GR expression was reduced to 50% of basal levels and MR expression was initially reduced to 25% of basal levels after six hours, but only 75% of basal levels after 24h (Fig. 2-4B). Stimulation with Pam_3CSK4 qualitatively had a similar inhibitory effect over time on both GR and MR expression in B-1 cells. After six hours, MR expression was more profoundly inhibited by Pam_3CSK4 than Kdo2-Lipid A (Fig. 2-4C). Notably, the expression level of MR in basal B-1 cells was strikingly higher than that observed in basal bone marrow derived macrophages (Supplemental Table 2-1) or peritoneal macrophages (data not shown).

The closely related GR and MR direct distinct, almost opposing transcriptional programs; generally GR activation is anti-inflammatory and MR activation is pro-inflammatory. Amazingly this occurs despite the fact that they bind the same DNA response elements (33). Furthermore, MR is equally sensitive to glucocorticoids and mineralocorticoids (34). The mechanism of MR or GR specificity is poorly understood, but on a cellular level it is accomplished by the activity of 11β-hydroxysteroid dehydrogenase type 2 (11HSD2), which inactivates glucocorticoids. Cells that strongly express 11HSD2 metabolize glucocorticoids and are therefore selectively sensitive to mineralocorticoids

through the MR (35). Interestingly, 11HSD2 is expressed at very low levels in resting or TLR-4 stimulated B-1 cells (data not shown). Given the facts that the concentration of free glucocorticoids are approximately 100-fold higher than mineralocorticoids in the plasma and that the expression of 11HSD2 is extremely low in B-1 cells, it is likely that circulating glucocorticoids activate the MR in B-1 cells. It remains to be investigated whether mineralocorticoids and glucocorticoids have distinct effects on B-1 cell function.

The function of MR in B-1 cells, as well as most other immune cells is completely unknown. However, both experimental and clinical data demonstrate that aldosterone promotes inflammatory damage to the heart, kidneys, and vasculature (36;37). Recent reports document that MR augments the capacity of dendritic cells to activate $CD8^+$ T cells and induce a Th17 phenotype on antigen-specific $CD4^+$ T cells and that myeloid MR controls macrophage polarization and promotes cardiac hypertrophy and fibrosis in various animal models (38-40). By contrast, the GR and glucocorticoids have well established anti-inflammatory and immunosuppressive effects. These effects are well documented in T cells and B cells in general, but no reports document specific effects of glucocorticoids on B-1 cells. Interestingly, stimulation of macrophages with the TLR-4 agonist LPS led to induction of GR expression after 4-8h (8), whereas TLR-4 stimulation in B-1 and B-2 cells had the opposite effect of moderately inhibiting GR expression (Figs. 2-4B & 2-5A). This suggests that the MR and GR may play a distinct role in modulating inflammation in macrophages versus B-1 and B-2 cells.

Although vitamin D is most commonly associated with calcium and phosphorous metabolism, it also modulates inflammation. In our B-1 cell culture system its expression increased dramatically over time in the absence

and presence of TLR stimuli (Fig. 2-4 D-F). Unstimulated B-1 cells demonstrated 4-fold to 10-fold increases in VDR expression over the 72h time period. Kdo2-Lipid A stimulated B-1 cells had a 10- to 15-fold increase in VDR expression at the 24h and 72h time points, whereas Pam_3CSK4 stimulation led to a consistent 6- to 7-fold increase in expression. In B-2 cells, TLR stimulation similarly induced VDR expression but to a more modest extent (Fig. 2-5C-D). It is postulated that vitamin D can act in a paracrine fashion in an immune or inflammatory setting (41). Macrophages stimulated by interferon-γ as well as other cytokines can rapidly produce vitamin D ($1\alpha,25\text{-}(OH)_2D_3$), which in turn is thought to modulate immune function at the site of inflammation (42;43). The locally produced $1\alpha,25\text{-}(OH)_2D_3$ is hypothesized to then act on activated T and B cells, as these cells (and not quiescent human T and B cells) have been shown to express VDR (44). Our finding that basal murine B-1 and B-2 cells as well as TLR-activated murine B cells express the VDR leads to the interesting hypothesis that mouse peritoneal B-1 and B-2 cells are sensitive to vitamin D immunoregulation regardless of activation status.

The AR was modestly expressed in B-2 cells, but not B-1 cells (Figs. 2-2 and 2-5A). The AR is activated by circulating testosterone, which is known to suppress most aspects of immune activity. Interestingly, castration of male mice results in increased B cell numbers in peripheral blood (45). Reports indicate that AR is expressed in developing B cells in the bone marrow but not in mature B cells in the spleen (46;47), consistent with our failure to observe AR in murine splenic B cells (Suppl. Fig 2-1). Purportedly, testosterone acting through the AR inhibits B cell development (48). The AR was expressed at relatively low levels (average Ct= 30.9) in unstimulated murine peritoneal B-2 cells (Fig. 2-5A-B), but KDO2-LipidA induced a near 2-fold increase in AR

mRNA levels by 6h, which then decreased over time to roughly 50% of baseline levels by 72h. Stimulation with Pam_3Cysk4 had a very similar effect at the 6h and 24h time points, but displayed approximately 150% higher expression levels at 72h compared to baseline (Fig. 2-5B). As B-1 cells (Fig. 2-2B) and splenic B cells (Suppl. Fig. 2-1) had very inconsistent and/or trace expression of AR it will be interesting to test if and how androgens affect peritoneal B-2 cells versus other B cell subsets.

ERα, but not ERβ, was expressed in both B-1 and B-2 cells, and to a greater degree in peritoneal B-2 cells (Figs 2-2 and 2-3). ERα is a high affinity cellular sensor for circulating estrogens, which are recognized as immunomodulatory hormones with specific effects on B cell development and function. Pregnancy has been shown to lead to a reduction in B lymphopoiesis that can be recapitulated by a single injection of estrogen (49). This effect appears to be at least in part mediated through progenitor B cells themselves as both ERα and ERβ are expressed in these cells (50). Interestingly, whereas estrogens appear to inhibit progenitor B cell development, they appear to enhance the immune response of mature B cells. This enhancement is manifest in human B cells by increased immunoglobulin production without affecting cell proliferation or viability (51). Additionally, estradiol has been observed to endow murine splenic B cells with a resistance to apoptosis induced by IgM-crosslinking (52). In contrast to reports documenting the expression of both ERα and ERβ in mature murine splenic B cells and human B cells (52;53), we report the expression of only the ERα in murine peritoneal B-1, B-2 and splenic B cells (Fig. 2-2C-D, Suppl. Fig. 2-1). Our failure to observe ERβ expression in mature B cells may be specific for the C57BL/6 mouse strain as B cells of Balb/c mice have been reported to express it (52). The expression of ERα was

quickly and consistently down regulated by both TLR4 and TLR2/1 in B-2 cells (Fig 2-5A-B). This is intriguing, as both ER activation and TLR signaling are reported to induce an increase in immunoglobulin production in B cells, with the difference being that TLR activation also induces a strong proliferative response. TLR stimulation seems to have less of an effect or at least a delayed inhibitory effect on ERα expression in B-1 cells (Fig. 2-4B-C). Comparing the mechanism of the ER versus TLR induced immunoglobulin response in B cells could potentially lead to a better understanding of the cellular mechanisms regulating this important process, and potential gender differences.

Thyroid hormones have pleiotropic effects on differentiation, growth, and metabolism (reviewed in (54)). The effects of these hormones are mediated by binding to TRα or TRβ, with virtually all body tissues expressing at least one splice variant of these receptors (55;56), whose relative expression level differ between tissues and cell types. TRα or TRβ have documented roles in the development and/or physiological function of bone, heart, fat, liver, pituitary, and brain tissue. There is a paucity of information regarding the expression and function of thyroid hormone receptors in specific cells of the immune system, including B cells. Both TRα and TRβ were expressed in peritoneal B-1 and B-2 cells (Fig. 2-2), with remarkably higher levels in B-2 cells, TRα > 4 fold, and TRβ >20 fold (Fig. 2-3). TRα was robustly expressed in unstimulated B-2 cells and was down regulated by the addition of KDO2-LipidA (Fig. 2-5C). Pam_3Cysk4 treatment also downregulated TRα expression, except at the 72h time point, where a 3-fold increase in expression above basal levels was observed (Fig. 2-5D). In response to TLR4 activation, TRβ expression was increased 2-fold, but appeared to be suppressed with more prolonged stimulation. TLR2/1 stimulated TRβ in a biphasic manner, increasing expres-

sion 2-fold at 6h and 72h compared to baseline (Fig. 2-5D). TLR stimulation had a very distinct effect on TRα and β expression patterns in B-1 cells, which consistently displayed a large peak in expression at the 72h time point (Fig. 2-4B-C). Studies exploring the effect of thyroid hormone on B cells are especially provocative in the context of autoimmune-mediated Graves' disease, in which activating auto-antibodies specific for the thyrotrophin receptor stimulate the thyroid gland, resulting in unregulated excessive production of thyroid hormone, producing thyrotoxicosis. It is unclear what effect the resultant high levels of thyroid hormone in turn have on the auto-antibody producing B cells.

The Adopted Orphan Receptor Family

Adopted orphan receptors respond to dietary lipids and their metabolic derivatives, such as fatty acids (PPARs), oxysterols (LXRs), and bile acids (FXR), as well as to xenobiotics (PXR and CAR) (23). They function as heterodimers with the retinoid x receptor (RXR) to regulate lipid metabolism, storage, transport, and elimination. Adopted orphan receptors bind their ligands with lower affinities than concentrations normally generated by dietary intake (>1 to 10μM), and for this reason they are thought to act as cellular lipid sensors, where their "specific" ligands are locally more concentrated (23). Importantly, various PPARs and LXRs have been found to be prominent regulators of inflammation.

Six adopted orphan receptors are expressed in the B-1 cell, including PPARα and δ, LXRα and β, and RXRα and β (Fig. 2-2A & B). PPARγ expression was barely detectable in about half of the samples and had an average Ct value of 34.1. Five adopted orphan receptors are expressed in peritoneal B-2 cells, including PPARα and δ, LXR β, and RXRα and β (Fig. 2-2C-D). Expres-

sion of LXRα and PPARγ were also detectable at trace levels. For mutually expressed receptors, basal levels were generally 1 to 3-fold higher in B-2 cells (Fig. 2-3).

PPARα is generally expressed in tissues with high mitochondrial and peroxisomal fatty acid beta-oxidation rates, such as heart and skeletal muscle, liver, kidney, and brown fat (57). Furthermore, expression of PPARα has been observed in many cells of the immune system, including lymphocytes (58;59). The activation of PPARα has a general anti-inflammatory effect, which in lymphocytes is manifested by decreased proliferation, altered cytokine production, and impaired immunoglobulin responses (60;61). PPARα is the primary mediator of the cholesterol and lipid modulating effects of the fibrate class of drugs. Fibrates oppose the effects of TLR activation by inducing the mRNA and protein expression of IκBα, which prevents NFκB inflammatory signaling by inhibiting its translocation to the nucleus (62). B-1 cells cultured without TLR stimulation displayed dynamic expression of PPARα; expression was slightly decreased after six hours but increased sharply to 3-fold baseline levels at the 24h time point and then fell to near baseline levels at the 72h time point (Fig. 2-6A). Treatment with either Kdo2-Lipid A or Pam_3CSK4 dramatically altered the temporal pattern of PPARα mRNA expression: Kdo2-Lipid A had an inhibitory effect until the 72h time point, at which point PPARα expression levels spiked to near 15-fold baseline (Fig. 2-6B). Pam_3CSK4 on the other hand had a moderate but consistent inhibitory effect on PPARα expression (Fig. 2-6C). This is the first evidence that PPARα may play a role in regulating the function of B-1 cells. Treatment of B-2 cells with TLR agonists induced slightly different temporal patterns of PPARα mRNA expression: KDO2-LipidA induced an oscillatory pattern of expression with peaks of 150% and 300% above baseline

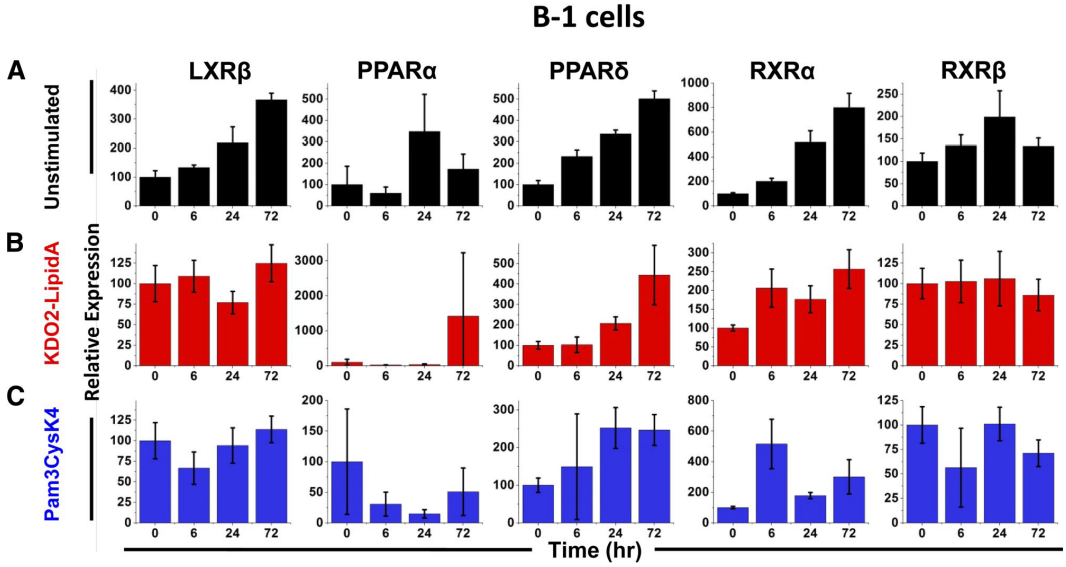


Fig.2-6 Basal and Stimulated Expression Profiles of B-1 Cell Adopted Orphan Nuclear Receptors. A, Adopted orphan receptors in basal peritoneal B-1 cells generally display increasing levels of expression over time. PPARα expression was dynamic. **B,** Kdo2-Lipid A enhanced the expression of PPARδ and RXRα, whereas LXRβ, PPARα, and RXRβ expression was generally unaffected. **C,** Pam3CSK4 induced the expression of PPARδ and RXRα, had minimal effect on LXRβ and RXRβ, and inhibited the expression of PPARα. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD.

at 6h and 72h respectively. Pam_3CSK4 on the other hand had a moderate but consistent inhibitory effect on PPARα expression until the 72h time point, at which time expression peaked to 250% above baseline (Fig. 2-7A-B). Interestingly, the level of PPARa is higher in B-1 cells than B-2 cell (Fig. 2-3). Additionally, it has been reported that PPARα is more highly expressed in splenic B cells than in T-lymphocytes (58;59), and remarkably, in our study, splenic B cells expressed > 10-fold higher levels of PPARα than peritoneal B-2 cells (Suppl. Fig. 2-2). It will be interesting to investigate whether fibrates affect splenic B cells preferentially, compared to other lymphocytes due to the high levels of expression PPARα in these cells.

PPARδ, unlike PPARα or γ, is almost ubiquitously expressed and has been shown to play a role in regulating fatty acid catabolism and thermogen-

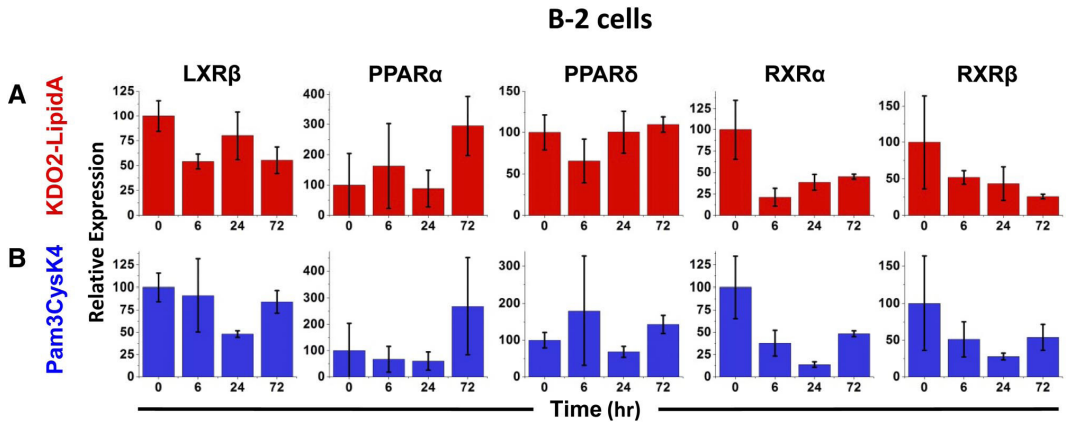


Fig.2-7 Stimulated Expression Profiles of B-2 Cell Adopted Orphan Nuclear Receptors. **A,** Treatment with KDO2-LipidA. Adopted orphan receptors in stimulated peritoneal B-2 cells displayed decreasing expression levels of LXRβ, RXRα, and RXRβ whereas PPARα expression was increased and PPARδ expression was unaffected. **B,** Pam3CSK4 treatment had the same general effect as KDO2-LipidA on all nuclear receptors except PPARδ which displayed dynamic expression. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD.

esis in adipose and skeletal muscle (63). Furthermore, the role of PPARδ in regulating inflammation and immunity is only now emerging, with studies recently showing that it modulates inflammation in skin as well as macrophages, adipose tissue, and muscle (63-66). Besides the macrophage, very little is known regarding PPARδ function in immune cells, such as lymphocytes or dendritic cells. PPARδ mRNA has been measured in human CD4+ and CD8+ T-cells and CD19+ B cells, but its distinct role in these cells is unknown (10). Using the human Jurkat T cell line, it was shown that a PPARδ ligand enhanced T-cell proliferation and inhibited apoptosis (67). We were able to detect the presence of PPARδ mRNA in both basal and TLR stimulated B-1 cells. Basal levels of PPARδ mRNA increased progressively during 72h in culture, and this increase was essentially unchanged by Kdo2-Lipid A stimulation and slightly attenuated by Pam_3CSK4 (Fig. 2-6A-C). Basal PPARδ mRNA levels in B-2 cells were about twice that of B-1 cells and TLR4 or TLR2/1 stimulation had minimal effect on PPARδ expression levels, except at the 6h time

point where PPARδ mRNA levels were slightly decreased by KDO2-LipidA and slightly increased by Pam_3CSK4 (Figs. 2-3 & 2-7A-B). What role if any PPARδ fulfills in B cells remains to be elucidated.

The Liver X receptors (LXR) function as cellular cholesterol sensors that respond to elevated oxysterol concentrations by regulating genes that control cholesterol transport into and out of the cell, thus impacting cellular cholesterol levels, as well as bile acid production, and fatty acid synthesis (reviewed in (23)). LXRα is expressed in the liver as well as other tissues including adipose, kidney, intestine, lung, adrenals, and macrophages, where-as LXRβ is expressed ubiquitously (8;68). The activation of LXRβ in murine splenic B and T cells has been shown to inhibit their proliferation by limiting cellular cholesterol levels, demonstrating that LXR signaling is important in the proliferative responses of lymphocytes (69). Mirroring what has been observed for murine splenic B-cells; B-1 cells express high levels of LXRβ (average Ct value of 23.6) and barely detectable levels of LXRα (average Ct value of 34.8). B-2 cells expressed slightly higher levels of LXRβ and slightly lower levels of LXRα. In unstimulated B-1 cells, LXRβ expression consistently increased over time, rising close to 4-fold above baseline by 72h (Fig. 2-6A). Interestingly, treatment of B-1 cells with Kdo2-Lipid A or Pam_3CSK4 did not appreciably alter the levels of LXRβ mRNA (Fig. 2-6B-C). TLR stimulation of B-2 cells led to a modest downward trend in LXRβ mRNA levels over time (Fig. 2-7A-B). In response to certain mitogenic stimuli, B-1 cells are capable of entering S phase much more rapidly than B-2 cells (70) . This leads to the interesting question of whether there are differences in LXR signaling between B cell subsets that in turn affect their mitogenic characteristics.

The Orphan Nuclear Receptor Family

B-1 cells expressed ten of the 25 orphan nuclear receptor (Fig. 2-2B). This includes six constitutive activators: estrogen-related receptor (ERR) 1, liver receptor homolog (LRH) 1, neuronal growth factor 1β (NGFIβ), neuron-derived orphan A receptor (NOR) 1, nuclear receptor-related (NURR) 1, and RAR-related orphan receptor (ROR) β; as well as four constitutive repressors: Rev-erb α and β (recently considered to be 'de-orphanized' based on their ability to bind heme (71)) and the testicular orphan receptors TR2 and TR4. The function of any of these orphan receptors in B-1 cells is entirely unknown. Peritoneal B-2 cells expressed the same ten orphan receptors (Fig. 2-2D).

In general, the basal levels of all these receptors were higher in B-2 cells than B-1 cells, and in particular, NURR1 and RORb were expressed >10-fold higher (Fig. 2-3). The NR4A orphan nuclear receptor subgroup consisting of NGFIb, NURR1, and NOR1 displayed a remarkable increase in expression levels from the zero to six hour time points with or without TLR stimulation in B-1 cells (Fig. 2-6A-C). NGFIb (also known as Nur77, TR3, NAK1, and eight other names) is an immediate early serum-induced gene (72), which is also upregulated in activated B- and T-cells (73;74). NGFIb has been shown to promote apoptosis independent of caspase activation through association with Bcl-2 proteins during translocation to the mitochondria (75). Notably, by promoting apoptosis, NGFIb is thought to play a prominent role in regulating T cell negative selection (76). Much less is known regarding the role of NGFIb in B cells, nevertheless, it has been shown that Bcl-B, a member of the apoptosis regulating Bcl-2 family of proteins, binds to NGFIb and is strongly expressed in follicular B cells (centrocytes and centroblasts) and plasma cells (77). NGFIb expression changed dramatically in unstimulated

B-1 cells, peaking at 6h with a 7 to 10-fold increase above baseline levels and then returning to near baseline levels at later time points; this pattern was observed in the absence or presence of Kdo2-Lipid A or Pam_3CSK4 stimulation (Fig. 2-8A-C). A very similar pattern was observed in TLR stimulated B-2 cells (Fig. 2-9A-B). Nor1 expression in unstimulated B-1 cells increased by a remarkable 50 to 80-fold above initial values and unlike other NR4A receptors, this dramatically increased level of expression was sustained for the duration of the experiment. Stimulation with Kdo2-Lipid A led to a small increase at 6h compared to unstimulated cells, but marked inhibition of Nor1 expression by 24h of incubation. Whereas Pam_3CSK4 treatment increased Nor1 expression 4 to 5-fold above unstimulated cells at the 6h time point (Fig. 2-8A-C), there was apparent suppression at 24h and 72h. Again, very similar patterns were observed in stimulated B-2 cells (Fig. 2-9A-B). Nurr1 mRNA levels may be lower than that of the other NR4A receptors as expression analysis resulted in a notably larger Ct value (Suppl. Table 2-1); nevertheless, in response to TLR4 activation it displayed a robust ~25-fold induction within 6h but resolving back toward basal by 3 days. In response to TLR2 activation a different pattern was seen with a remarkable 150-fold increase in levels at 24h, which then returned to basal levels by 72h. Unlike the other NR4A receptors Nurr1 had a unique expression pattern in stimulated B-2 cells, highlighted by a biphasic induction by TLR stimulation of 20-fold and 40-fold after 6h and 72h, respectively (Fig. 2-9A-B). It has been proposed that in response to extracellular signals, NGFIβ and other NR4A receptors interact with NFκB and glucocorticoid signaling cascades to function as modulators of cell fate decisions to affect activation, differentiation, or cell death through apoptotic or non-apoptotic pathways (78).

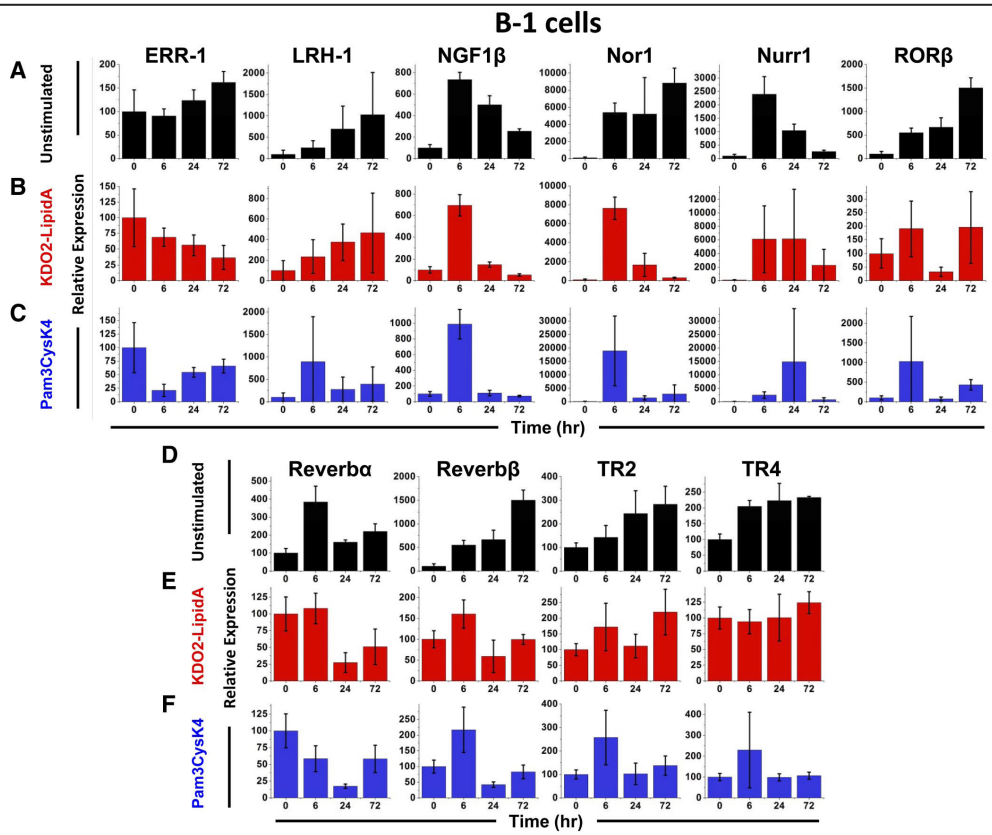


Fig.2-8 Basal and Stimulated Expression Profiles of B-1 Cell Orphan Nuclear Receptors. A, Orphan receptors activators in basal peritoneal B1 cells generally display dynamic expression. LRH-1 and NOR-1 increased expression over time. NGF1-β and NURR-1 had dramatically increased expression at 6h followed by decreasing expression. ERR-1 expression had minimal change over time. **B,** Kdo2-LipidA caused modest down regulation of ERR-1 expression and induced expression of LRH-1. It strongly induced expression of NGF1-β, NOR-1, and NURR-1 at 6h followed by decreasing expression. **C,** Pam3CSK4 induced many of the same general effects as Kdo2-Lipid A on the expression of ERR-1, NGF1-β, and NOR-1. LRH-1 expression was only strongly induced at 6h time point and induction of NURR-1 was delayed until the 24h time point. **D,** Orphan receptors repressors in basal peritoneal B-1 cells generally display increasing expression over time, Reverb-α expression peaked at 6h and decreased thereafter. **E,** Kdo2-Lipid A inhibited Reverbα and β expression at later time points and had minimal effect on TR2 and TR4 expression. **F,** Pam3CSK4 induced many of the same general effects as Kdo2-Lipid A on the expression of Reverb-α, –β, TR2 and TR4. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD.

RAR-related receptor (ROR) beta is reported to have a restricted expression pattern that is limited to certain regions of the brain and retina (79;80). Nevertheless, we report the expression of RORβ in peritoneal B-1 cells (Figs. 2-2 & 2-8), although its function in B-1 cells is entirely unknown. RORβ purportedly plays an important role in early rod photoreceptor fate decision during retinal development (81). The expression pattern of RORβ in the brain suggests that it may be involved in regulating cells that process sensory information (82). RORβ null mice exhibit motor defects, neurological reflex impairment, olfactory deficits, as well as several behavioral changes (83). Remarkably, unstimulated B-1 cells displayed a large induction of RORβ expression over time in culture, peaking at 72h with a 15-fold increase over baseline (Fig. 2-8A). Compared to the basal time course, stimulation with either Kdo2-Lipid A or Pam_3CSK4 led to a biphasic response in which RORβ expression increased at 6h and 72h, but decreased at 24h (Fig 2-8B-C). Unstimulated B-2 cells express 13-fold higher levels of RORβ than B-1 cells (Fig. 2-3) and this high level of expression is dramatically reduced by TLR stimulation (Fig. 2-9A-B). The novel findings of robust expression of RORβ in B cells, which increases dramatically over time in culture in the absence of activation (at least in B-1 cells) as well as the complex patterns of expression in response to TLR 4 and 2 activation suggest an important role for this nuclear receptor in B-1 and B-2 cell function. It will be of great interest to determine what function(s) it might play in these cells.

Rev-erb α and β are thought to act exclusively as constitutive repressors of transcription as they lack the interaction domain responsible for association with coactivator molecules (84). Both receptors are involved in circadian regulation and are important in lipid metabolism (reviewed in (71)). Reports

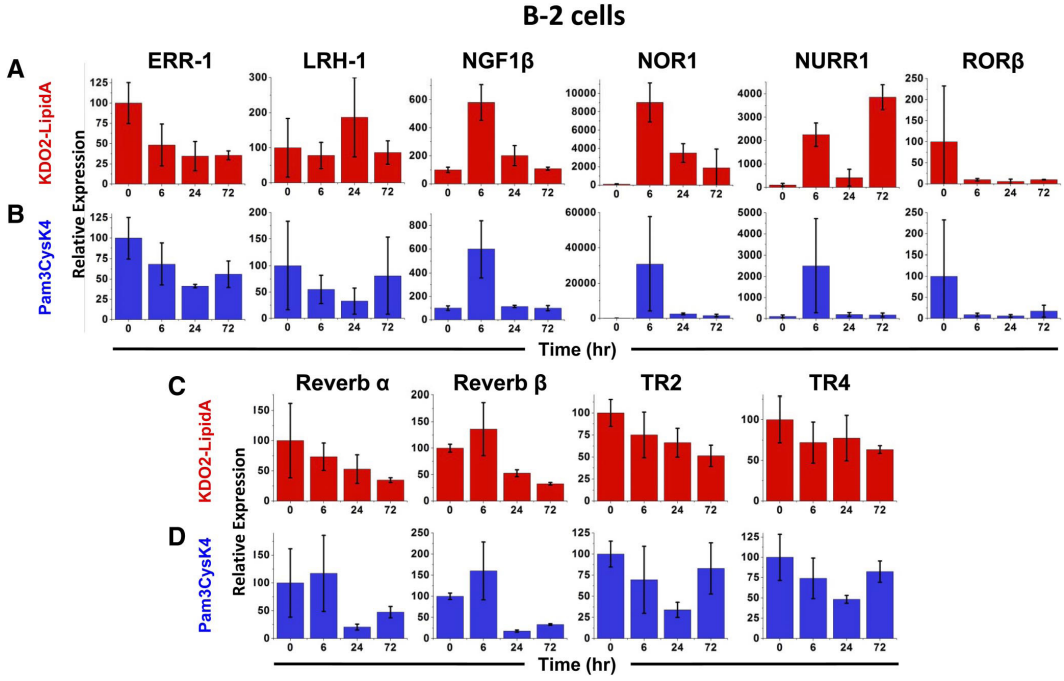


Fig.2-9 Stimulated Expression Profiles of B-2 Cell Orphan Nuclear Receptors. A,C, Treatment with KDO2-LipidA. Orphan receptors in stimulated peritoneal B2 cells displayed dynamic expression. ERR-1, Reverb-α, Reverb-β, ROR-β, TR2, TR4 displayed decreasing levels of expression overtime. LRH-1, NGF1-β, NOR-1, and NURR-1 showed peaks of expression at subsequent time points. **B,D,** Treatment with Pam3CSK4 induced many of the same general effects as KDO2-LipidA on the expression of most of the orphan nuclear receptors. LRH-1 expression was not induced at the 24h time point and NURR-1 expression did not peak at 72h. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD.

document the expression of Rev-erb α in mouse and human B cells and Rev-erb β in human B cells (10;85). Concordantly, we report that both Rev-erb α and β are expressed in both B-1 and B-2 cells, with slightly greater expression in B-2 cells (Fig. 2-3). Within B-1 cells, Rev-erb α and β showed marked increases in expression in culture even in the absence of stimulation, particularly Reverb β, which increased 15-fold. In response to TLR stimulation there was a general decrease in expression over time (Fig 2-8D-F). Similarly, in B-2 cells, the expression of both Rev-erb receptors was inhibited by TLR stimulation over the 72h time course (Fig. 2-9 C-D). There is evidence for a role for Rev-erb nuclear receptors in regulation of inflammation as Rev-erb α over

expression in smooth muscle cells induces NF-κB signaling and proinflammatory cytokine expression (86). Additionally, it has been demonstrated that Rev-erb α in a negative feedback loop represses the induction of TLR4 expression by LXR signaling in human macrophages (87). It remains to be investigated whether Rev-erb activity can modulate inflammatory signaling and immune responses in B cells.

ERR1 has pleiotropic effects on metabolism and is a major effector of the coactivators PGC-1α/β in specific tissues such as the heart, brown adipose tissue, macrophages, and others (reviewed in (88)). Interestingly, it has also been implicated in immune function: as a controller of metabolism it plays an important role in the anti-bacterial immune response of macrophages stimulated with interferon-γ (IFN-γ) (89). In B lymphocytes, using a yeast two hybrid screen it was found that human ERR1 interacts with the Epstein bar virus nuclear antigen leader protein and was important in virus induced cellular immortalization (90). It has been demonstrated that IFN-γ and LPS induce a 4-fold and 8-fold increase respectively in ERR1 expression in macrophages (8). ERR1 expression in these LPS stimulated macrophages was unchanged until the 8h time point where it suddenly peaked and then declined to near baseline levels by 24h. Both B-1 and B-2 cells expressed ERR1 and in both cell types there was a suppression of expression in response to TLR activation (Figs 2-8A-C & 2-9A-B). It is plausible that ERR1 expression could have increased between the 6h and 24h time points (or any other time points) examined in this study, so it is unclear whether ERR1 is differentially regulated in B cells versus macrophages. Moreover, the functional role of ERR1 in B cells remains to be studied.

Nuclear Receptor Expression in Splenic B cells

In addition to peritoneal B-2 cells, splenic B cells were isolated from the same experimental mice and exposed to the identical treatment protocol. Expression of the 49 murine nuclear receptors was assessed in a basal state and over the same 72h time course using a high-throughput qPCR platform in parallel to the analysis of the B-2 cells as shown in Fig. 2-1. Here we report the basal levels of expression of nuclear receptors in splenic B cells and compare these to values in peritoneal B-2 cells (Suppl Figs.2-1 & 2-2).

Qualitatively, splenic B cells express a similar cohort of nuclear receptors as peritoneal B-2 cells, with a few prominent exceptions (Suppl Fig. 2-1). Splenic B cells express 23 of the 49 murine nuclear receptors, including eight endocrine receptors, five adopted orphan receptors, and ten orphan receptors, including six constitutive activators and four constitutive repressors. Compared to peritoneal B-2 cells, splenic B cells lacked consistent expression of both the AR and RARβ. Interestingly, it has been reported that B-2 cells from the peritoneal cavity display unique properties relative to splenic B-2 cells (follicular). Many of the unique peritoneal B-2 cell characteristics are B-1 cell-like, including: a unique cell surface phenotype, enhanced *in vitro* survival, and enhanced *in vitro* secretion of IgM (91). Therefore, it is not surprising to find that splenic B cells, compared to peritoneal B-2 cells, express quantitatively dissimilar levels of mRNA transcripts for many if not most of the nuclear receptors (Suppl. Fig. 2-2). Furthermore, splenic B cells are a heterogeneous population, comprised of 80-90% follicular B cells, 5-10% marginal zone B cells, and 2-3% B-1 cells (92;93). The splenic B cell population used in these experiments was depleted of CD43+ cells (a marker of B-1 like cells in the spleen) and therefore contained very few B-1 cells and was a mixture of pre-

dominantly follicular B cells and a few marginal zone B cells (93). Therefore, the disparate nuclear receptor expression levels observed between peritoneal B-2 and splenic B cells may be due to both inherent differences between B-2 cells from different locations within the body and the contribution of marginal zone B cells to the splenic B cell population.

We also present the expression profiles of the 23 nuclear receptors observed over the 72h time course of TLR4 and TLR2 stimulation. While we think these profiles offer valuable information for future studies, these observations should be viewed with the caveat noted above that this represents a heterogeneous population of splenic B cells (Suppl. Figs. 2-3, 2-4, 2-5).

Discussion

In this study, we have ascertained the complete complement of nuclear receptors expressed in basal and TLR activated B-1 and B-2 cells, as well as similar comparative profiles of splenic B cells. These data provide valuable information that should be highly heuristic for understanding the molecular events involved in the differential regulation and unique biology of B-1 and B-2 cells. Utilizing quantitative PCR, we identified 24 nuclear receptors expressed in B-1 cells and report that they are dynamically expressed in both the unstimulated as well as in TLR 4 or TLR 2/1 stimulated conditions. Notably, the expression of most nuclear receptors was quite dynamic over 72h in culture even in the unstimulated state, showing substantial increases or decreases in expression in our *in vitro* culture system. The addition of Kdo2-Lipid A or Pam_3CysK4 to the cell culture medium often had profound and unique effects on the expression profiles of expressed nuclear receptors. Another remarkable finding was the quantitative dissimilarity of expression levels between B-1

and B-2 cells for most nuclear receptors. These findings offer clues as to the identity of nuclear receptors responsible for regulating B-1 and or B-2 cells.

This Atlas provides a basal set of information to allow study of the mechanisms by which nuclear receptors participate in controlling B-1 and B-2 cell function. Careful analysis of the temporal patterns of expression of the receptors especially under inflammatory stimuli can provide useful information. For example, MR expression levels drop dramatically in both B-1 and B-2 cells at the initial time point following TLR 4 or TLR 2/1 stimulation (Figs. 2-4B-C & 2-5A-B). B cells respond to TLR stimulation by increased proliferation and differentiation, which perhaps is normally antagonized by MR (and GR) mediated signaling. Conversely, many receptors display dramatic increases in expression after stimulation with TLR agonists and may participate in the proliferative or differentiation response of these cells. As many of the nuclear receptors indentified in B-1 and B-2 cells have known ligands and in some cases pharmaceutical antagonists, this work highlights the potential therapeutic role of these receptors in B cells. As B cells are critically involved in both innate and adaptive immune functions, as well as in various autoimmune or inflammatory diseases, this Atlas provides a critical foundation for new roles of nuclear receptor signaling in B cells.

