THE ROLE OF MACROPHAGE LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN IN THE ACTIVATION OF INVARIANT NATURAL KILLER T CELLS

By

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TABLE OF CONTENTS

ABBREVIATIONS

α2M: alpha-2-macroglobulin

AD: Alzheimer's Disease

αGC: alpha-galactosyl ceramide

APC: antigen presenting cell

apoE: apolipoprotein E

β2M: beta-2-microglobulin

BbGL-II: galactosyl diacylglycerol

βGC: beta-galactosyl ceramide

BMDC: bone marrow-derived dendritic cells

CM: chylomycrons

EM: extracellular matrix

ER: endoplasmic reticulum

GD3: disialogangloside-3

GGC: galactosyl (α1-2) galactosyl ceramide

GSL-1: α-glucuronosylceramide

$hDTR^{tg}$: human diphtheria toxin receptor transgenic

HEL: hen egg lysozyme

HHSC: human hepatic stellate cells

HSP: heat shock protein

IFN-γ: interferon gamma

iGb3: isoglobohexosylceramide

iNKT: invariant natural killer T cell

KO: Knockout

LDLr: low density lipoprotein receptor

LPG: lipophosphoglycan

LRP: low density lipoprotein receptor related protein

LRPmr: low density lipoprotein receptor related protein mini receptor

MΦ: macrophage

MHC: major histocompatibility complex

MTP: microsomal triglyceride transfer protein

MZ: marginal zone

OVA: ovalbumin

PEA: pseudomonas exotoxin A

PIM-2: phosphatidyl-innositol dimmanoside

pMΦ: peritoneal macrophage

PRR: pattern recognition receptor

Siglec-1: sialic acid binding immunoglobulin-like lectin 1

SRA: scavenger receptor

TLR: Toll-like receptor

TG: triglyceride

TNFα: tumor necrosis factor alpha

tPA: tissue plasminogen activator

VLDL: very low density lipoprotein receptor

WT: wild type

LIST OF FIGURES

LIST OF TABLES

LIST OF PUBLICATIONS

1. Covarrubias R, Wilhelm AJ, Major AS (2014). Specific deletion of LDL Receptor-Related Protein on macrophages has skewed in vivo effects on cytokine production by invariant natural killer T Cells. PLoS One.

2. Mendez-Fernandez Y V, Stevenson BG, Diehl CJ, Braun NA, Wade NS, Covarrubias R, Van Leuven S, Witztum JL, Major AS (2011). The inhibitory FcγRIIb modulates the inflammatory response and influences atherosclerosis in male apoE (-/-) mice. Atherosclerosis 214: 73–80.

3. Braun NA, Mendez-Fernandez Y V, Covarrubias R, Porcelli SA, Savage PB, et al. (2010). Development of spontaneous anergy in invariant natural killer T cells in a mouse model of dyslipidemia. Arteriosclerosis Thrombosis and Vascular Biology

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CHAPTER I

INTRODUCTION

Immunological protection by the innate and adaptive immune system

The mammalian immune system is divided in two main categories: the innate and adaptive immune system. The interplay between both is constant and complex, but there are clear distinctions that highlight the abilities of each to protect the host. The innate immune system is always active and is the first line of protection against environmental insults. This branch of the immune system is composed of anatomical barriers and a subset of cells that provide non-specific responses against pathogens. The main cells of the innate immune system are phagocytes, neutrophils, mast cells, eosinophils and natural killer (NK) cells. All these cells express receptors that are encoded in the germline and lead to expression of pattern recognition receptors (PRRs).

A classic example of PRRs is the toll-like receptors (TLRs). TLRs are present in phagocytes that recognize diverse molecules such as bacterial cell surface lipopolysaccharides, lipoproteins, viral double-stranded RNA, etc. Binding of TLRs initiates a signaling cascade that culminates in the activation of phagocytes, cytokine release and inflammation of tissues [1]. All of these are produced in concert to contain and eliminate infection in its early stages. An example of another prominent cell of the immune system is NK cells. NK cells are cytotoxic lymphocytes that express surface receptors without antigen specificity, yet through expression of Fc receptors can target and destroy any cellular structure bound by an antibody [2].

Therefore, receptors particular to the innate immune system provide a cellular response that is immediate, but non-specific as it reacts with similar potency upon repeated rounds of stimulation [3].