Acknowledgements

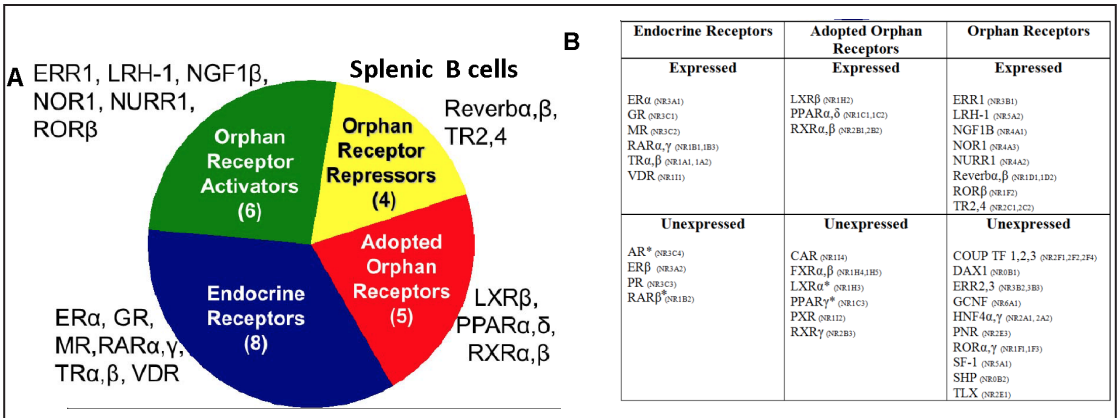
Chapter 2, in full, is a reprint of the material with only slight formatting changes from how it appears in Research Resource: Comparative Nuclear Receptor Atlas: Basal and Activated Peritoneal B-1 and B-2 Cells in Molecular Endocrinology 2011. Diehl, Cody J; Barish, Grant D; Downes, Michael; Chou, Meng-Yun; Heinz, Sven; Glass, Christopher K; Evans, Ronald M; Witztum, Joseph L. Molecular Endocrinology 25 (3): 529. The dissertation author was the primary investigator and author of this paper.

Supplemental Material

Presented below are the supplemental table and figures that were published online in conjunction with the manuscript presented above.

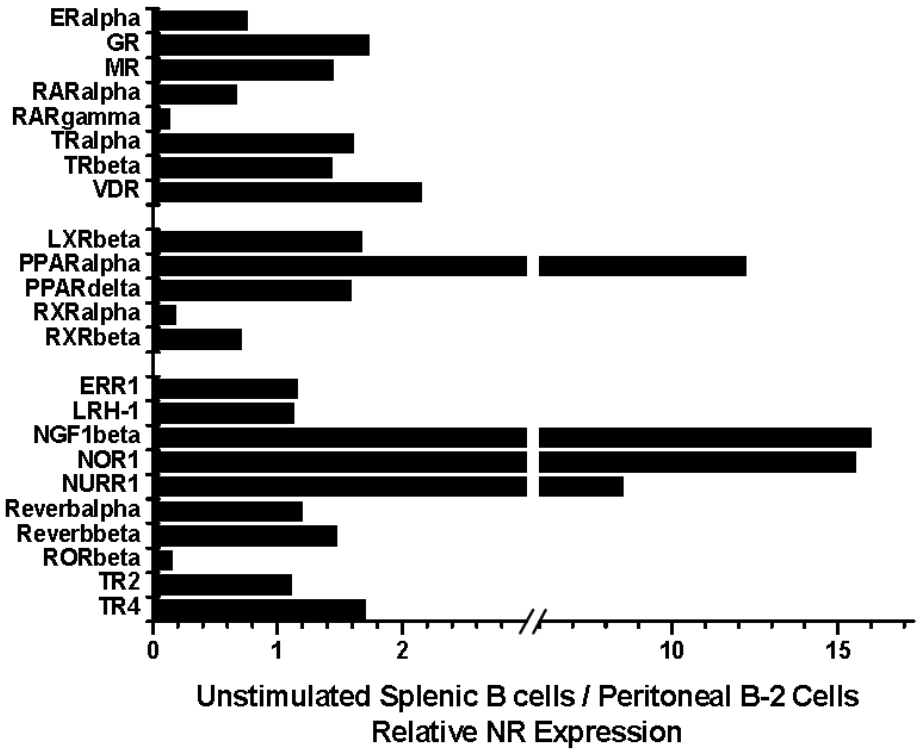
Suppl.Table 2-1. Average Cycle Threshold Values of Expressed Nuclear Receptors among select Leukocytes. Mean cycle threshold (Ct) values from qPCR reactions using cDNA derived from basal peritoneal B-1 and B-2 cells, splenic B cells, and basal bone marrow derived macrophages (BMMΦ) for all expressed nuclear receptors. Note that one cannot use Ct values to rank expression levels among different nuclear receptors as the amplification threshold is calculated uniquely for each target and primer efficiencies differ between qPCR reactions.

	AR	ERα	ERR1	GR	LRH1	LXRα	LXRβ	MR	NGF1β	NOR1	NURR1	PPARα	PPARδ	PPARγ	PPARγ2	PR	RARα	RARβ	RARγ	Reverbα	Reverbββ	RORβ	RXRα	RXRβ	SHP	TR2	TR4	TRα	TRβ	VDR
B1 cell	trace	27.7	25.6	24.9	29.4	34.8	23.6	19.8	25.5	29.9	32.7	29.7	27.3	trace	-	-	21.2	trace	24.3	26.2	21.8	29	24.6	25.8	-	26.1	20.1	24.1	32.2	31.3
B2 cell	30.9	27.2	25.7	24.7	28.7	trace	23.9	23.0	24.9	30.5	33.9	32.7	27.2	trace	-	trace	23.6	27.8	25.2	27.5	27.2	28.3	24.4	25.3	-	25.6	20.2	23.1	29.3	30.4
Splenic B cell	-	28.4	26.3	25.0	29.7	-	24.0	23.1	21.5	27.5	31.3	30.3	27.3	-	-	trace	22.2	-	29.6	22.5	22.5	29.8	27.1	26.4	-	26.1	20.4	23.3	29.1	29.8
BMMφ	-	30.2	28.4	27.5	27.7	28.3	25.8	32.0	31.2	33.5	trace	trace	28.8	31.1	31.8	-	25.2	-	27.0	30.7	26.8	27.0	26.9	28.8	34.6	26.7	26.5	27.2	31.5	32.1

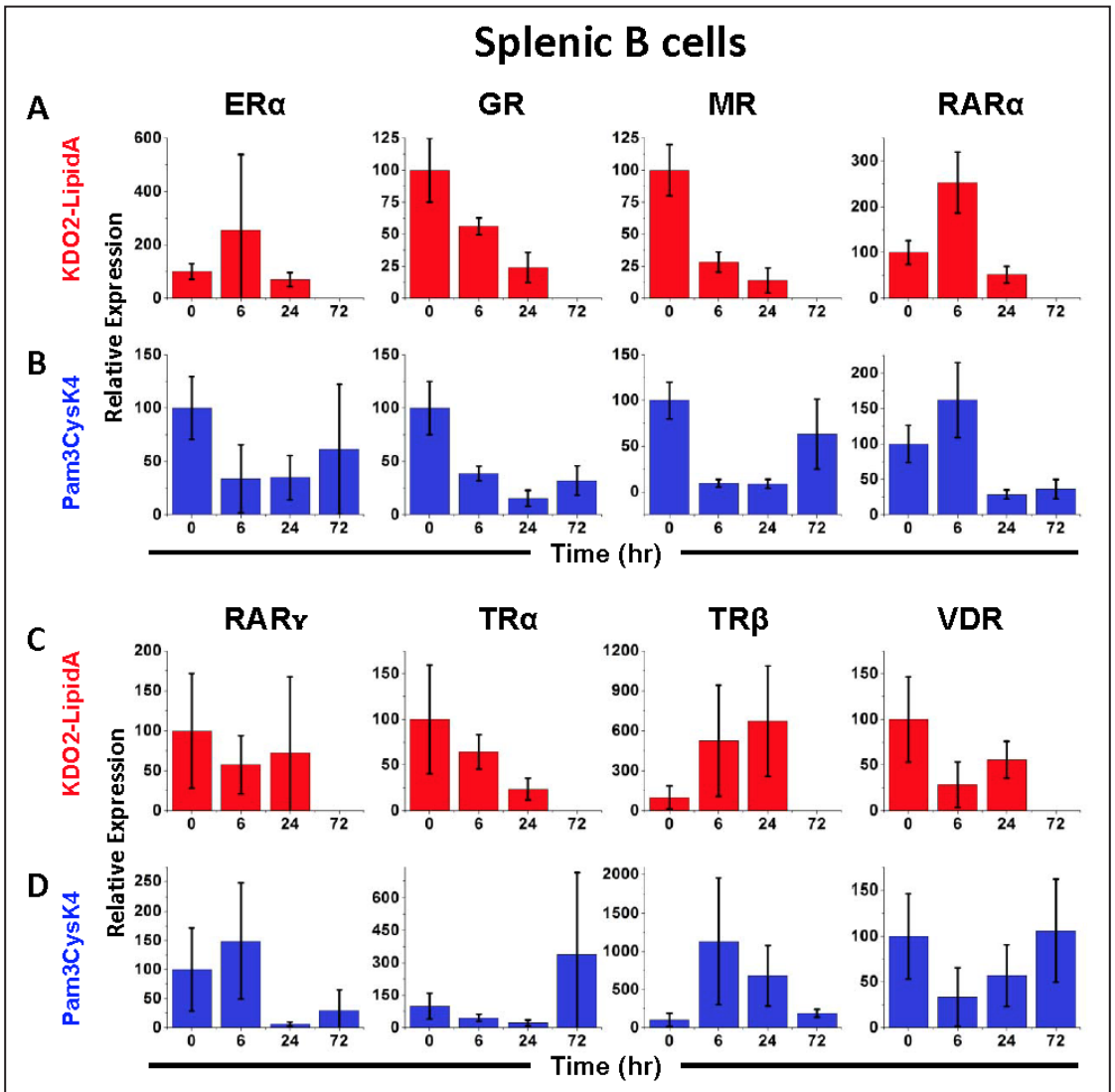


Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Expressed	**Expressed**	**Expressed**
ERα (NR3A1) GR (NR3C1) MR (NR3C2) RARα,γ (NR1B1,1B3) TRα,β (NR1A1, 1A2) VDR (NR1I1)	LXRβ (NR1H2) PPARα,δ (NR1C1,1C2) RXRα,β (NR2B1,2B2)	ERR1 (NR3B1) LRH-1 (NR5A2) NGF1B (NR4A1) NOR1 (NR4A3) NURR1 (NR4A2) Reverbα,β (NR1D1,1D2) RORβ (NR1F2) TR2,4 (NR2C1,2C2)
Unexpressed	**Unexpressed**	**Unexpressed**
AR* (NR3C4) ERβ (NR3A2) PR (NR3C3) RARβ* (NR1B2)	CAR (NR1I4) FXRα,β (NR1H4,1H5) LXRα* (NR1H3) PPARγ* (NR1C3) PXR (NR1I2) RXRγ (NR2B3)	COUP TF 1,2,3 (NR2F1,2F2,2F4) DAX1 (NR0B1) ERR2,3 (NR3B2,3B3) GCNF (NR6A1) HNF4α,γ (NR2A1, 2A2) PNR (NR2E3) RORα,γ (NR1F1,1F3) SF-1 (NR5A1) SHP (NR0B2) TLX (NR2E1)

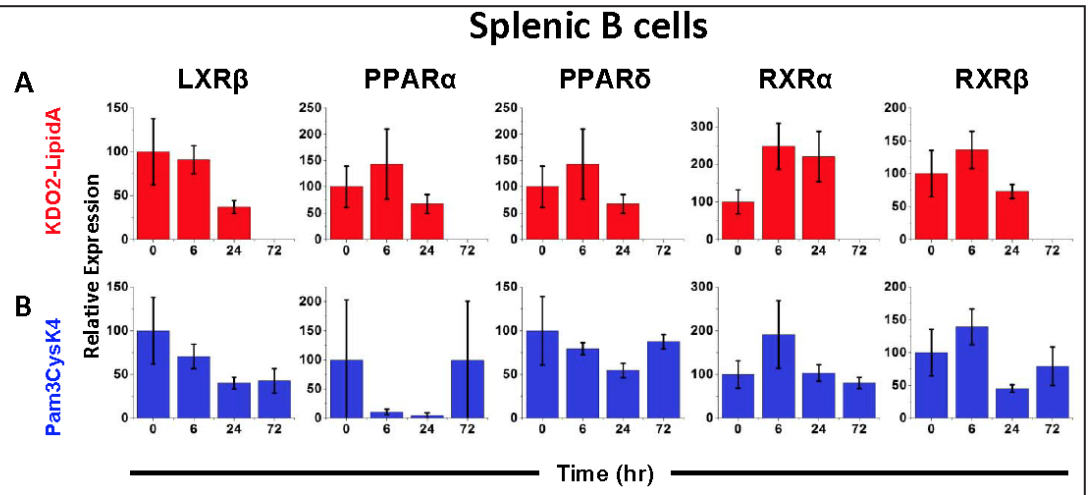
Suppl. Fig. 2-1. The Composition of Nuclear Receptors Expressed in Splenic B cells. **A,** Twenty-three of 49 known nuclear receptors are expressed in the splenic B cell. These include eight endocrine receptors, five adopted orphan receptors that bind low-affinity dietary lipids, and ten orphan receptors, including six constitutive activators and four constitutive repressors. **B,** Tabular listing of nuclear receptors expressed or non-expressed in peritoneal B-2 cells with their unified nomenclature system names listed in parentheses (94). Receptors were deemed unexpressed if cycle threshold (Ct) values exceeded 35. The actual Ct values are given in Supplementary Table 1 in comparison to simultaneously determined Ct values for the same receptors in peritoneal B-1 and B-2 cells and bone marrow derived macrophages. *Trace or inconsistent expression detectable.



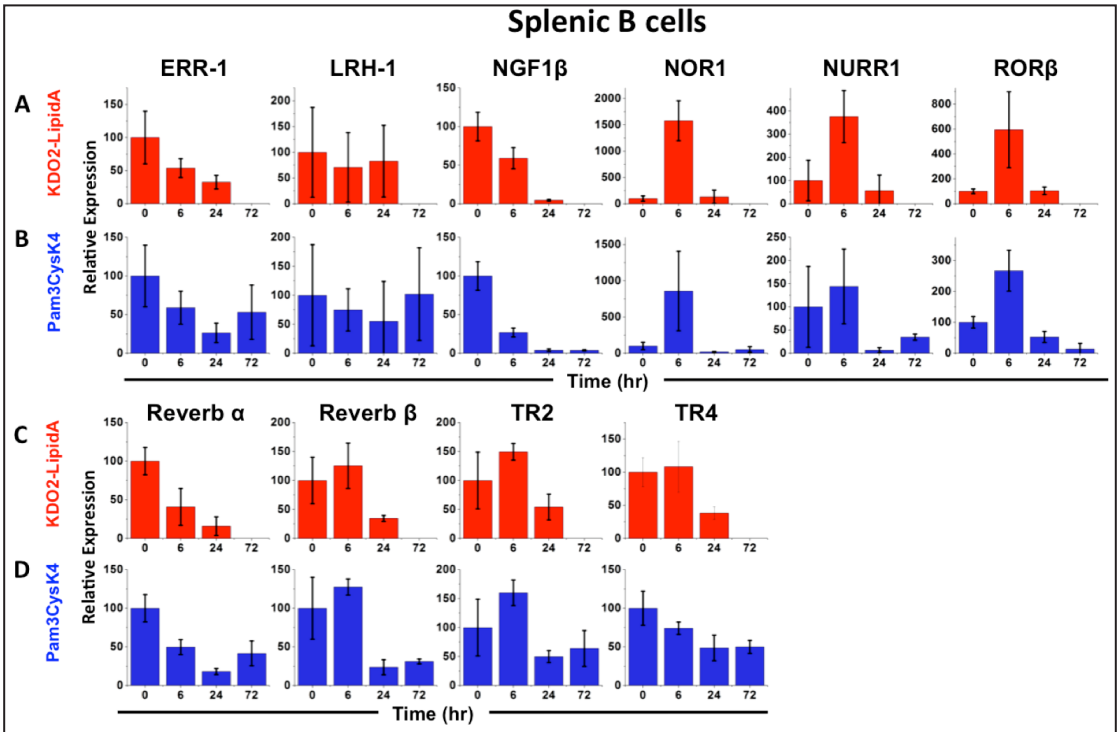
Suppl.Fig. 2-2. Relative Nuclear Receptor Expression in Basal splenic B cells versus peritoneal B-2 cells. Basal expression at time zero (normalized to 36B4) in splenic B cells relative to peritoneal B-2 cells. The NR4A subgroup (NGF1-β, NOR-1, NURR-1) as well as PPARα nuclear receptors are expressed at 8 to 15-fold higher levels in splenic B cells. RARγ, RXRα, and RORβ are expressed at drastically lower levels in splenic B cells than in peritoneal B-2 cells.



Suppl. Fig. 2-3. Stimulated Expression Profiles of Splenic B Cell Endocrine Nuclear Receptors. A,C, Treatment of splenic B cells with KDO2-LipidA, a TLR4 ligand. Note that splenic B cells did not survive in adequate numbers to assess expression at the 72h time point. **B,D,** Treatment with Pam3CSK4, a TLR2/1 ligand. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD



Suppl. Fig. 2-4. Stimulated Expression Profiles of Splenic B Cell Adopted Orphan Nuclear Receptors. A, Treatment of splenic B cells with KDO2-LipidA, a TLR4 ligand. Note that splenic B cells did not survive in adequate numbers to assess expression at the 72h time point. **B, T**reatment with Pam3CSK4, a TLR2/1 ligand. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD



Suppl. Fig. 2-5 Stimulated Expression Profiles of Splenic B Cell Orphan Nuclear Receptors. A,C, Treatment of splenic B cells with KDO2-LipidA, a TLR4 ligand. Note that splenic B cells did not survive in adequate numbers to assess expression at the 72h time point. **B,D,** Treatment with Pam3CSK4, a TLR2/1 ligand. Basal expression at time zero (normalized to 36B4) was assigned an expression value of 100, and subsequent time points are relative to time zero. Error bars represent SD

References

1. Martin F, Kearney JF 2000 B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a “natural immune memory”. Immunological Reviews 175:70-79

2. Baumgarth N, Tung JW, Herzenberg LA 2005 Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Seminars in Immunopathology 26:347-362

3. Shaw PX, Hörkkö S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, Witztum JL 2000 Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. Journal of Clinical Investigation 105:1731-1740

4. Bouaziz JD, Yanaba K, Tedder TF 2008 Regulatory B cells as inhibitors of immune responses and inflammation. Immunological Reviews 224:201-214

5. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA 1983 The Ly-1 B-Cell Subpopulation in Normal, Immunodefective, and Autoimmune Mice. Journal of Experimental Medicine 157:202-218

6. Murakami M, Honjo T 1995 B-1 Cells and Autoimmunity. Annals of the New York Academy of Sciences 764:402-409

7. Viau M, Zouali M 2005 B-lymphocytes, innate immunity, and autoimmunity. Clinical Immunology 114:17-26

8. Barish GD, Downes M, Alaynick WA, Yu RT, Ocampo CB, Bookout AL, Mangelsdorf DJ, Evans RM 2005 A nuclear receptor atlas: Macrophage activation. Mol Endocrinol 19:2466-2477

9. Szatmari I, Nagy L 2008 Nuclear receptor signalling in dendritic cells connects lipids, the genome and immune function. Embo Journal 27:2353-2362

10. Schote AB, Turner JD, Schiltz J, Muller CP 2007 Nuclear receptors in human immune cells: Expression and correlations. Molecular Immunology 44:1436-1445

11. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T 2007 TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. Journal of Immunology 178:7779-7786

12. Akira S, Takeda K 2004 Toll-like receptor signalling. Nature Reviews Immunology 4:499-511

13. Miller YI, Choi S, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, Boullier A, Gonen A, Diehl CJ, Que X, Montano E, Shaw PX, Tsimikas S, Binder CJ, Witztum JL 2011 Oxidation-Specific Epitopes Are Danger-Associated Molecular Patterns Recognized by Pattern Recognition Receptors of Innate Immunity. *Circulation Research In Press* ed.

14. Raetz CR, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC, Jr., Ribeiro AA, Murphy RC, Ulevitch RJ, Fearns C, Reichart D, Glass CK, Benner C, Subramaniam S, Harkewicz R, Bowers-Gentry RC, Buczynski MW, Cooper JA, Deems RA, Dennis EA 2006 Kdo2-Lipid A of Escherichia coli, a defined endotoxin that activates macrophages via TLR-4. J Lipid Res 47:1097-1111

15. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, Zychlinsky A 1999 Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285:736-739

16. Murakami M, Tsubata T, Shinkura R, Nisitani S, Okamoto M, Yoshioka H, Usui T, Miyawaki S, Honjo T 1994 Oral-Administration of Lipopolysaccharides Activates B-1 Cells in the Peritoneal-Cavity and Lamina Propria of the Gut and Induces Autoimmune Symptoms in an Autoantibody Transgenic Mouse. Journal of Experimental Medicine 180:111-121

17. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Bäckhed F, Miller YI 2009 Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. J Clin Invest 119:1335

18. Savitsky D, Calame K 2006 B-1B lymphocytes require Blimp-1 for immunoglobulin secretion. Journal of Experimental Medicine 203:2305-2314

19. Barr TA, Brown S, Ryan G, Zhao J, Gray D 2007 TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells. European Journal of Immunology 37:3040-3053

20. Borsutzky S, Kretschmer K, Becker PD, Muhlradt PF, Kirschning CJ, Weiss S, Guzman CA 2005 The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. Journal of Immunology 174:6308-6313

21. Mandler R, Finkelman FD, Levine AD, Snapper CM 1993 Il-4 Induction of Ige Class Switching by Lipopolysaccharide-Activated Murine B-Cells Occurs Predominantly Through Sequential Switching. Journal of Immunology 150:407-418

22. Pone EJ, Zan H, Zhang J, Al-Qahtani A, Xu Z, Casali P 2010 Toll-like receptors and B-cell receptors synergize to induce immunoglobulin class-switch DNA recombination: relevance to microbial antibody responses. Critical reviews in immunology 30:1-29

23. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ 2001 Nuclear receptors and lipid physiology: opening the X-files. Science 294:1866-1870

24. Arpin C, Pihlgren M, Fraichard A, Aubert D, Samarut J, Chassande O, Marvel J 2000 Effects of T3R alpha 1 and T3R alpha 2 gene deletion on T and B lymphocyte development. Journal of Immunology 164:152-160

25. Dorshkind K, Horseman ND 2000 The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: Insights from genetic models of hormone and hormone receptor deficiency. Endocrine Reviews 21:292-312

26. Balmer JE, Blomhoff R 2002 Gene expression regulation by retinoic acid. Journal of Lipid Research 43:1773-1808

27. Stephensen CB 2001 Vitamin A, infection, and immune function. Annu Rev Nutr 21:167-192

28. Ballow M, Wang XC, Xiang SN, Allen C 2003 Expression and regulation of nuclear retinoic acid receptors in human lymphoid cells. Journal of Clinical Immunology 23:46-54

29. Ertesvag A, Naderi S, Blomhoff HK 2009 Regulation of B cell proliferation and differentiation by retinoic acid. Semin Immunol 21:36-41

30. Chen Q, Ross AC 2007 Retinoic acid promotes mouse splenic B cell surface IgG expression and maturation stimulated by CD40 and IL-4. Cell Immunol 249:37-45

31. Sherr E, Adelman DC, Saxon A, Gilly M, Wall R, Sidell N 1988 Retinoic acid induces the differentiation of B cell hybridomas from patients with common variable immunodeficiency. J Exp Med 168:55-71

32. Ballow M, Wang W, Xiang S 1996 Modulation of B-cell immunoglobulin synthesis by retinoic acid. Clin Immunol Immunopathol 80:S73-S81

33. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM 1987 Cloning of Human Mineralocorticoid Receptor Complementary-Dna - Structural and Functional Kinship with the Glucocorticoid Receptor. Science 237:268-275

34. Pascual-Le Tallec L, Lombes M 2005 The mineralocorticoid receptor: A journey exploring its diversity and specificity of action. Mol Endocrinol 19:2211-2221

35. Funder JW, Pearce PT, Smith R, Smith AI 1988 Mineralocorticoid Action - Target Tissue-Specificity Is Enzyme, Not Receptor, Mediated. Science 242:583-585

36. Hsueh WA, Do YS, Jeyaseelan R 1998 Angiotensin II and cardiac remodeling. Mt Sinai J Med 65:104-107

37. Pu Q, Neves MF, Virdis A, Touyz RM, Schiffrin EL 2003 Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. Hypertension 42:49-55

38. Herrada AA, Contreras FJ, Marini NP, Amador CA, Gonzalez PA, Cortes CM, Riedel CA, Carvajal CA, Figueroa F, Michea LF, Fardella CE, Kalergis AM 2010 Aldosterone Promotes Autoimmune Damage by Enhancing Th17-Mediated Immunity. Journal of Immunology 184:191-202

39. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schntz G,

Lumeng CN, Mortensen RM 2010 Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. J Clin Invest 120:3350

40. Rickard AJ, Morgan J, Tesch G, Funder JW, Fuller PJ, Young MJ 2009 Deletion of mineralocorticoid receptors from macrophages protects against deoxycorticosterone/salt-induced cardiac fibrosis and increased blood pressure. Hypertension 54:537

41. Hayes CE, Nashold FE, Spach KM, Pedersen LB 2003 The immunological functions of the vitamin D endocrine system. Cell Mol Biol 49:277-300

42. Gyetko MR, Hsu CH, Wilkinson CC, Patel S, Young E 1993 Monocyte 1 alpha-hydroxylase regulation: induction by inflammatory cytokines and suppression by dexamethasone and uremia toxin. Journal of Leukocyte Biology 54:17

43. Reichel H, Koeffler HP, Norman AW 1990 Production of 1 alpha,25-dihydroxyvitamin D3 by hematopoietic cells. Prog Clin Biol Res 332:81-97

44. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC 1983 1,25-dihydroxyvitamin D3 receptors in human leukocytes. Science 221:1181-1183

45. Wilson CA, Mrose SA, Thomas DW 1995 Enhanced production of B lymphocytes after castration. Blood 85:1535-1539

46. Kumar N, Shan LX, Hardy MP, Bardin CW, Sundaram K 1995 Mechanism of androgen-induced thymolysis in rats. Endocrinology 136:4887

47. Viselli SM, Reese KR, Fan J, Kovacs WJ, Olsen NJ 1997 Androgens alter B cell development in normal male mice. Cellular Immunology 182:99-104

48. Ellis TM, Moser MT, Le PT, Flanigan RC, Kwon ED 2001 Alterations in peripheral B cells and B cell progenitors following androgen ablation in mice. International Immunology 13:553-558

49. Medina KL, Smithson G, Kincade PW 1993 Suppression of B lymph-

opoiesis during normal pregnancy. Journal of Experimental Medicine 178:1507

50. Igarashi H, Kouro T, Yokota T, Comp PC, Kincade PW 2001 Age and stage dependency of estrogen receptor expression by lymphocyte precursors. Proceedings of the National Academy of Sciences of the United States of America 98:15131

51. Kanda N, Tamaki K 1999 Estrogen enhances immunoglobulin production by human PBMCs. Journal of Allergy and Clinical Immunology 103:282-288

52. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B 2002 Estrogen alters thresholds for B cell apoptosis and activation. Journal of Clinical Investigation 109:1625-1633

53. Suenaga R, Evans MJ, Mitamura K, Rider V, Abdou NI 1998 Peripheral blood T cells and monocytes and B cell lines derived from patients with lupus express estrogen receptor transcripts similar to those of normal cells. Journal of Rheumatology 25:1305-1312

54. Yen PM 2001 Physiological and molecular basis of thyroid hormone action. Physiological Reviews 81:1097-1142

55. Falcone M, Miyamoto T, Fierrorenoy F, Macchia E, Degroot LJ 1992 Antipeptide Polyclonal Antibodies Specifically Recognize Each Human Thyroid-Hormone Receptor Isoform. Endocrinology 131:2419-2429

56. Hodin RA, Lazar MA, Chin WW 1990 Differential and Tissue-Specific Regulation of the Multiple Rat C-Erba Messenger-Rna Species by Thyroid-Hormone. Journal of Clinical Investigation 85:101-105

57. Auboeuf D, Rieusset J, Fajas L, Vallier P, Frering V, Riou JP, Staels B, Auwerx J, Laville M, Vidal H 1997 Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: no alteration in adipose tissue of obese and NIDDM patients. Diabetes 46:1319-1327

58. Cunard R, Ricote M, DiCampli D, Archer DC, Kahn DA, Glass CK, Kelly CJ 2002 Regulation of cytokine expression by ligands of peroxisome

proliferator activated receptors. J Immunol 168:2795-2802

59. Jones DC, Ding X, Daynes RA 2002 Nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha) is expressed in resting murine lymphocytes. The PPARalpha in T and B lymphocytes is both transactivation and transrepression competent. J Biol Chem 277:6838-6845

60. Cunard R 2005 The potential use of PPARalpha agonists as immunosuppressive agents. Curr Opin Investig Drugs 6:467-472

61. Cunard R, DiCampli D, Archer DC, Stevenson JL, Ricote M, Glass CK, Kelly CJ 2002 WY14,643, a PPAR{alpha} Ligand, Has Profound Effects on Immune Responses In Vivo. J Immunol 169:6806-6812

62. Delerive P, Gervois P, Fruchart JC, Staels B 2000 Induction of Ikappa-Balpha expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor-alpha activators. J Biol Chem 275:36703-36707

63. Bishop-Bailey D, Bystrom J 2009 Emerging roles of peroxisome proliferator-activated receptor-beta/delta in inflammation. Pharmacol Ther 124:141-150

64. Tan NS, Michalik L, Noy N, Yasmin R, Pacot C, Heim M, Fluhmann B, Desvergne B, Wahli W 2001 Critical roles of PPAR beta/delta in keratinocyte response to inflammation. Genes Dev 15:3263-3277

65. Lee CH, Chawla A, Urbiztondo N, Liao D, Boisvert WA, Evans RM, Curtiss LK 2003 Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. Science 302:453-457

66. Barish GD, Narkar VA, Evans RM 2006 PPAR : a dagger in the heart of the metabolic syndrome. Journal of Clinical Investigation 116:590-597

67 al YN, Romanowska M, Krauss S, Schweiger S, Foerster J 2008 PPAR-delta is a type 1 IFN target gene and inhibits apoptosis in T cells. J Invest Dermatol 128:1940-1949

68. Lu TT, Repa JJ, Mangelsdorf DJ 2001 Orphan nuclear receptors as

eLiXiRs and FiXeRs of sterol metabolism. J Biol Chem 276:37735-37738

69. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P 2008 LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell 134:97-111

70. Tanguay DA, Colarusso TP, Pavlovic S, Irigoyen M, Howard RG, Bartek J, Chiles TC, Rothstein TL 1999 Early induction of cyclin D2 expression in phorbol ester-responsive B-1 lymphocytes. J Exp Med 189:1685-1690

71. Burris TP 2008 Nuclear Hormone Receptors for Heme: REV-ERB{alpha} and REV-ERB{beta} Are Ligand-Regulated Components of the Mammalian Clock. Mol Endocrinol 22:1509-1520

72. Zhang J, DeYoung A, Kasler HG, Kabra NH, Kuang AA, Diehl G, Sohn SJ, Bishop C, Winoto A 1999 Receptor-mediated apoptosis in T lymphocytes. Cold Spring Harb Symp Quant Biol 64:363-371

73. Mittelstadt PR, DeFranco AL 1993 Induction of early response genes by cross-linking membrane Ig on B lymphocytes. J Immunol 150:4822-4832

74. Liu ZG, Smith SW, McLaughlin KA, Schwartz LM, Osborne BA 1994 Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. Nature 367:281-284

75. Lin B, Kolluri SK, Lin F, Liu W, Han YH, Cao X, Dawson MI, Reed JC, Zhang XK 2004 Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. Cell 116:527-540

76. Calnan BJ, Szychowski S, Chan FKM, Cado D, Winoto A 1995 A Role for the Orphan Steroid-Receptor Nur77 in Apoptosis Accompanying Antigen-Induced Negative Selection. Immunity 3:273-282

77. Krajewska M, Kitada S, Winter JN, Variakojis D, Lichtenstein A, Zhai D, Cuddy M, Huang X, Luciano F, Baker CH, Kim H, Shin E, Kennedy S, Olson AH, Badzio A, Jassem J, Meinhold-Heerlein I, Duffy MJ, Schim-

mer AD, Tsao M, Brown E, Sawyers A, Andreeff M, Mercola D, Krajewski S, Reed JC 2008 Bcl-B expression in human epithelial and nonepithelial malignancies. Clin Cancer Res 14:3011-3021

78. Castro-Obregon S, Rao RV, del RG, Chen SF, Poksay KS, Rabizadeh S, Vesce S, Zhang XK, Swanson RA, Bredesen DE 2004 Alternative, nonapoptotic programmed cell death: mediation by arrestin 2, ERK2, and Nur77. J Biol Chem 279:17543-17553

79. Schaeren-Wiemers N, Andre E, Kapfhammer JP, Becker-Andre M 1997 The expression pattern of the orphan nuclear receptor RORbeta in the developing and adult rat nervous system suggests a role in the processing of sensory information and in circadian rhythm. Eur J Neurosci 9:2687-2701

80. Andre E, Gawlas K, Becker-Andre M 1998 A novel isoform of the orphan nuclear receptor RORbeta is specifically expressed in pineal gland and retina. Gene 216:277-283

81. Jia L, Oh EC, Ng L, Srinivas M, Brooks M, Swaroop A, Forrest D 2009 Retinoid-related orphan nuclear receptor RORbeta is an early-acting factor in rod photoreceptor development. Proc Natl Acad Sci U S A 106:17534-17539

82. Jetten AM 2009 Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. Nucl Recept Signal 7:e003

83. Masana MI, Sumaya IC, Becker-Andre M, Dubocovich ML 2007 Behavioral characterization and modulation of circadian rhythms by light and melatonin in C3H/HeN mice homozygous for the RORbeta knockout. Am J Physiol Regul Integr Comp Physiol 292:R2357-R2367

84. Dumas B, Harding HP, Choi HS, Lehmann KA, Chung M, Lazar MA, Moore DD 1994 A new orphan member of the nuclear hormone receptor superfamily closely related to Rev-Erb. Mol Endocrinol 8:996

85. Hastings ML, Milcarek C, Martincic K, Peterson ML, Munroe SH 1997 Expression of the thyroid hormone receptor gene, erbAalpha, in B lymphocytes: alternative mRNA processing is independent of differentiation but correlates with antisense RNA levels. Nucleic acids research

25:4296

86. Migita H, Morser J, Kawai K 2004 Rev-erb [alpha] upregulates NF-[kappa] B-responsive genes in vascular smooth muscle cells. FEBS letters 561:69-74

87. Fontaine C, Rigamonti E, Pourcet B, Duez H, Duhem C, Fruchart JC, Chinetti-Gbaguidi G, Staels B 2008 The Nuclear Receptor Rev-erb{alpha} Is a Liver X Receptor (LXR) Target Gene Driving a Negative Feedback Loop on Select LXR-Induced Pathways in Human Macrophages. Mol Endocrinol 22:1797-1811

88. Villena JA, Kralli A 2008 ERR[alpha]: a metabolic function for the oldest orphan. Trends in Endocrinology & Metabolism 19:269-276

89. Sonoda J, Laganiore J, Mehl IR, Barish GD, Chong LW, Li X, Scheffler IE, Mock DC, Bataille AR, Robert F 2007 Nuclear receptor ERR and coactivator PGC-1 are effectors of IFN-gamma -induced host defense. Genes & Development 21:1909

90. Igarashi M, Kawaguchi Y, Hirai K, Mizuno F 2003 Physical interaction of Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) with human oestrogen-related receptor 1 (hERR1): hERR1 interacts with a conserved domain of EBNA-LP that is critical for EBV-induced B-cell immortalization. Journal of General Virology 84:319

91. Hastings WD, Tumang JR, Behrens TW, Rothstein TL 2006 Peritoneal B-2 cells comprise a distinct B-2 cell population with B-1b-like characteristics. Eur J Immunol 36:1114-1123

92. Oliver AM, Martin F, Kearney JF 1999 IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J Immunol 162:7198-7207

93. Stall AM, Wells SM, Lam KP 1996 B-1 cells: unique origins and functions. Semin Immunol 8:45-59

94. Laudet V, Auwerx J, Gustafsson JA, Wahli W 1999 A unified nomenclature system for the nuclear receptor superfamily. Cell 97:161-163

CHAPTER 3

Exploring *In Vitro* the Function of Various Nuclear Receptors in B-1 Cells.

Abstract

In vitro studies were undertaken to explore the role in B-1 cells of expressed nuclear receptors that have a known role in regulating immune and inflammatory function in other immune cells. The receptors studied in this chapter include the mineralocorticoid (MR), glucocorticoid (GR), and liver X receptors (LXR). Primary B-1 cells were cultured in vitro with nuclear receptor agonists and/or antagonists. A prominent phenotype for MR activation could not be identified using these methods. It was found that GR activation strongly inhibits the production of antibodies and induces apoptosis in B-1 cells. LXR activation on the other hand did not induce apoptosis but did inhibit B-1 cell proliferation. Furthermore, LXR knockout mice had increased IgM titers indicating that LXRs potentially play a role in inhibiting natural antibody production from B-1 cells.

Introduction

After finding that 24 of the 49 mouse nuclear receptors were expressed in B-1 cells, the goal was to test the hypothesis that some of these nuclear receptors are impacting the unique biology of B-1 cells and controlling their characteristic functions, most notably the production of natural antibodies (1). Based on literature and on the findings of the expressed nuclear receptors identified in B-1 cells outlined in Chapter 2, my strategy was to identify specific nuclear receptors that have unique expression patterns in B-1 cells and/or a reported prominent role in controlling other immune cells, particularly other B cells. My attention was immediately drawn to three nuclear receptors: The mineralocorticoid receptor (MR) because of its uniquely high expression in B-1 cells relative to B-2 cells. The glucocorticoid receptor (GR) because of its

close identity in many aspects with MR. The Liver X receptors (LXR) as they had recently been identified to play a prominent role in controlling the proliferative response of various lymphocytes (2).

The Mineralocorticoid Receptor has an Emerging Pro-inflammatory Role.

The role of the mineralocorticoid receptor in B-1 cells is completely unknown, in fact its role in B cells or lymphocytes in general is unknown. However, aldosterone, the physiological ligand for MR, has been shown to induce inflammatory injury to the heart, kidney, and vasculature (3,4). Furthermore, various recent studies have found a role for MR in various innate immune myeloid cells. Usher et al. (5) report that a macrophage specific knockout of MR results in macrophages that possess an 'alternative activation' phenotype- a more anti-inflammatory phenotype. Additionally, treatment of wild type macrophages with an MR antagonist (eplerenone) reduces the production of proinflammatory cytokines (eg. Tnfα, IL12, IL16, MCP1, RANTES). Another recent report has demonstrated the proinflammatory capacity of MR, but in dendritic cells in this case (6). They demonstrate that the CD8+ T cell response is enhanced by aldosterone and that this effect is dependent on dendritic cells (DC). They also show that aldosterone treatment of DCs induces proinflammatory cytokines (IL-6 &TGFβ) and promotes DC-dependent CD4+ T cell polarization toward Th17 cells, which can exacerbate disease in a mouse model of autoimmune encephalomyelitis. These studies provide strong evidence that aldosterone and in turn its receptor MR are strongly proinflammatory in macrophages/dendritic cells. Nevertheless, an additional study recently showed that aldosterone can have an anti-inflammatory effect and inhibit NFκB signaling and TNFα production in human neutrophils (7).