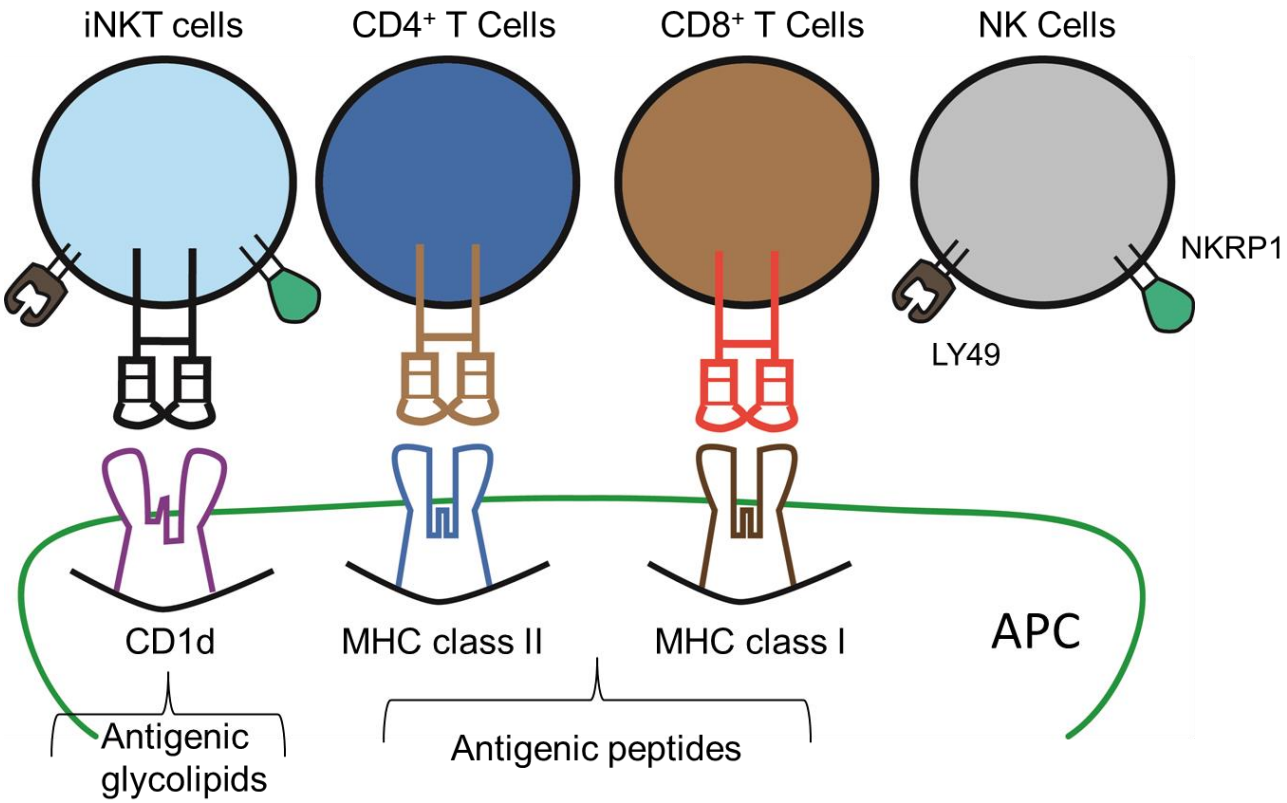
In contrast to the immediate response by the innate immune system, the adaptive immune system is slower, but with a more potent and specific response. Cells of the adaptive immune system are composed by conventional T cells ($CD4^+$ and $CD8^+$) and antibody producing B cells. T cells are subdivided into many categories (T helper, regulatory, cytotoxic, etc), but their determining property is the presence of a surface T cell receptor (TCR), which is ligated by peptide antigens bound to major histocompatibility complex (MHC). Naïve T cells encounter antigens in lymphoid organs where antigen presenting cells (APCs) reside. After ligation of the TCR, along with proper co-stimulatory signals by APCs, naïve T cells re-enter the cell cycle and divide to produce large copies of themselves, a process known as clonal expansion. Further steps in T cell maturation involve proliferation, differentiation and expression of surface proteins that allow them to travel to site of infection to promote effector functions. Because of this delay, the T cell arm of the immune system acts slower than the immediate response provided by the innate immune system. However, once established an adaptive T cell response is highly specific and more potent upon secondary encounters with the same antigen [3].

B cells protect the host in a different manner than T cells, yet the timing of their response resembles that of T cells. B cells produce immunoglobulin (Ig), which serves as an antigen receptor when found on the cell surface. Ig has specificity for peptide antigens and does not require presentation in the context of MHC. However,

their activation depends upon signals given by $CD4^+$ helper T cells. After activation, B cells undergo changes that lead to structural alterations of Igs that produce highly specific and large quantities capable of neutralizing pathogens. Just like T cells, this process requires time and a delay that leads to a robust and rapid response and immunological memory upon a secondary exposure to the same antigen. In addition to the distinct lineages of the innate and adaptive immune system, a separate compartment of immune cells exists that can bridge the immune system between the innate and adaptive response. One such cell is the invariant natural killer T (iNKT) cell that expresses a surface TCR, yet produces a rapid and robust immune response. INKT cells are hybrids of T lymphocytes with innate-like qualities that protect the host with the specificity of a T lymphocyte and the robustness of an innate lymphocyte. The contents of this dissertation explore a novel mechanism that is essential in the activation of iNKT cells.

Invariant Natural Killer T cells

Invariant natural killer T (iNKT) cells are a specialized subset of lymphocytes that recognize lipid antigens in the context of major histocompatibility complex (MHC) class I-like molecule CD1d. Despite activation occurring through an $\alpha\beta$ T cell receptor, iNKT cells possess innate qualities due to the rapid and robust secretion of immunoregulatory cytokines such as IFN-γ, IL-4, IL-13, IL-17 and IL-10 [4]. INKT cell share receptor with conventional T cells and natural killer cells (Figure 1), but unlike



Adapted from [15]
Abbreviations (NK: natural killer cell, MHC: major histocompatibility complex, APC: antigen presenting cell)

Figure 1: iNKT cells share receptors with conventional T cells ($CD4^+$ and $CD8^+$) and NK cells. $CD4^+$ T cells and $CD8^+$ T cells respond to a diverse repertoire of antigens presented in the context of major histocompatibility complex II and I respectively. In contrast, iNKT cells respond to glycolipid antigens loaded in the context of CD1d. The activation of iNKT cells and T cells requires the presence of the presentation molecules by antigen presenting cells (B cells, dendritic cells or macrophages). Additionally, iNKT cells also share receptors with innate immune cell known as an NK cell. These receptors are the inhibitory LY49 and the activating receptor NKRP1.

$CD4^+$ and $CD8^+$ T cells that require 4-6 days for activation, differentiation and clonal expansion after encountering TCR specific antigen, iNKT cells secrete cytokines as early as 90 minutes following ligation of the iNKT cell receptor [5]. This allows iNKT cells to affect a wide range of immune processes that can impact human health [6] such as microbial immunity [7,8], cancer [9–11], autoimmunity [12–15] and atherosclerosis [16,17].

The understanding of iNKT cell biology has been aided by the fact that mice and humans have similar components that make up this immune compartment [18]. Both species express a similar iNKT cell receptor as evidenced by the homology in their α chains (Vα14Jα18 in mice and Vα24Jα18 in humans) and semi-invariant β chains (Vβ2, Vβ7, Vβ8.2 in mice and Vβ11 in humans) [19]. Despite these parallels, iNKT cell distribution differs between species. The frequency of iNKT cells is lower in humans than mice, and a wide normal distribution has also been observed in humans. For example, a screen of thymus and blood from children undergoing corrective cardiac surgery showed a large distribution of iNKT cell frequency in the thymus (0.0005% to 0.01%) and blood (0.003% to 0.78%) [20]. Additionally, there was no correlation between thymus iNKT cell frequency and blood iNKT cell frequency in individual patients, suggesting that peripheral pools of iNKT cells are not maintained by development of thymic iNKT cells. This suggests that some iNKT cell therapies may prove to have more efficacies in select populations with ideal number of iNKT cells.

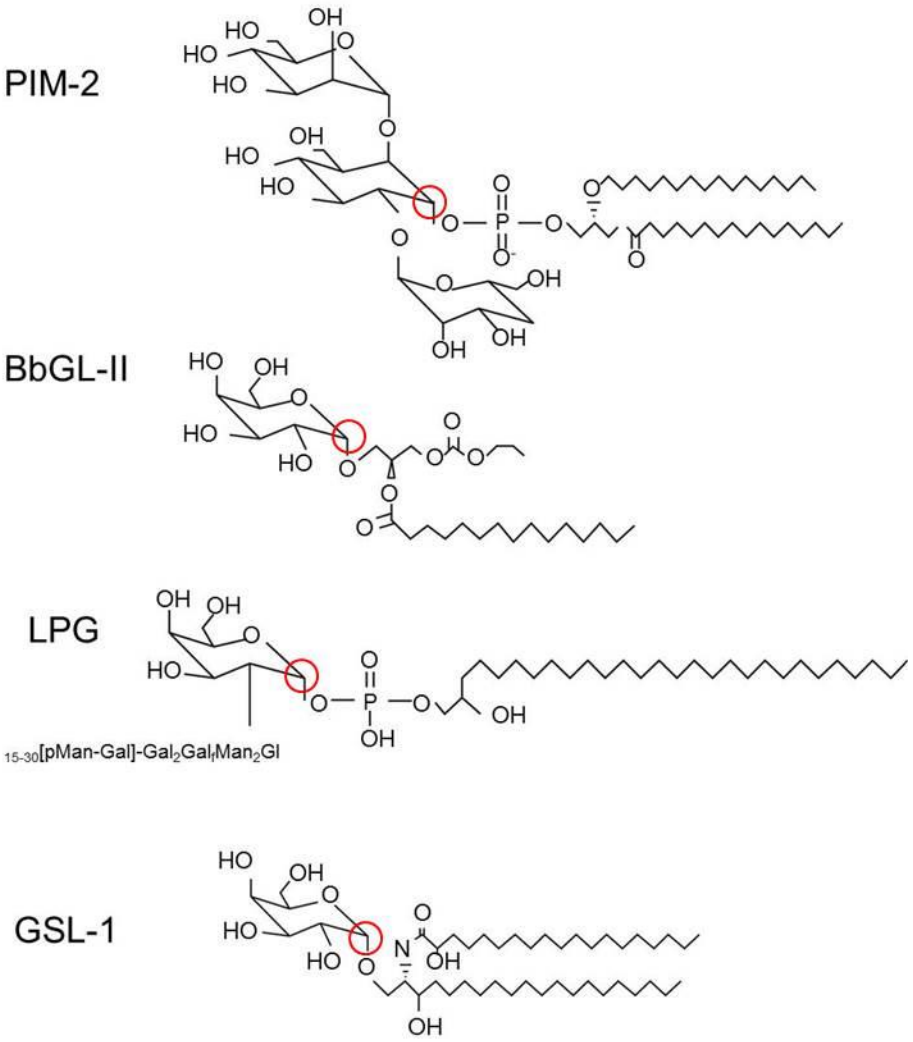
Successful treatment of mouse models of human disease by activating iNKT cells has led to a number of clinical trials using the iNKT cell agonist α-

galactosylceramide (αGC) to modify progression of neoplastic disorders including glioma, melanoma and cancers of the liver, lung, breast, kidney, prostate and head and neck [21–23]. Tolerance and toxicity of αGC has been measured to be optimal for human therapy, but the clinical results have been unable to provide a therapeutic advantage via iNKT cell activation. Several explanations for this phenomena have been proposed including advanced stage of disease, distinct methods of αGC delivery [24,25] and the undesirable characteristic of iNKT cells to undergo anergy, a state in which iNKT cells can no longer respond to further lipid stimulation [26]. The development of anergy in iNKT cells is thought to occur in response to a strong TCR ligation by the αGC/CD1d complex [27]. After a primary challenge with αGC *in vivo*, iNKT cells downregulate expression of interleukin-2 receptor making iNKT cells unresponsive to IL-2 stimulation and hindering their ability to proliferate and to produce IFN-γ. Moreover, little is known about the cellular factors that promote lipid transport and targeting to CD1d for activation of iNKT cells. Therefore, efficacy of adjuvants to modify immune function will depend on the knowledge of these mechanisms. The work presented in this dissertation is aimed at elucidating cellular factors that influence iNKT cell activation to harness their ability to impact human health.