The studies described in this chapter were designed to assess whether activation of MR in B-1 cells leads to cellular activation or inhibition. My hypothesis was that pretreatment of B-1 cells with an MR agonist prior to activation with an immune stimulant (such as a TLR ligand) would enhance their antibody response. An important consideration in the studies above and in all studies regarding the mineralocorticoid receptor is the impact the closely related glucocorticoid receptor may be playing.

The Glucocorticoid Receptor is Anti-inflammatory

The MR and GR are highly related, with significant amino acid identity in their DNA and ligand binding domains (8). They are known to bind the same DNA-response elements as well as many of the same ligands, but with differing affinities (9,10). Amazingly, despite this high similarity these receptors are known to exert nearly polar opposite effects in immune cells. As explained above, MR activation is predominantly considered pro-inflammatory whereas GR activation is known to be profoundly anti-inflammatory. In fact, there is a vast literature detailing the kaleidoscope of anti-inflammatory effects that GR exerts upon virtually every cell of the immune system and it is widely used clinically for its profound anti-inflammatory properties (11). Even so, there are no reports detailing the effects of GR in B-1 cells and more surprisingly, very few for B cells in general. Those reports that do exist with respect to GR activation and B cells are indirect or simply observational. The studies described below endeavored to separate and distinguish the effect of MR activation and GR activation in B-1 cells.

Liver X Receptors Control Innate Immune Inflammation and Lymphocyte Proliferation.

LXRα and LXRβ together when activated regulate a series of tissue-specific transcriptional programs that regulate whole-organism cholesterol homeostasis (12). Besides their classical role in regulating cholesterol metabolism LXRs have become known as important regulators of inflammatory gene expression. They have been shown to antagonize inflammatory signaling from TLR4 (but not TLR3), TNFα, or IL-1β activation (13,14). Additionally, the loss of LXR seems to severely handicap innate immune function, as LXR knockout mice are more susceptible to *Listeria monocytogenes* infection (15). More recent data has emerged linking the capacity of LXRs to control cholesterol efflux from peripheral cells and immunity (2). Activated LXRs are able to limit the proliferative capacity of T cells and splenic B cells by limiting the endogenous supply of cholesterol, which is needed for membrane synthesis, by inducing genes responsible for cholesterol efflux. In summary, LXRs provide a link between metabolic control of cholesterol homeostasis and inflammation and immunity.

Results

The Mineralocorticoid Receptor is Expressed at High Levels in B-1 cells.

As noted in Chapter 2, MR was highly expressed in B-1 cells (see Fig 2-4). To determine the relative level of expression of MR in various cell types, I utilized qPCR to compare MR expression in bone marrow derived macrophages, resident peritoneal macrophages, and peritoneal B-1 cells (Fig 3-1A) or in total kidney tissue and peritoneal B-1 cells (Fig 3-1B). B-1 cells expressed >20-fold higher levels of MR than macrophages and about a 7-fold

higher level than that found in the kidney. This is a remarkable finding as the kidney is the classical target tissue for MR expression and supports the idea that MR may be active in B-1 cells as it is active in kidney tissue at much lower expression levels. Additionally, MR is known to be active in macrophages even though its expression is much lower than in B-1 cells (5).

MR has equal binding affinity for aldosterone as well as glucocorticoids such as the stress hormone cortisol (10). Cortisol is normally present at 10- to 1000- fold higher concentrations than aldosterone in the plasma (16). Spec-

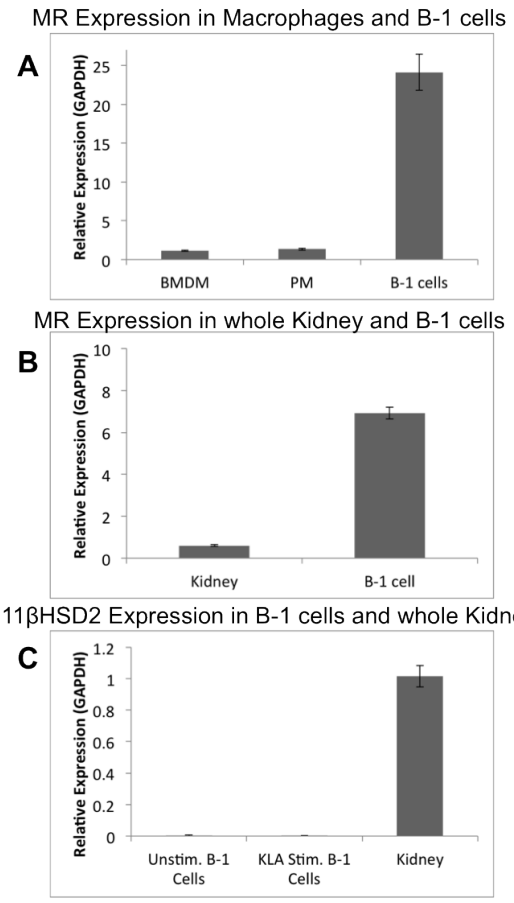


Fig.3-1 Mineralocorticoid Receptor and 11β Hydroxysteroid Dehydrogenase type 2 Expression in B-1 cells. **A.** QPCR expression of MR in bone marrow derived (BMDM) or peritoneal macrophages (PM) compared to B-1 cells. **B.** QPCR expression of MR in whole kidney tissue compared to B-1 cells. **C.** QPCR expression of 11βHSD2 in unstimulated or Kdo2-LipidA (a TLR4 ligand) stimulated B-1 cells compared to whole kidney tissue. Values are means and standard deviation of triplicate biological replicates.

ificity for aldosterone in tissues such as the kidney is gained by pre-receptor metabolism of cortisol by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). This enzyme functions intracellularly to selectively metabolizes cortisol (and not aldosterone) and renders it inactive (17). As shown in figure 3-1C 11βHSD2 is expressed at much higher (>200 fold) levels in kidney tissue than in B-1 cells. Therefore, the near absence of expression of 11βHSD2 in B-1 cells implies that circulating cortisol will not be metabolized in B-1 cells and therefore would be the most likely ligand to be bound to MR in B-1 cells in vivo.

Activating the Mineralocorticoid Receptor in B-1 cells *In Vitro*

In order to test the hypothesis that MR is active in B-1 cells and regulates innate immune functions, primary peritoneal B-1 cells were isolated and

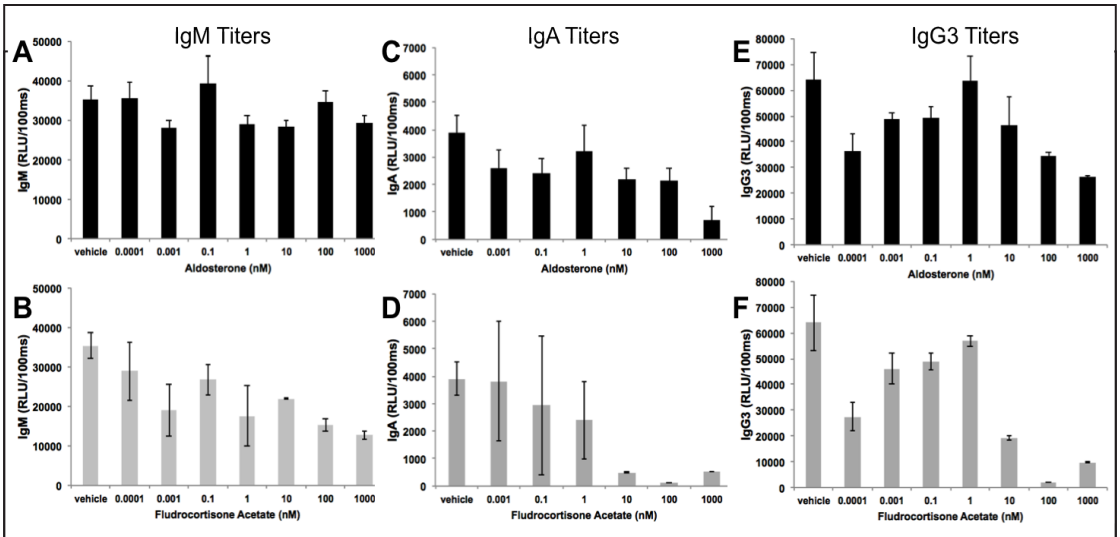


Fig.3-2 Total IgM, IgA, and IgG3 Titers from in vitro B-1 cell Cultures Treated with Mineralocorticoid Receptor Agonists
A-B. Total IgM titers from B-1 cell cultures treated with aldosterone (A) or fludrocortisone acetate (B). **C-D.** Total IgA titers from B-1 cell cultures treated with aldosterone (C) or fludrocortisone acetate (D). **E-F.** Total IgG3 titers from B-1 cell cultures treated with aldosterone (E) or fludrocortisone acetate (F). 100,000 primary B-1 cells were cultured per well in the presence of 300ng/mL Pam3cysK4, a TLR 2/1 ligand for 4 days. Antibody titers were measured using sandwich ELISA techniques. Values represent means and standard deviations of triplicate biological samples.

cultured in vitro with various MR ligands including aldosterone and a potent synthetic ligand fludrocortisone in the presence of a TLR 2/1 ligand (Pam-3CysK4). After culturing for 4 days, cells and supernatants were harvested and assessed for viability and antibody levels respectively (Fig 3-2 and Fig 3-3). Fludrocortisone and not aldosterone had a consistent dose-dependent inhibitory effect upon IgM and IgG3 production (Fig 3-2A-B, E-F). Both aldosterone and fludrocortisone inhibited IgA production but fludrocortisone did so more potently (Fig 3-2 C-D). However, because baseline IgA levels were very low and not significantly above background threshold levels, I am not confident that aldosterone does in fact inhibit IgA production from B-1 cells. In a separate experiment, I attempted to induce higher levels of IgA production from B-1

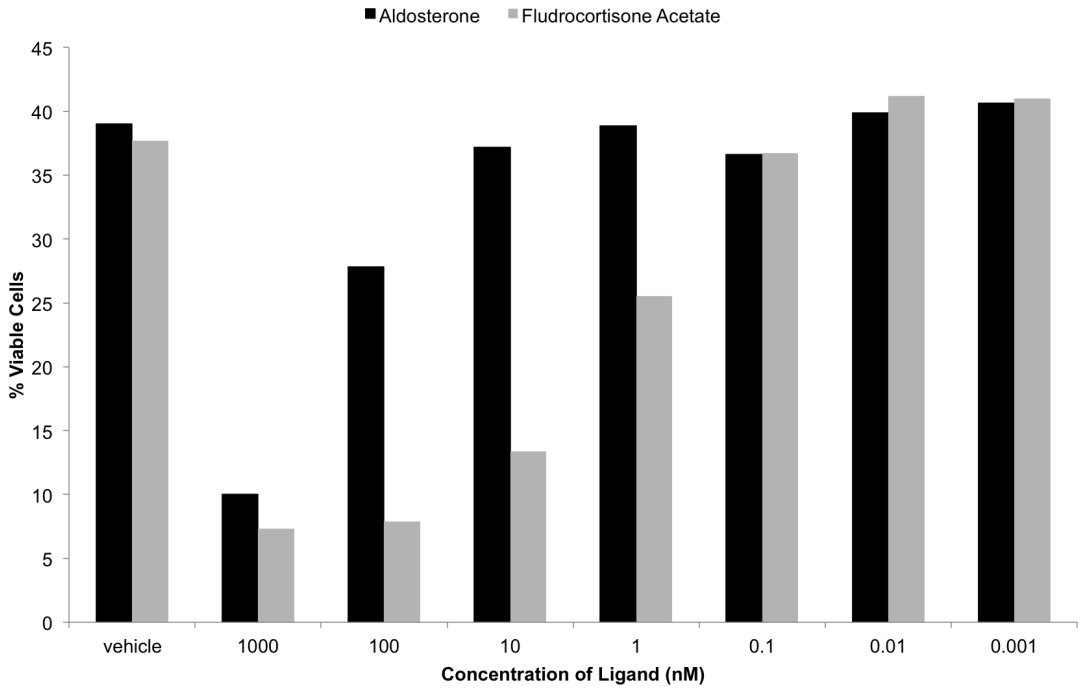


Fig.3-3 B-1 cell Viability after Treatment with Mineralocorticoid Agonists. 100,000 primary B-1 cells were cultured per well in the presence of the indicated mineralocorticoid agonists as well as 300ng/mL Pam3cysK4, a TLR 2/1 ligand for 4 days. Cell viability was assessed using Annexin-V PE and 7-aminoactinomycin D, viable cells were negative for both stains as assessed using flow cytometry. Triplicate samples were combined in single tube for flow cytometry.

cells by adding a cocktail of anti-CD40 antibody and IL-5, which are reported to induce IgA production from B-2 cells, but this did not occur with B-1 cells and I therefore could not validate the effect of aldosterone on IgA production (data not shown).

Fludrocortisone had a much more potent effect upon cell viability than aldosterone (Fig 3-3). Only the highest dose of aldosterone (1uM) seemed to appreciably reduce the number of viable cells whereas fludrocortisone seemed to reduce viability at concentrations as low as 10nM. Cell viability was assessed using Annexin-V, and 7-aminoactinomycin D staining, viable cells are negative for both stains.

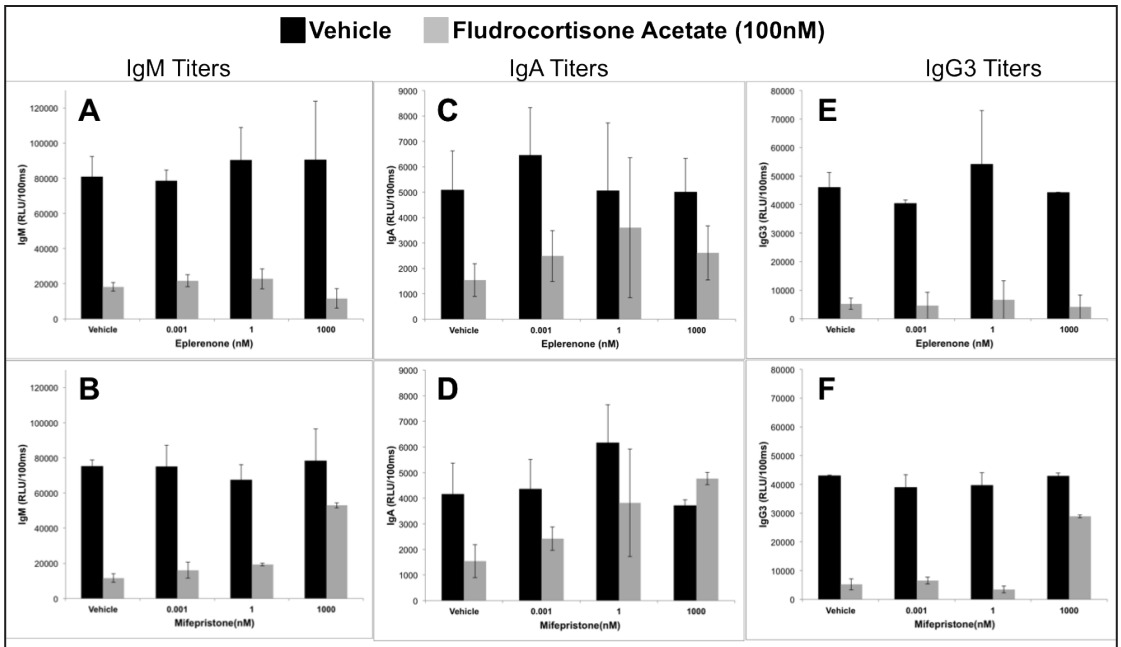


Fig.3-4 Affect of Eplerenone or Mifepristone upon Fludrocortisone acetate mediated inhibition of in vitro antibody production from stimulated B-1 cells. A-B. Total IgM titers from B-1 cell cultures treated with Eplerenone (A) or Mifepristone (B). **C-D.** Total IgA titers from B-1 cell cultures treated with aldosterone Eplerenone (C) or Mifepristone (D). **E-F.** Total IgG3 titers from B-1 cell cultures treated with Eplerenone (A) or Mifepristone (B). 100,000 primary B-1 cells were cultured per well in the presence of 100nM Fludrocortisone acetate as well as 300ng/mL Pam3cysK4, a TLR 2/1 ligand for 4 days. Antibody titers were measured using sandwich ELISA techniques. Values represent means and standard deviations of triplicate biological samples.

Both aldosterone and fludrocortisone have the ability to bind to both MR and GR but have much higher affinity for MR (10). Fludrocortisone, though it does have much higher affinity for MR compared to GR, nevertheless possesses a strong binding affinity to GR, even stronger than that of cortisol (10). Therefore the effects described above regarding inhibition of antibody production and induction of apoptosis could have occurred by activation of MR, GR, or both. To gain insight into which receptor may be responsible, inhibitors specific for MR (eplerenone) or GR (mifepristone) were added prior to the addition of fludrocortisone to the B-1 cell culture (Fig 3-4). Mifepristone (GR antagonism) and not eplerenone (MR antagonism) was able to rescue the inhibitory effect of fludrocortisone on antibody secretion. Therefore, the 'MR ligand' fludrocortisone appears to have actually activated GR and inhibited antibody production from B-1 cells in a GR-dependent mechanism.

To further test whether MR activity has any affect upon B-1 cells, the MR ligand deoxycorticosterone acetate (DOCA) was employed. DOCA has

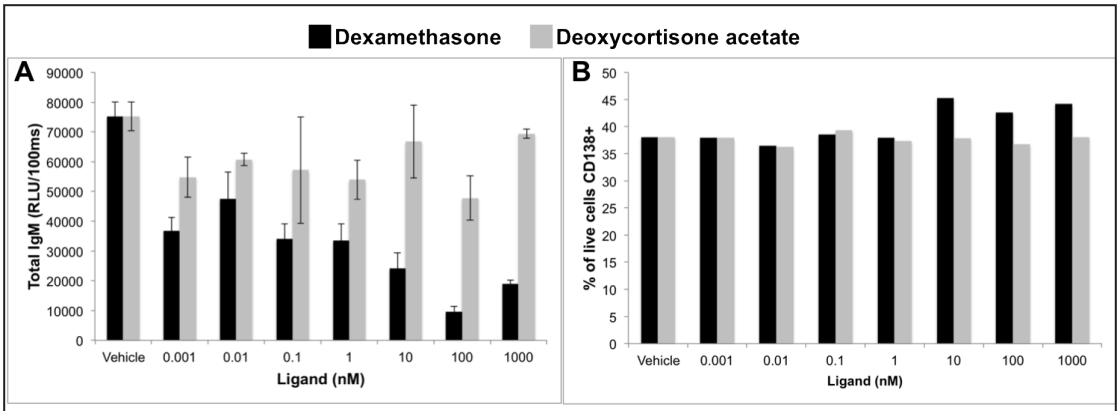


Fig.3-5 Affect of Dexamethasone and Deoxycortisone acetate upon in vitro production of IgM antibodies or expression of CD138 by B-1 cells. A. 100,000 primary B-1 cells were cultured per well in the presence of indicated ligands as well as 300ng/mL Pam3cysK4, a TLR 2/1 ligand for 4 days. Antibody titers were measured using sandwich ELISA techniques. Values represent means and standard deviations of triplicate biological samples. **B.** Same B-1 cells cultures as used in (A) were harvested, biological replicates were pooled and stained with a phycoerythrin conjugagted antibody specific for CD138, a plasma cell marker. Staining intensity was analyzed for flow cytometry.

weaker MR binding than aldosterone or fludrocortisone, but unlike these two ligands it does not have appreciable GR activity (10). In parallel, the GR specific ligand dexamethasone (Dex) was used to confirm that GR activation does in fact inhibit antibody production from B-1 cells. As shown in figure 3-5 Dex and not DOCA was able to inhibit IgM production from B-1 cells in a dose dependent manner. I additionally tested whether DOCA or Dex could affect the proportion of cells expressing CD138 (Fig 3-5B). CD138 is a marker for antibody producing plasma cells and is therefore a surrogate for B-1 cell differentiation (18). Neither DOCA nor Dex had any appreciable effect upon B-1 cell plasma cell differentiation. In summary, treatment of B-1 cells with MR ligands initially appeared to inhibit B-1 cell production of antibodies. Upon further evaluation, these ligands were most likely also activating the GR and it was the GR activation that was responsible for inhibiting antibody secretion. Treatment with more specific ligands (DOCA and Dex) with less potential for GR-MR crosstalk further cemented the finding that GR activation inhibits antibody production from B-1 cells. A summary of the finding from in vitro treatment of B-1 cells with MR and GR ligands can be found in table 3-1.

Table 3-1 Summary of Effects of In Vitro Treatment of B-1 Cells with MR or GR Ligands

	MR ligand	GR ligand
IgM Production	No effect	↓↓
IgA Production	↓?	↓↓?
IgG3 Production	↓	↓↓
Apoptosis	↑	↑↑
Plasma cell differentiation	No effect	No effect

Activating the Liver X Receptors in B-1 cells *In Vitro*

As discussed previously, LXRs have been implicated to play a prominent role in atherosclerosis and recent studies have shown that their activation can inhibit the proliferation of adaptive immune splenic B cells (2). LXRα was minimally expressed in B-1 cells, though LXRβ was expressed at much higher levels (data not shown). In analogous experiments conducted with MR or GR activation described above, B-1 cells were treated with the LXR ligands TO-901317 (abbreviated T1317) or GW3965 (Fig 3-6). In both cases viability of the cells was not affected. Similar to the effect documented in splenic B cells and in T cells (2), activation of LXR by T1317 had a dose-dependent inhibitory effect upon stimulated B-1 cell proliferation (Fig 3-6 C-D). Proliferation was measured by staining cells with the intracellular dye CFSE, which is diluted each time a cell divides and becomes successively more dilute and less fluorescent with each division. B-1 cells were stimulated with a TLR4 ligand, Kdo2-LipidA (KLA), which is the active component of LPS (19). Strangely, treatment of B-1 cells with a high concentration of GW3965 did not have the same effect (data not shown).

Effect of Liver X Receptor Deficiency upon B-1 cell Antibody Titers

In a collaborative effort with the Tontonoz lab at UCLA, I analyzed various antibody titers from the plasma of various LXR knockout mice. In the first study, mice that were double knockouts for ApoE and either LXRα or LXRβ were used. Titers for Total IgM, EO6 (a protoypic natural antibody with specificity for phosphocholine), IgM against MDA-LDL, and IgM against CuOx-LDL were measured (Fig 3-7 A, C, E, G). There was no significant difference between wild type, LXRα, or LXRβ for any of these titers except EO6. LXRβ knockout mice had significantly higher titers for EO6 (Fig 3-7 C). Though it

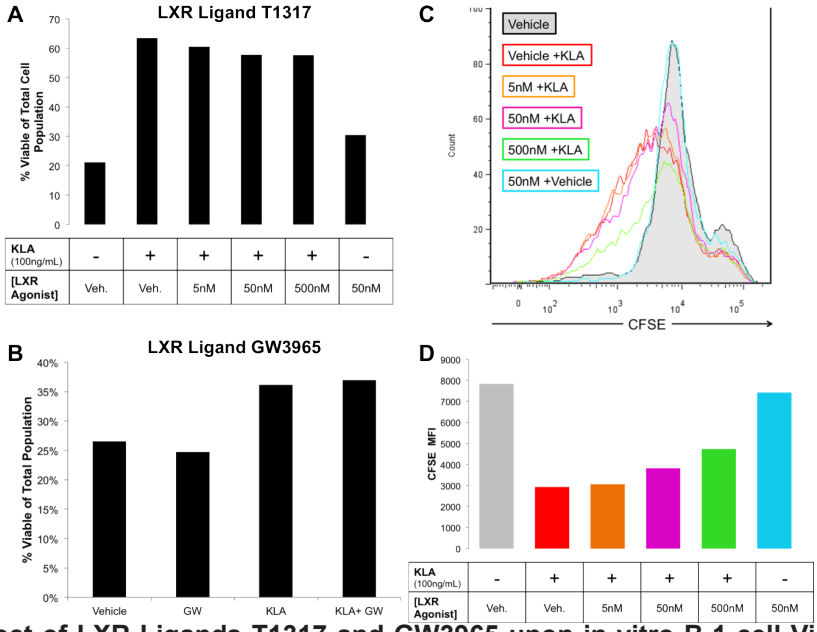


Fig.3-6 Affect of LXR Ligands T1317 and GW3965 upon in vitro B-1 cell Viability and Proliferation. A. 100,000 primary B-1 cells were cultured per well in the presence of the LXR ligand TO-901317 as well as 100ng/mL Kdo2LipidA (KLA), a TLR 4 ligand for 4 days. Cell viability as described in Fig 3-3. Biological replicates were pooled for flow cytometry analysis. **B.** Same protocol as (A) except 1μM of the LXR ligand GW3965 was used. **C-D.** Prior to culturing, primary B-1 cells were stained with carboxyfluorescein succinimidly ester (CFSE), cells were cultured as in (A). (C) CFSE fluorescence was measured by flow cytometry in pooled replicate samples and (D) the mean fluorescence intensity (MFI) was calculated by Flowjo.

should be pointed out that in this experiment only five LXRβ-KO animals were used, two had very high titers for E06 and three had normal titers (Fig 3-7C). In a second study, mice that were double knockouts for LXRα and LXRβ and on a western diet (high fat and high cholesterol) were used. Generally, the knockout of both LXRα/β resulted in significantly increased titers for total IgM and IgM against MDA-LDL (Fig 3-7 B, H). When mice were exposed to the stress of a western diet the titers increased and this increase was further exacerbated by the deficiency of LXRα/β. In fact, LXR double knockout mice on western diet had significantly higher titers than WT mice on western diet for all antibodies measured (Fig 3-7 B, D, F, H).

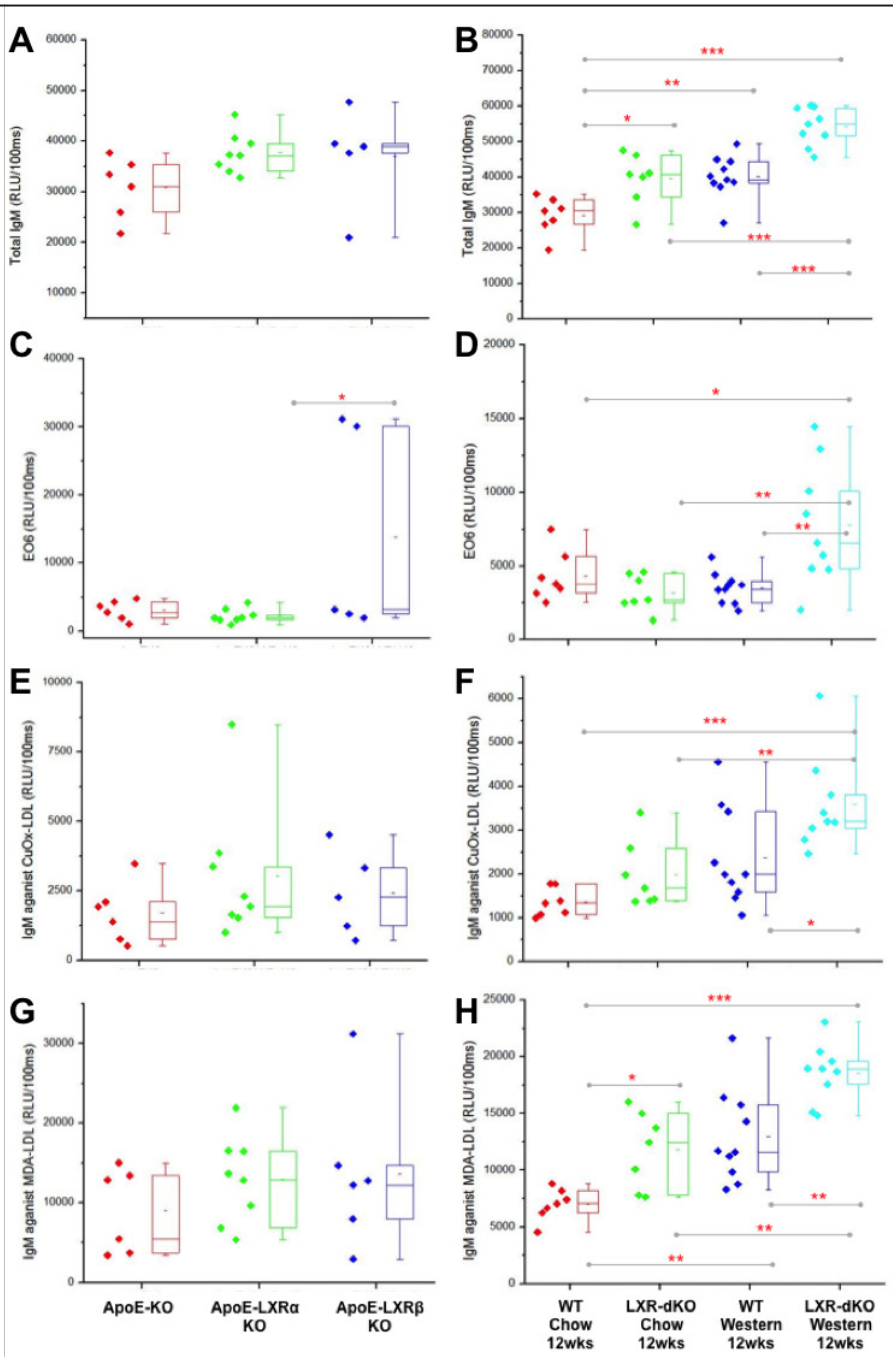


Fig.3-7 Effect of LXR Deficiency and High Fat Diet upon Various IgM Antibody Titers. **A,B.** Total plasma IgM measured using sandwich ELISA. **C,D.** Plasma EO6 levels measured using anti-idiotype sandwich ELISA. **E,F.** ELISA plates coated with LDL which had been oxidized with $CuSO_4^{3+}$ (CuOxLDL). Plasma IgM binding to CuOxLDL assessed using ELISA. **G,H.** ELISA plates coated with LDL modified with MDA, MDA-LDL. Plasma IgM binding to MDA-LDL was assessed using ELISA. Mice used in A,C,E,G were 4wk old. Mice used in B,D,F,H were 10wk old when started on western diet and were on diet for 12 wk. Values are means with SD. * p-value <0.05, **p-value<0.01, ***p-value<0.001 by one-way ANOVA and Tukey post-hoc multiple comparison test.

Discussion

The mineralocorticoid receptor's function has classically been confined to the regulation of salt and water balance in the kidney and colon. More recent and exciting studies have shown that it plays a critical pathophysiological function in cardiac fibrosis (20,21). Its role in immune cells, particularly innate immune cells, is just now being elucidated and defined. The evidence I have presented for the presence and remarkably high expression of MR in B-1 cells imply that it is carrying out an important function in B-1 cells in particular. Of interest, it is also expressed in B-2 cells, but only at about one quarter the levels of B-1 cells (see figure 2-3).

In kidney cells, the enzyme 11βHSD2 is highly expressed and essentially deactivates cortisol so that it cannot bind to MR. Thus, aldosterone becomes the major ligand for MR in this cell type, effecting regulation of sodium and water transport. The fact that B-1 cells essentially lacked all expression of 11βHSD2 means that both MR and GR ligands can bind to and potentially activate MR in B-1 cells. Because glucocorticoids are present in the plasma at greatly elevated levels relative to mineralocorticoids, this indicates that glucocorticoids are likely the physiological ligands for B-1 cell MR.

In vitro studies were designed to determine what role MR was fulfilling in regulating B-1 cell biology. These studies failed to find a prominent phenotype for MR activation or antagonism on antibody secretion, cell death, or cell differentiation. I also performed preliminary studies and could not find a phenotype for MR activity on IL-10 production or expression of the B-1 cell migration marker CD9 (data not shown). Future studies should utilize transcriptomic techniques to ascertain what genes are being regulated by MR activation. One challenge to MR transcriptomic studies is that MR is a weak transcription-

al activator compared to GR and it has been historically difficult to separate the MR response from that of GR (10). I am currently producing B cell specific GR knockout mice that could potentially be used to study the transcriptome of MR in B-1 and B-2 cells. Since the GR will be deleted (or mostly deleted) from these cells there will be no cross-talk between ligands or competition for response element binding and the MR transcriptome could conceivably be more easily defined in these cells. Ideally the converse would also be performed, and the MR would be deleted from additional mice and the GR transcriptome could be ascertained in like manner. This would give clear evidence for the unique and shared genes under the control of MR and GR. Additionally, there is an open question regarding the mechanisms whereby MR and GR can regulate unique genes when they both bind to the same DNA response elements and bind very similar ligands. The hypothetical studies I have mentioned above would begin to elucidate these mechanisms.

The fact that GR activation directly inhibits B-1 cell antibody production and induces apoptosis may not seem that surprising as the current paradigm suggests that GR is anti-inflammatory and pro-apoptotic for lymphocytes. However, a quick search of the literature will reveal that no research has been carried out to directly test the effects or mechanism whereby GR activation affects B-cells in general or B-1 cells in particular (recently reviewed (11)). The studies described in chapter 4 were designed to begin to define the role and mechanism of action of GR in B-1 and B-2 cells. Of interest, it is notable that an inhibition of IgM antibody secretion was detected at much lower concentrations of glucocorticoids (Fig 3-2B and Fig 3-5A) than was a change in apoptosis (Fig 3-3). This implies that glucocorticoid induced apoptosis is not solely responsible for the reduced production of antibodies from B-1 cells and

that another mechanism may also be at play. The fact that glucocorticoids at such low levels can impact so dramatically the production of IgM implies that B-1 cells are quite sensitive to glucocorticoids and this gives credence to the idea that this phenomenon should be studied in vivo. Glucocorticoids are administered very regularly in the clinic and if they do significantly impact natural antibody production in vivo this should be taken under consideration when they are prescribed.

As expected, LXR activation in vitro had very little effect upon the viability of TLR4 stimulated cultured primary B-1 cells. Pretreatment of B-1 cells with the LXR ligand T1317 had a dose dependent effect upon inhibiting cell proliferation, which is consistent with what has been reported by Bensinger et al. for conventional B cells (2). The effect observed in B-1 cells does appear to be less pronounced than that reported in primary T cells or splenic B cells. This may be due to the reduced capacity of B-1 cells to proliferate, at least when their BCRs are crosslinked (22). The size and consistency of the impact of LXR ablation *in vivo* on increasing various plasma IgM antibody titers was surprising. The current understanding is that LXR functions to curtail lymphocyte proliferation and therefore the differences in antibody titers observed may be due to increased proliferation of specific B-1 cell clones. It is an unknown question whether or not proliferation is necessary for antibody production from B-1 cells *in vivo*. The evidence presented here implies that it may be necessary. In conclusion, it appears that LXR is functioning in a similar manner in B-1 cells as it does in adaptive immune cells.

Acknowledgement

The plasma from various LXR knockout mice was kindly provided by Ayaka Ito from Peter Tonotonoz's lab at UCLA. Dr. Ayelet Gonen of the Witztum lab helped perform a number of the ELISA assays to measure antibody titers from the LXR knockout mice presented in figure 3-7.

References

1. Diehl CJ, Barish GD, Downes M, Chou MY, Heinz S, Glass CK, Evans RM, Witztum JL. Research Resource: Comparative Nuclear Receptor Atlas: Basal and Activated Peritoneal B-1 and B-2 Cells. Mol Endocrinol. 2011.

2. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell. 2008;134(1):97-111.

3. Hsueh WA, Do YS, Jeyaseelan R. Angiotensin II and cardiac remodeling. MtSinai JMed. 1998;65(2):104-107.

4. Pu Q, Neves MF, Virdis A, Touyz RM, Schiffrin EL. Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. Hypertension. 2003;42(1):49-55.

5. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Sch++tz G, Lumeng CN, Mortensen RM. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. The Journal of Clinical Investigation. 2010;120(9):3350-3364.

6. Herrada AA, Contreras FJ, Marini NP, Amador CA, Gonzalez PA, Cortes CM, Riedel CA, Carvajal CA, Figueroa F, Michea LF, Fardella CE, Kalergis AM. Aldosterone Promotes Autoimmune Damage by Enhancing Th17-Mediated Immunity. Journal of Immunology. 2010;184(1):191-202.

7. Bergmann A, Eulenberg C, Wellner M, Rolle S, Luft F, Kettritz R. Aldosterone Abrogates Nuclear Factor {kappa} B-Mediated Tumor Necrosis Factor {alpha} Production in Human Neutrophils via the Mineralocorticoid Receptor. Hypertension. 2010;55(2):370.

8. Funder JW. Mineralocorticoid receptors: distribution and activation. Heart failure reviews. 2005;10(1):15-22.

9. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. Cloning of Human Mineralocorticoid Receptor Complementary-Dna - Structural and Functional Kinship with the Glucocorticoid Receptor. Science. 1987;237(4812):268-275.

10. Rupprecht R, Reul JMHM, van Steensel B, Spengler D, Söder M, Berning B, Holsboer F, Damm K. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. European Journal of Pharmacology: Molecular Pharmacology. 1993;247(2):145-154.

11. Zen M, Canova M, Campana C, Bettio S, Nalotto L, Rampudda M, Ramonda R, Iaccarino L, Doria A. The kaleidoscope of glucorticoid effects on immune system. Autoimmun Rev. 2011;10(6):305-310.

12. Bensinger SJ, Tontonoz P. Integration of metabolism and inflammation by lipid-activated nuclear receptors. Nature. 2008;454(7203):470-477.

13. Ogawa S, Lozach J, Benner C, Pascual G, Tangirala RK, Westin S, Hoffmann A, Subramaniam S, David M, Rosenfeld MG, Glass CK. Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. Cell. 2005;122(5):707-721.

14. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nature Medicine. 2003;9(2):213-219.

15. Joseph SB, Bradley MN, Castrillo A, Bruhn KW, Mak PA, Pei L, Hogenesch J, O'Connell R M, Cheng G, Saez E, Miller JF, Tontonoz P. LXR-dependent gene expression is important for macrophage survival and the innate immune response. Cell. 2004;119(2):299-309.

16. Pascual-Le Tallec L, Lombes M. The mineralocorticoid receptor: A journey exploring its diversity and specificity of action. Molecular Endocrinology. 2005;19(9):2211-2221.

17. Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid Action - Target Tissue-Specificity Is Enzyme, Not Receptor, Mediated. Science. 1988;242(4878):583-585.

18. Wijdenes J, Vooijs WC, Clement C, Post J, Morard F, Vita N, Laurent P, Sun RX, Klein B, Dore JM. A plasmocyte selective monoclonal antibody (B-B4) recognizes syndecan-1. Br J Haematol. 1996;94(2):318-323.