CD1d and iNKT cell activation

In order for iNKT cells to be activated, antigen presenting cells (APCs) have to access and load lipids onto CD1d. Humans express five different isoforms of CD1 (a-e), while mice express the single isoform CD1d [28]. While this allows for

detection of a larger repertoire and diversity of lipids in humans, the conserved nature of both human and mouse CD1d allows for study of iNKT cell activation in mice with application to human biology. The identity of lipids known to bind CD1d can be broken down into two categories: exogenous and endogenous [29]. The exogenous glycolipids belong to those found in cell walls of pathogenic bacteria and are known to play a role in the activation of the immune system. Figure 2 shows examples of the exogenous lipids phosphatidyl-inositoldimannoside (PIM-2) [30], galactosyl diacylglycerol (BbGL-II) [31], lipophosphoglycan (LPG) [32] and α-glucuronosylceramide (GSL-1) [29] present in *Mycobacterium tuberculosis, Borrelia burgdorferi, Leishmania donovani* and *Pseudomonas aeruginosa,* respectively [33] . Given their bacterial origin, glycolipids like PIM-2 haven been studied for their adjuvant efficacy and shown to establish robust adaptive immune responses to ovalbumin [34] and hepatitis C viral antigens in mice [35]. Additionally, synthetic analogs of glycolipid BbGL-II are being studied as vaccine candidates to prevent *B. bugdorferi's* infection that leads to Lyme disease [36] as well as LPG-conjugates to induce protection against *L. donovani* [37]. The common motif amongst the exogenous glycolipids observed in Figure 1 that allow them to bind CD1d is the α-conformation of the sugar head group and the ceramide backbone that yields long aliphatic carbon tails capable of burying into the deep hydrophobic pockets of CD1d. How the different head groups of these glycolipids affect CD1d binding and subsequent iNKT cell activation is not known.

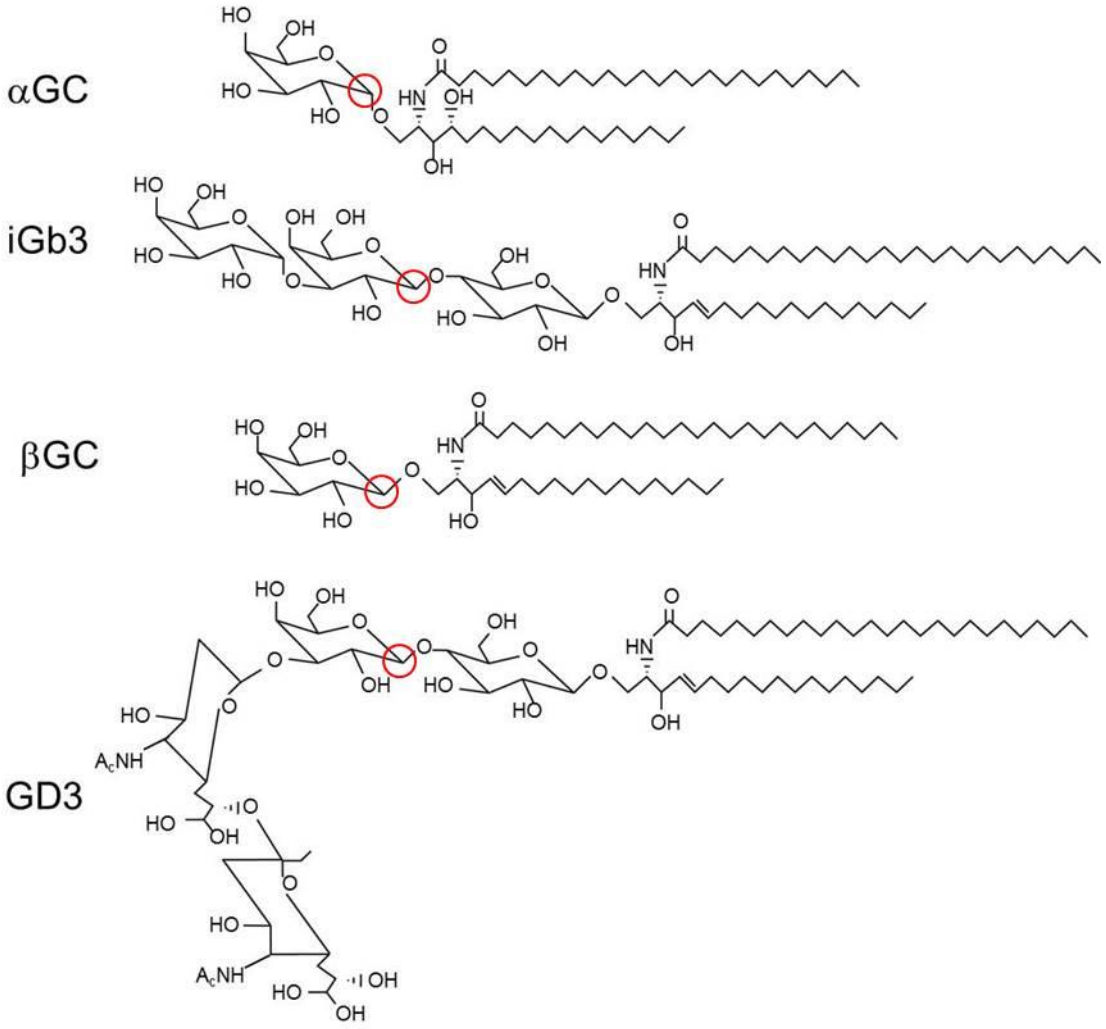


Adapted from [33]

Abbreviations (PIM-2: phosphatidyl-inositoldimannoside. BbGL-II: galactosyl diacylglycerol. LPG: liphophosphoglycan. GSL-1: α-glucuronosylceramide.

Figure 2: Bacterial glycolipids activate iNKT cells Exogenous glycolipid ligands derived from bacteria that bind CD1d contain aliphatic lipid chains attached to a sugar head group in the α anomeric conformation. The α-carbon is highlighted in each molecular structure with a red circle.