19. Raetz CR, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC, Jr., Ribeiro AA, Murphy RC, Ulevitch RJ, Fearns C, Reichart D, Glass CK, Benner C, Subramaniam S, Harkewicz R, Bowers-Gentry RC, Buczynski MW, Cooper JA, Deems RA, Dennis EA. Kdo2-Lipid A of Escherichia coli, a defined endotoxin that activates macrophages via TLR-4. JLipid Res. 2006;47(5):1097-1111.

20. Beggah AT, Escoubet B, Puttini S, Cailmail S, Delage V, Ouvrard-Pascaud A, Bocchi B, Peuchmaur M, Delcayre C, Farman N, Jaisser F. Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. ProcNatlAcadSciUSA. 2002;99(10):7160-7165.

21. Rickard AJ, Morgan J, Tesch G, Funder JW, Fuller PJ, Young MJ. Deletion of mineralocorticoid receptors from macrophages protects against deoxycorticosterone/salt-induced cardiac fibrosis and increased blood pressure. Hypertension. 2009;54(3):537.

22. Morris DL, Rothstein TL. Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes. The Journal of experimental medicine. 1993;177(3):857-861.

CHAPTER 4

Genome-wide Effects of Dexamethasone: Exploring Epigenetic Mechanisms Controlling B-1 and B-2 Cells

Abstract

The Glucocorticoid Receptor (GR) is robustly expressed in B-1 cells and especially in B-2 cells. GR is a ligand inducible transcription factor capable of inducing or repressing expression of target genes. As demonstrated in Chapter 3, Dexamethasone (Dex), a synthetic GR ligand, has potent *in vitro* effects upon B-1 cells. Studies in this chapter describe the transcriptomes for basal and stimulated B-1 and B-2 cells treated with Dex. A surprisingly distinct transcriptional response to Dex treatment was observed in B-1 and B-2 cells. Also, genome-wide binding patterns for GR in B-1 and B-2 cells were quite unique and may explain, at least in part, the distinct transcriptional response to Dex in these cells. Cell-type specific enhancers have been reported to dictate the cell specific binding patterns of signal dependent transcription factors such as GR. H3K4Me2 is a histone mark, that when present at promoter distal genomic sites is a marker for enhancers. B-1 and B-2 cells each had large numbers of H3K4Me2 regions unique to their cell type. Motifs for various transcription factors such as NFAT for B-1 cells and Mef2c for B-2 cells were enriched at these cell-specific enhancer sites. These transcription factors are hypothesized to collaborate with PU.1 to help establish cell-type specific enhancers.

Introduction

In vitro studies have demonstrated that the biological outcome of treatment of B-1 cells with Dex is decreased antibody production and cell proliferation as well as increased cell death. Dex is a synthetic ligand for the Glucocorticoid Receptor and when bound causes it to translocate to the nucleus where it regulates gene transcription. GR activation is often associated with anti-inflammatory and immunosuppressive actions.

Effect of Glucocorticoid Receptor Activation on B cells

GR is expressed ubiquitously, including within the cells of the immune system (1). GR activation is known to affect many if not all immune cells and does so in a non-uniform fashion between cell types (2). Overall, GR activation is immunosuppressive and is able to exert this function by either potently inhibiting immune cells or by activating regulatory immune cells such as T regulatory lymphocytes (2,3). The role and mechanism of GR in many immune cells, particularly in T cells, has been extensively studied. Surprisingly, however, this is not true for B cells. Though glucocorticoids (GC) have long been used in B cell dependent diseases, the effects of and mechanisms of actions of GCs in B cells have not been rigorously studied. Even so, there are a small number of mostly observational studies documenting the effect of GCs on B cells.

Chronic administration of GCs has been observed to reduce the number of B cells in secondary lymphoid tissues such as the spleen and lymph nodes (4). In a separate study using human pre-B cell leukemia cell lines, GCs inhibited cellular proliferation through a mechanism postulated to involve c-Myc repression and induced apoptosis via a mechanism involving BCL-2 (5). In a study done using human tonsillar B cells, it was shown that pretreatment with GCs inhibited the expression of early activation markers and proliferation of stimulated cells (6). Low doses of GCs seem to have minor effects upon circulating levels of antibodies while high doses of GCs can potently decrease immunoglobulin levels (2). The initial reduction in antibody levels observed with high-doses of GC treatment is due to increased catabolism of circulating antibodies and was B cell independent, but overtime antibody production was inhibited. Additionally, in mice, high doses of GCs can reduce the circulating

levels of the B cell activating factor (BAFF) cytokine (7). BAFF is critical for conventional B cell development and survival (8,9). However, BAFF is not required for the development and survival of B-1 cells (10,11).

An early study performed in atopic asthmatic patients showed that administration of GCs for just 15 days lead to a 22% decrease in IgG, 10% drop in IgA, while IgM was unchanged (12). Interestingly, they found that initially IgE levels were increased 18% by GC treatment but that their levels dropped back below baseline levels following intervention. Another study also showed that GCs could promote IgE isotype switching in a mechanism dependent on CD40 ligand (13). Overall, studies to date indicate that GCs inhibit B cell proliferation and activation, reduce most circulating antibody titers, except for IgE, which seems to be induced by GCs, and induces apoptosis of B cells. None of these studies explored the effects of GR activation on B-1 cells or other B cell subsets.

Mechanism of Glucocorticoid Induced Apoptosis in Lymphocytes

As detailed in chapter 3, a major result of *in vitro* treatment of B-1 cells with Dex is promotion of apoptosis. And as discussed immediately above, one of the few studies performed directly exploring the effects of GCs on B cells has shown that apoptosis is induced in an apparent BCL-2 dependent manner in at least one human pre-leukemic B cell line (5). The mechanism of GC induced apoptosis in B cells or any other immune cell for that matter is poorly understood and is a topic of active inquiry, especially since GCs are often used as a therapy in lymphoid malignancies (14). There is strong evidence however that the transactivation capacity of GR to induce gene expression is important in GC-induced apoptosis (15). There are a large number of GR

regulated genes from various systems that have been implicated with a role in lymphocyte apoptosis (16). In an analysis of 8 microarray studies of GC treated cells, researchers uncovered 31 genes that were most consistently regulated across most of the 8 cell types (16). Interestingly, three of the cells included in this meta-analysis were human B cells, including two pre-B cell lines and B-cell acute lymphocytic leukemia cells. The authors of this study grouped the genes into three categories: 1) genes with known roles in directly controlling cell death and survival decisions (such as Bim), 2) genes with functions that may lead to cellular distress that could result in induction of apoptosis, and 3) genes without a known causal role in apoptosis (16). This list can serve as a baseline for understanding what genes may be playing a role in B-1 and B-2 cells and in understanding the general mechanism of apoptosis induction in these cells. The decision to undergo Dex induced apoptosis can be broken down into three steps; an initiation stage, where the GR receptor induces changes in gene expression that push a cell toward cell death, a decision stage, wherein the balance of pro- and anti-apoptotic factors dictate whether a cell undergoes programmed cell death, and finally the execution stage, where the classical apoptotic machinery, such as caspases, proteases, and endonucleases become active and carry out the apoptotic program. The focus of many of the studies described in this chapter were to understand which genes regulated by GR are potentially leading to the induction of apoptosis in B-1 and B-2 cells.

Transcriptional Mechanisms of the Glucocorticoid Receptor

In the absence of ligand, GR is almost entirely maintained in the cytoplasm by physical association with various chaperone proteins that mask

the nuclear location signals (NLS) on GR. These chaperones include various heat shock proteins (Hsp) such as Hsp90 and Hsp70 as well as various other proteins such as FK506-binding protein (FKBP) (17,18). Upon ligand binding, GR undergoes a conformational change that causes it to disassociate from its chaperones thus uncovering the NLS, which in turn ultimately leads to its nuclear translocation (18). Once in the nucleus, GR can act through various mechanisms to positively or negatively affect the transcription of numerous genes (19,20). These mechanisms are graphically represented in figure 4-1, which is an illustration from a recent review published by Beck et al. (21).

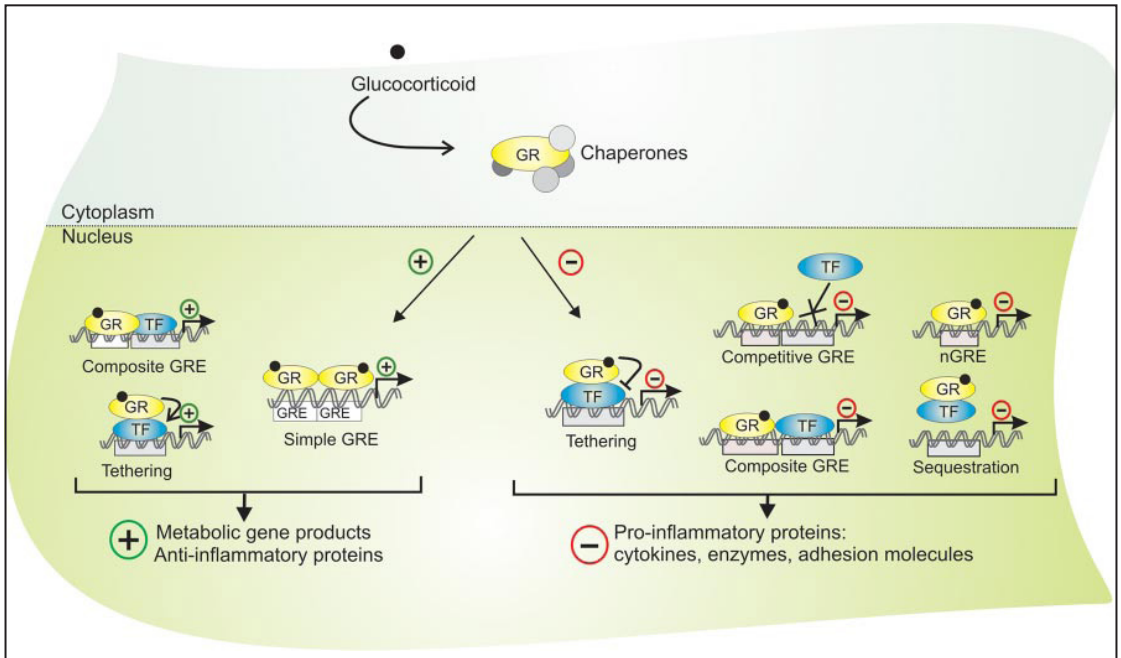


Fig. 4-1 Activation and Nuclear Actions of the Glucorticoid Receptor. This figure is from a recent review by Beck et. al (21).

GR can induce or transactivate the expression of genes using a variety of different mechanisms. In the classical GR transactivation mechanism, a homodimer of GR binds to the major groove of DNA via zinc finger DNA binding domains that specifically target the consensus glucocorticoid response element sequence (GRE) (21). The GRE is an imperfect palindrome made up

of the following sequence, where ‘n’ represents any nucleotide: 5’ GGTACAnnnTGTTCT 3’ (22). Once bound to DNA, GR associates with transcriptional co-activator complexes and induces the increased expression of a nearby genes. Interestingly many genes, which are known to respond to glucocorticoid treatment, do not possess consensus GREs in or near their promoters and are thought to not require dimers of GR for their response. It is assumed that in these cases GR can form a composite GRE or heterodimer with other transcription factors and together they can regulate the transcription of target genes (23). Finally, it has been reported that activated GR can tether to other DNA bound transcription factors, including among others STAT5, and thereby increase transcription of specific genes without directly binding to DNA (24,25).

The GR has gained notoriety because when it is bound to its ligand it can inhibit or transrepress the expression of specific genes, including importantly many pro-inflammatory mediators such as various cytokines, enzymes, adhesion molecules, etc. (21). In particular, intense research has endeavored to elucidate both the cellular and molecular mechanisms for the potent anti-inflammatory effects of GC treatment (2,21). As was observed for GR transactivation, the mechanism of GR transrepression can also include binding to a composite GRE with another transcription factor and the tethering of GR to other transcription factors. GR is known to inhibit most but not all NFκB-driven gene expression through these mechanisms (20). Furthermore, negative GREs (nGRE) have been described in which GR binds to DNA as a monomer in proximity to a gene promoter and inhibits its transcription. However, the sequence motif of a nGRE has not been well characterized (21). Other described mechanisms of GR mediated transrepression include competition for DNA binding with transactivating transcription factors and sequestration of

activating transcription factors thereby preventing their binding to DNA (21). Many if not all of the above mentioned mechanisms for GR regulation of gene transcription are cell and context dependent and have not been directly studied within B cells. Of interest in the studies undertaken herein, I hypothesized that GR would have unique transcriptional effects within B-1 and B-2 cells to both up- and down-regulate unique sets of genes both in the quiescent and activated conditions.

Cell-type Specific Enhancers Establish Cell-type Identity

In addition to exploring the genome-wide effect of GR activation in B-1 and B-2 cells, an overarching question to these studies is what controls the unique transcriptional responses and therefore biology of B-1 versus B-2 cells. This of course leads to the more basic questions of what regulates cellular identity in general. Collaborators in the laboratory of Dr. Christopher Glass at UCSD have established through a comparison of mature macrophages and mature splenic B-2 cells a general transcriptional model for how cell identity is established and maintained (26,27). They propose that small numbers of lineage determining transcription factors (LDTFs) collaborate to establish unique patterns of open chromatin in distinct cell types. These unique regions of open chromatin gain specific histone marks (e.g. mono- or di-methylation of lysine 4 on histone 3, H3K4me1/2), which are indicative of active cis-regulatory elements. These regions describe a large fraction of the enhancer-like regions that regulate unique cell-specific transcription factor binding and therefore gene expression patterns found in different cell types. An enhancer is a region of DNA capable of increasing the transcription of neighboring genes independent of directionality. The Glass lab recently published an elegant

paper in which they used natural genetic variation in gene expression between mice strains in response to a given stimulus to provide support for this model. They showed that genetic variation between mice strains in the binding sites for LDTFs results in unique enhancer landscapes and in turn unique signal dependent transcription factor binding and therefore strain-specific gene expression patterns between different strains of mice (28). I propose to apply this model to compare mature B-1 and B-2 cells and hypothesize that B-1 and B-2 cells will have cell-type specific enhancers, which in turn will provide a basis for explaining their unique cellular identities.

Results

The Glucocorticoid Receptor Protein is Expressed and Transcriptionally Active in B-1 and B-2 cells.

Evidence presented in Chapters 2 and 3 established that mRNA for GR is robustly expressed in B-1 and B-2 cells and that treatment with a GR agonist has profound inhibitory effects upon B-1 cells. Of interest, GR was expressed more highly in resting B-2 cells than in B-1 cells (Fig. 2-3). To verify that the GR protein, in addition to GR mRNA, is expressed in both B-1 and B-2 cells, a western blot analysis was performed, in which GR expression was compared between B-1 and B-2 cells as well as in bone marrow derived macrophages (BMDM) (Fig. 4-2A). Corroborating the GR mRNA data in B-1 and B-2 cells, GR protein levels were noticeably higher in B-2 cells than in B-1 cells. Notably, BMDMs had very low levels of GR expression relative to B-1 and B-2 cells, even though it has been reported that GR mRNA is expressed in BMDMs (29).

To verify that GR is transcriptionally active in B cells, B-1 cells were

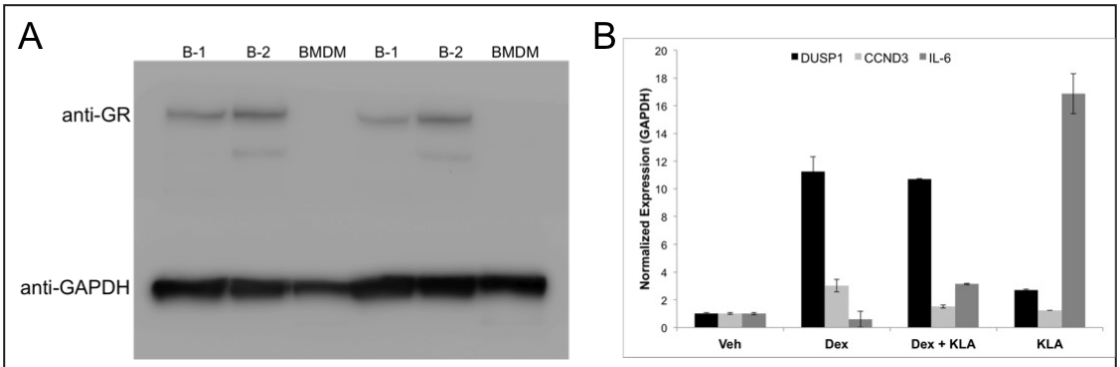


Fig. 4-2 GR Protein Expression and Transcriptional Activity. A. GR protein expression in untreated B-1 and B-2 cells as well as bone marrow derived macrophages (BMDM). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control. **B.** QPCR analysis of Dual specificity protein phosphatase 1 (DUSP1), Cyclin D3 (CCDN3), and Interleukin 6 (IL-6) mRNA levels in response to 2hr of 1uM Dexamethasone (Dex) and/or 100ng/mL Kdo2lipidA (KLA, a TLR-4 ligand) treatment. Values are means and standard deviations of triplicate biological replicates.

cultured *in vitro* for 4hr with 1μM Dexamethasone (Dex) after which qPCR analysis was performed to assess mRNA levels for known GR sensitive genes including Dual Specificity Protein Phosphatase 1 (DUSP1) and Cyclin D3 (CCND3). Both DUSP1 and CCND3 were upregulated when B-1 cells were exposed to Dex (Fig. 4-2B). Additionally, as discussed earlier, when ligated, GR can act as a transcriptional repressor, especially of inflammatory genes. To test if GR is active as a transrepressor, B-1 cells were stimulated with a TLR4 agonist (Kdo2LipidA, KLA) alone, which induced IL-6 expression, or were pretreated with Dex before stimulation with KLA, which potently transrepressed the KLA induced expression of IL-6 (Fig.4-2B). These data were the first evidence that GR can both actively regulat transcription as well as mediate transrepression in B-1 cells.

Exploring the Transcriptome of Dexamethasone and/or Kdo2LipidA Treated B-1 and B-2 cells

To profile the genes being regulated through transactivation and tran-

srepression by GR, as well as the genes regulated by TLR4 stimulation, a whole-transcriptome study of mRNA levels was undertaken. Primary mouse B-1 and B-2 cells were cultured for 4hr with 1μM Dex to assess and compare the genes regulated by GR activation (Fig.4-3). To assess genes regulated by transrepression, B-1 and B-2 cells were pretreated for 1hr with 1μM Dex before being stimulated with the TLR4 ligand KLA for an hour (2hr total of Dex treatment). B-1 and B-2 cells were also stimulated for 1hr with KLA alone to serve as a comparator for the transrepressed cells as well as a reference for the early B-1 and B-2 cell inflammatory response (Fig.4-3).

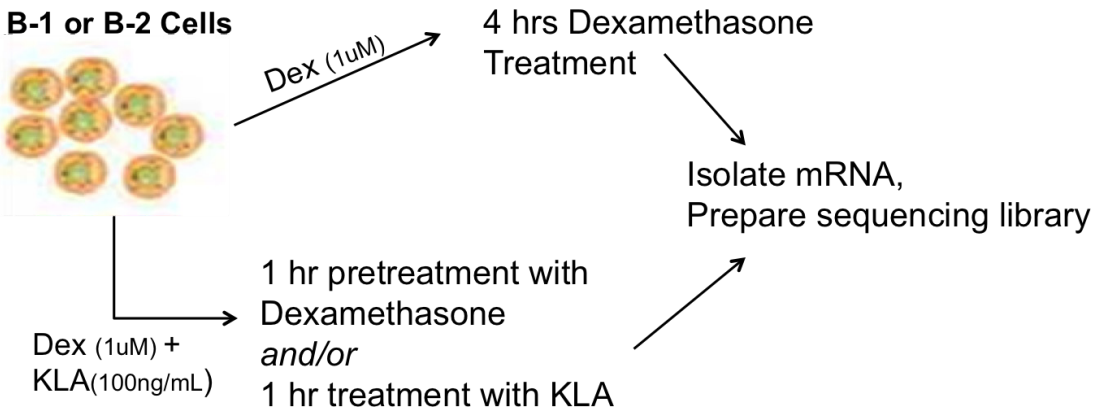


Fig. 4-3 Experimental Design for RNA-seq Evaluation of GR Transactivation or Transrepression. B-1 or B-2 cells were cultured overnight in RPMI media containing 10% charcoal stripped serum. They were then treated for 4hr with 1uM Dex to evaluate transactivation or for 1hr with 1uM Dex followed by 1hr 100ng/mL KLA or with 100ng/mL KLA alone for 1hr to evaluate transrepression of inflammatory genes. After treatment mRNA was isolated and sequenced.

B-1 cells treated with Dex had a nearly equal number of up- versus down-regulated genes (592up vs. 566down)(Fig.4-4A). Most of these genes had only modest changes in expression, with a few notable exceptions. Genes that were most strongly and significantly upregulated by Dex include mitogen-activated protein kinase kinase kinase 6 (Map3k6), DNA-damage-inducible transcript 4 (Ddit4), and sestrin 1 (Sesn1). These genes all have

known involvement in apoptosis; in fact Map3k6 is also referred to as apoptosis kinase (30-32). Whereas, the most strongly down regulated genes were colony stimulating factor 1 , sphingosine-1-phosphate receptor 1 (S1PR1), Chemokine receptor 9 (CCR9), and marginal zone B and B1 cell-specific protein 1 (Mzb1). A gene ontology analysis was performed using the bioinformatic resource Database for Annotation, Visualization, and Integrated Discovery (DAVID) to view common signaling pathways and functions that were enriched among the Dex regulated genes (Table 4-1) (33,34). Genes upregulated by Dex in B-1 cells were significantly associated with gene ontology terms related to cell signaling, phosphorylation, or phosphate metabolism. On the other hand, down regulated genes were significantly and commonly associated with inflammatory and immune responses. To determine what regulatory elements may be playing a role in the Dex mediated up- or down-regulated genes between B cell subsets, a motif discovery analysis was carried out using the program suite Hypergeometric Optimization of Motif Enrichment (HOMER) (26). In this particular analysis the sequences surrounding the promoters (-300 to +50bp of the transcriptional start site) of the regulated genes were compared to the DNA sequences surrounding all other promoters and analyzed for enrichment of 8, 10, or 12bp length DNA motifs. Once a motif is found to be statistically enriched, it is aligned to all known regulatory elements (i.e. transcription factor binding sites). Figure 4-4 D-F reports the results of *de novo* motif analysis in which the discovered motifs are displayed as well as the name of the most closely aligned known motif. For Dex up-regulated genes, motifs similar to Ets1, STAT6, RUNX, and FOXO3 were statistically enriched (p-value < 1e-11) in their promoters (Fig.4-4D). There were no statistically significant motifs found in the promoters of genes down-regulated by Dex.

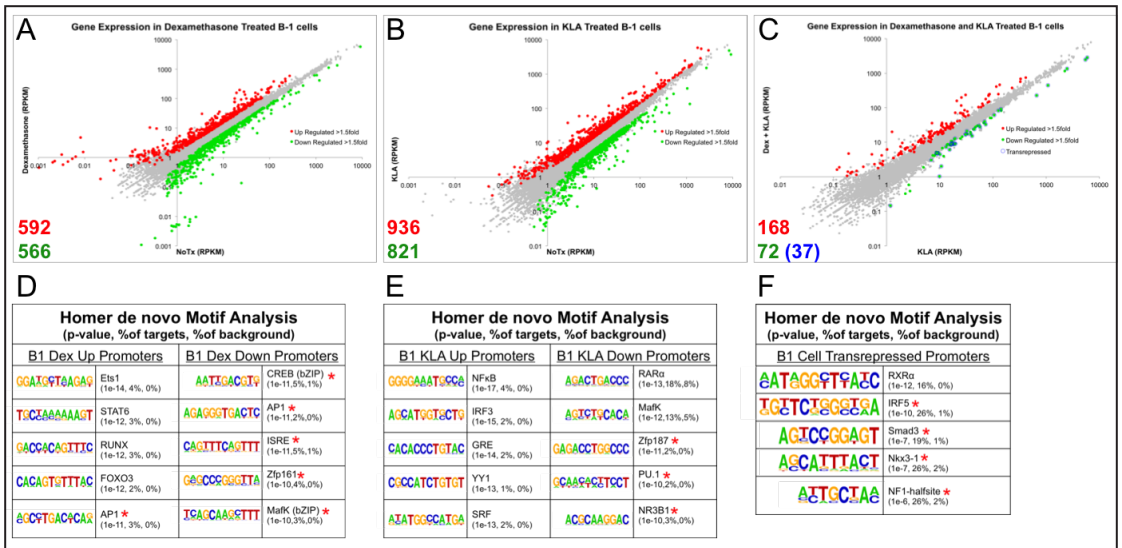


Fig. 4-4 RNA-Seq Analysis of B-1 Cells. A-C Normalized gene expression, reads per kilobase of exon per million mapped reads (RPKM) from B-1 cells. Red data points represent genes expressed >1.5 fold higher with an adjusted p-value of <0.1 in **A.** B-1 cells treated 4hr with 1μM Dex versus no treatment, **B.** B-1 cells treated 1hr with 100ng/mL KLA versus no treatment, **C.** B-1 cells pretreated 1hr with 1μM Dex and then 1hr with 100ng/mL KLA versus 1hr with 100ng/mL KLA alone. Green data points represent the inverse of red data points , genes expressed >1.5 fold higher with an adjusted p-value <0.1 in the opposite conditions. **D-F.** De Novo Motif analysis of the promoters (-300 to +50bp from transcriptional start site) of the genes up- or down-regulated by D. Dex versus no treatment , E. KLA versus no treatment , or F. Dex + KLA versus KLA alone performed using the computational program HOMER. Sequence motif diagrams represent enriched recurring patterns of DNA sequence where the size of each nucleotide corresponds to its frequency at each position in the motif. Also shown are the name of a known motif that aligned most closely to the found motif shown as well as the p-value for the enrichment of the motif in the promoters of the regulated genes relative to background sequences taken from all other promoters along with the frequency with which the motif was found in the promoters of regulated genes and in background promoters. Red asterisks indicate motifs that HOMER designated as possible false positives based upon the p-value of enrichment.

The B-1 cell response to 1hr of KLA treatment was noticeably more robust than the Dex response as 936 genes were up-regulated and 821 were down-regulated (Fig.4-4B). Notable genes that were strongly up-regulated by KLA include Fosb, Jun-d, and myeloid-associated differentiation marker (Myadm). Downregulated genes of note include: transforming growth factor beta receptor 1, heme oxygenase 1, and apolipoprotein e. Gene ontology analysis revealed that KLA upregulated genes associated with the immune response, cell proliferation, and cell activation (Table 4-1). Whereas, KLA

Table 4-1 Gene Ontology Analysis of Dexamethasone and/or Kdo2LipidA Up and Down Regulated Genes in B-1 and B-2 cells. Gene Ontology analysis using the online bioinformatic suite DAVID. Lists of regulated genes were compared to a background list of random genes. In parenthesis are G.O. terms followed by P-values, which represent the enrichment of the indicated G.O. term among the regulated genes versus background genes.

	Vehicle vs. Dexamethasone		Vehicle vs. Kdo2-LipidA		Kdo2LipidA vs. Dexamethasone+ Kdo2LipidA	
	B-1 Cell	B-2 Cell	B-1 Cell	B-2 Cell	B-1 Cell	B-2 Cell
Up Regulated (>1.5 fold)	protein amino acid phosphorylation (7.22E-06)	regulation of lymphocyte activation (1.49E-04)	immune response (7.21E-06)	immune response (4.01E-05)	cell cycle arrest (0.005243927)	immune response (7.92E-07)
	phosphate metabolic process (1.22E-05)	regulation of leukocyte activation (3.25E-04)	cytokine-mediated signaling pathway (4.33E-05)	regulation of cell proliferation (1.13E-04)	positive regulation of catabolic process (0.00878202)	nucleosome assembly (6.10E-06)
	phosphorus metabolic process (1.22E-05)	regulation of cell activation (3.76E-04)	regulation of cell proliferation (4.45E-05)	defense response (1.89E-04)	brown fat cell differentiation (0.013759626)	chromatin assembly (8.48E-06)
	intracellular signaling cascade (1.69E-05)	positive regulation of programmed cell death (7.64E-04)	hemopoiesis (8.43E-05)	positive regulation of cell proliferation (3.54E-04)	cell death (0.037150377)	protein-DNA complex assembly (9.96E-06)
	phosphorylation (6.31E-05)	positive regulation of cell death (8.47E-04)	cell activation (1.89E-04)	regulation of leukocyte activation (8.96E-04)	regulation of catabolic process (0.038200753)	nucleosome organization (9.96E-06)
Down Regulated (>1.5 fold)	regulation of cytokine production (3.74E-08)	cell activation (3.02E-08)	membrane invagination (2.34E-04)	homophilic cell adhesion (-2.25E-05)	inflammatory response (3.64E-06)	translation (2.58E-24)
	immune response (4.10E-08)	cholesterol biosynthetic process (3.48E-08)	endocytosis (2.34E-04)	translation (-2.66E-05)	response to wounding (6.28E-06)	generation of precursor metabolites and energy (4.50E-05)
	defense response (7.51E-07)	sterol biosynthetic process (3.59E-08)	membrane organization (0.001523838)	electron transport chain (-7.35E-05)	defense response (2.93E-04)	cell division (6.72E-05)
	response to wounding (1.67E-06)	immune response (1.45E-07)	cortical actin cytoskeleton organization (0.002182251)	generation of precursor metabolites and energy (-5.34E-04)	immune response (3.97E-04)	cell activation (6.30E-04)
	inflammatory response (3.95E-06)	regulation of cytokine production (3.08E-07)	cholesterol efflux (0.002182251)	oxidation reduction (-7.14E-04)	regulation of system process (0.002800182)	ion transmembrane transport (6.81E-04)

down-regulated genes associated with various membrane related processes such as endocytosis and cholesterol efflux. Not surprisingly the most significantly enriched motif in the promoters of the KLA up-regulated genes was that of NFκB (Fig. 4-4E). Other significantly enriched motifs were also found in the promoters of these genes including motifs similar to IRF3, GRE, YY1, and SRF. In contrast, the promoters of the genes downregulated by KLA treatment had enriched motifs similar to those of RARα and MafK.

In B-1 cells very few genes were differentially regulated when comparing treatment with KLA alone versus pretreatment with Dex followed by KLA stimulation (Fig. 4-4C). Only 168 genes were upregulated by the pretreatment with Dex and only 72 were expressed at lower levels. Among those 72 genes about 50% (37genes) were transrepressed, meaning that they were also upregulated by KLA alone but when pretreated with Dex this induction was inhibited (Fig. 4-4B). Several notable genes were profoundly transrepressed

by Dex, including interleukin 1 α and β (IL-1a and IL-1b), Cxcl2, inhibitor of DNA binding 3 (Id3), and prostaglandin endoperoxide synthase 2 (Cox-2). The entire list of GR transrepressed genes associated significantly with gene ontology terms relating to various aspects of immunity and inflammation, including the wound response, cell migration, and cytokine production (Table 4-2). The motif of RXRα alone was modestly yet significantly enriched in the promoters of these 37 transrepresed genes (Fig. 4-4F).

B-2 cells exhibited an overall more robust response to both Dex , KLA, and especially the combination thereof (Fig. 4-5). Compared to changes in B-1 cells, Dex treatment resulted in the levels of about twice as many mRNA transcripts being increased (1182) and decreased (1136). Though more genes were differentially regulated in B-2 cells, like B-1 cells, most fold-changes in gene expression levels were modest. Some of the most significantly upregulated genes in B-2 cells include: DUSP1, CD69, Period circadian clock 1 (Per1), and Apobec1. Notable downregulated genes include: LDLR, Id3, and complement receptor 2 (Cr2). Gene ontology analysis illustrates that upregulated genes in B-2 cells associate with either cell activation or cell death (Table 4-1). Down-regulated genes are involved in various processes including cell activation, cholesterol synthesis, and immune or inflammatory responses. In the B-2 cells, only one motif was found to be significantly enriched in the promoters of Dex up-regulated genes (Fig. 4-5D). This motif was similar to that of Smad3. Various motifs were modestly but significantly enriched in the promoters of genes downregulated by Dex in B-2 cells (Fig 4-5D). Many of these did not align very significantly with known motifs (data not shown).

The B-2 cell response to KLA resulted in roughly the same number of genes being differentially regulated as compared to B-1 cells (Fig. 4-4B &

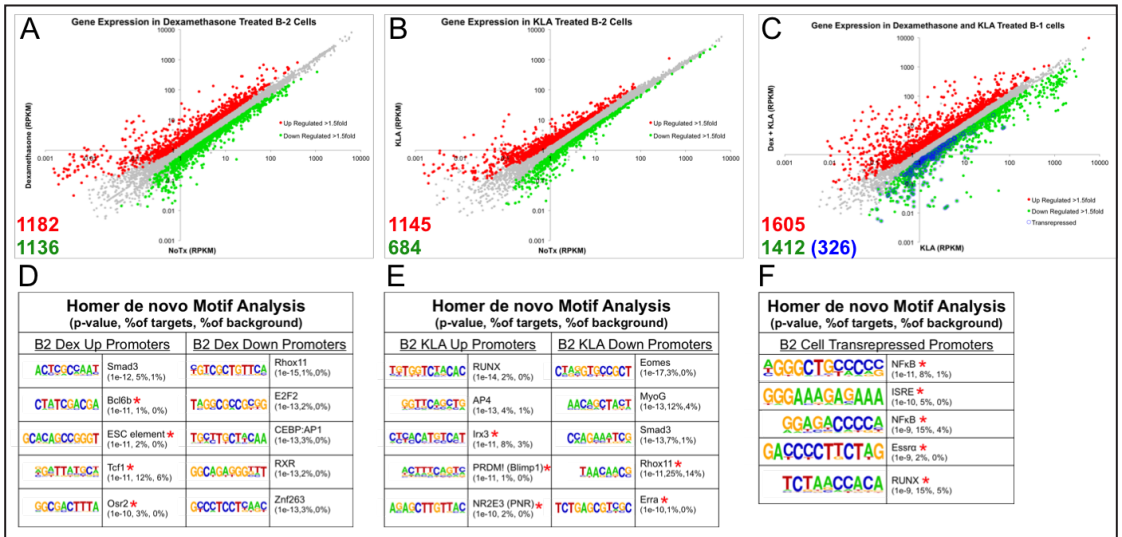


Fig. 4-5 RNA-Seq Analysis of B-2 Cells. A-C Normalized gene expression, reads per kilobase of exon per million mapped reads (RPKM) from B-2 cells. Red data points represent genes expressed >1.5 fold higher with an adjusted p-value of <0.1 in **A**. B-2 cells treated 4hr with 1μM Dex versus no treatment, **B.** B-2 cells treated 1hr with 100ng/mL KLA versus no treatment, **C.** B-2 cells pretreated 1hr with 1μM Dex and then 1hr with 100ng/mL KLA versus 1hr with 100ng/mL KLA alone. Green data points represent the inverse of red data points , genes expressed >1.5 fold higher with an adjusted p-value <0.1 in the opposite conditions. **D-F.** De Novo Motif analysis of the promoters (-300 to +50bp from transcriptional start site) of the genes up- or down-regulated by **D.** Dex versus no treatment , **E.** KLA versus no treatment, or **F.** Dex + KLA versus KLA alone performed using the computational program HOMER. See Fig. 4-4 for a detailed description of de novo motif analysis. Red asterisks indicate motifs that HOMER designated as possible false positives based upon the p-value of enrichment.

Fig4-5B). The upregulated genes associated with very similar gene ontologies as B-1 cells and were related to the immune response and cellular proliferation (Table 4-1). On the other hand, the KLA downregulated genes associated with different gene ontologies that related to basic cellular functions including cell adhesion, translation, and electron transport chain. Noteworthy genes significantly induced by KLA treatment include NFκB inhibitor zeta (Nfkbiz), myelocytomatosis oncogene (Myc), and Tumor Necrosis Factor (Tnf). While interesting and significantly downregulated genes include: glutathione peroxidase1 (Gpx1) and hydroxysteroid 17-beta dehydrogenase 4 (Hsd17b4). Several motifs were found to be in enriched in the promoters of both up- and down-regulated KLA genes in B-2 cells (Fig. 4-5E). Notably absent from this

list is the motif for NFκB that was found in the promoters of KLA upregulated genes in B-1 cells (Fig. 4-4E).

Surprisingly, pretreatment with Dex followed by KLA stimulation resulted in a very robust response in B-2 cells relative to B-1 cells. When comparing the expression of KLA alone with Dex + KLA treatment, 1605 genes had significantly higher levels when pretreated with Dex in B-2 cells compared to only 168 in B-1 cells. Similarly, KLA alone treatment resulted in 1412 genes with lower mRNA levels compared to cells that were pretreated with Dex. B-2 cells had 326 genes that were transrepressed by Dex pretreatment, whereas B-1 cells had only 37. Some of the same genes that were transrepressed in B-1 cells were also transrepressed in B-2 cells including IL-1β, Tnf and

Table 4-2 Gene Ontology Analysis of Kdo2LipidA Upregulated Genes Transrepressed by Dexamethasone. Analysis performed using the online bioinformatic suite DAVID. Lists of transrepressed genes from B-1 cells (37 genes from Fig. 4-4C) and B-2 cells (326 genes from Fig.4-5C) genes were compared to a background list of random genes. P-values in parenthesis represent the enrichment of the indicated G.O. term among the regulated genes versus background genes.

B-1 Cells	B-2 Cells
response to wounding (1.53E-04)	**defense response** (2.44E-04)
inflammatory response (3.51E-04)	**immune response** (0.004104787)
leukocyte migration (0.001977287)	**inflammatory response** (0.00476115)
positive regulation of cytokine production (0.004068185)	**negative regulation of glucose transport** (0.005216613)
defense response (0.004467069)	**homeostatic process** (0.007523972)
immune response (0.005335822)	**dephosphorylation** (0.00973157)
positive regulation vascular endothelial growth factor production (0.006168288)	**negative regulation of MAP kinase activity** (0.014314209)
fever (0.006168288)	**regulation of glucose transport** (0.014314209)
regulation of vascular endothelial growth factor production (0.006168288)	**response to bacterium** (0.014953984)
heat generation (0.010770707)	**positive regulation of cell proliferation** (0.015949676)

Csf1 though to a more modest degree. Other interesting genes that were significantly transrepressed in B-2 cells were chemokine receptor 2 (CXCR2) and DUSP4. Like B-1 cells, the genes that were transrepressed in B-2 cells strongly associated with the immune response but there was also an association with gene ontology terms related to glucose transport (Table 4-2). Even though there were more genes transrepressed in B-2 cells than B-1 cells, there were no motifs significantly enriched in transrepressed genes in B-2 cells (Fig. 4-5F). However, the motif that was most enriched was that of NFκB (p-value of 1e-11).

One of the more interesting observations noted above is that the cellular processes significantly associated with the genes up- or down-regulated by Dex exposure in B-1 and B-2 cells were remarkably different (Table 4-1). There were also differences in the motifs enriched in the promoters of the Dex regulated genes (Figs 4-4 D & 4-5D). Dex treatment in B-1 cells led to increased levels of gene transcripts associated with phosphorylation, many of which were kinases and phosphatases such as Map3K6, DUSP1, serum/glucocorticoid regulated kinase 3 (Sgk3), etc. On the other hand, genes upregulated by Dex in B-2 cells associated predominately with the processes of cell activation and apoptosis (Table 4-1).

One of the most remarkable findings of these studies was that GR led to the activation or transrepression of essentially distinct sets of genes in B-1 cells vs. those of B-2 cells. A quantitative comparison of whether the genes being regulated are the same or distinct between B-1 cells and B-2 cells is shown in Fig 4-6A. Of the 1158 or 2318 genes differentially regulated by Dex in B-1 cells and B-2 cells respectively, only 388 were the same genes (Fig 4-6A). 66% of the Dex regulated genes in B-1 cells and 83% in B-2 cells were unique

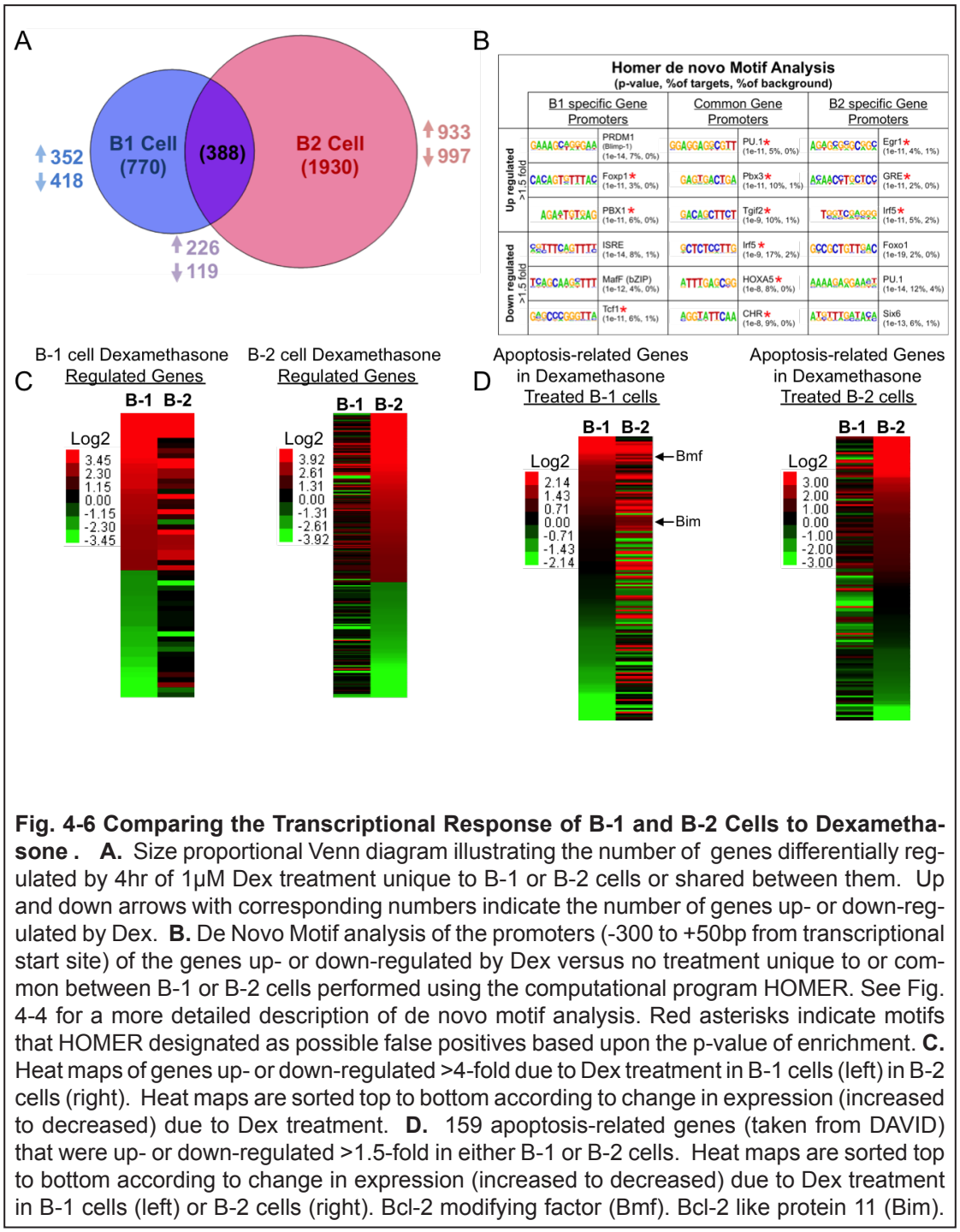


Fig. 4-6 Comparing the Transcriptional Response of B-1 and B-2 Cells to Dexamethasone . **A.** Size proportional Venn diagram illustrating the number of genes differentially regulated by 4hr of 1μM Dex treatment unique to B-1 or B-2 cells or shared between them. Up and down arrows with corresponding numbers indicate the number of genes up- or down-regulated by Dex. **B.** De Novo Motif analysis of the promoters (-300 to +50bp from transcriptional start site) of the genes up- or down-regulated by Dex versus no treatment unique to or common between B-1 or B-2 cells performed using the computational program HOMER. See Fig. 4-4 for a more detailed description of de novo motif analysis. Red asterisks indicate motifs that HOMER designated as possible false positives based upon the p-value of enrichment. **C.** Heat maps of genes up- or down-regulated >4-fold due to Dex treatment in B-1 cells (left) in B-2 cells (right). Heat maps are sorted top to bottom according to change in expression (increased to decreased) due to Dex treatment. **D.** 159 apoptosis-related genes (taken from DAVID) that were up- or down-regulated >1.5-fold in either B-1 or B-2 cells. Heat maps are sorted top to bottom according to change in expression (increased to decreased) due to Dex treatment in B-1 cells (left) or B-2 cells (right). Bcl-2 modifying factor (Bmf). Bcl-2 like protein 11 (Bim).

to each cell type. Also, of note is that the most strongly regulated genes in each cell type were not regulated similarly between B-1 and B-2 cells. For example, heat maps showing only the genes with changes in expression greater than 4-fold for either Dex treated B-1 or B-2 cells reveal that the same genes were often not qualitatively or quantitatively similarly up or down regulated in both cell types (Fig 4-6C). Motif analysis of the promoters of genes specific to B-1 or B-2 cells reveal that binding sites for unique transcription factors such as Blimp-1 for B-1 cells were slightly enriched (Fig. 4-6B). A gene ontology analysis of the genes commonly or specifically regulated in either B-1 or B-2 cells again demonstrated that unique cellular processes are being regulated (Table 4-3). Interestingly, apoptosis or cell death related gene ontology terms almost only appeared for the genes commonly regulated by both B-1 and B-2 cells. Of note, most of the gene ontologies associated with B-2 cell specific regulated genes related to cellular activation whereas those specific to B-1

Table 4-3 Gene Ontology Analysis of Dexamethasone Regulated Genes. Analysis performed using the online bioinformatic suite DAVID. Lists of Dex up- or down-regulated genes specific to B-1 cells and B-2 cells were compared to a background list of random genes. See Fig. 4-5A for numbers of genes in each gene list. P-values in parenthesis represent the enrichment of the indicated G.O. term among the regulated genes versus background genes.

	B1 Cell Specific Genes		Common Genes		B2 Cell Specific Genes	
Up Regulated (>1.5fold)	phosphorylation	7.10E-05	negative regulation of cellular process	2.83E-06	regulation of lymphocyte activation	3.45E-06
	regulation of immune system process	7.44E-05	intracellular signal transduction	6.30E-06	regulation of leukocyte apoptotic process	7.67E-06
	regulation of insulin receptor signaling pathway	9.11E-05	regulation of cell death	1.17E-05	positive regulation of lymphocyte apoptotic process	1.28E-05
	immune system process	0.00014236	negative regulation of biological process	1.40E-05	positive regulation of lymphocyte activation	3.79E-05
	cellular response to interferon-beta	0.00016148	regulation of apoptotic process	1.69E-05	regulation of leukocyte activation	4.20E-05
Down Regulated (>1.5fold)	response to stress	2.60E-09	positive regulation of response to stimulus	2.57E-08	leukocyte activation	1.15E-13
	response to wounding	2.81E-09	regulation of immune system process	3.69E-08	immune system process	8.26E-13
	immune system process	5.76E-09	regulation of response to stimulus	4.58E-08	cell activation	4.30E-12
	inflammatory response	1.99E-08	regulation of immune effector process	5.01E-08	lymphocyte activation	5.41E-11
	regulation of immune system process	3.86E-08	immune system process	6.59E-08	cholesterol biosynthetic process	9.15E-11

cells were more generally related to the regulation of the immune response.

The *in vitro* experiments detailed in chapter 3 demonstrate that apoptosis was a predominant phenotype observed when B-1 cells were cultured with Dex. Surprisingly, gene ontology terms related to cell death were not as common as expected in Dex treated B-1 or B-2 cells, nevertheless they were consistently observed (Table 4-1 & Table 4-3). A curated list of all the genes (980) that DAVID associates with apoptosis and cell death was used to assess the affect of Dex treatment on the expression levels of these genes (Fig. 4-6D). For both B-1 and B-2 cells, a large number of apoptosis-related genes were up or down regulated by Dex, but in many cases these were not the same genes. Figure 4-6D portrays expression heat maps of the 159 genes from the 980 apoptosis-related genes that were differentially regulated by Dex treatment in either B-1 or B-2 cells. This list of genes is sorted either according to change in expression level in B-1 cells (left) or B-2 cells (right). These data again emphasize what appears to be fundamentally different responses of B-1 and B-2 cells to Dex exposure.

Comparing the Cistrome of the Glucocorticoid Receptor Between Various B cell Subsets

The surprisingly unique transcriptomes of B-1 and B-2 cells especially in response to Dex treatment led me to hypothesize that GR would bind to unique locations in the genomes of B-1 and B-2 cells. To characterize the genome-wide bind binding of GR in these cells, I performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) in primary B-1 and B-2 cells. Because ChIP-seq often requires large numbers of cells, I decided to optimize the technique and protocol for GR ChIP-seq using BCL-1 cells. BCL-

1 cells are a mouse B cell line derived 40 years ago from a spontaneous tumor in a 2-year old Balb/c mouse (35). More recent evidence has demonstrated that BCL-1 cells most likely originated from B-1a cells (36). I reasoned that these would offer a valid approximation of primary B-1 cells and would allow me to optimize the ChIP-seq protocol without sacrificing more mice than necessary in order to harvest large numbers of primary B-1 cells. Before utilizing BCL-1 cells for GR ChIP-seq I confirmed that GR was expressed and active in these cells. Using qPCR methods I first compared the expression of GR from quiescent BCL-1 cells and B-1 cells from Balb/c mice. As shown in Figure 4-7A, BCL-1 cells did express GR but only at about 20% the levels observed

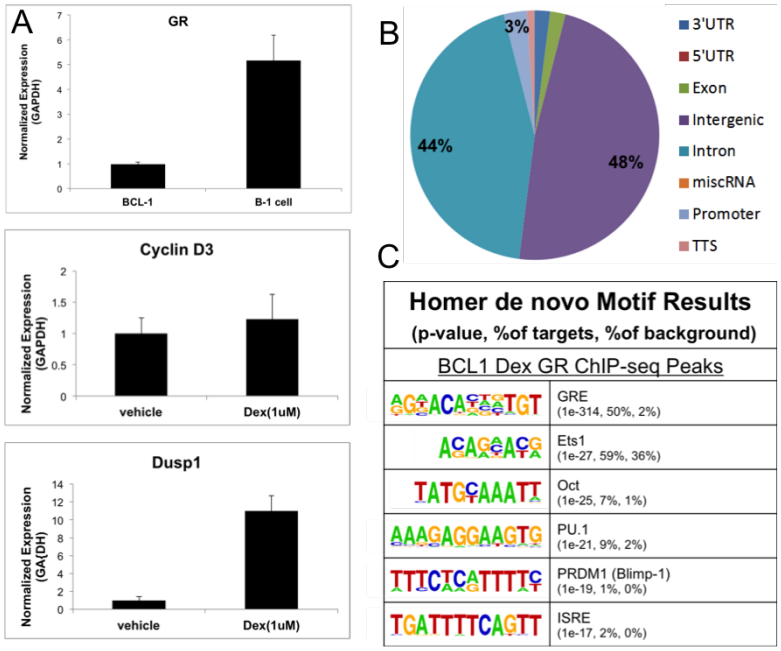


Fig. 4-7 Exploring the Function of GR in BCL-1 Cells. A. GR (top panel), Cyclin D3 (middle panel) and Dusp1 (bottom panel) expression as measured by qPCR and normalized to GAPDH. GR expression measured in B-1 cells from Balb/c mice as BCL-1 cells were originally derived from Balb/c mice. **B.** Genomic annotation of all 680 GR ChIP-seq binding peaks from BCL-1 cells. Untranslated Region (UTR). Transcriptional termination site (TTS). miscRNA include known non-coding RNAs, microRNAs, ribosomal RNAs. **C.** De Novo Motif analysis of 200bp (±100bp) from the center of all 680 GR ChIP-seq peaks from BCL-1 cells performed using HOMER. See Fig. 4-4 for more detailed description of de novo motif analysis, here peaks are analyzed and p-values for enrichment and motif frequencies are relative to random background sequences.

in primary B-1 cells. Next, I cultured BCL-1 cells for 4hr with 1μM Dex and then harvested the mRNA and performed qPCR analysis for the known GR target genes CCND3 and DUSP1. Only DUSP1 was appreciably and dramatically upregulated by Dex treatment (Fig. 4-7A). Next I used protocols that had been developed for macrophages, but found that considerable modifications were needed to optimize the buffer system and sonication protocols necessary for an efficient immunoprecipitation of GR in the BCL-1 cells. Eventually, I was able to successfully immunoprecipitate GR from BCL-1 cells and isolate the GR-bound DNA and sequence it. However, only 680 GR binding locations (peaks) were found. Peaks represent genomic locations with enrichment for GR bound DNA fragments. In other words, they are putative GR binding locations. Figure 4-7 illustrates the results of the ChIP-seq experiment in BCL-1 cells. These peaks were predominantly intergenic or intronic and very few peaks were found in the promoter regions of genes (Fig 4-7B). The GRE was found to be the most significantly enriched motif near these peaks. This verifies that GR is binding directly to DNA in BCL-1 cells and that this GR ChIP-seq experiment was valid.

Once I had successfully performed ChIP-seq in BCL-1 cells, I isolated large numbers (~25million) of primary B-1 and B-2 cells from the same cohorts of mice and cultured them in media containing charcoal-stripped serum, which is depleted of endogenous glucocorticoids and other lipid-based hormones, for 4hr with Dex before crosslinking the cells with formaldehyde and disuccinimidyl glutarate (DSG). DSG is a crosslinker with a 7.7 angstrom linker arm which serves to further stabilize the structure of proteins and protein-protein adducts (Fig. 4-8A) (37). In B-1 cells, 3867 GR binding peaks were identified and in B-2 cells 2376 peaks were found (Fig. 4-8B).

A comparison of GR peak locations in B-1 and B-2 cells revealed a notable dissimilarity in GR binding (Fig.4-8B). B-1 and B-2 cells only shared GR binding at 1109 genomic locations. This represents only 29% of the B-1 cell GR peaks and 47% of the B-2 cell peaks. The number of DNA sequencing tags that map in the vicinity of each peak is an indirect measure of GR occupancy frequency. Figure 4-8C depicts the number of sequence tags within 200bp of each GR peak from B-1 and/or B-2 cells. Interestingly, the peaks with the highest surrounding tag density were those that were found in both B-1 and B-2 cells. Also of note, peaks unique to B-1 cells tended to have more

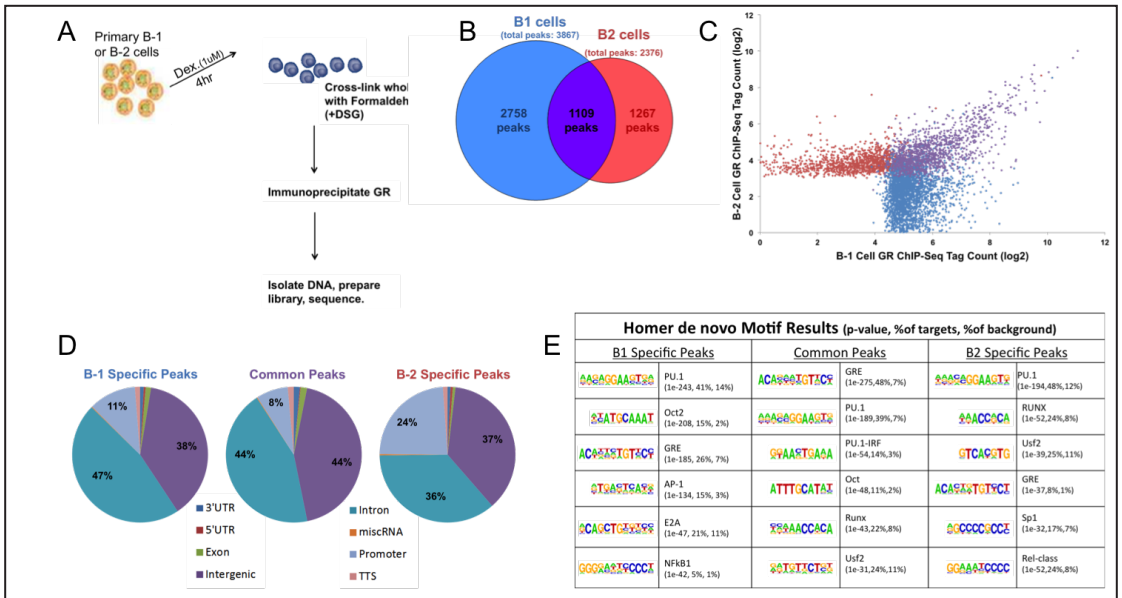


Fig. 4-8 GR Cistrome in Primary B-1 and B-2 Cells. **A.** Experimental Design for analyzing the genome wide binding of GR in primary B-1 and B-2 cells. B-1 or B-2 cells were cultured overnight in RPMI media containing 10% charcoal stripped serum. They were then treated for 4hr with 1uM Dex to induce GR nuclear translocation. After treatment cells were crosslinked with 1% formaldehyde and disuccinimidyl glutarate (DSG). GR was then immunoprecipitated and bound DNA was isolated and sequenced. **B.** Size proportional Venn diagram illustrating common and unique GR binding locations (if peaks were within 200bp they were considered to be shared) in B-1 and B-2 cells. **C.** Scatterplot of Log2 GR ChIP-seq sequencing tag counts within 200bp of all peaks in B-1 or B-2 cells. Colors correspond to those shown in (B). **D.** Genomic annotation of all GR ChIP-seq binding peaks from unique to or shared between B-1 and B-2 cells. See Fig. 4-6B for more detailed description of annotations. **E.** De Novo Motif analysis of 200bp (±100bp) from the center of all GR ChIP-seq peaks unique to or shared between B-1 and B-2 cells performed using HOMER. See Fig. 4-4 for more detailed description of de novo motif analysis, here peaks are analyzed and p-values for enrichment and motif frequencies are relative to random background sequences.

surrounding tag counts than those unique to B-2 cells. This may indicate that these peaks are in fact 'real' GR binding sites and not simply experimental artifacts. The majority of GR binding locations in B-1 and B-2 cells were found either intronically or intergenically (Fig. 4-8D). Peaks unique to B-2 cells were found considerably more often in promoters (24%) than peaks unique to B-1 cells (11%) or peaks common to both cell types (8%). There were very few peaks found in other genomic locations such as exons, 3' or 5' untranslated regions, transcription termination sites, etc. To both verify that known GR DNA binding motifs are found in the vicinity of GR peaks and to determine if there is enrichment for DNA motifs of other transcription factors in their vicinity a *de novo* motif analysis was performed (Fig. 4-8E). The bona fide GR motif, the Glucocorticoid response element (GRE), was the most enriched and significantly found motif near the peaks common to both B-1 and B-2 cells and was found within 100bp of 48% of the common peaks. The GRE was found near 26% of the B-1 cell specific peaks and only in 8% of the B-2 cell specific peaks. Motifs for the transcription factor PU.1 were very strongly enriched in both B-1 and B-2 cells. There were various other transcription factor motifs that were commonly found in both cell types including NFκB, Oct2, and Runx. Importantly, there were also motifs that were uniquely found in each cell type including AP-1 and E2A for B-1 cells and Sp1 for B-2 cells.

Comparing the Dexamethasone Transcriptome and GR Cistrome in B-1 and B-2 cells

Up to this point the genome-wide comparisons performed comparing B-1 and B-2 cells has revealed a remarkable disparity in the genes up- or down-regulated by Dex treatment and in the genome wide binding patterns of

GR. To get an understanding of whether the unique binding patterns of GR in B-1 and B-2 cells can at least in part explain the disparate gene expression regulation observed during Dex treatment, a comparison was carried out to evaluate changes in gene expression due to Dex treatment and GR binding. In this analysis, each GR binding peak was assigned to the transcriptional start site of the gene that was linearly the closest in the genome. If multiple GR peaks assigned to one gene, then that gene was only counted once in the analysis. Although B-1 cells had many more GR peaks (3867 vs. 2376) these only related to 2354 genes, whereas GR peaks (2376) in B-2 cells assigned to 1772 genes. In other words, B-1 cells had more peaks assigned to each gene (peaks/gene: 1.64) than B-2 cells (peaks/gene: 1.34).

Next, I compared the genes that had differential gene expression when treated with Dex with the list of genes with nearby GR binding peaks in B-1 cells (Fig. 4-9A) and B-2 Cells (Fig. 4-9B). Of the 1158 up or down regulated genes in B-1 cells, only 289 (25%) had GR binding peaks assigned to them. This represents only 12% of the 2354 genes assigned GR binding peaks (Fig. 4-9A). B-2 cells had more overall genes (347) that were differentially regulated with nearby GR binding peaks than B-1 cells (289). These 347 genes in B-2 cells represent only 15% of all the differentially regulated genes and about 20% of the genes assigned GR binding peaks (Fig. 4-9B). A gene ontology analysis was performed for all the genes that were differentially regulated with nearby GR binding peaks for B-1 cells (Fig. 4-9C) and B-2 cells (Fig. 4-9D). Interestingly, the gene ontology terms for these genes were quite unique for both cell types and quite statistically significant considering the small number of genes in the analysis. For example, the upregulated genes from B-1 cells associated strongly with G.O. terms related to cell signaling and programmed

cell death whereas in B-2 cells regulation of lymphocyte activation was a common theme (Fig. 4-9 C-D). Motif analysis was carried out for the sequences surrounding the GR peaks that were assigned to differentially Dex regulated genes in B-1 and B-2 cells (Fig. 4-9 E-F). The GRE and PU.1 motifs were the most significantly enriched motifs in both cell types. A motif quite similar to that of myeloid enhancer factor 2c (Mef2c) was uniquely enriched in B-2 cells.

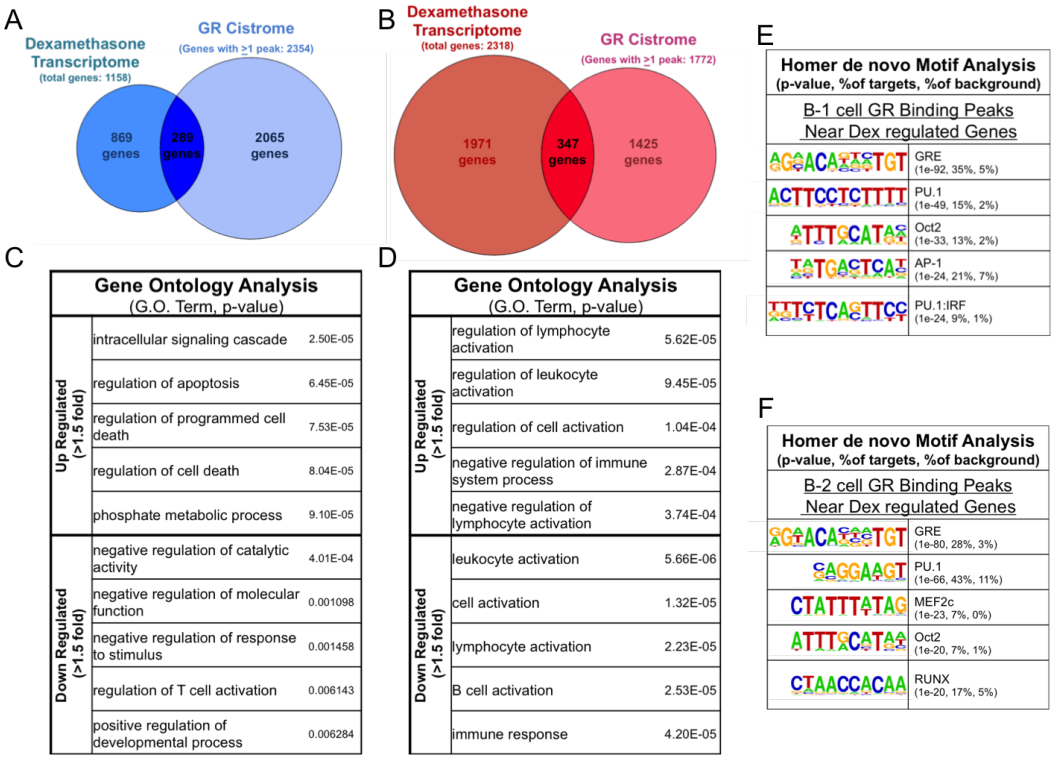


Fig. 4-9 Comparing the Dexamethasone Transcriptome and GR Cistrome in B-1 and B-2 cells. A-B. Size proportional Venn diagrams illustrating in B-1 cells (A) or B-2 cells (B) the number of genes differentially regulated by Dex which also had at least one nearby GR binding peak (assigned by linear proximity to transcriptional start site). **C-D.** Gene Ontology analysis performed using the online bioinformatic suite DAVID. Dex regulated genes with nearby GR binding peaks (overlap section of Venn diagrams) from B-1 cells (C) and B-2 cells (D) were compared to a background list of random genes. P-values represent the enrichment of the indicated G.O. term among the indicated genes versus background genes. **E-F.** De Novo Motif analysis of 200bp (±100bp) from the center of all GR ChIP-seq peaks assigned to Dex regulated genes from B-1 (E) and B-2 cells (F) performed using HOMER. See Fig. 4-4 for more detailed description of de novo motif analysis, here peaks are analyzed and p-values for enrichment and motif frequencies are relative to random background sequences.

Comparing the Enhancer Landscape of B-1 and B-2 cells

As detailed in great depth in Chapter 1, B-1 and B-2 cells possess remarkably disparate phenotypes. Perhaps unsurprisingly the transcriptomic response to Dex and the cistrome of GR are very unique between B-1 and B-2 cells. The mechanism for the unique GR binding and therefore the unique transcriptional response to Dex and ultimately the unique phenotypes of B-1 and B-2 cells and in fact cell type differences in general is hypothesized to be due to cell-type specific enhancers (26). To test if B-1 and B-2 cells possess unique enhancer landscapes across their genomes, I performed ChIP-seq for the post-translational histone mark of di-methylation of lysine 4 on histone 3 (H3K4Me2) from B-1 and B-2 cells in which the non-nucleosomal DNA had been digested by micrococcoal nuclease (MNase) (Fig. 4-10A). H3K4Me2 is an histone marker commonly considered to be present on promoter distal enhancers (38). As expected, B-1 and B-2 cells had near equal numbers of peaks or regions with enrichment for H3K4Me2 and most of these regions were found in both cells (Fig. 4-10B). H3K4Me2 marks were distributed across the genome in similar proportions in B-1 and B-2 cells and were found predominantly intronically (50%) or intergenically (35%) and to a lesser extent in promoters (9%) (Fig.4-10C). A motif analysis was performed for intergenic H3K4Me2 peaks unique to B-1 or B-2 cells or common to both cells (Fig. 4-10D). There were various transcription factor motifs that were common for both cell types, including among others PU.1, Oct, and Runx1. There were also a few transcription factor motifs that were unique to B-1 cells such as NFAT or for B-2 cells Mef2c.

To begin to answer whether the enhancers unique to B-1 or B-2 cells

influenced the unique GR cistromes in these cells, the number of H3K4Me2 sequencing tags was counted in the vicinity of each GR binding peak. This metric serves as a measure of GR binding proximity to enhancers. Plotted in blue in Figure 4-10E are those GR binding peaks that had 2-fold greater H3K4Me2 tag counts in B-1 cells than in B-2 cells and in red the converse is plotted. It is readily apparent that GR peaks with greater numbers of nearby H3K4Me2 tags in B-1 cells tended to be those peaks that are unique to B-1 cells. On the other hand, those GR peaks with greater numbers of nearby

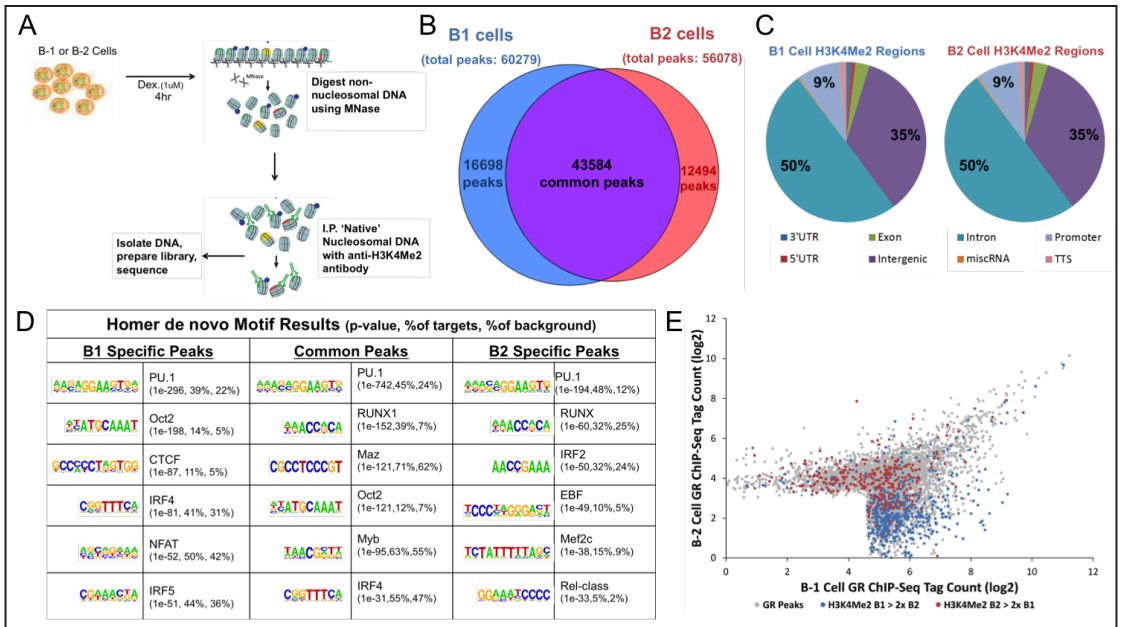


Fig. 4-10 H3K4Me2 ChIP-seq from B-1 and B-2 cells. A. Experimental Design for analyzing the genome wide prevalence of H3K4Me2 in primary B-1 and B-2 cells. B-1 or B-2 cells were treated for 4hr with 1uM Dex and then for 5 min with micrococcal nuclease (MNase). After treatment nucleosomes possessing the H3K4Me2 mark were immunoprecipitated and bound DNA was isolated and sequenced. **B.** Size proportional Venn diagram illustrating common and unique H3K4Me2 regions (shared peaks within 1000bp were considered to be shared) in B-1 and B-2 cells. **C.** Genomic annotation of all H3K4Me2 ChIP-seq binding peaks for B-1 and B-2 cells. See Fig. 4-7B for more detailed description of annotations. **D.** De Novo Motif analysis of 200bp (±100bp) from the nucleosome free (nfr) defined 'center' of intergenic (>5000 from TSS and not intronic) H3K4Me2 ChIP-seq regions unique to or shared between B-1 and B-2 cells performed using HOMER. See Fig. 4-4 for more detailed description of de novo motif analysis, here peaks are analyzed and p-values for enrichment and motif frequencies are relative to random background sequences. **E.** Scatterplot of Log2 GR ChIP-seq sequencing tag counts within 200bp of all peaks in B-1 or B-2 cells. Data points in blue correspond to GR peaks with surrounding (±200bp) H3K4Me2 tag densities two times > in B-1 cells versus B-2 cells. Red data points are same as blue but with higher surrounding H3K4Me2 density in B-2 cells.

H3K4Me2 tags in B-2 cells were those that are both unique to B-2 cells or common to both cell types.

Discussion

Studies described in this Chapter and in Chapters 2 and 3 have established that GR mRNA and protein are expressed and transcriptionally active in both in B-1 and B-2 cells. Both GR mRNA and protein are expressed at higher levels in B-2 cells. The transcriptional response to GR activation is surprisingly different between B-1 and B-2 cells. More genes are both transactivated and transrepressed in Dex treated B-2 cells. Surprisingly, even the most profoundly up- or down-regulated Dex genes often displayed different responses between B-1 and B-2 cells. The biological consequences of these unique transcriptional responses to Dex between B-1 and B-2 cells have not been established. Chapter 3 details the response of B-1 cells treated *in vitro* with Dex. Dex reduced antibody production in B-1 cells, which was probably due in large part to the potent induction of apoptosis. Future studies should establish in a similar *in vitro* culture system what the biological consequences of Dex treatment are in B-2 cells. However, these studies would be challenging as B-2 cells undergo rapid cell death in an *in vitro* culture system.

I hypothesize that B-2 cells would exhibit a unique phenotype to Dex activation relative to B-1 cells as the gene ontologies enriched by both GR up- or down-regulated genes were unique between B-1 and B-2 cells. Up-regulated genes in B-1 cells associated with phosphorylation and cell-signaling whereas in B-2 cells cell activation and cell death were commonly enriched gene ontologies. Dex down-regulated genes in B-2 cells uniquely associated with cholesterol biosynthesis related genes. The biological differences

of these unique effects are unknown. I am currently establishing an *in vivo* system in which to explore the biological consequences of GR activation on B cells. Mice possessing GR floxed alleles were gifted to our lab from Jonathan Ashwell (39). These have been crossed with CD19-Cre mice to generate mice with a B-cell specific knockout of GR ($GR^{fl/fl}/CD19^{Cre/+}$ mice). In order to explore and establish the effect of GR activation in B-1 versus B-2 cells the B-1 cells from these mice will be adoptively transferred into $Rag1^{-/-}$ mice, which lack B- and T-cells. Adoptively transferred B-1 cells will reconstitute the B-1 cell population of $Rag1^{-/-}$ mice (40). The effect of GR deletion upon the proliferative response that occurs during reconstitution will be measured. Furthermore, if GR-null B-1 cells are able to reconstitute the B-1 cell pool of $Rag1^{-/-}$ mice these mice can then be treated with Dex and compared to mice that received wild-type B-1 cells. Additionally, homozygous CD19-Cre ($CD19^{Cre/Cre}$) mice have been shown to lack B-1 cells (41). This phenomenon can be utilized to explore the effect of Dex treatment on B-2 cells specifically by comparing wild type and GR-null B-2 cells in these mice. Therefore, two mouse models, in which either B-1 or B-2 cells are exclusively present, can be utilized to compare the effect of GR activation *in vivo.* This is especially useful as B-2 cells cannot readily be cultured *in vitro.* Additionally, exploring the effects of *in vivo* treatment of Dex upon B cells is of clinical importance as glucocorticoids are one of the most often prescribed drug treatments. It will be important to establish what effects Dex and other glucocorticoids have upon B cells and their antibody titers and repertoires. This may be especially important in the context of IgG isotypes secreted by B-2 cells, and the IgM natural antibodies generated by B-1 cells. For example, IgM appear to play an atheroprotective role in murine models of atherosclerosis. The doses and time courses of Dex

administration that most profoundly impact B cells would be an important consideration when deciding whether to administer glucocorticoids to a patient. Additionally, the in depth genome-wide studies detailed in this chapter have the potential to improve understanding of the mechanisms whereby GR activation leads to the phenotypes observed *in vitro* and *in vivo* for B-1 and B-2 cells.

A motif analysis of the promoters of Dex or KLA up- or down-regulated genes did not result in the identification of any motifs that were profoundly enriched. Most motifs were near the threshold of significance (p-value of 1e-11 is HOMER's cut-off for possible false positives). Nevertheless, some interesting highlights emerged, that may be informative biologically. For example, the promoters of up-regulated genes for B-1 cells treated with KLA had the most significant enrichment, which included motifs similar to NFκB (p-value 1e-17), IRF3 (p-value 1e-15), and the GRE (p-value 1e-14) (Fig. 4-4 E). Considering that GR does not bind to the promoter very often in B-1 cells (Fig. 4-8D) it was surprising to find its response element motif enriched, even modestly, in the promoters of KLA up-regulated genes. I would've expected to find it enriched in the promoters of the Dex up-regulated genes. However, a recent report has shown that GR can bind to classical GREs in close association to NFκB binding elements and mediate either activation or repression in LPS stimulated cells (42). Another surprising example came from the comparison of motif enrichment of transrepressed genes between B-1 and B-2 cells. B-1 cells only had 37 transrepressed genes whereas B-2 cells had 326. Despite having so few gene promoters in the analysis, B-1 cells had one significantly enriched motif whereas B-2 cell had none. RXRα motif enrichment was barely above the threshold to avoid being labeled a possible false positive, but even so it is interesting that it was enriched at all. RXRα binds as a heterodimer with

various other nuclear receptors to regulate lipid metabolism, the retinoic acid response, etc (43,44). It is unknown whether RXR plays any role in GR mediated transrepression, though RXR when bound as a heterodimer with other nuclear receptors in the absence of ligand is known to mediate repression (45).

Even though apoptosis was potently induced in B-1 cells in vitro (see Chapter 3), none of the top 5 enriched gene ontology terms for Dex up- or down-regulated genes in B-1 cells were related to apoptosis (Table 4-1). This was not the case for B-2 cells as several G.O. terms associated with cell death were enriched among the Dex up-regulated genes. Though it bears mentioning that among the genes that were commonly up-regulated by Dex between B-1 and B-2 cells there was enrichment for several G.O. terms associated with apoptosis (Table 4-3). Furthermore, a heatmap of a curated list of apoptosis-associated genes reveal that many apoptosis-related genes are indeed regulated by Dex in B-1 and B-2 cells (Fig. 4-6D). However, these genes were regulated quite differently between B-1 and B-2 cells. This along with the unique G.O. term enrichment between these cells for apoptosis-related terms implies that Dex-induced apoptosis is regulated differently in B-1 and B-2 cells. The fact that known apoptosis related cellular processes are not the most enriched gene ontologies in B-1 cells may indicate that Dex most strongly induces genes or networks of genes in B-1 cells that don't directly induce apoptosis but rather induce cellular distress that then leads to apoptosis. Interestingly, the most significantly up-regulated gene statistically in B-1 cells is Sestrin-1, which is up-regulated 8-fold by Dex. It is also up-regulated in B-2 cells but to a lesser extent (5-fold). Sestrin-1 is up-regulated by p53 in response to DNA-damage and functions to regulate the scavenging of intracellular hydro-

gen peroxide and thereby inhibit apoptosis (32,46). This implies that p53 may be potently induced in B-1 cells and that it may be regulating apoptosis. The fact that Sestrin-1 also acts to scavenge oxidants in the cell also suggests that oxidative stress may be part of the cellular distress inducing apoptosis.