The prototypical iNKT cell ligand αGC is a glycosyl ceramide that produces one of the highest binding affinities in a TCR/presentation:complexes known to date (K_d =100nM) [38]. The physiological relevance of αGC has been questioned because of its marine origin [39] and the fact that mammalian cells do not contain enzymes capable of generating glycosyl ceramides in the α conformation [40]. However a recent report has shown that endogenous levels of glycosylceramides in the 0.5-0.1% range belong to the α conformation with strong evidence that one of these glycosylceramides present in the thymus is αGC [41]. The mystery of how these lipids are generated in mammalian cells remains to be solved and confirmation by other research groups needs to be reported, however this and future work detailing iNKT cell activation by αGC will have to take into consideration that this ligand can be endogenously generated. Therefore in this work, αGC is presented in Figure 3 as part of the collection of endogenous lipids known to activate iNKT cells. Another lipid that has been typically associated with activating iNKT cells is the isoglobotrihexosylceramide (iGb3) which has been eluted and crystallized in complex with CD1d [42]. Upon its discovery, iGb3 was hailed as the endogenous ligand that promotes iNKT cell activation and thymic selection *in vivo*. However, the presence of iGb3 has not been documented in immune/lymphoid tissues by sensitive detection methods [43] and mouse studies with global deficiency of iGb3 led to normal development and function of iNKT cells. Another glycolipid also found to activate iNKT cells and bind iNKT cell receptor when complexed with CD1d is the β-galactosyl ceramide depicted in Figure 3. This glycolipid has been shown to activate iNKT cells *in vivo,* but with a diminished ability to induce secretion of IFN-γ,



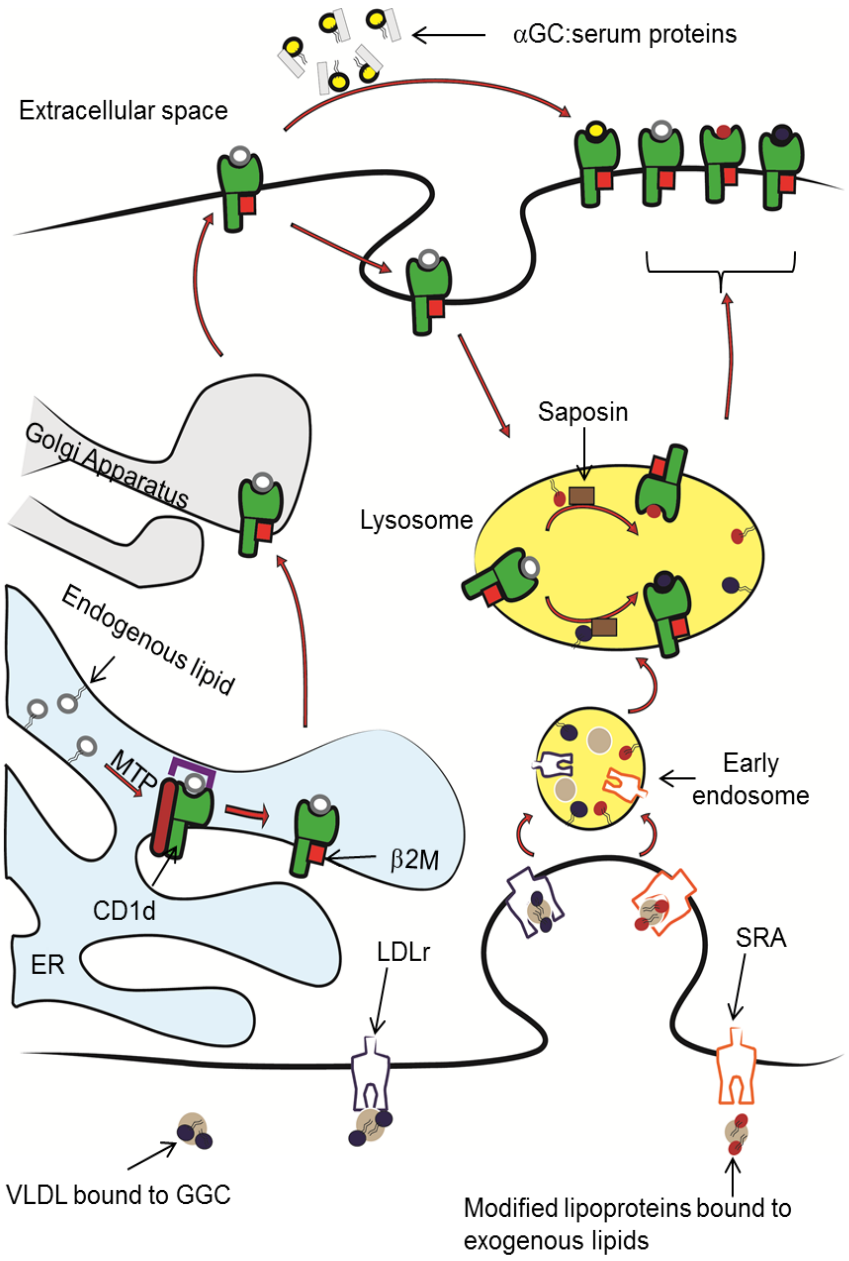
Adapted from [33]

Abbreviations (αGC: alpha-galactosyl ceramide. iGb3: isoglobotrihexosylceramide. βGC: beta-galactosylceramide. GD3: disialoganglioside 3).

Figure 3: Endogenous glycolipids can activate iNKT cells. With the exception of αGC, endogenous iNKT cell ligands contain sugar moieties in the β-conformation attached to a ceramide group that contains two aliphatic lipid tails capable of binding CD1d. Conformation of this carbon is noted with red circle in the diagram.

IL-4 and TNF-α when compared to αGC [44,45]. A contrasting example of endogenous glycolipids that bind CD1d and activate iNKT cells is that provided by disialoganglioside (GD3). This ganglioside was initially shown to be expressed in human melanoma and to produce an iNKT cell response in mouse splenocytes when APCs were cultured *in vitro* by measuring production of IFN-γ, IL-4 and IL-10 [46]. This was thought to be a mechanism for iNKT cells to recognize components from tumorigenic tissues, however recent reports have shown that GD3 binding to CD1d cells can inhibit αGC mediated activation of iNKT cells *in vivo* and *in vitro*. GD3, is therefore thought to be a ganglioside that can bind CD1d, but instead of activating, it limits the ability of iNKT cells to become activated by other glycolipids [47]. It has been suggested that these gangliosides are produced as a way for tumors to avoid immune surveillance by iNKT cells and elicit immunosuppression in the host. All these examples of iNKT ligands illustrate the diversity of endogenous and exogenous glycolipids and the complex mechanisms regulating iNKT cell selection and activation.