Although many of the apoptosis-related genes were regulated differentially in B-1 and B-2 cells, there were a number that were regulated in a similar fashion (Fig. 4-6D). Many of these were classic Dex target genes including DUSP1, Ddit4, and Sgk3. Interestingly, at least two members of the Bcl-2 family of apoptosis regulating genes, Bcl-2 modifying factor (BMF) and Bcl-2 like protein 11 (BCL2L11 or Bim) were induced in both B-1 and B-2 cells. (Fig. 4-6D). BMF and Bim are both pro-apoptotic and bind to Bcl-2 as well as other apoptosis regulating proteins (47,48). It has been reported that Bim protein expression is induced by Dex in HF28RA cells, a human follicular lymphoma cell line (48). Furthermore, microarray studies of acute lymphoblastic leukemic (ALL) cells isolated from children demonstrated that glucocorticoid treatment induced both pro- and anti-apoptotic BCL-2 family members, which varied between patients (49). However, the authors of this study conclude that BMF and Bim induction dominated the response of ALL cells to Dex and that both but especially Bim were necessary and sufficient for Dex induced induction of apoptosis (49). Additional studies need to be performed to establish whether Bim and BMF regulate the Dex mediated induction of apoptosis in primary B-1 and B-2 cells and whether there are differences between these cells.

Due to the drastic differences in Dex-regulated gene expression between B-1 and B-2 cells, I decided to carry out a ChIP-seq experiment for GR in these cells. The hypothesis put forth before performing this experiment was that GR would have unique binding patterns in B-1 and B-2 cells and that

this would correlate with their unique gene expression patterns. GR did in fact bind in many unique genomic locations in B-1 and B-2 cells (Fig. 4-8B). B-2 cells had fewer GR binding peaks than B-1 cells despite showing higher mRNA and protein expression and a more robust transcriptional response to Dex. This is most likely a technical issue and not a biological phenomenon. I plan to repeat the ChIP-seq study for GR in B-2 cells and predict that more GR binding peaks will be found. This technical issue may also account for the lower number of sequencing tag counts near B-2 cell specific peaks relative to B-1 cells (Fig. 4-8C). The fact that the GRE was the most enriched motif for the common peaks between B-1 and B-2 cells with a strong significance (p-value 1e-275) confirms that both the B-1 and B-2 cell GR ChIP-seq data sets were valid. Interestingly, the common peaks and not the cell-specific peaks between B-1 and B-2 cells had the highest nearby sequencing tag counts (Fig. 4-9C). This implies that the common peaks between B-1 and B-2 cells are the sites most often bound by GR. Even so, it was unexpected to observe that the B-2 cell specific peaks were found so often in the promoters of genes (Fig. 4-8D). Perhaps this unique promoter enrichment for B-2 cell specific peaks can account for some of the unique transcriptional effects of Dex in B-2 cells. Even so, GR bound predominantly intronically or intergenically in both cell types (Fig. 4-8D). This promoter distal binding implies that GR may be carrying out much of its regulatory effect in enhancers and other cis-regulatory elements.

All the GR ChIP-seq peaks from B-1 and B-2 cells were assigned to the closest transcriptional start site (based on linear proximity). This yielded 2354 genes for B-1 cells and 1772 for B-2 cells. An astonishingly small proportion of these genes were differentially regulated by Dex (289 genes for

B-1 cells and 347 genes for B-2 cells). The number of genes that were both regulated by Dex and had nearby GR ChIP-seq peaks was probably so low because there is currently no easy way to properly associate bound transcription factors with the genes that they are regulating. Associated transcription factor binding to the most proximal gene will undoubtedly lead to miss-assignments. This is probably especially true for those GR binding sites that are at very large distances from genes. Dex regulated genes with assigned GR ChIP-seq binding peaks were 32% or 26% closer on average in B-1 and B-2 cells, respectively, to the nearest transcriptional start site than all the genes assigned GR peaks. This suggests perhaps that GR binding in closer proximity to a gene will increase the likelihood that that gene will be regulated by GR. However, the average distance from a GR binding peak to the TSS of the nearest Dex regulated gene was ~27,000bp or ~31,000bp in B-1 and B-2 cells, respectively. A technique that could potentially be employed to better associate the regions of GR binding with the genes that they're regulating would be chromatin conformation capture or some derivative thereof, such as Hi-C. These methods can powerfully demonstrate long-range intra-chromosomal interactions that are probably occurring between cis-regulatory elements and Dex regulated genes.

A gene ontology analysis was carried out for the 289 genes from B-1 cells or the 347 genes from B-2 cells that were both differentially regulated and possessed assigned GR binding peaks. Remarkably, statistically significant G.O. terms were enriched in these small groups of genes. This supports the idea that common biological pathways and functions are being carried out by these groups of genes in their respective cell types. In B-1 cells, cell signaling and cell death were significantly up-regulated G.O terms whilst cell activation

appears to be a commonly down-regulated cellular function (Fig. 4-9C). In B-2 cells, G.O. terms associated with cellular activation were significantly enriched for both up- and down-regulated genes (Fig. 4-9D). Though there are undoubtedly other Dex regulated genes that were directly regulated by GR, those that were assigned at least one nearby GR binding peak by the crude methods mentioned above seem to regulate common cellular processes.

Motif analysis was performed for the 200bp surrounding all the peaks assigned to Dex regulated gene for both B-1 and B-2 cells (Fig. 4-9 E&F). The GRE was the most potently enriched motif for both cell types followed by PU.1, and the Oct2 motif was also enriched in both cell types. Interestingly, motifs for several transcription factors were enriched uniquely in each cell type, these included AP-1 and the composite motif of PU.1:IRF for B-1 cells and MEF2c and RUNX for B-2 cells. These unique cell-type specific motifs represent transcription factors that potentially were bound or could bind in close proximity to GR binding locations and therefore could play a role in dictating the unique GR binding patterns between B-1 and B-2 cells.

PU.1 has been shown to collaborate with cell-type specific LDTFs to establish cis-regulatory elements in either B-cells or macrophages, which then dictate the cell-type specific binding pattern of other signal dependent transcription factors as well as more generally the identity of the cell (26). The motif for PU.1 was significantly enriched near the GR binding sites in both B-1 and B-2 cells (Figs 4-8E, 4-9E-F). Additionally, motifs for known B-cell LDTFs such as Oct2 and E2A were also observed. I hypothesized that PU.1 would collaborate with unique LDTFs in B-1 and/or B-2 cells to establish unique cis-regulatory elements between these cell types. A ChIP-seq experiment analyzing the nucleosomal regions of the genome marked by H3K4Me2 was car-

ried out to begin to address this hypothesis. B-1 and B-2 cells possess large numbers of common regions enriched for H3K4Me2 but they also each posses large numbers of unique regions (Fig. 4-10B). A motif analysis was performed for the intergenic H3K4Me2 regions unique or common to B-1 or B-2 cells (Fig. 4-10D). As expected, PU.1 was the most significantly enriched motif. Interestingly however, several other motifs were enriched uniquely in B-1 or B-2 cells. A motif similar to NFAT was enriched only in B-1 cells. NFATc1 has been shown to be required for normal B-1a cell development (50). This represents a possible candidate LDTF specific for B-1 cells. The motif for Mef2c was uniquely enriched at B-2 cell specific H3K4Me2 regions. Additionally, as mentioned earlier, the Mef2c motif was also enriched near B-2 cell GR peaks associated with Dex regulated genes (Fig. 4-9F). Mef2c therefore represents a potential B-2 cell LDTF. Interestingly, Mef2c has recently been specifically knocked out from the B cell lineage and overall numbers of immature splenic and bone marrow as well as mature bone marrow B cells (B220+) were significantly decreased (51). Interestingly the authors did not evaluate the effect of Mef2c deficiency upon B-1 versus B-2 cells but did report that numbers of marginal zone B cells were increased. Marginal zone B cells are similar to B-1 cells in various respects. Additionally, the loss of Mef2c altered the expression of many B cell specific genes and notably reduced the expression of CD23 on splenic B cells. CD23 is a cell surface marker that distinguishes B-2 cells from B-1 cells. Further studies are needed to corroborate and validate the hypothesis that NFAT and Mef2c are LDTFs for B-1 and B-2 cells, respectively. Currently the ChIP-seq for PU.1 is being performed in B-1 and B-2 cells. These ChIP-seq data should shed further light on which transcription factors bind in the vicinity of PU.1 binding sites and whether there are unique motifs near

PU.1 binding sites in B-1 and B-2 cells. Additionally, the transcription factor Early B Cell Factor 1 (EBF1) has been reported to uniquely control B-1 cell development (52). A comparison of ChIP-seq binding patterns of EBF1 will be carried out for B-1 and B-2 cells. It is expected that EBF1 will bind near B-1 cell specific enhancers defined by the enrichment of H3K4Me2 at intergenic regions.

Once the identity of potential LDTFs has been established through the ChIP-seq experiments for PU.1 and potentially EBF1 described above, then the role of these factors in B-1 cell development and maintenance will be tested. If these factors are important in B-1 and/or B-2 cell maintenance and/or development then they should be expressed in adult and/or progenitor cells, respectively. The expression levels of the putative LDTFs will evaluated by both qPCR and western blot in adult and progenitor B-1 and B-2 cells. B-1 cell progenitors were recently reported to reside predominantly within the fetal liver but can also be found in the adult spleen and have the following cell surface phenotype $Lin^{-}CD45R^{lo\text{-}neg}CD19^{+}$ that can be used for isolation by FACS (53,54). The developmental cascade of B-2 cells is much more well defined and progresses within the adult bone marrow from the pre-pro B-2 cell ($CD45R^{+}CD43^{+}AA4.1^{+}CD19^{-}Ly\text{-}6C^{-}$), to the pro-B-2 cell ($CD45R^{+}CD19^{+}CD43^{+}AA4.1^{+}$), and finally to the pre-B-2 cell ($CD45R^{+}CD19^{+}$) stage. All progenitor B-2 cell populations will be isolated by FACS and analyzed for the expression of the transcription factor(s) in question. Additionally, ChIP-seq experiments for the putative LDTFs will be carried out. For example, if the motif for Mef2c in confirmed by PU.1 ChIP-seq to again be near unique B-2 cell binding locations then ChIP-seq will be performed for this factor in both B-1 and B-2 cells. Other investigators have already performed ChIP-seq for

Mef2c in a B cell line but not in primary B-1 or B-2 cells (51). It would be expected that Mef2c binding would occur in close association with PU.1 at B-2 cell specific enhancers. Finally, a knock-out model for the putative LDTFs for B-1 and B-2 cell will be utilized to evaluate the importance of the factors in establishment and maintenance of stable B-1 and B-2 populations. Specifically, the impact of the knock-out(s) on the population size of various progenitor B cells will be evaluated using the cell populations and markers described above. We will also extensively explore the impact of the knockout on antibody production. Additionally, our lab has previously employed adoptive transfer experiments using B-1 cells, wherein B-1 cells are isolated and injected into the peritoneal cavity of $Rag1^{-/-}$ mice, which are devoid of B or T cells, and through homeostatic proliferation and self-replenishment the donor B-1 cells repopulate the spleen and replenish the plasma IgM compartment (40). This model will be useful in directly evaluating the impact of transcription factor gene ablation on B-1 cell survival, proliferation, self-replenishment, and spontaneous NAb secretion.

Acknowledgements

Dr. Nathan Spann, Dr. Minna Kaikkonen and various other members of Dr. Chris Glass' laboratory have provided extensive assistance and mentorship in performing and analyzing the RNA-seq and ChIP-seq experiments described in this chapter.

References

1. Heng TS, Painter MW, Immunological Genome Project C. The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol. 2008;9(10):1091-1094.

2. Zen M, Canova M, Campana C, Bettio S, Nalotto L, Rampudda M, Ramonda R, Iaccarino L, Doria A. The kaleidoscope of glucorticoid effects on immune system. Autoimmun Rev. 2011;10(6):305-310.

3. Suarez A, Lopez P, Gomez J, Gutierrez C. Enrichment of CD4+ CD25high T cell population in patients with systemic lupus erythematosus treated with glucocorticoids. Annals of the rheumatic diseases. 2006;65(11):1512-1517.

4. Cupps TR, Gerrard TL, Falkoff RJ, Whalen G, Fauci AS. Effects of in vitro corticosteroids on B cell activation, proliferation, and differentiation. J Clin Invest. 1985;75(2):754-761.

5. Alnemri ES, Fernandes TF, Haldar S, Croce CM, Litwack G. Involvement of BCL-2 in glucocorticoid-induced apoptosis of human pre-B-leukemias. Cancer research. 1992;52(2):491-495.

6. Bowen DL, Fauci AS. Selective suppressive effects of glucocorticoids on the early events in the human B cell activation process. J Immunol. 1984;133(4):1885-1890.

7. Zhu XJ, Shi Y, Sun JZ, Shan NN, Peng J, Guo CS, Qin P, Hou M. High-dose dexamethasone inhibits BAFF expression in patients with immune thrombocytopenia. J Clin Immunol. 2009;29(5):603-610.

8. Thompson JS, Schneider P, Kalled SL, Wang L, Lefevre EA, Cachero TG, MacKay F, Bixler SA, Zafari M, Liu ZY, Woodcock SA, Qian F, Batten M, Madry C, Richard Y, Benjamin CD, Browning JL, Tsapis A, Tschopp J, Ambrose C. BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. J Exp Med. 2000;192(1):129-135.

9. Batten M, Groom J, Cachero TG, Qian F, Schneider P, Tschopp J, Browning JL, Mackay F. BAFF mediates survival of peripheral immature B lymphocytes. J Exp Med. 2000;192(10):1453-1466.

10. Shulga-Morskaya S, Dobles M, Walsh ME, Ng LG, MacKay F, Rao SP, Kalled SL, Scott ML. B cell-activating factor belonging to the TNF family acts through separate receptors to support B cell survival and T cell-independent antibody formation. J Immunol. 2004;173(4):2331-2341.

11. Lentz VM, Hayes CE, Cancro MP. Bcmd decreases the life span of B-2 but not B-1 cells in A/WySnJ mice. J Immunol. 1998;160(8):3743-3747.

12. Settipane GA, Pudupakkam RK, McGowan JH. Corticosteroid effect on immunoglobulins. J Allergy Clin Immunol. 1978;62(3):162-166.

13. Jabara HH, Brodeur SR, Geha RS. Glucocorticoids upregulate CD40 ligand expression and induce CD40L-dependent immunoglobulin isotype switching. J Clin Invest. 2001;107(3):371-378.

14. Gaynon PS, Carrel AL. Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia. Adv Exp Med Biol. 1999;457:593-605.

15. Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ. 2002;9(1):6-19.

16. Ploner C, Schmidt S, Presul E, Renner K, Schrocksnadel K, Rainer J, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia. J Steroid Biochem Mol Biol. 2005;93(2-5):153-160.

17. Grad I, Picard D. The glucocorticoid responses are shaped by molecular chaperones. Mol Cell Endocrinol. 2007;275(1-2):2-12.

18. Pratt WB, Galigniana MD, Morishima Y, Murphy PJ. Role of molecular chaperones in steroid receptor action. Essays in biochemistry. 2004;40:41-58.

19. De Bosscher K, Haegeman G. Minireview: latest perspectives on antiinflammatory actions of glucocorticoids. Mol Endocrinol. 2009;23(3):281-291.

20. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev. 2003;24(4):488-522.

21. Beck IM, Vanden Berghe W, Vermeulen L, Yamamoto KR, Haegeman G, De Bosscher K. Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases.

Endocr Rev. 2009;30(7):830-882.

22. La Baer J, Yamamoto KR. Analysis of the DNA-binding affinity, sequence specificity and context dependence of the glucocorticoid receptor zinc finger region. Journal of molecular biology. 1994;239(5):664-688.

23. Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science. 1990;249(4974):1266-1272.

24. De Martino MU, Bhattachryya N, Alesci S, Ichijo T, Chrousos GP, Kino T. The glucocorticoid receptor and the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II interact with and mutually affect each other's transcriptional activities: implications for intermediary metabolism. Mol Endocrinol. 2004;18(4):820-833.

25. Doppler W, Windegger M, Soratroi C, Tomasi J, Lechner J, Rusconi S, Cato AC, Almlof T, Liden J, Okret S, Gustafsson JA, Richard-Foy H, Starr DB, Klocker H, Edwards D, Geymayer S. Expression level-dependent contribution of glucocorticoid receptor domains for functional interaction with STAT5. Mol Cell Biol. 2001;21(9):3266-3279.

26. Heinz S, Benner C, Spann N, Bertolino E, Lin Y, Laslo P, Cheng J, Murre C, Singh H, Glass C. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Molecular cell. 2010;38(4):576-589.

27. Heinz S, Glass CK. Roles of lineage-determining transcription factors in establishing open chromatin: lessons from high-throughput studies. Curr Top Microbiol Immunol. 2012;356:1-15.

28. Heinz S, Romanoski CE, Benner C, Allison KA, Kaikkonen MU, Orozco LD, Glass CK. Effect of natural genetic variation on enhancer selection and function. Nature. 2013.

29. Barish GD, Downes M, Alaynick WA, Yu RT, Ocampo CB, Bookout AL, Mangelsdorf DJ, Evans RM. A nuclear receptor atlas: Macrophage activation. Molecular Endocrinology. 2005;19(10):2466-2477.

30. Takeda K, Shimozono R, Noguchi T, Umeda T, Morimoto Y, Naguro I, Tobiume K, Saitoh M, Matsuzawa A, Ichijo H. Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase kinase in a heteromeric complex with ASK1. J Biol Chem. 2007;282(10):7522-7531.

31. Shoshani T, Faerman A, Mett I, Zelin E, Tenne T, Gorodin S, Moshel Y, Elbaz S, Budanov A, Chajut A, Kalinski H, Kamer I, Rozen A, Mor O, Keshet E, Leshkowitz D, Einat P, Skaliter R, Feinstein E. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. Mol Cell Biol. 2002;22(7):2283-2293.

32. Budanov AV, Sablina AA, Feinstein E, Koonin EV, Chumakov PM. Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. Science. 2004;304(5670):596-600.

33. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research. 2009;37(1):1-13.

34. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.

35. Slavin S, Strober S. Spontaneous murine B-cell leukaemia. Nature. 1978;272(5654):624-626.

36. Koganei S, Ito M, Yamamoto K, Matsumoto N. B-1a cell origin of the murine B lymphoma line BCL1 characterized by surface markers and bacterial reactivity of its surface IgM. Immunology Letters. 2005;98(2):232-244.

37. Nowak DE, Tian B, Brasier AR. Two-step cross-linking method for identification of NF-kappaB gene network by chromatin immunoprecipitation. Biotechniques. 2005;39(5):715-725.

38. Zentner GE, Scacheri PC. The chromatin fingerprint of gene enhancer elements. J Biol Chem. 2012;287(37):30888-30896.

39. Mittelstadt PR, Monteiro JP, Ashwell JD. Thymocyte responsiveness to

endogenous glucocorticoids is required for immunological fitness. J Clin Invest. 2012;122(7):2384-2394.

40. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, B„ckhed F, Miller YI. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. The Journal of Clinical Investigation. 2009;119(5):1335.

41. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature. 1995;376(6538):352-355.

42. Uhlenhaut NH, Barish GD, Yu RT, Downes M, Karunasiri M, Liddle C, Schwalie P, Hubner N, Evans RM. Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. Mol Cell. 2013;49(1):158-171.

43. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. Science. 2001;294(5548):1866-1870.

44. Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. Journal of Lipid Research. 2002;43(11):1773-1808.

45. Hu X, Lazar MA. Transcriptional repression by nuclear hormone receptors. Trends in endocrinology and metabolism: TEM. 2000;11(1):6-10.

46. Velasco-Miguel S, Buckbinder L, Jean P, Gelbert L, Talbott R, Laidlaw J, Seizinger B, Kley N. PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. Oncogene. 1999;18(1):127-137.

47. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. Bim: a novel member of the Bcl-2 family that promotes apoptosis. The EMBO journal. 1998;17(2):384-395.

48. Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DC, Strasser A. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science. 2001;293(5536):1829-1832.

49. Ploner C, Rainer J, Niederegger H, Eduardoff M, Villunger A, Geley S, Kofler R. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. Leukemia. 2008;22(2):370-377.

50. Berland R, Wortis HH. Normal B-1a cell development requires B cell-intrinsic NFATc1 activity. Proc Natl Acad Sci U S A. 2003;100(23):13459-13464.

51. Debnath I, Roundy KM, Pioli PD, Weis JJ, Weis JH. Bone marrow-induced Mef2c deficiency delays B-cell development and alters the expression of key B-cell regulatory proteins. Int Immunol. 2013;25(2):99-115.

52. Vilagos B, Hoffmann M, Souabni A, Sun Q, Werner B, Medvedovic J, Bilic I, Minnich M, Axelsson E, Jaritz M, Busslinger M. Essential role of EBF1 in the generation and function of distinct mature B cell types. J Exp Med. 2012;209(4):775-792.

53. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1B cell-specified progenitor. Nature Immunology. 2006;7(3):293-301.

54. Ghosn EE, Sadate-Ngatchou P, Yang Y, Herzenberg LA. Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen. Proc Natl Acad Sci U S A. 2011;108(7):2879-2884.

CHAPTER 5

Collaborative Studies Investigating the Role of the Humoral Immune Response in Various Models of Atherosclerosis

Abstract

Dr. Witztum's laboratory has been a pioneer in demonstrating that the immune system plays a significant role in modulating the development of atherosclerosis. Immune cells and immune mediators can either promote or inhibit the atherogenic process. Detailed in this chapter are five collaborative studies exploring the impact of the immune system on the development of atherosclerosis. The first study used a knockout of the invariant chain to investigate the role of antigen presentation in disease progression. A second study considered the role of the transcriptional repressor BCL-6, which has been shown to potently antagonize NFκB, in atherogenesis. The third study analyzed the impact of a B- and T-cell specific immunosuppressant drug upon disease severity. The fourth collaboration analyzed the impact of the inhibitory FcγRIIb on atherosclerosis. And finally, the influence of vitamin D administration in an accelerated atherosclerosis model was evaluated. In each of these studies I carried out analyses to evaluate the impact of the disease model upon either B-1 cells or on plasma levels of circulating natural and/or adaptive immune antibodies. In all studies, except for vitamin D treatment, B cells and/or antibody levels were significantly impacted by the intervention and therefore potentially carried out a significant role in the athero-phenotypes observed.

Introduction

B cells, and the antibodies they produce are known to potently impact the development of atherosclerosis (1). Described below are five atherosclerosis studies carried out in collaboration with various laboratories in which I assessed the impact of a genetic or pharmacologic intervention upon B cells and/or antibody levels.

Function of Invariant Chain and its Role in Atherosclerosis.

The Invariant chain, other wise called CD74, is a type II transmembrane protein that plays a critical role in proper antigen processing and presentation by antigen presenting cells (APC) (2). CD74 functions as a chaperone for the major histocompatibility complex II (MHC-II) and helps it to assemble properly. When CD74 expression is ablated, MHC-II $A\alpha^b$ and $A\beta^b$ chains are mostly free and not properly assembled (3). Additionally, CD74 binds in the peptide binding cleft of the MHC-II complex intracellularly as it is being processed and prevents the inappropriate binding and therefore presentation of other peptides (2). CD74 when bound to MHC-II also functions to target the complex to lysosomes and endosomes, where it is removed by proteases (2). Once CD74 is removed this allows MHC-II to be properly loaded with antigenic peptides that can then be presented to CD4 T-cells on the cell surface (4). Loss of proper CD74 expression or proteolytic processing results in major disturbances in antigen presentation and immune homeostasis in general. APCs of mice lacking cathepsin S, which plays a role in the proteolytic breakdown of CD74, present a 10kDa CD74 fragment as opposed to antigenic peptides on their cell surfaces (4). This results in failure to activate T cells and fewer IgG2a and IgG3 antibodies even though T and B cell numbers are normal. CD74 knockout mice have a drastic reduction in numbers of CD4 T cells, greater CD8 T cells, and reduced numbers of B cells (3,5-7). However, it appears that B cell development is not dependent on CD74, as double knockout of the CD74 and MHC-II genes did not affect B cell numbers (7).

APCs and T Cells are found in atherogenic plaques and are considered to be proatherogenic (8). T-cell ablation reduces atherosclerotic plaque burden and reconstitution restores the disease phenotype. In collaboration with

the laboratory of Guo-Ping Shi at Harvard, the hypothesis was tested whether the loss of antigen presentation in the absence of CD74 results in reduced T cell activation in the context of atherogenesis, and the impact of this on atherogenesis (9). *Cd74-/-* mice were crossed with *Ldlr*$^{-/-}$ mice and given an atherogenic diet and were found to have reduced atherosclerosis. Compared to control mice CD74-/- mice had reduced levels of plasma IgG1, IgG2b, and IgG2c against malondialdehyde-modified LDL (MDA-LDL), indicating that T cell mediated adaptive responses against disease-specific oxidation specific epitopes of oxidized LDL were blunted. These data are consistent with a wide body of literature that T cell specific responses against oxidation specific epitopes are proatherogenic. This interpretation however is complicated by the interesting observation that these mice had increased titers of innate IgM and IgG3 natural antibodies against MDA-LDL as well as increased numbers of B-1 and Marginal zone B cells. As discussed in Chapter 1, B-1 cells are considered to be atheroprotective. Therefore the loss of CD74 and antigen presentation may not only impact atherosclerosis by reducing the T cell dependent effects but also by directly modulating B cell biology. These data are also interesting as they suggest that there is some kind of feedback regulation between adaptive and innate immune humoral responses.

B-cell Lymphoma 6, Atherosclerosis, and Antibody Responses.

B-cell lymphoma 6 (BCL-6) is a sequence specific transcriptional repressor (10). It is known to play an intrinsic role in B cell regulation but is also known to be important in T cells and macrophages (11,12). In B cells expression of BCL-6 is restricted to the germinal center reaction where it is known to repress plasma cell differentiation and is thought to function in a

reciprocal inhibitory regulatory loop with Blimp-1 (13). BCL-6 may function in a unique manner in B-1 cells as its expression is lower than in B-2 cells, yet Blimp-1 expression is not dramatically induced as would be expected (14). As its name suggests BCL-6, when genetically damaged, is implicated in various malignancies (10). BCL-6 represses target gene expression through interaction with various co-repressors including SMRT, NCoR, BCorR, HDACS, and others (10). In an acute or chronic inflammatory state, the expression of many inflammatory genes are up-regulated by various transcriptional inflammatory regulators, typified by NFκB. Dr. Grant Barish, an investigator in the laboratory of Dr. Ron Evans, has shown that BCL-6 can inhibit acute TLR induced inflammation in macrophages by binding in the proximity of NFκB binding sites (15). Dr. Barish hypothesized that BCL-6 functions in macrophages to curb chronic inflammation in the context of atherosclerosis. To test this hypothesis, bone marrow transplantation studies were carried out using bone marrow deficient in BCL-6 (16). It was found that mice on an atherogenic diet that received BCL-6 deficient bone marrow had enhanced atherogensis as well as xanthomatous tendonitis compared to mice that received wild type bone marrow. BCL-6, through its repressor activity in conjunction with SMRT and NCoR was atheroprotective. Whether these mice also had changes in antibody titers that could also contribute to the changes in atherogenesis was unknown. As BCL-6 plays a prominent role in inhibiting B-cell differentiation in the germinal center it was hypothesized that antibody titers would be affected in BCL-6 bone marrow transplantation atherosclerotic mice.

Immunosuppression with Mycophenolate Mofetil, Atherosclerosis, and Antibody Responses.

Atherosclerosis is characterized as a chronic inflammatory response that involves the activation of inflammatory leukocytes (17). Both myeloid and lymphoid cells have been demonstrated to play intrinsic or important roles in the pathophysiology of atherosclerosis (18). Unfortunately however the use of systemic immunosuppressants has resulted in varied and often detrimental effects with regard to atherosclerosis (19). Various classes of immunosuppressive agents can negatively impact various cardiovascular risk factors. For example, prolonged administration of glucocorticoids is known to increase hypertension, levels of circulating lipids, and especially incidence of diabetes mellitus (19). In animal studies, inhibition of calcineurin through treatment with cyclosporine A, which prevents lymphocytes from becoming active, has been shown to enhance atherosclerosis (20). Conversely, inhibition of sphingosine 1-phosphate receptor signaling, by administration of Fingolimod, which sequesters lymphocytes in lymph nodes, has been shown to reduce atherosclerosis in several mouse models but also to increase levels of circulating lipids (21-23).

Mycophenolic acid and the pro-drug, Mycophenolate Mofetil (MMF), are immunosuppressive agents that act as purine antagonist and block guanosine nucleotide generation specifically in proliferating T and B cells (24,25). Compared to other commonly used immunosuppresants, MMF has been reported to have advantageous results with regard to vascular disease and cardiovascular risk factors, though it has not been studied as extensively as other agents (19). Dr. Sibylle von Vietinghoff of Dr. Klaus Ley's lab explored whether MMF would inhibit atherosclerosis in a mouse model of atherosclerosis

(26). They administered 30mg/kg of daily MMF to apolipoprotein-E-deficient (*Apoe-/-)* mice. MMF was given to young *ApoE-/-* mice for 12 weeks or old *ApoE-/-* mice for 3 weeks while on a high fat diet. It was found that MMF moderately decreased atherosclerosis without significantly impacting cholesterol levels. I measured and analyzed the antibody titers from the young 12-week MMF treated *ApoE-/-* mice and the results, in part, were published in Figure 2 A-D of their paper (26).

Biology of the Inhibitory FcγRIIb and its Effect on Atherogenesis

Various immune cells such as B cells, macrophages, neutrophils, mast cells, natural killer cells, and dendritic cells express Fcγ receptors (27). Fcγ receptors recognize the non-variable Fc portion of antigen bound IgG antibodies and can be either activating or inhibitory (27,28). Activating receptors such as FcγRI, FcγRIIa, or FcγIII have high affinity for IgG and possess intracellular tyrosine activating motifs (ITAM) that can initiate and augment inflammatory signaling (27). The inhibitory receptor FcγRIIb has an intracellular tyrosine inhibitory motif (ITIM) that can decrease immune cell activation when bound to IgG immune complexes (27).

Activating Fcγ receptors appear to promote atherosclerosis while inhibitory receptors may inhibit disease progression (1). *Apoe*$^{-/-}$ mice that express only the inhibitory receptor FcγRIIB due to knockout of the Fc γ-chain develop significantly less atherosclerosis (29). This reduction in disease severity was associated with reduced numbers of macrophages and T cells in the atherosclerotic lesions (29). Another study also found greatly reduced lesion size in *Ldlr*$^{-/-}$ mice deficient for the activating FcγRIII (30). This reduction occurred in the face of increased plasma total cholesterol. Additionally, these mice exhib-

ited elevated IgG1 and IgG2c plasma titers against MDA-LDL and CuOx-LDL, while total plasma IgG levels were not different (30). The inhibitory FcγRIIB, on the other hand has been shown to protect mice from several autoantibody-mediated diseases (31). A recent bone marrow transplantation experiment from mice deficient for FcγRIIB into *Ldlr*$^{-/-}$ mice fed a high fat diet resulted in increased atherosclerosis (32). Splenic B and T cells from these mice possessed enhanced proliferative capacity and demonstrated elevated cytokine and antibody levels, all demonstrative of a de-repressed and hyperactivated immune system.

In a collaborative study with the lab of Amy Major, mice deficient for both the FcRγIIB receptor and ApoE were generated and used to further study its role in atherosclerosis (33). Male FcRγIIB receptor deficient mice were observed to develop enhanced atherosclerosis, especially at an early time point. I assessed the levels of specific IgM, IgG1, and IgG2c titers to MDA-LDL and CuOx-LDL as well as IgM and IgG immune complexes and the levels of the natural antibody EO6. All of these data are published except the EO6 titers (see Figure 4 of (33)).

Vitamin D in the Immune System.

Vitamin D classically regulates calcium homeostasis and therefore bone health; however new roles for vitamin D in immune regulation are emerging. Vitamin D as consumed or produced in the skin as a consequence of UV exposure is inactive and must undergo two hydroxylation events to become active; first in the liver to become 25-OH Vitamin D3 and then in the kidney by 1-α-hydroxylase to become active 1,25-OH Vitamin D3 (1,25-VitD3). 1,25-VitD3 binds to the vitamin D receptor (VDR), which is a nuclear receptor, that

pairs with RXR and binds to DNA response elements in gene promoters to regulate gene expression. I and others have demonstrated that VDR is expressed in B cells (34). It is also expressed in T cells, dendritic cells, and macrophages (35). TLR4 or TLR2-1 activation of B-1 and B-2 cells further increased the expression levels of the VDR, this effect has also been reported for other immune cells (34,36,37). Additionally, extra-renal expression of 1-α-hydroxylase has been reported in B-cells, T-cells, and macrophages (35). The local expression of this enzyme is postulated to regulate localized 1,25-VitD3 production that would then act in an autocrine or paracrine fashion. However, RNA-seq data for peritoneal B-1 and splenic B-2 cells did not demonstrate expression of 1-α-hydroxylase (data not shown). Furthermore, microarray data from the Immunological Genome Project also demonstrates very low or background levels of expression of 1-α-hydroxylase in a wide variety of lymphoid and myeloid immune cells including most B cell subsets (38).

Vitamin D deficiency has been reported to contribute to various autoimmune conditions, including multiple sclerosis, rheumatoid arthritis, systemic erythematosus (SLE), and others (39). Persons with low levels of vitamin D have been shown to have greater rates of infection and susceptibility to pathogens (35). Vitamin D and its receptor have been reported to inhibit B cell proliferation, differentiation, and immunoglobulin secretion (40,41). Yet, other reports have found very little effect for vitamin D exposure upon B cells (42). In collaboration with Dr. Rajendra Tangirala at UCLA, the effect of Vitamin D treatment on various antibody titers from *Ldlr-/-* mice given a high fat diet and angiotensin II was evaluated. Angiotensis II is a proinflammatory agent and accelerates the development of atherosclerosis (43). It was hypothesized that vitamin D treatment would inhibit the normal B cell response to the atherogen-

ic treatment and that antigen specific antibody titers would be inhibited in a dose dependent manner.

Results

Intrinsic Effect of CD74 Deficiency upon B-1 cell Proliferation and Antibody Production.

Cd74-/-, Ldlr-/- mice on an atherogenic diet have increased numbers of B-1 and Marginal zone B cells as well increased titers of IgM and IgG3 antibodies against MDA-LDL compared to *Ldlr-/-* control mice (9). To test whether this effect is potentially intrinsic to B cells, B-1 cells were isolated by FACS from C57BL/6J wild-type mice or *Cd74-/-* knock-out mice and stimulated with the Toll-like Receptor 4 ligand Kdo2LipidA (KLA) or vehicle control for 3 days *in vitro* to induce proliferation and antibody secretion (Fig. 5-1A). KLA induced increased antibody production from both WT and *Cd74 -/-* B-1 cells (Fig. 5-1B). However, the titers for total IgM, IgM against MDA-LDL, copper-oxidized LDL (CuOx-LDL), native LDL, and the prototypic natural antibody epitope α1,3-dextran. Titers for the IgM natural antibody EO6 were only detectable in culture supernatants from *Cd74-/-* B-1 cells stimulated with KLA. Additionally, total IgG3 titers were greatly increased in stimulated *Cd74-/-* B-1 cell cultures.

The proliferative capacity of stimulated *Cd74-/-* B-1 cells was compared to that of WT B-1 cells by staining the cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) before stimulation and 3-day culture. CFSE passively diffuses into cells and becomes fluorescent upon acetate cleavage by intracellular esterases. When cells divide, the quantity of CFSE is divided between daughter cells and CFSE fluorescent intensity decreases. In the cell culture conditions used, only KLA stimulated *Cd74-/-* B-1 cell proliferated to a

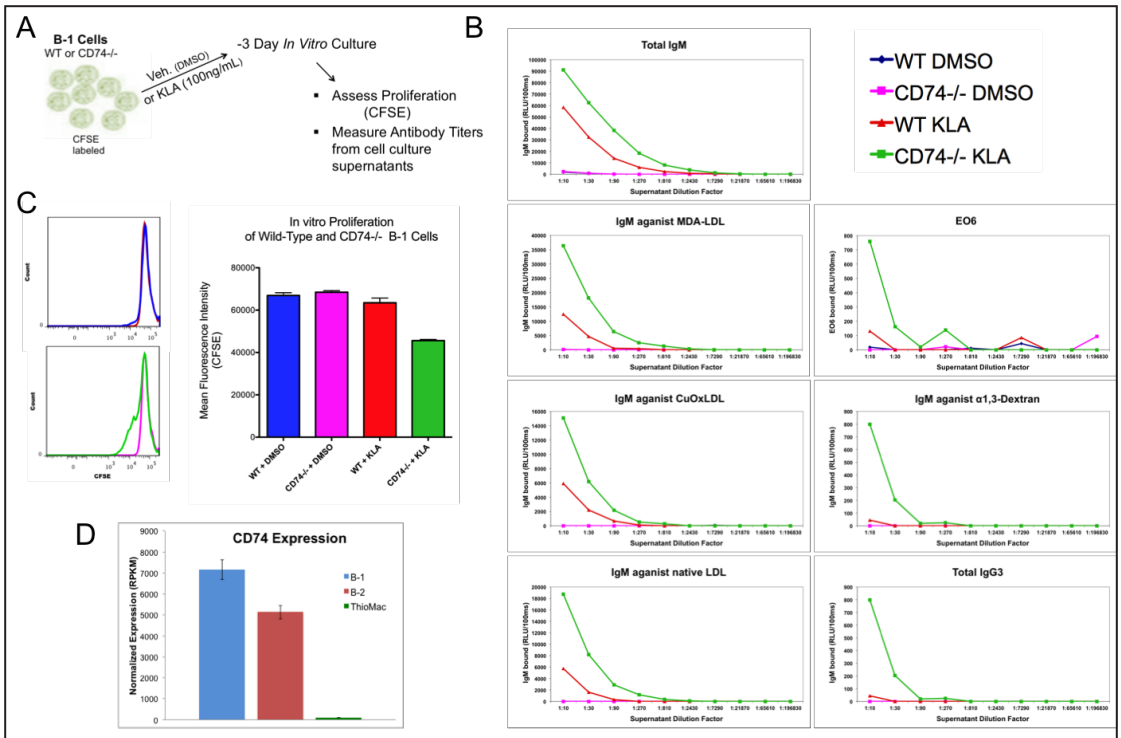


Fig. 5-1 In vitro analysis of effect of invariant chain deficiency upon B-1 cell proliferation and antibody production. A. Experimental design for analyzing B-1 cell intrinsic effects of CD74 deficiency. B-1 cells from WT or CD74-/- mice were isolated by FACS, stained with carboxyfluorescein succinimidyl ester (CFSE) and cultured for 3-days with the TLR-4 ligand Kdo2-LipidA (KLA). Cells were harvested and assessed for proliferation using flow cytometry and antibody titers were measured from cell culture supernatants using Enzyme Linked Immunosorbent Assay (ELISA). **B.** ELISA analysis of antibody titers from B-1 cell culture supernatants from WT and CD74-/- mice. MDA-LDL malondialdehyde modified- low density lipoprotein. CuOx-LDL copper oxidized LDL. EO6 is a natural antibody with specificity for phosphocholine. α1,3 dextran is a known natural antibody antigen. **C.** Flow cytometry analysis of CFSE fluorescent intensity of B-1 cell cultures from KLA stimulated WT and CD74-/- mice. **D.** RNA-seq analysis of CD74 expression levels in B-1 and B-2 cells and thioglycollate elicited macrophages (ThioMac). Values are means of normalized mRNA expression values (reads per kilobase of exon per million sequencing reads, RPKM) and standard deviation of expression levels from various CD74 splice variants.

detectable degree (Fig. 5-1C). Of interest, in RNA-seq experiments described in Chapter 4 it was observed that CD74 was the most highly expressed gene in B-1 cells (as measured by sequencing reads per kilobase of exon per million sequencing reads, RPKM). B-1 cells also expressed significantly higher levels CD74 mRNA than B-2 cells, and B cells in general had much higher levels (> 60-fold) of CD74 mRNA than thioglycollate elicited macrophages (Fig.

5-1D).

Effect of Bone-Marrow BCL-6 Deficiency on Antibody Titers of Atherosclerotic Mice.

BCL-6 deficiency from the bone marrow was shown to increase atherosclerosis but what role B cells and antibodies may have played in this effect is

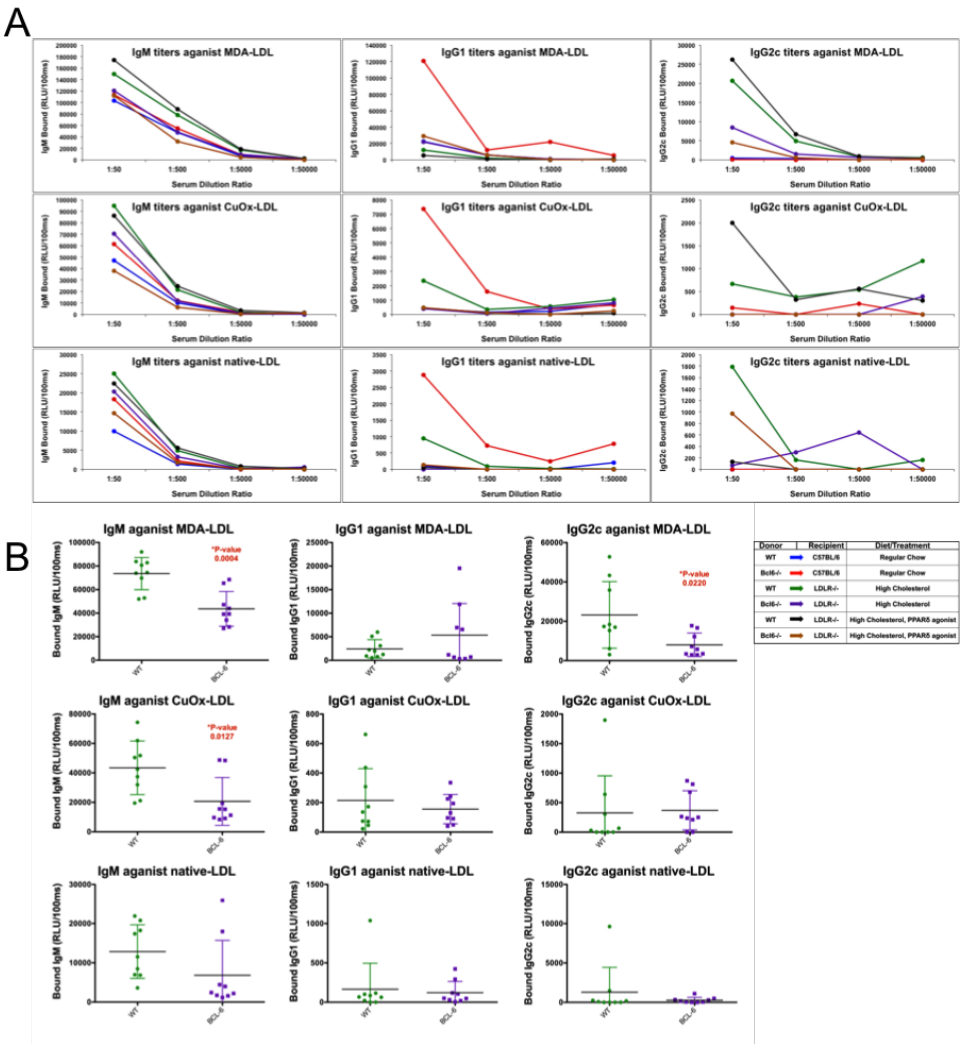


Fig. 5-2 Effect of Bcl-6 deficiency and high cholesterol diet on circulating antibody titers. A. Specific antibody titers against the antigens indicated from mouse serum. Samples from multiple mice (8-10 per group) were pooled. **B.** Specific mouse serum antibody titers against the antigens indicated from 18 individual mice, 9 LDLR-/- mice that received bone marrow transplant from C57BL/6 WT mice and 9 LDLR-/- mice that received BCL6-/- bone marrow, all mice were on a high cholesterol diet. Vertical scatterplots represent the titer for each individual mouse as well as the mean and SD. * P-value <0.05 from Student's t test.

unknown (16). To determine what effect if any bone marrow BCL-6 deficiency had on antibody titers from wild-type or atherogenic mice, plasma samples from cohorts (8-10 per group) of mice were pooled and assessed (Fig. 5-2A). IgM, IgG1, and IgG2c titers to MDA-LDL, CuOx-LDL, and native LDL were measured. Additionally, plasma samples from *Bcl-6-/-* bone marrow mice that received a PPARδ agonist were included. PPARδ is able to exert anti-inflammatory effects that were hypothesized to counteract the proinflammatory loss of BCL6-/-. IgM and IgG2c titers to MDA-LDL and CuOx-LDL were lower in BCL-6-/- recipients on a high cholesterol diet. There didn't appear to be differences for the mice on the normal chow diet or for mice that received the PPARδ agonist. IgG1 titers were the same for all cohorts of mice except for C57BL/6 mice that received BCL6-/- bone marrow. These mice had greatly elevated IgG1 titers to MDA- , CuOx-, and native-LDL.

Because differences in antibody titers were observed in the cohorts of mice, we decided to analyze the same antibody titers from individual atherosclerotic *Ldlr-/-* mice that received either WT or *Bcl6-/-* bone marrow (Fig. 5-2B). Unlike the pooled cohorts of mice, these individual mice had no differences in IgG1 titers. The *Bcl6-/-* recipients did however have significantly lower IgM titers to MDA-LDL (p-value 0.0004) and CuOx-LDL (p-value 0.0127). As well as lower IgG2c titers to MDA-LDL (p-value 0.0220). Titers to native-LDL were the same for all isotypes between groups. There was great variability in the IgG1 titers observed.

Effect of Mycophenolate Mofetil Treatment on Plasma Antibody Titers.

MMF treatment of ApoE mice on high fat diet decreased atherosclerosis (26). As MMF is thought to act by inhibiting proliferation of B and T cells spe-

cifically the total IgM, IgG1, and IgG2c antibody levels were evaluated in these mice (Fig. 5-3A). There was not a significant change in total IgM antibody levels, but MMF treatment tended to decrease IgM levels. IgG2c was also not significantly affected by MMF treatment. However, IgG1 levels were slightly but significantly decreased (p-value < 0.05) in mice treated with MMF. Titers of IgG1 and IgG2c to MDA-, CuOx-, or native-LDL were unaffected by MMF treatment (Fig. 5-3 B-D). Interestingly, specific IgM titers to MDA-LDL (p-value 0.0071) and CuOx-LDL (p-value 0.016) but not native-LDL (p-value 0.0825) were significantly decreased by MMF administration (Fig. 5-3E). These data

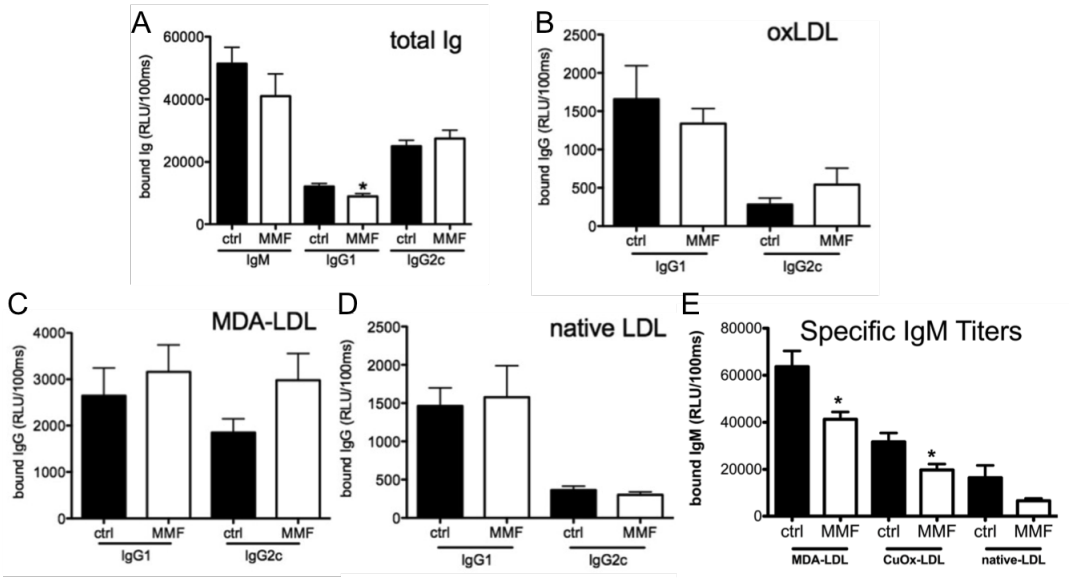


Fig. 5-3 Plasma Immunoglobulin Levels in Control and MMF-treated ApoE-/- Mice. Plasma immunoglobulin (Ig) levels after 12 weeks of high-fat diet (starting age 7 weeks) were assessed by ELISA. **A.** Total IgM and IgG2c were unchanged; IgG1 slightly decreased in MMF-treated mice**. B-D.** No significant changes were observed in IgG1 or IgG2c against either MDA-, CuOx-, or native-LDL. **E.** IgM titers to MDA- and CuOx-LDL but not native-LDL were significantly lower in MMF-treated mice. n=10 per group from 3 independent experiments. P-value <0.05 by Student's t test.

suggest that disease specific B-1 cells, as well as Th2-biased disease specific B-2 cell proliferation was decreased.

FcRγIIB Receptor Deficiency Increases Various Antibody Measures in a Model of Atherosclerosis.

Plasma was taken from 10-, 17-, or 34-week old *FcRγIIB-/-, Apoe-/-* double knockout mice on a normal chow diet and assessed for IgM, IgG1, and IgG2c titers against MDA-LDL or CuOx-LDL (Fig. 5-4 A-F). At 34-weeks IgM titers to MDA- and CuOx-LDL were significantly elevated, but also showed a tendency toward elevation in the 17-week but not the 10-week old mice (Fig. 5-4 A,D). IgG1 titers to the same antigens were also dramatically and significantly higher in *FcRγIIB-/-* mice at 17- and 34-weeks of age as well as at 10-weeks of age in the case of MDA-LDL (Fig. 5-4 B,E). The difference in

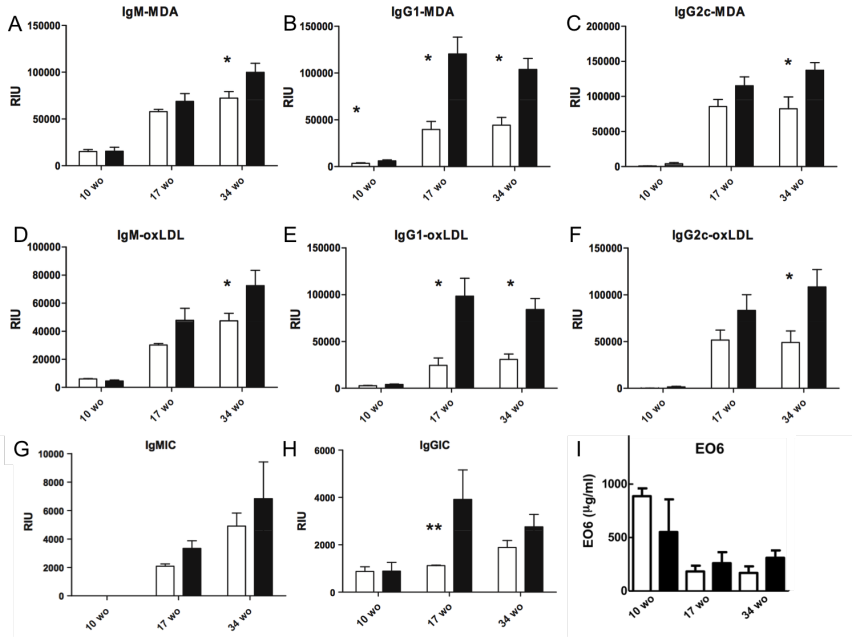


Fig. 5-4 Increased B cell Response to modified LDL in apoE/FcγRIIb-/- mice. A-F. Serum was analyzed for the presence of IgM, IgG1, and IgG2c binding to MDA-LDL, CuOx-LDL by ELISA. **G-H** IgM immune complexes (IgMIC) and IgG immunce complexes (IgGIC). n= at least three mice per group. *P-value <0.05 using Student's t test. **p<0.05 using a Mann Whitney analysis.

IgG2c titers between *FcR*γ*IIB-/-* mice and controls was less dramatic but still significant at 34-weeks of age for both antigens (Fig. 5-4 C,F).

There were not significant differences in the levels of IgM immune complexes between animal cohorts but the *FcR*γ*IIB-/-* mice tended to have higher but more variable levels (Fig. 5-4G). 17-week old *FcR*γ*IIB-/-* mice had substantially and statistically significant elevation of IgG immune complexes. Finally, in unpublished data, it was observed that levels of EO6 were unaffected by *FcR*γ*IIB* deficiency but were markedly elevated in both animal groups at 10-weeks of age.

Vitamin D Treatment of Atherosclerotic Mice Did Not Affect Total or Specific Antibody Levels.

A synthetic Vitamin D analog (paricalcitol), which potently activates the vitamin D receptor, was given in two doses to *Ldlr-/-* mice on an atherogenic regimen of high fat diet and angiotensin II. Paricalcitol was administered daily through intraperitoneal injection in doses of either 0.033 or 0.1 μg/kg body weight for either two or four weeks. These doses were verified to affect expression of known VDR target genes as well as atherogenic genes (data not shown). Total IgM, IgG1, and IgG2c plasma levels were measured and compared between treatment groups and to a cohort of age-matched baseline *Ldlr-/-* mice that received a normal chow diet without any treatment. In total there were eight treatment groups with 5-10 mice per group for a total of 55 mice. Plasma IgM, IgG1, and IgG2c titers to the atherosclerosis antigens of MDA-LDL and CuOx-LDL as well as native LDL were also measured.

Using one-way analysis of variance (ANOVA) to compare between all 8 treatment groups for the12 antibody titers assessed, there were very few

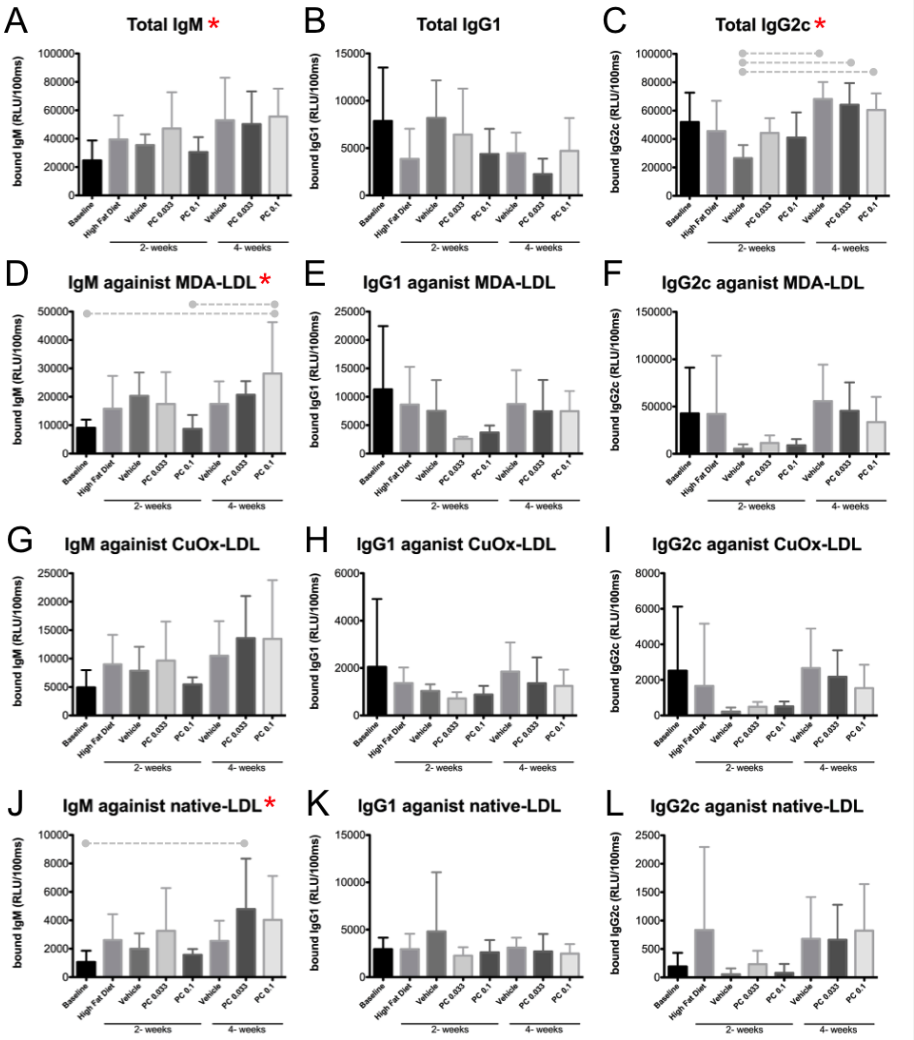


Fig. 5-5 Plasma Immunoglobulin Levels in Vitamin D or Control Treated Mice. Plasma immunoglobulin levels were measured by ELISA from the following cohorts of mice: Baseline- 14 week-old LDLR-/- mice on chow diet. High Fat diet- LDLR-/- mice on a high fat western diet for 2-weeks. Vehicle- daily IP injections of vehicle for 2- or 4- weeks on HFD with angiotensin II, PC 0.033 or 0.1- daily IP injections of 0.033 or 0.1 μg/kg body weight paricalcitol for 2- or 4-weeks on HFD with angiotensin II, **A-C.** Total IgM, IgG1, or IgG2c plasma titers. **D-F.** MDA-LDL specific IgM, IgG1, or IgG2c plasma titers. **G-I.** CuOx-LDL specific IgM, IgG1, or IgG2c plasma titers. **J-L.** native-LDL specific IgM, IgG1, or IgG2c plasma titers. Values are means with standard deviation. *p-value <0.05 using one-way ANOVA. Gray dashed lines represent pair-wise p-value < 0.05 using post-hoc Tukey multiple comparison test.

measurements that had significance differences among the groups (Fig. 5-5). Those that had statistically significant (95% confidence) differences between groups were: Total IgM (p-value 0.03), IgM against MDA-LDL (p-value 0.0069) and native-LDL (p-vaule 0.0234) as well as total IgG2c (p-value 0.0004). In

post-hoc analysis comparing individual groups using Tukey's multiple comparison test there were however very few statistically significant pairwise comparisons (marked with dashed horizontal lines in Fig. 5-5 C-D,J). Those that were statistically different were most likely the result of the High Fat diet, e.g. IgG2c reflective of enhanced Th1 biased responses known to occur in this setting. Whether these had any biological relevant effects are not clear however. It is likely that the time frame of the intervention was too short to evaluate the impact on the humoral responses. In summary, under these conditions, vitamin D treatment did not consistently affect the levels of total or specific antibodies circulating in the plasma of atherosclerotic *Ldlr-/-* mice.

Discussion

The surprising findings from the collaborative study with the Guo-Ping Shi lab that B-1 cell numbers and IgM antibodies levels were increased in CD74-/- knockout mice was mirrored *in vitro* by culturing and stimulating B-1 cells (9). This strongly suggests that CD74 plays an intrinsic role in B-1 cells to potentially inhibit their activation, proliferation, and/or antibody secretion. This is very interesting, as the invariant chain has been reported to induce B cell maturation and differentiation (44,45). Also, very recent studies have further shown that proper proteolytic processing is important for conventional B cell development and survival (46-48). Therefore, one would've predicted its loss in B-1 cells would've decreased their numbers similar to what has been observed in conventional B cells.

The dramatic increases in antibody titers observed for *in vitro* cultured B-1 cells (Fig. 5-1B) appear to not solely be due to increased proliferation of these cells. CD74-/- B-1 cells when stimulated proliferated more than WT con-

trols, but only to a moderate extent. The magnitude of increased antibody levels appears to be larger than the increased proliferation of B-1 cells, although, the sensitivity of the CFSE proliferation assay may have been too low to properly assess the extent of proliferation. It appears that only a small proportion of only the CD74-/- B-1 cells proliferated. In other experiments, WT B-1 cells stimulated with KLA normally proliferate to a moderate extent, but their proliferation was essentially undetectable in this assay. Therefore, the extent of CD74-/- B-1 cell proliferation may in fact be much greater than reported in Figure 5-1. It would be interesting to perform an ELISPOT assay to assess if antibody production per CD74-/- B-1 cell is comparable to WT B-1 cells. The data presented in Figure 5-1 seems to contradict the results presented in the literature for the role of invariant chain in B cells, but this has only been studied in B-2 cells to our knowledge. It is unknown what could account for these diametrically opposite effects, but this again highlights the remarkable unique regulation of B-1 versus B-2 cells.

It is also of interest that B-1 cells express CD74 so abundantly (RPKM of ~7000) and to a significantly higher level than B-2 cells. What role this unique expression may play is unknown. However, CD74 has been shown to not only be a MHC-II chaperone but also a cell-surface survival receptor in B cells (49). This study utilized splenic B cells, which are predominately B-2 cells. Perhaps, CD74 only functions as a survival factor for B-2 cells and its loss therefore uniquely affects B-2 cells. Unfortunately cell survival was not assessed in the studies performed. It is also surprising to note the much greater expression levels of CD74 in B cells than in peritoneal macrophages (Fig. 5-1D). This finding is corroborated by the data available from the Immunological Genome Project, B-1 cells, Marginal zone B cells, and immature B-2 cells

have high CD74 expression whereas some but not all thioglycollate elicited macrophage populations had low expression (38). This may account for the report that B-1 cells and not macrophages are the major antigen presenting cell from the peritoneal cavity (50).

BCL-6-/- deficiency from bone marrow leads to wide spread inflammation and increases atherosclerosis (16). As BCL-6 acts as a 'brake' on inflammatory signaling, it would be predicted that loss of BCL-6 from bone marrow cells would increase many markers of inflammation, including antibody titers. This is not the case however; in fact the opposite seems to occur. Specific antibody titers of IgM and IgG2c to atherosclerotic antigens (MDA-LDL and CuOx-LDL) tended to be lower in BCL-6-/- recipients on a high cholesterol diet compared to WT recipients. High cholesterol diet induces an inflammatory state and is known to increase IgM titers to MDA-LDL and CuOx-LDL as was observed in Figure 5-2A. This increase was lost for BCL-6-/- recipients and was not inhibited by treatment with a PPARδ agonist. Though PPARδ is expressed in both B-1 and B-2 cells its effect upon these cells is unknown (34). Dr. Barish concluded that the increased atherosclerosis in BCL-6-/- bone marrow recipient mice was a result of unrestrained inflammation (16). However, the lower titers of IgM antibodies to MDA-LDL and CuOx-LDL, which have been shown to be atheroprotective, could also be mechanistically involved in this effect.

The high titers of IgG1 antibodies observed in the pooled plasma of the cohort of WT mice that received BCL-6-/- bone marrow is expected as BCL-6-/- has been observed to increase the levels of T helper type 2 (Th2) cytokines which are known to promote IgG1 production (Fig. 5-2A) (51). This effect was however not significantly recapitulated in the analysis of individual

mice (Fig. 5-2B). The individual mice were observed in some cases to have high variability in their IgG1 titers. This may account for the high titers observed in the pooled cohorts as several mice may have had especially high IgG1 titers that skewed the results. The fact that the IgG1 response was not dependably observed is consistent with the atherogenic phenotype observed in BCL-6-/- mice, as this is an indicator of a Th2 response, which has been found to be atheroprotective in some settings (52).

Immunosuppression using MMF had surprisingly little effect upon total or specific antibody titers from ApoE mice on a high fat diet. As MMF is a purine antagonist that selectively affects proliferating B and T cells this implies that antibody levels and therefore B cells were relatively unaffected by MMF because these cells were not proliferating. IgG1 titers were however slightly decreased. As discussed earlier, IgG1 antibody titers are a product of Th2 T cell help. This decrease in IgG1 may be due to less stimulation from Th2 cells. However, the levels of the Th2 cytokine IL-4 were below the detection limit and other Th2 cytokines such as IL-5 or IL-13 were not measured (26). Interestingly, the plasma levels of the Th17 cytokine IL-17a were decreased by MMF and was shown to play an important role in the atheroprotective function of MMF (26).

Unlike IgG2c titers, IgM titers were moderately inhibited by MMF treatment. Whereas total IgM levels tended to be decreased by MMF treatment, the titers specific to the disease specific antigens of MDA-LDL and CuOx-LDL were significantly decreased. This strongly indicates that disease-specific antigen specific B cells were suppressed by MMF. As B-1 cells are known to produce much but probably not all of the IgM specific to these antigens, it would be interesting to measure numbers and the in vivo proliferative state

of the B-1 cells in the MMF mice. Titers of IgM against MDA-LDL and CuOx-LDL are thought to be atheroprotective (53). The fact that they are lowered by MMF treatment is one potential detrimental effect of MMF treatment with regard to vascular disease. However, atherosclerosis was still decreased by MMF treatment despite the lowering of potentially beneficial antibody titers through other mechanisms involving inhibition of T cells and IL-17 mediated macrophage accumulation in artery walls (26).

ApoE/FcRγIIB double knockout mice had accelerated atherosclerosis despite not having significant differences in lipid levels as compared to age-matched *Apoe-/-* control mice. This implies that functional differences in the immune system due to FcRγIIB ablation were potentially responsible for the enhanced disease phenotype. As B cell activation can be modified by FcRγIIB it was decided that antibody titers for disease relevant antigens would be measured (54). The dramatic increases in the IgG1 isotype are indicative of a skewed Th2 adaptive immune response in the *Apoe/FcR*γ*IIB-/-* mice. Similarly, the enhanced levels of IgG immune complexes to modified LDL indicate a Th2 response. These changes in antibody titer were observed in the absence of any specific change in the proportion of splenic B cells or T cells (data not shown). These results suggest that FcRγIIB is modulating the response of B cells in these mice to at least the disease specific antigens measured. As FcRγIIB has an important role in inhibiting B cell activation, proliferation, and survival the inhibition of these responses were potentially lost in this study. Therefore, more antigen specific B cells would be activated and induced to proliferate and form memory and plasma cells. Follow up studies could be carried out to test these hypotheses by directly measuring the activation status, proliferation, and differentiation of B cells. The moderate changes in IgM

titers in the *Apoe/FcRγIIB-/-* mice may be due to changes in B-1 cells. B-1 and B-2 cells express comparable mRNA levels of FcRγIIB (as assessed by RNA-seq, data not shown). Therefore, it is conceivable that innate like B-1 cells are also dramatically impacted by the loss of FcRγIIB. However, levels of the atheroprotective IgM Natural antibody EO6 were not affected by loss of FcRγIIB. Additional studies could address this question by assessing the numbers of B-1 cells and whether they have an enhanced activation status in FcRγIIB null mice.

It was surprising to observe that treatment of atherosclerotic mice with a vitamin D analog had very little effect upon total or disease specific antibody levels. Interestingly, antibody titers to disease-specific antigens (MDA-LDL or CuOx-LDL) were not changed by the high fat diet or angiotensin II treatment, but the short duration of the interventions may be insufficient to induce such changes. These data imply that B-cells were not activated *in vivo* in this short term study, which may account for the absence of an effect due to vitamin D treatment. Notably, B cells taken from patients with SLE and treated *in vi-tro* with vitamin D had decreased proliferation and when activated had more apoptosis (40). If this study were to be repeated, splenic B cells should be isolated and analyzed for markers of activation or for markers of plasma cell differentiation to assess the degree to which the atherogenic regimen induced an immune response. Additionally, perhaps an effect of vitamin D treatment would be observable if an atherosclerosis regimen was used that more potent-ly activated B cells. Specifically, the treatment protocol used in this study was very short, a more long term model should be employed to adequately assess if vitamin D affects B cells and antibody titers in atherosclerosis.

Acknowledgements

Chapter 5, in part, is a reprint of results to which the dissertation author contributed in the following publications: S. von Vietinghoff, E. K. Koltsova, J. Mestas, C. J. Diehl, J. L. Witztum, K. Ley, Mycophenolate mofetil decreases atherosclerotic lesion size by depression of aortic T-lymphocyte and interleukin-17-mediated macrophage accumulation. *J Am Coll Cardiol* 57, 2194-2204 (2011). Y. V. Mendez-Fernandez, B. G. Stevenson, C. J. Diehl, N. A. Braun, N. S. Wade, R. Covarrubias, S. van Leuven, J. L. Witztum, A. S. Major, The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. *Atherosclerosis* 214, 73-80 (2011) The dissertation author was a co-author on these papers.

References

1. Tsiantoulas D, Diehl C, Witztum J, Binder CJ. B Cells and Humoral Immunity in Atherosclerosis. Circulation Research. 2014;in press.

2. Pieters J. MHC class II-restricted antigen processing and presentation. Adv Immunol. 2000;75:159-208.

3. Bikoff EK, Huang LY, Episkopou V, van Meerwijk J, Germain RN, Robertson EJ. Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and CD4+ T cell selection in mice lacking invariant chain expression. J Exp Med. 1993;177(6):1699-1712.

4. Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, Chapman HA. Cathepsin S required for normal MHC class II peptide loading and germinal center development. Immunity. 1999;10(2):197-206.

5. Kenty G, Bikoff EK. BALB/c invariant chain mutant mice display relatively efficient maturation of CD4+ T cells in the periphery and secondary proliferative responses elicited upon peptide challenge. J Immunol.

1999;163(1):232-241.

6. Viville S, Neefjes J, Lotteau V, Dierich A, Lemeur M, Ploegh H, Benoist C, Mathis D. Mice lacking the MHC class II-associated invariant chain. Cell. 1993;72(4):635-648.

7. Maehr R, Kraus M, Ploegh HL. Mice deficient in invariant-chain and MHC class II exhibit a normal mature B2 cell compartment. Eur J Immunol. 2004;34(8):2230-2236.

8. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011;12(3):204-212.

9. Sun J, Hartvigsen K, Chou MY, Zhang Y, Sukhova GK, Zhang J, Lopez-Ilasaca M, Diehl CJ, Yakov N, Harats D, George J, Witztum JL, Libby P, Ploegh H, Shi GP. Deficiency of Antigen-Presenting Cell Invariant Chain Reduces Atherosclerosis in Mice. Circulation. 2010;122(8):808-820.

10. Basso K, Dalla-Favera R. BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. Adv Immunol. 2010;105:193-210.

11. Barish GD, Yu RT, Karunasiri M, Ocampo CB, Dixon J, Benner C, Dent AL, Tangirala RK, Evans RM. Bcl-6 and NF-kappaB cistromes mediate opposing regulation of the innate immune response. Genes Dev. 2010;24(24):2760-2765.

12. Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, Martin-Orozco N, Wang Y, Chang SH, Esplugues E, Flavell RA, Tian Q, Dong C. Bcl6 expression specifies the T follicular helper cell program in vivo. J Exp Med. 2012;209(10):1841-1852, S1841-1824.

13. Basso K, Dalla-Favera R. Roles of BCL6 in normal and transformed germinal center B cells. Immunol Rev. 2012;247(1):172-183.

14. Tumang JR, Frances R, Yeo SG, Rothstein TL. Spontaneously Ig-secreting B-1 cells violate the accepted paradigm for expression of differentiation-associated transcription factors. J Immunol. 2005;174(6):3173-3177.

15. Barish G, Yu R, Karunasiri M, Ocampo C, Dixon J, Benner C, Dent A, Tangirala R, Evans R. Bcl-6 and NF- B cistromes mediate opposing regulation of the innate immune response. Genes & Development. 2010;24(24):2760.

16. Barish GD, Yu RT, Karunasiri MS, Becerra D, Kim J, Tseng TW, Tai LJ, Leblanc M, Diehl C, Cerchietti L, Miller YI, Witztum JL, Melnick AM, Dent AL, Tangirala RK, Evans RM. The Bcl6-SMRT/NCoR cistrome represses inflammation to attenuate atherosclerosis. Cell Metab. 2012;15(4):554-562.

17. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol. 2008;8(10):802-815.

18. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res. 2002;91(4):281-291.

19. Miller LW. Cardiovascular toxicities of immunosuppressive agents. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2002;2(9):807-818.

20. Roselaar S, Schonfeld G, Daugherty A. Enhanced development of atherosclerosis in cholesterol-fed rabbits by suppression of cell-mediated immunity. Journal of Clinical Investigation. 1995;96(3):1389.

21. Keul P, Tolle M, Lucke S, von Wnuck Lipinski K, Heusch G, Schuchardt M, van der Giet M, Levkau B. The sphingosine-1-phosphate analogue FTY720 reduces atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2007;27(3):607-613.

22. Nofer JR, Bot M, Brodde M, Taylor PJ, Salm P, Brinkmann V, van Berkel T, Assmann G, Biessen EA. FTY720, a synthetic sphingosine 1 phosphate analogue, inhibits development of atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation. 2007;115(4):501-508.

23. Klingenberg R, Nofer JR, Rudling M, Bea F, Blessing E, Preusch M, Grone HJ, Katus HA, Hansson GK, Dengler TJ. Sphingosine-1-phosphate analogue FTY720 causes lymphocyte redistribution and hyper-

cholesterolemia in ApoE-deficient mice. Arterioscler Thromb Vasc Biol. 2007;27(11):2392-2399.

24. Hedstrom L, Gan L. IMP dehydrogenase: structural schizophrenia and an unusual base. Current opinion in chemical biology. 2006;10(5):520-525.

25. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. 2000;47(2-3):85-118.

26. von Vietinghoff S, Koltsova EK, Mestas J, Diehl CJ, Witztum JL, Ley K. Mycophenolate mofetil decreases atherosclerotic lesion size by depression of aortic T-lymphocyte and interleukin-17-mediated macrophage accumulation. J Am Coll Cardiol. 2011;57(21):2194-2204.

27. Nimmerjahn F, Ravetch JV. Fc-receptors as regulators of immunity. Adv Immunol. 2007;96:179-204.

28. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity. 2005;23(1):41-51.

29. Hernandez-Vargas P, Ortiz-Munoz G, Lopez-Franco O, Suzuki Y, Gallego-Delgado J, Sanjuan G, Lazaro A, Lopez-Parra V, Ortega L, Egido J, Gomez-Guerrero C. Fcgamma receptor deficiency confers protection against atherosclerosis in apolipoprotein E knockout mice. Circ Res. 2006;99(11):1188-1196.

30. Kelly JA, Griffin ME, Fava RA, Wood SG, Bessette KA, Miller ER, Huber SA, Binder CJ, Witztum JL, Morganelli PM. Inhibition of arterial lesion progression in CD16-deficient mice: evidence for altered immunity and the role of IL-10. Cardiovasc Res. 2010;85(1):224-231.

31. Smith KG, Clatworthy MR. FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. Nat Rev Immunol. 2010;10(5):328-343.

32. Zhao M, Wigren M, Duner P, Kolbus D, Olofsson KE, Bjorkbacka H, Nilsson J, Fredrikson GN. FcgammaRIIB inhibits the development of atherosclerosis in low-density lipoprotein receptor-deficient mice. J Im-

munol. 2010;184(5):2253-2260.

33. Mendez-Fernandez YV, Stevenson BG, Diehl CJ, Braun NA, Wade NS, Covarrubias R, van Leuven S, Witztum JL, Major AS. The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. Atherosclerosis. 2011;214(1):73-80.

34. Diehl CJ, Barish GD, Downes M, Chou MY, Heinz S, Glass CK, Evans RM, Witztum JL. Research Resource: Comparative Nuclear Receptor Atlas: Basal and Activated Peritoneal B-1 and B-2 Cells. Mol Endocrinol. 2011.

35. Aranow C. Vitamin D and the immune system. Journal of investigative medicine : the official publication of the American Federation for Clinical Research. 2011;59(6):881-886.

36. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, Kamen DL, Wagner M, Bals R, Steinmeyer A, Zugel U, Gallo RL, Eisenberg D, Hewison M, Hollis BW, Adams JS, Bloom BR, Modlin RL. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science. 2006;311(5768):1770-1773.

37. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JW, Mader S, White JH. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol. 2004;173(5):2909-2912.

38. Heng TS, Painter MW, Immunological Genome Project C. The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol. 2008;9(10):1091-1094.

39. Adorini L. Intervention in autoimmunity: the potential of vitamin D receptor agonists. Cell Immunol. 2005;233(2):115-124.

40. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J Immunol. 2007;179(3):1634-1647.

41. Lemire JM, Adams JS, Sakai R, Jordan SC. 1 alpha,25-dihydroxyvitamin D3 suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells. J Clin Invest. 1984;74(2):657-661.

42. Knippenberg S, Smolders J, Thewissen M, Peelen E, Tervaert JW, Hupperts R, Damoiseaux J. Effect of vitamin D(3) supplementation on peripheral B cell differentiation and isotype switching in patients with multiple sclerosis. Multiple sclerosis. 2011;17(12):1418-1423.

43. Weiss D, Sorescu D, Taylor WR. Angiotensin II and atherosclerosis. Am J Cardiol. 2001;87(8A):25C-32C.

44. Matza D, Kerem A, Medvedovsky H, Lantner F, Shachar I. Invariant chain-induced B cell differentiation requires intramembrane proteolytic release of the cytosolic domain. Immunity. 2002;17(5):549-560.