CD1d is synthesized in the endoplasmic reticulum where it binds chaperones calnexin, calreticulin and Erp57 [48]. After formation of disulfide bonds by Erp57 between CD1d's heavy chains, CD1d associates with β_2-microglobulin prior to transport through the Golgi network to the outer membrane [49]. Figure 4 illustrates this process, showing that endogenous lipids can be loaded to the hydrophobic groove of CD1d to facilitate assembly and provide stabilization to nascent CD1d molecules [50]. Following assembly in the ER, CD1d travels through the trans-Golgi network to the plasma membrane, where it can bind exogenous lipids directly on the



Abbreviations (ER: endoplasmic reticulum, β2M: beta-2-microglobulin, LDLr: low density lipoprotein receptor, VLDL: very low density lipoprotein, MTP: microsomal triglyceride transfer protein, SRA: scavenger receptor A). Adapted from [256]

Figure 3: CD1d acquires lipids from multiple cellular compartments. CD1d is assembled in the ER where it associates with endogenous lipids and β2M as a chaperoning complex. Loading of endogenous lipids in ER is mediated by MTP. CD1d is then transported to cellular membrane through Golgi transport where it can directly bind lipids such as αGC. Additionally, CD1d can also be internalized to late endosomal and lysosomal compartments where exchange of the endogenous to exogenous lipids occurs. This process is mediated by the lipid transport proteins saposins. Exogenous lipids gain entry to the lysosome through associating with serum lipoproteins and/or modified

cell surface [51]. This striking feature has been investigated with tail truncated mutants of CD1d unable to recycle through the lysosome. These CD1d-tail mutants unable to acquire lipids in the endosome can still activate iNKT hybridoma DN32.D3 when high concentrations of αGC are used to load cells in culture [52]. In addition to acquiring exogenous lipids directly from the extracellular membrane, CD1d can be internalized to late endosomal or lysosomal compartments where it can survey for additional self or foreign lipids [53]. Exogenous lipids can reach the endosome through multiple pathways such as VLDL binding through the LDL-R or modified lipoproteins to scavenger receptor A. The exchange of lipids in the endosome occurs through action of lipid transport proteins saposins which catalyze extraction of lipids from biological membranes. After this exchange of lipids occurs in the lysosome, CD1d is shuttled to the cell membrane where it can bind to the iNKT cell receptor or undergo multiple rounds of endocytosis to the lysosome to survey for other lipids. In summary, CD1d can bind lipids from distinct cellular locations allowing for a wide range of mechanisms that can alter the pathways by which lipids activate iNKT cells.

Lipid homeostasis proteins and iNKT cell activation

Proteins that are known to be associated with lipid homeostasis have also been shown to play a role in iNKT cell activation. An example of this is microsomal triglyceride transfer protein (MTP) whose primary function in hepatocytes is to transfer phospholipids, triglycerides and cholesterol obtained from diet to newly formed apolipoprotein (apo) B-containing lipoproteins. This process is essential for formation of particles leading to transport of lipids obtained from diet to the rest of

the body [54]. However, MTP has also been shown to play a prominent role in iNKT cell activation as MTP was first shown to directly associate with CD1d in the ER of hepatocytes [55]. Furthermore, genetic deletion of the *mttp* gene was shown to disrupt cellular distribution and decrease surface expression of CD1d leading to decreased iNKT cell activation [56]. Additionally, chemical inhibition of MTP in mouse APCs such as bone marrow derived dendritic cells (BMDCs) leads to decreased secretion of IL-2 by iNKT hybridoma DN32.D3 [55]. The exact mechanism by which MTP modulates CD1d function in APCs has not been elucidated, but the association of MTP and CD1d in the ER and the ability of MTP to transfer phospholipids to CD1d *in vitro* [57] suggest a scenario where MTP can promote loading of endogenous lipids to CD1d as a way to induce stable folding and formation of CD1d. In fact, a similar involvement of MTP has recently been shown in adipocytes, as these cells can activate iNKT cells [58] and require expression of MTP to function as APCs [59]. These reports highlight dual functions for MTP in lipoprotein assembly and lipid loading to CD1d, suggesting that lipid homeostasis and lipid antigen presentation are intimately related.

The low-density lipoprotein receptor (LDLr) plays a role in lipid homeostasis and delivery of lipids for activation of iNKT cells. LDLr is mainly known for its ability to bind cholesterol rich apolipoprotein (apo) B and apo E containing lipoproteins. The function and expression of LDLr is linked to progression of atherosclerosis, as serum-borne LDL particles are the primary contributors of lipid deposition to sub-intimal space leading to cardiovascular disease [60,61]. However, Van Den Elzen, et al. reported that LDLr expression in human dendritic cells (DCs) can impact iNKT

[62] and B cell activation [63]. This study showed that apoE plays a critical role in exogenous lipid antigen uptake leading to enhancement of iNKT cell activation. To show that LDLr binding to apoE supports antigenic lipid entry to the endosome, the authors used galactosyl (α1-2) galactosyl ceramide (GGC) to stimulate human CD1d-restricted T cells. GGC requires access to the endosome and cleavage of a galactosyl group prior to binding to CD1d and activation of iNKT cells. Therefore, GGC can be used as a tool to study lipid trafficking and processing prior to iNKT cell activation. Incubation of serum with GGC followed by fraction separation through fast protein liquid chromatography showed that the antigenic activity of GGC was only present in the very low density lipoprotein (VLDL) portion of serum which contains both apoE and apoB cointaining lipoproteins. Additionally, when neutralizing antibodies were used in human serum to deplete apoE, the ability to stimulate CD1d restricted T cells by VLDL was lost, suggesting that cellular transport of GGC by VLDL is mediated through binding of apoE-containing lipoproteins to the LDLr. While these experiments laid the groundwork for understanding how lipid homeostasis mechanisms can also impact iNKT cell immunity, a number of issues were raised by this study. First, the readout used to investigate iNKT cell activation was based on secretion of IFN-γ. While this is an acceptable parameter to determine iNKT cell activation, measuring other cytokines known to be secreted by iNKT cells such as interleukin (IL)-4, IL-5 and TNF-α [64,65] can provide a working model of the full immunoregulatory potential of LDLr. Second, the relevance of human iNKT cells sorted and expanded *in vitro* [66] by exogenous IL-2 has been questioned previously [67]. Third, while Van Den Elzen et al. exploited the need of GGC to enter the

endosome, the contribution of apoE containing lipoproteins associating with other antigenic lipids such as αGC was not addressed. In fact, a recent report examined this issue and found a slight decrease in efficiency in the activation of iNKT cells by loading mouse LDLr$^{-/-}$ BMDCs with αGC [68]. Lastly, multiple receptors involved in lipoprotein metabolism such as the VLDL receptor (VLDLr) and LDL receptor-related protein (LRP) have been shown to bind apoE, albeit with different binding affinities [69–72], and the contribution that VLDLr and LRP binding apoE-containing lipoproteins loaded with GGC and enhancing iNKT cell activation was only explored briefly by Van Den Elzen *et al.* in this report (Discussed in detail in section "LRP as a modulator of lipid function"). Therefore, multiple mechanisms need to be explored in order to understand how different cellular factors involved in lipid homeostasis can impact iNKT cell activation.

Scavenger receptors and iNKT cell activation

Scavenger receptors (SRs) are PRRs known to detect a wide variety of molecules of both host and foreign origin and target lipids to CD1d for iNKT cell activation. SRs include a large repertoire of surface and endosome-localized molecules mainly expressed in macrophages (MΦs) and dendritic cells (DCs) and play a wide range of functions in physiologic processes such as clearance of apoptotic cells [73,74], recognition of bacterial components [75], skewing adaptive T cell responses [76] and transfer of antigen between different types of APCs [77]. Classical SRs were initially described for their ability to recognize modified lipoproteins [78]. In particular, CD36 and scavenger receptor A (SRA) have been

studied extensively due to their contribution to atherosclerotic processes [79,80]. Recently, SRs were described as PRRs capable of targeting lipids to CD1d for activation of iNKT cells [68]. Using mouse BMDCs, Freigang *et al.* demonstrated that activation of iNKT cell hybridomas by αGC and *Sphingomonas*-derived lipid GSL-1 depended on expression of SRA. This was determined by using IL-2 secretion by iNKT cell hybridomas as a measure of activation. Mice deficient in SRA ($Sra^{-/-}$) were challenged with soluble αGC resulting in complete absence of IL-4 and delayed IFN-γ responses measured in serum, suggesting that expression of SRA directs glycolipids to activate iNKT cells *in vivo* . Additionally, $LDLr^{-/-}$ mice showed only diminished IL-4 and WT although delayed levels of IFN-γ in the serum and a complete dependence of LDLr to initiate an iNKT cell response to GGC. These results suggested that *in vivo* iNKT responses to soluble lipids are dependent on expression of multiple receptor pathways and absence of SRA and LDLr can have profound effects on iNKT cell activation. In this study Freigang *et al.* used global knockouts of SRs, which do not allow identification of the type of the APC affected by the absence of SRs and responsible for the perturbed iNKT cell responses. This matter is further complicated by reports showing that hyperlipidemia in $LDLr^{-/-}$ mice can modify iNKT cell responses to αGC [81] and serum from hypercholesterolemic $LDLr^{-/-}$ mice is sufficient to activate iNKT cells *in vitro* [82]. Conditional knockouts of SRs in different APCs by cre-loxP systems could be used to determine which cell type is responsible for targeting lipids via SRs to CD1d for presentation to iNKT cells. However, the results presented by Freigang *et al.* show the need for multi-ligand receptors for proper iNKT cell function.

LRP and its ligands

LRP is a multi-ligand receptor classically described to be a SR due to its ubiquitous expression and diversity of ligands. Initially described as two distinct receptors, LRP-1 (also known as CD91) and α2-macroglobulin receptor (α2MR) were later shown to share homology and function [83,84]. At the time, LRP-1 was known to bind the serum protease inhibitor α2M [85], tissue plasminogen activator (tPA) [86], and apoE containing lipoproteins [87]. Subsequently, in 2001, Herz *et al.* provided a list detailing 30 known LRP extracellular ligands and 12 scaffolding proteins known to interact with the intracellular domain of LRP [88]. However, since this list was compiled, a number of reports have been published linking LRP to a diverse array of ligands capable of modulating a number of physiological processes. Table 1 is a comprehensive list of these newly found ligands and cellular cofactors known to associate with LRP.