45. Matza D, Lantner F, Bogoch Y, Flaishon L, Hershkoviz R, Shachar I. Invariant chain induces B cell maturation in a process that is independent of its chaperonic activity. Proc Natl Acad Sci U S A. 2002;99(5):3018-3023.

46. Bergmann H, Yabas M, Short A, Miosge L, Barthel N, Teh CE, Roots CM, Bull KR, Jeelall Y, Horikawa K, Whittle B, Balakishnan B, Sjollema G, Bertram EM, Mackay F, Rimmer AJ, Cornall RJ, Field MA, Andrews TD, Goodnow CC, Enders A. B cell survival, surface BCR and BAFFR expression, CD74 metabolism, and CD8- dendritic cells require the intramembrane endopeptidase SPPL2A. J Exp Med. 2013;210(1):31-40.

47. Schneppenheim J, Dressel R, Huttl S, Lullmann-Rauch R, Engelke M, Dittmann K, Wienands J, Eskelinen EL, Hermans-Borgmeyer I, Fluhrer R, Saftig P, Schroder B. The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. J Exp Med. 2013;210(1):41-58.

48. Beisner DR, Langerak P, Parker AE, Dahlberg C, Otero FJ, Sutton SE, Poirot L, Barnes W, Young MA, Niessen S, Wiltshire T, Bodendorf U, Martoglio B, Cravatt B, Cooke MP. The intramembrane protease Sppl2a is required for B cell and DC development and survival via cleavage of the invariant chain. J Exp Med. 2013;210(1):23-30.

49. Starlets D, Gore Y, Binsky I, Haran M, Harpaz N, Shvidel L, Becker-Herman S, Berrebi A, Shachar I. Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. Blood. 2006;107(12):4807-4816.

50. Bamba H, Ishigaki H, Ishida H, Kajino K, Fujiyama Y, Ogasawara K. B1-B cells are the main antigen presenting cells in CpG-ODN-stimulated peritoneal exudate cells. Microbiology and immunology. 2005;49(1):89-95.

51. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science. 1997;276(5312):589-592.

52. Mallat Z, Taleb S, Ait-Oufella H, Tedgui A. The role of adaptive T cell immunity in atherosclerosis. J Lipid Res. 2009;50 Suppl:S364-369.

53. Ravandi A, Boekholdt SM, Mallat Z, Talmud PJ, Kastelein JJ, Wareham NJ, Miller ER, Benessiano J, Tedgui A, Witztum JL, Khaw KT, Tsimikas S. Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. J Lipid Res. 2011;52(10):1829-1836.

54. Ravetch JV, Bolland S. IgG Fc receptors. Annu Rev Immunol. 2001;19:275-290.

CHAPTER 6

Conclusions and Future Directions

There is general agreement that B-1 cells, through the production of IgM natural antibodies (NAbs) are atheroprotective, whereas the role of adaptive immune B-2 cells in atherosclerosis is controversial. Studies have shown that they can either promote, have no effect on, or inhibit atherosclerosis depending on the experimental context (1). B-1 cell derived NAbs bind to oxidation-specific epitopes on oxidized LDL (OxLDL) and at least in vitro can prevent the uptake of OxLDL into macrophages and thereby prevent foam cell formation (2). NAb production occurs even in the absence of apparent exogenous antigenic stimuli and therefore must be regulated differently from the induction of adaptive specific Abs as carried out by B-2 cells during infection (3-5). In fact, many aspects of B-1 and B-2 cell biology are regulated differently, including their development, antigen selection, B cell receptor assembly, cell-surface protein expression, body localization, renewal, and so on (Table 1-1). Most of the studies described in this dissertation center on the question of what controls the unique biology of B-1 versus B-2 cells with the underlying goal of discovering ways to manipulate B-1 cells and safely increase NAb production and thereby potentially ameliorate inflammation and associated atherosclerosis.

The first experiments I undertook were to test the hypothesis that the expression of members of the nuclear receptor (NR) family of transcription factors would be expressed differentially between basal and stimulated B-1 and B-2 cells. Nuclear receptors are a super-family of ligand activated transcription factors that as a group are capable of regulating diverse processes ranging from development to the immune response and of responding to many circulating lipophilic extracellular hormones and intracellular metabolites (6,7). At the time I began my studies, very little was known of NR expression in B-2

cells, and virtually nothing in B-1 cells. Therefore, my first task was to determine the basal and stimulated expression of NRs in both B-1 and B-2 cells (Fig. 2-2). From this profiling of NR expression in B-1 and B-2 cells, I identified candidate NRs with the potential capacity to influence and contribute to the differential biology of B-1 and B-2 cells.

In this study, I ascertained using quantitative PCR the complete repertoire of NR expressed in basal and TLR-4 or TLR-2/1 activated B-1 and B-2 cells, as well as similar comparative profiles of splenic B cells. Utilizing quantitative PCR, I identified 24 nuclear receptors expressed in B-1 cells and report that most of them are dynamically expressed in both unstimulated as well as the TLR stimulated conditions. The addition of Kdo2-Lipid A or Pam_3CysK4 to the cell culture medium often had profound and unique effects on the expression profiles of expressed nuclear receptors. B-2 cells expressed 25 of the 49 mouse nuclear receptors. B-1 and B-2 cells mutually expressed 23 nuclear receptors. B-1 cells alone expressed LXRα while B-2 cells uniquely expressed the androgen receptor and the retinoic acid receptor β. Notably, there was a remarkable quantitative dissimilarity of expression levels between B-1 and B-2 cells for most nuclear receptors. Many of the mutually expressed NRs had higher levels in B-2 cells. The mineralocorticoid receptor (MR) was one exception and had 4-fold higher levels in B-1 cells.

The observation that the MR, which classically responds to circulating aldosterone and regulates salt and water reabsorption in the kidney and colon, is expressed in B cells and that expression levels were considerably higher in B-1 cells prompted great curiosity and further investigation. This was the first report of MR expression in any lymphocyte including B cells. Its role in B cells is completely unknown, however, several recent reports have documented

effects of MR in immune myeloid cells including macrophages, neutrophils, and dendritic cells (8-10). In almost all of these studies MR activation was shown to be pro-inflammatory. Studies were designed to assess whether activation of MR in B-1 cells leads to cellular activation or inhibition. My hypothesis was that pretreatment of B-1 cells with an MR agonist prior to activation with an immune stimulant (such as a TLR ligand) would enhance their NAb response. B-1 cells were purified from wild-type mice and pretreated with MR agonists and/or antagonists before TLR stimulation. These studies failed to find a prominent phenotype for MR activation or antagonism on NAb secretion, as well as on proliferation, differentiation to NAbs secreting cells and/or cell death. I also performed preliminary studies and could not find a phenotype for MR activity on IL-10 production or expression of the B-1 cell migration marker CD9. The role of MR in B-1 cells remains a mystery.

While performing the MR studies detailed above, it became necessary to also evaluate the impact of glucocorticoid receptor (GR) activation or inhibition on B-1 cells. GR is closely related to MR and shares high sequence identity in its ligand and especially DNA binding domains (11). MR and GR can bind to many of the same natural or synthetic ligands, albeit with differing affinities, and both are understood to bind to the same DNA response element. The enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11βHSD2) is highly expressed in cells that are sensitive to the MR ligand aldosterone and acts to metabolize corticosteroids and render them incapable of MR activation (12). 11βHSD2 was expressed at very low levels in B-1 cells; therefore, corticosteroids would not be inactivated in B-1 cells and along with mineralocorticoids would be capable of activating MR. Treatment of B-1 cells with fludrocortisone acetate, a mineralocorticoid that can also bind to the GR, caused a dose-de-

pendent decrease in IgM NAb production and induction of apoptosis. This effect was inhibited by mifepristone, a GR specific antagonist, and not by eplerenone, a MR specific antagonist. Therefore, GR activation had potent inhibitory effects upon B-1 cell NAb production. This was further corroborated by treating B-1 cells with dexamethasone (Dex), which is a potent glucocorticoid with only minimal mineralocorticoid effects (13). Dex potently induced apoptosis and inhibited antibody production *in vitro* from stimulated B-1 cells.

This finding seemed unremarkable at first as Dex and other synthetic GR ligands are frequently given clinically to treat B cell dependent malignancies and autoimmune conditions (14,15). However, a survey of the literature revealed that the mechanisms whereby steroids inhibit B cells have not been extensively studied (16). Additionally, there is no published data regarding the effect of GR activation in different B cell subsets, including B-1 cells. Studies using T cells have shown that the transactivation activity of GR is necessary for steroid induced apoptosis (17). Moreover, an analysis of microarray data from various cell types, including several human B cell lines, undergoing steroid induced apoptosis failed to discover any universal gene expression signatures responsible for GR regulated apoptosis, but that many genes with known direct or indirect roles in apoptosis regulation were induced (18).

I designed studies to compare the transcriptional response of B-1 and B-2 cells to Dex treatment in order to test the hypothesis that genes related to apoptosis would be induced in both cell types. Furthermore, these RNA-seq analyses would be used to compare the transcriptomes of B-1 and B-2 cells in response to Dex treatment as well as in the basal and stimulated states. Many genes with known roles in apoptosis were differentially regulated by Dex in both B-1 and B-2 cells. However, the actual genes regulated were quite

different in each cell type. However, two pro-apoptotic BCL-2 family members, Bim and Bmf were upregulated in both cell types. These proteins are likely to play an integral role is regulating the Dex-induced apoptosis in both cell types.

The transcriptome of B-1 and B-2 cells not only for apoptotic-related genes but for all Dex-regulated genes as well as for genes regulated by a TLR-4 agonist were remarkably different between B-1 and B-2 cells. For example, for cells cultured for 4 hrs with Dex there were 1158 genes differentially regulated in B-1 cells and 2318 gene in B-2 cells of these only 388 were regulated in both cell types. Similar disparity was observed when cells were activated with a TLR-4 agonist or when cells were pretreated with Dex before TLR-4 stimulation. This surprising disparity prompted me to question if the genome-wide binding of GR would follow a similar pattern and bind in many unique locations and account for the unique Dex dependent transcriptome between B-1 and B-2 cells. Chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) was carried out for GR in Dex treated B-1 and B-2 cells. As expected, GR bound in largely unique locations in B-1 and B-2 cells; there were 3867 GR binding peaks in B-1 cells and 2376 in B-2 cells and only 1109 peaks shared between them. A motif analysis was performed and it was found that the sequence for the glucocorticoid response element (GRE) was the most significantly enriched motif in the vicinity of the peaks shared between B-1 and B-2 cells. However, for the peaks unique to either B-1 or B-2 cells, the most statistically significant enriched motif was that of PU.1. PU.1 is a transcription factor that plays an important role in the development of both myeloid and lymphoid cells (19). Collaborators in Chris Glass' lab have shown that PU.1 in collaboration with small sets of either lymphoid-specific or myeloid-specific lineage determining transcription factors (LDTF) establish

cell-specific binding sites that are associated with histone marks for enhancers (20). These enhancer regions then serve as cell-specific landing pads for signal dependent transcription factors such as nuclear receptors.

I hypothesized that B-1 and B-2 cells would each possess significant numbers of enhancer regions that were unique to each cell type. And that these cell-specific enhancer regions would have motif enrichment not only for PU.1 but also for potential B-1 versus B-2 cell LDTFs. This hypothesis was put to the test by performing ChIP-seq for the enhancer histone mark of di-methylation on lysine 4 of histone 3 (H3K4Me2) after non-nucleosomal DNA has been digested by exposure to a micrococcal nuclease. It was found that both B-1 and B-2 cells possessed large numbers of genomic regions enriched for H3K4Me2 that were mostly shared between these cells. Nevertheless there were 16,693 B-1 cell specific H3K4me2 peaks and 12,494 B-2 cell specific H3K4Me2 peaks. A motif analysis showed as expected that PU.1 motifs were enriched in the sequences surrounding both the shared and unique H3K4Me2 peaks. PU.1 motifs were found near 39% and 48% of the B-1 or B-2 cell specific peaks, respectively and near 45% of the shared peaks. Also several enriched motifs were found to be unique to B-1 or B-2 cell-specific H3K4Me2 regions, such as NFAT for B-1 cells and Mef2c for B-2 cells. There is convincing evidence that NFATc1 is important for B-1a cell development and/or survival and therfore it may continue to play a role in maintaining the identity of B-1 cells (21). Myeloid enhancer factor 2 c or Mef2c has also very recently been implicated with a role in B cell development as a B cell specific knockout of Mef2c reduced the numbers of various immature B cell subpopulations (22). However, it was not investigated whether the B-1 versus B-2 cells population sizes or phenotypes were affected. Therefore, both the cell specific enhancer

motif analysis and the literature hint that NFAT and/or Mef2c may respectively be B-1 or B-2 cell lineage-determining transcription factors. Also, RNA-seq data demonstrated that Mef2c is one of the transcription factors most highly expressed in B-2 cells relative to B-1 cells. Finally, it was also demonstrated that cell specific GR binding peaks tended to have cell-specific enrichment for H3K4Me2 in their vicinity. This further demonstrates that cell-specific GR binding is correlated with cell-specific enhancers.

Future Directions

Studies are currently underway to perform ChIP-seq for PU.1 in B-1 and B-2 cells. PU.1 has been shown to work in a collaborative manner to establish cell-specific enhancers with cell-specific lineage determining factors that control the identity of B cells versus macrophages and motifs for PU.1 have been found in the vicinity of cell specific GR binding locations and H3K4Me2 regions of B-1 and B-2 cells (20). It is hypothesized that motifs for B-1 versus B-2 cell lineage determining factors will be enriched in the vicinity of B-1 or B-2 cell specific PU.1 peaks. It is expected that the results of PU.1 ChIP-seq will either further cement NFAT for B-1 cells and Mef2c for B-2 cells as potential LDTFs or identify other possible candidates.

Once strong candidates for B-1 and/or B-2 cell lineage determining transcription factors (LDTFs) are identified studies will be undertaken to test the hypothesis that the factor in question does in fact control all or some of the unique biological properties of B-1 versus B-2 cells. For example, if this putative factor is important in B-1 and/or B-2 cell maintenance and/or development then it should be expressed in adult and/or progenitor cells, respectively. The expression levels of the putative LDFs will evaluated by both qPCR and west-

ern blot in adult and progenitor B-1 and B-2 cells. B-1 cell progenitors reside predominantly within the fetal liver but can also be found in the adult spleen and have the following cell surface phenotype $Lin^{-}CD45R^{lo\text{-}neg}CD19^{+}$ that can be used for isolation by FACS (23,24). The developmental cascade of B-2 cells is much more well defined and progresses within the adult bone marrow from the pre-pro B-2 cell ($CD45R^{+}CD43^{+}AA4.1^{+}CD19^{-}Ly\text{-}6C^{-}$), to the pro-B-2 cell ($CD45R^{+}CD19^{+}CD43^{+}AA4.1^{+}$), and finally to the pre-B-2 cell ($CD45R^{+}CD19^{+}$) stage. All progenitor B-2 cell populations will be isolated by FACS and analyzed for the expression of the transcription factor(s) in question. Additionally, ChIP-seq experiments for the putative cell-type specific LDTFs will be carried out. For example, if the motif for Mef2c is confirmed by PU.1 ChIP-seq to again be enriched near unique B-2 cell binding locations, then ChIP-seq will be performed for Mef2c in both B-1 and B-2 cells. Other investigators have already performed ChIP-seq for Mef2c in a B cell line but not in primary B-1 or B-2 cells (22). It would be expected that Mef2c binding would occur in close association with PU.1 at B-2 cell specific enhancers. Finally, a knock-out model for the putative LDTF for B-1 versus B-2 cells will be utilized to evaluate the importance of the factor(s) in establishment and maintenance of stable B-1 and B-2 populations. Specifically, the impact of the knock-out(s) on the population size of various progenitor B cells will be evaluated using the cell populations and markers described above. We will also extensively explore the impact of the knockout on antibody production. Additionally, our lab has previously employed adoptive transfer experiments, wherein B-1 cells are isolated and injected into the peritoneal cavity of $Rag1^{-/-}$ mice, which are devoid of B or T cells, and through homeostatic proliferation and self-replenishment the donor B-1 cells repopulate the peritoneal cavity and spleen and replenish the

plasma IgM compartment (25). This model will be useful in directly evaluating the impact of transcription factor gene ablation on B-1 cell survival, proliferation, self-replenishment, and spontaneous NAb secretion.

Additionally, in the course of the studies performed in this dissertation various ideas for experiments have arisen that have not yet been performed but would serve to increase the understanding of B-1 and B-2 cell regulation. B-1 and B-2 cells were shown to have not only unique transcriptional responses to Dex treatment but also to TLR-4 activation. One of the principal transcriptional mediators of the TLR-4 response is NFκB. A ChIP-seq experiment for p65, which together with p50 constitute the most abundant form of NFκB, will be performed in quiescent, Dex pretreated, and/or TLR-4 stimulated B-1 and B-2 cells. These experiment would be hypothesized to demonstrate cell-type specific binding of yet another signal dependent transcription factor besides GR between B-1 and B-2 cells. Additionally, insights could also be gained into the mechanism whereby GR can transrepresses NFκB signaling in B-cells as was recently demonstrated in macrophages by the Evans laboratory (26).

Additionally, I have created a B cell specific knockout of GR by crossing GR-floxed mice with mice that express the Cre recombinase under the influence of the CD19 promoter. I envision using these mice for various experiments to test the effect of GR upon B cell biology. First, the effect of GR deficiency upon B cell development and maintenance will be assessed. I will assess the effect of GR deficiency on the population sizes of the various B cell subsets (B-1, B-2, marginal zone, and follicular B cells) compared to WT controls. There should not be an effect upon B cell development or selection as GR signaling was not essential to T cell development (27). However, if there is

an effect upon the populations of mature B cell subsets, I will then assess the phenotype of immature and developing B-1 and B-2 cell subsets using the cell surface markers described earlier. Additionally, GR-deficient B-1 cells will be adoptively transferred into Rag1-/- mice as previously described. The kinetics of B-1 cell proliferation and therefore reconstitution of the B-1 cell compartment would be assessed by monitoring plasma IgM titers over time.

An additional experiment with clinical importance would be to assess *in vivo* the effect of GR signaling in B-1 cells. Dex and other glucocorticoids are given very regularly in the clinic and yet the effect of these drugs upon B-1 cells and levels of NAbs generated by these cells are unknown. In this regard, the $GR^{fl/fl}$/CD19-Cre mice would serve as an important control to assess the B-1 cell intrinsic effects of Dex treatment. Wild-type or $GR^{fl/fl}$/CD19-Cre mice would be administered clinically relevant regimens of Dex. I will then determine the effect of Dex administration upon NAb titers, such as IgM, IgA and IgG3, as well as on adaptive IgG isotypes, and will also assess the impact on B-1 cell population size and B cell repertoire. Additionally, B-1 cell specific effects of *in vivo* Dex treatment will be assessed by adoptively transferring WT or $GR^{fl/fl}$/CD19-Cre B-1 cells into Rag1-/- mice and then treating these mice with Dex.

Finally, despite not finding an *in vitro* effect of MR activation in B-1 cells my strong curiosity for understanding what role MR is fulfilling in these cells and in B cells in general has not waned. MR is a weak transcriptional activator relative to GR and therefore it has been difficult to definitively define MR target genes (13). As MR and GR are essentially always co-expressed and have very similar ligand binding affinities, treatment with a ‘MR Ligand’ will undoubtedly also activate GR to some extent and because GR more potently activates

transcription, the effect of GR activation has not been completely deconvoluted from that of MR. In this regard, the B cell specific knockout of GR offers an attractive experimental system in which to assess the transcriptional effect of MR activation independent of GR activation. The lack of GR expression in B cells from these mice will allow me to perform RNA-seq on B cells that have been treated with high levels of the MR ligand aldosterone. Additionally, B cells express high levels of MR even in comparison to the kidney- the classical MR target tissue. This exclusive and large expression of MR will allow the identification of MR target genes despite its weak transactivational properties. This will undoubtedly lead to better identification of MR target genes and will allow insights into the role of MR in regulating B cell biology.

References

1. Tsiantoulas D, Diehl C, Witztum J, Binder CJ. B Cells and Humoral Immunity in Atherosclerosis. Circulation Research. 2014;in press.

2. Boullier A, Gillotte KL, Horkko S, Green SR, Friedman P, Dennis EA, Witztum JL, Steinberg D, Quehenberger O. The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. J Biol Chem. 2000;275(13):9163-9169.

3. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. European Journal of Immunology. 1997;27(6):1557-1563.

4. Hooijkaas H, Benner R, Pleasants JR, Wostmann BS. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered “antigen-free” diet. Eur J Immunol. 1984;14(12):1127-1130.

5. Bos NA, Kimura H, Meeuwsen CG, De Visser H, Hazenberg MP, Wost-

mann BS, Pleasants JR, Benner R, Marcus DM. Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. Eur J Immunol. 1989;19(12):2335-2339.

6. McKenna NJ, O'Malley BW. SnapShot: Nuclear receptors II. Cell. 2010;142(6):986 e981.

7. McKenna NJ, O'Malley BW. SnapShot: Nuclear receptors I. Cell. 2010;142(5):822-822 e821.

8. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Sch++tz G, Lumeng CN, Mortensen RM. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. The Journal of Clinical Investigation. 2010;120(9):3350-3364.

9. Herrada AA, Contreras FJ, Marini NP, Amador CA, Gonzalez PA, Cortes CM, Riedel CA, Carvajal CA, Figueroa F, Michea LF, Fardella CE, Kalergis AM. Aldosterone Promotes Autoimmune Damage by Enhancing Th17-Mediated Immunity. Journal of Immunology. 2010;184(1):191-202.

10. Bergmann A, Eulenberg C, Wellner M, Rolle S, Luft F, Kettritz R. Aldosterone Abrogates Nuclear Factor {kappa} B-Mediated Tumor Necrosis Factor {alpha} Production in Human Neutrophils via the Mineralocorticoid Receptor. Hypertension. 2010;55(2):370.

11. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. Cloning of Human Mineralocorticoid Receptor Complementary-Dna - Structural and Functional Kinship with the Glucocorticoid Receptor. Science. 1987;237(4812):268-275.

12. Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid Action - Target Tissue-Specificity Is Enzyme, Not Receptor, Mediated. Science. 1988;242(4878):583-585.

13. Rupprecht R, Reul JMHM, van Steensel B, Spengler D, Söder M, Berning B, Holsboer F, Damm K. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. European Journal of Pharmacology: Molecular Pharmacology.

1993;247(2):145-154.

14. Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ. 2002;9(1):6-19.

15. Fan H, Morand EF. Targeting the side effects of steroid therapy in autoimmune diseases: the role of GILZ. Discovery medicine. 2012;13(69):123-133.

16. Zen M, Canova M, Campana C, Bettio S, Nalotto L, Rampudda M, Ramonda R, Iaccarino L, Doria A. The kaleidoscope of glucorticoid effects on immune system. Autoimmun Rev. 2011;10(6):305-310.

17. Ramdas J, Harmon JM. Glucocorticoid-induced apoptosis and regulation of NF-kappaB activity in human leukemic T cells. Endocrinology. 1998;139(9):3813-3821.

18. Ploner C, Schmidt S, Presul E, Renner K, Schrocksnadel K, Rainer J, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia. J Steroid Biochem Mol Biol. 2005;93(2-5):153-160.

19. Friedman AD. Transcriptional control of granulocyte and monocyte development. Oncogene. 2007;26(47):6816-6828.

20. Heinz S, Benner C, Spann N, Bertolino E, Lin Y, Laslo P, Cheng J, Murre C, Singh H, Glass C. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Molecular cell. 2010;38(4):576-589.

21. Berland R, Wortis HH. Normal B-1a cell development requires B cell-intrinsic NFATc1 activity. Proc Natl Acad Sci U S A. 2003;100(23):13459-13464.

22. Debnath I, Roundy KM, Pioli PD, Weis JJ, Weis JH. Bone marrow-induced Mef2c deficiency delays B-cell development and alters the expression of key B-cell regulatory proteins. Int Immunol. 2013;25(2):99-115.

23. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a

B-1B cell-specified progenitor. Nature Immunology. 2006;7(3):293-301.

24. Ghosn EE, Sadate-Ngatchou P, Yang Y, Herzenberg LA. Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen. Proc Natl Acad Sci U S A. 2011;108(7):2879-2884.

25. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, B„ckhed F, Miller YI. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. The Journal of Clinical Investigation. 2009;119(5):1335.

26. Uhlenhaut NH, Barish GD, Yu RT, Downes M, Karunasiri M, Liddle C, Schwalie P, Hubner N, Evans RM. Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. Mol Cell. 2013;49(1):158-171.

27. Purton JF, Boyd RL, Cole TJ, Godfrey DI. Intrathymic T cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling. Immunity. 2000;13(2):179-186.

CHAPTER 7

Materials and Methods

FACS Isolation of Peritoneal B-1 and B-2 Cells

Peritoneal exudate cells (PECs) from 16- to 20-week-old naïve wild-type female C57BL/6 mice (Charles River, Wilmington, MA) were harvested by peritoneal lavage using ice-cold PBS supplemented with 1% heat-inactivated FCS (Invitrogen, Carlsbad, CA). PECs were incubated with an anti-Fcγ receptor mAb (clone 2.4G2: BD Biosciences-Pharmingen, San Diego, CA) for 15 minutes at 4^{o}C to block nonspecific binding before being stained with fluorescently labeled monoclonal antibodies (mAbs). PECs were stained with R-PE labeled anti-CD19 (clone 1D3), FITC-labeled anti-CD23 (clone B3B4), and PE-Cy5-labeled anti-CD3 (clone 145-2C11) (all from BD Biosciences-Pharmingen, San Diego, CA). B-1 and B-2 cells were sorted to greater than 98% and 97% respective purity using a MoFlo cell sorter (Dako, Glostrup, Denmark). B-1 cells were sorted into culture medium containing 20% heat-inactivated FCS as the $CD3^{-}$, $CD19^{+}$, and $CD23^{-}$ population and B-2 cells as the $CD3^{-}$, $CD19^{+}$, and $CD23^{+}$ population.

Magnetic Bead Isolation of Peritoneal B-1 cells and Splenic B cells

PECs were isolated in same manner as described above. PECs were stained with anti-CD23-biotin (clone B2B4, BD Biosciences) followed by streptavidin microbeads (Miltenyi Biotec, Germany). The stained cells were run through a MS column (Miltenyi Biotec, Germany) and the flow through cells (CD23-) were harvested, stained with CD19 microbeads (Miltenyi Biotec, Germany) and the cells were run through two MS columns in sequence. CD19+ cells were eluted from the columns. Typical isolations have 90-95% pure CD19+, CD23- B-1 cells. Splenic B cells were isolated by a single step magnetic depletion of CD43- and CD11b-expressing cells to 97% purity

(Miltenyi Biotech). Anti-CD43 microbeads (Miltenyi Biotec) and anti-CD11b-biotin (clone M1/70, eBioscience, San Diego, CA).

Kdo2-Lipid A and Pam3CSK4 Stimulations for Nuclear Receptor Atlas

Purified B-1, B-2, or splenic B cells were seeded at 5 x 10^5 cells per well in 48-well flat-bottom plates in culture medium (RPMI 1640 medium containing 10% heat-inactivated FCS, 10mM HEPES buffer, 2mM L-glutamine, 0.05 mM 2-mercaptoethanol, 50µg/mL gentamicin). For time course studies, sorting medium (culture medium with 20% FCS) was replaced with fresh culture medium (for time point of zero and control cells) or fresh culture medium containing 100ng/mL Kdo2-Lipid A (Avanti Polar Lipids, Alabaster, AL) or 300ng/mL Pam_3CSK4 (Invivogen, San Diego, CA). Cells were maintained at 37°C in a 5% CO_2 incubator over the experimental time course.

qPCR Procedures for Assessing Nuclear Receptor Expression

All treatments were performed in triplicate. For time course studies, cells were harvested at each of the indicated time points of 0, 6, 24 and 72 h. Upon harvesting, media and cells were centrifuged and media were aspirated and cells were lysed using RLT buffer from RNeasy mini kit (Qiagen, Valencia, CA). Total RNA was extracted from the lysed cells using the RNeasy mini kit. RNA was amplified and cDNA synthesized using the WT-Ovation RNA Amplification System (Nugen, San Carlos, CA). A 384-well microtiter dish format was used for quantitative polymerase chain reactions (qPCRs) with SYBR green (Sigma, St. Louis, MO), and final reaction volumes were 10µL. High-throughput processing was achieved using a semi-automated Beckman (Fullerton, CA) liquid handler, an ABI Prism 7900HT (Applied Biosystems, Foster City,

CA), and sequence detection system software. For each biological sample, qPCRs were performed in triplicate and expression was normalized to 36B4 expression. Bar graphs represent the average relative expression of the triplicate biological samples and SD, with the initial time point assigned a relative expression of 100 for each transcript.

Primer sequences for all nuclear receptors assessed in this study are available on the NURSA web site at www.nursa.org.

Experimental Animals for Nuclear Receptor Atlas Study

C57BL/6 mice were used as donors for B-1, B-2, and splenic B cells. All animal experimentation described in this work was conducted in accordance with a University of California, San Diego approved Institutional Animal Use and Care Committee protocol.

qPCR Expression Analysis

Mineralocorticoid Receptor expression was assessed from quiescent peritoneal B-1 cells isolated as described above. Bone marrow derived macrophages and peritoneal macrophages were a gift from Soo-Ho Choi and were isolated as described (1,2). Kidney tissue was isolated and homogenized in RLT buffer from RNeasy RNA isolation kit (Qiagen, Valencia, CA). Total RNA was isolated using RNeasy easy columns (Qiagen, Valencia, CA), treated with DNAse, and reverse transcribed using olig-dT and a First Strand Synthesis kit (Life Technologies, Carlsbad, CA). Real time qPCR analysis was performed using Rotor Gene Q (Qiagen). Taqman qPCR for MR (TaqMan assay Mm01241596_m1), GR (TaqMan assay Mm00433832_m1), 11HSD2 (TaqMan assay 01251104_m1), Cyclin D 3 (TaqMan assay Mm01612362_m1), DUSP1

(TaqMan assay Mm00457274_g1), IL-6 (TaqMan assay Mm00446190_m1), and GAPDH (TaqMan assay Mm99999915_g1) as a loading control were performed using reagents from Applied Biosystems (Life Technologies, Carlsbad, CA).

In Vitro B-1 cell Treatment with Nuclear Receptor Ligands

B-1 cells were isolated using magnetic beads as described above. They were seeded at $1x10^5$ cells/well in 96 well flat bottom plates in cell culture media as described above but with 10% fetal calf serum that has been charcoal dextran stripped to remove all residual steroid-like compounds. NR ligand was added: aldosterone dissolved in ethanol (Sigma, Saint Louis, MO), Fludrocortisone Acetate dissolved in ethanol (Sigma, Saint Louis, MO), Eplerenone dissolved in dimethyl sulfoxide (DMSO) (Tocris Bioscience, Bristol, UK), Mifepristone dissolved in ethanol (Sigma, Saint Louis, MO), Dexamethasone (Dex) dissolved in DMSO (Sigma, Saint Louis, MO), Deoxycorticosterone acetate dissolved in ethanol (Sigma, Saint Louis, MO), T0901317 dissolved in DMSO (Tocris Bioscience, Bristol, UK), or GW3965 hydrochloride dissolved in DMSO (Tocris Bioscience, Bristol, UK). Ethanol and DMSO were used at maximal 1:5000 dilution in cell culture media. Normal in vitro treatment with B-1 cells consisted of adding nuclear receptor (NR) ligands to cell culture shortly after adding cells, and then 24hrs later adding 5uL of Pam3CysK4 or Kdo2Lipid A as described above. Cells and/or supernatants were harvested on day 4 after plating.

Measurement of Antibody Titers

Specific antibody (Ab) titers to given antigens in plasma or cell culture

supernatants were determined by chemiluminescent ELISA as previously described (3,4). Purified rat anti-mouse IgM (clone II/41; BD Biosciences-Pharmingen), was used as capture Ab to measure total IgM levels. AP-labeled goat anti-mouse IgM (μ chain specific) and anti-mouse IgG (γ chain specific) (Sigma-Aldrich, Saint Louis, MO) were used as detection Abs, as well as biotinylated rat anti-mouse IgM (R6-60.2; BD Biosciences-Pharmingen). To detect other Ig isotypes, rat anti-mouse IgG1 (A85-3), IgG2a/c (R11-89), and IgA (C10-3) were used as capture Abs; biotin-conjugated rat anti-mouse IgG1 (A85-1), IgG2a/c (R19-15), IgG2b (R12-3), IgG3 (R40-82), and IgA (C10-1) (all from BD Biosciences-Pharmingen) were used as secondary Abs. To detect the levels of E06, a T15-specific anti-idiotype Ab (AB1-2) (5) was used as capture Ab, followed by incubation with AP-labeled goat anti-mouse IgM. Biotin-conjugated Abs were then detected with AP-conjugated neutravidin (Pierce, Thermo Scientific, Rockford, IL). Mouse anti-human IgG (G18-145) and IgM (G20-127; BD Biosciences-Pharmingen) were used as capture Abs to measure total IgM and IgG levels in humans. AP-labeled goat anti-human IgG and IgM (A3187 and A3437; Sigma-Aldrich) were used as detection Abs. The following antigens were prepared as described previously (6): copper sulfate–oxidized LDL (CuOx-LDL), MDA-LDL, and native-LDL were prepared from human LDL. α1,3-dextran was a gift from John F. Kearney (University of Alabama at Birmingham, Birmingham, Alabama, USA).

Assessing Cell Proliferation and Viability

B-1 cell proliferation was assessed using carboxyfluorescein diacetate succinmidyl ester (CFSE) staining. Prior to culturing B-1 cells were stained with 2μM CFSE for 20min. (Cell Trace, Invitrogen, Carlsbad, CA). CFSE

stained B-1 cells were cultured as described above. After culturing, B-1 cells were analyzed on BD FACSCanto II (BD Biosciences-Pharmingen). Cultured B-1 cells were assessed for cellular viability by incubating cells with annexin V and 7-AAD (BD Biosciences-Pharmingen), for 15 minutes and immediately analyzed by flow cytometry.

Western Blot for Glucocorticoid Receptor

Protein expression levels of glucocorticoid receptor (GR) were analyzed using SDS-PAGE and western blotting according to standard protocols as previously described in our lab (1). Anti-GR antibody (clone sc-1004, Santa Cruz, Santa Cruz, CA) was used at 1:1000 dilution.

RNA-seq from B-1 and B-2 cells

Magnetic bead purified B-1 or B-2 cells were cultured overnight in media containing charcoal stripped fetal calf serum (to allow for the *in vivo* induced GR expression to equalize) and then were treated for 4 hrs with 1μM Dex or pretreated for 1 hr with 1μM Dex before stimulation with Kdo2LipidA. Cells were also stimulated for 1 hr with Kdo2LipidA alone. See descriptions above for more detail about Dex and KLA treatments in vitro. Total RNA was isolated as described above. RNA-Seq was performed on poly(A)+ RNA after fragmentation, decapping, 3' and 5' adaptor ligation and reverse transcription. RNA-seq libraries were sequenced for 51 cycles on a HiSeq 2000 sequencer (Ilumina, San Diego, CA). Reads were mapped to the mm9 reference mouse genome. Data were normalized, reads were counted in exonic regions using the HOMER package (7). Differential expression and adjusted p-values were calculated using R scripts for limma available from Bioconductor (8).

ChIP-seq

ChIP-Seq was performed based on published protocols on either native chromatin after MNase digestion (H3K4me2) or fixed, sonicated chromatin (GR) (7). ChIP-Seq libraries were sequenced for 51 cycles on a HiSeq 2000 sequencer (Illumina). Reads were mapped to the mm9 reference mouse genome. Data were normalized, ChIP-Seq peaks identified, and motifs analyzed using the HOMER package. GR ChIP was performed in BCL-1 cells (a gift from Dr. Christoph Binder) and in primary magnetic bead purified B-1 and B-2 cells using two anti-GR antibodies in tandem, anti-human GR from Diagenode (clone mAB-010-050, Denville, NJ) or anti-mouse GR from Santa Cruz (clone sc-1004x, Santa Cruz, CA). anti-H3K4Me2 (polyclonal, Millipore, Temecula, CA).

Animal models used in collaborative studies

A description of CD74 knockout mice, BCL-6 deficient bone marrow transplant model mice, Mycophenolate mofetil treated mice, and ApoE/FcγRIIb double knockout mice have previously been published (9-12). A vitamin D analog paricalcitol was given IP daily to *Ldlr-/-* mice on a high fat diet along with angiotensin II.

References

1. Choi SH, Harkewicz R, Lee JH, Boullier A, Almazan F, Li AC, Witztum JL, Bae YS, Miller YI. Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake. Circ Res. 2009;104(12):1355-1363.

2. Montano EN, Boullier A, Almazan F, Binder CJ, Witztum JL, Hartvigsen K. Development and application of a nonradioactive binding assay of

oxidized low-density lipoprotein to macrophage scavenger receptors. J Lipid Res. 2013;54(11):3206-3214.

3. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nature Medicine. 2003;9(6):736-743.

4. Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. JImmunolMethods. 1985;77(2):305-319.

5. Kearney JF, Barletta R, Quan ZS, Quintans J. Monoclonal vs. heterogeneous anti-H-8 antibodies in the analysis of the anti-phosphorylcholine response in BALB/c mice. Eur J Immunol. 1981;11(11):877-883.

6. Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. JClinInvest. 1989;84(4):1086-1095.

7. Heinz S, Benner C, Spann N, Bertolino E, Lin Y, Laslo P, Cheng J, Murre C, Singh H, Glass C. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Molecular cell. 2010;38(4):576-589.

8. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical applications in genetics and molecular biology. 2004;3:Article3.

9. Sun J, Hartvigsen K, Chou MY, Zhang Y, Sukhova GK, Zhang J, Lopez-Ilasaca M, Diehl CJ, Yakov N, Harats D, George J, Witztum JL, Libby P, Ploegh H, Shi GP. Deficiency of Antigen-Presenting Cell Invariant Chain Reduces Atherosclerosis in Mice. Circulation. 2010;122(8):808-820.

10. Barish GD, Yu RT, Karunasiri MS, Becerra D, Kim J, Tseng TW, Tai LJ, Leblanc M, Diehl C, Cerchietti L, Miller YI, Witztum JL, Melnick AM, Dent AL, Tangirala RK, Evans RM. The Bcl6-SMRT/NCoR cis-

trome represses inflammation to attenuate atherosclerosis. Cell Metab. 2012;15(4):554-562.

11. von Vietinghoff S, Koltsova EK, Mestas J, Diehl CJ, Witztum JL, Ley K. Mycophenolate mofetil decreases atherosclerotic lesion size by depression of aortic T-lymphocyte and interleukin-17-mediated macrophage accumulation. J Am Coll Cardiol. 2011;57(21):2194-2204.

12. Mendez-Fernandez YV, Stevenson BG, Diehl CJ, Braun NA, Wade NS, Covarrubias R, van Leuven S, Witztum JL, Major AS. The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. Atherosclerosis. 2011;214(1):73-80.