Numerous reports of molecules not shown to interact directly with LRP can still affect its cellular expression and function. One example is nerve growth factor, which initiates transcription and translation of LRP in a rat neural cell line (PC12), leading to a concomitant increase in protein content [89]. Expression and function of LRP in brain neurons is critical as its expression has been linked to multiple pathways that can lead to Alzheimer's disease such as clearance of amyloid precursor protein (APP) and multiple isoforms of apoE [90,91]. Another example of a molecule involved in LRP function without direct binding is insulin, which in contrast to NGF, causes a sharp decrease in protein content of LRP [92]. To make matters more complex, previous work has shown that infection by parasite *Trypanosoma*

Table 1. Ligands and co-factors known to bind or associate with LRP

LRP ligand	LRP expressing cell type	Physiological process affected	Citation
SHP-2	Human WI-38 fibroblasts	Vascular remodeling	[93]
GULP-1	Mouse neuroblastoma neuro 2A cells	Changes in trafficking of nuclear proteins	[94]
IVIg	Mouse fibroblastic MEF and PEA	Influence the ability of IVIg to cross the blood brain barrier	[95]
VacA	Gastric epithelial cell line AZ-521	Apoptosis and autophagy	[96]
CD44	FTC-133 human follicular thyroid carcinoma	Adhesive properties of tumor cells	[97]
CCN2	Human chondrocytic cell line HCS-2/8	Endochondral ossification	[98]
Cathepsin D	Human mammary fibroblasts	Growth of fibroblasts in tumor microenvironment	[99]
Decorin	Mouse skeletal muscle cell line C2C12	Proliferation, differentiation and extracellular matrix signaling	[100]
sorLA/LR11	Mouse neuroblastoma neuro 2A cells	Trafficking of proteins from perinuclear compartments	[101]
Alpha2ML1	Primary human keratinocytes	Protease activity in the serum	[102]
Plasma derived factor V	Megakaryocyte-like cell line CMK and ex-vivo derived megakaryocytes	Endocytosis of protein involved in coagulation	[103]
Transglutaminase	Human (MRC-5, WI-38, U251). Mouse NIH3T3	Cell matrix adhesion and endocytosis	[104]
Melanotransferrin	Human U87 glioblastoma and mouse MEFs	Modulates uptake of plasminogen in conjunction with LRP	[105]
DSPAalpha1	Rat brain cells	Shuttles DSPAalpha1 across the blood brain barrier	[106]
Heparanase precursors	MEFs, HEK 293-T cells and CHO K1 cells.	Regulation of cell growth, migration and differentiation	[107]
Presenilin	Human neuroblastoma, human embryonic kidney and MEFs	Clearance of amyloid precursor protein which has been linked to Alzheimer's disease	[108]
β-2 integrin	HUVECs, human histiocytic lymphoma U937 cell line.	Leukocyte adhesion	[109]
Ceramide	J774 macrophages HepG2 cells	Enhances uptake of lipid particles	[110]
Plasminogen activator inhibitor 1	Human smooth muscle, endothelial, and dermal microvascular cells.	Stimulates cell migration and chemotactic effects	[111]
MafB	HEK 293	Modulates transcriptions of proteins involved in brain development	[112]
Neuroserpin	MEFs and mouse PEA13	Co-factor necessary for neuroserpin internalization	[113]
Connective tissue growth factor	Rat hepatic stellate cells	Modulates fibrotic processes	[114]
Leptin	Mouse brain cells	Expression in brain for energy required for leptin signaling	[115]

cruzi can have differential effects on LRP expression in distinct strains of mice [116]. Livers and hearts derived from C3H mice showed an increase in LRP mRNA when infected by *T. cruzi* while tissues from C57BL/6 mice downregulated levels of LRP. Interestingly, this resulted in an overall increase of total α2M serum levels and decreased parasite load in C3H mice. Further research showed that a cysteine protease, cruzipain, secreted by *T. cruzi* essential for parasitic infection, binds extracellular α2M followed by endocytosis through LRP [117]. Mice deficient in α2M are increasingly susceptible to *T. cruzi* infection resulting in heart fibrosis and inability to mount a proper immune response to clear this pathogen [116]. Therefore, the increased levels of LRP and α2M can directly influence the pathogenicity of *T. cruzi* and serve as a defense mechanism capable of disabling infectious molecules. All this information shows that LRP is a receptor that can protect the host from infection and whose expression can be modulated in response to diverse physiological stimuli.

LRP as a modulator of lipid function

LRP has been shown to modulate lipid function by facilitating clearance of chylomicron (CM) remnants in the liver. CMs are apoA-I and apoC containing lipoprotein particles synthesized in the small intestine that are mainly composed of triglycerides (TGs) (85-92%). The remaining content of CMs is made up of phospholipids (6-12%) and cholesterol (1-3%) [118]. CMs have been described to be as the primary vessels of dietary fatty acid transport to peripheral tissues from the digestive tract through the circulation [119]. When CMs travel through endothelial

capillaries the action of lipoprotein lipase (LPL) hydrolyzes TGs on the surface of CMs, and catalyzing the exchange of fatty acids from CM to tissues such as heart, muscle, liver and brain [120–122]. Once the majority of the TGs found in CMs have been hydrolyzed, the CM remnant loses apoA-I, apoC and acquires additional apoE and apoB-48. These changes target CM to be recaptured by the LDLr and LRP expressed in the liver and the remaining contents of CM remnants (cholesteryl esters, phospholipids) are either released through the bile excretion pathway or recycled and loaded with TGs. Due to their depletion of TGs, CM remnants are enriched in cholesterol and their accumulation in the blood has been linked to the development of atherosclerosis in humans [123,124].

Both LDLr and LRP have been shown to work synergistically and their expression in the liver leads to rapid removal of CM remnants. Initial evidence of this mechanism was shown *in vivo* when WT mice were treated with LDLr neutralizing Ab or LRP inhibitor RAP, which decreased uptake of ^{125}I radiolabeled CM remnants by 50% and 15%, respectively [125]. In addition to this, the use of mice with a genetic deletion of LDLr and LRP inhibitor RAP introduced by adenoviral vector eliminated the binding of LDLr and decreased LRP expression in the liver (75%) respectively. This combination led to an accumulation of CM remnants an order of magnitude higher than the independent conditions (LDLr deletion of adenoviral expression of RAP) [126]. Another contributing factor of LRP to CM remnant metabolism is the knowledge that it can independently bind the lipoprotein lipases responsible for hydrolysis of TGs [127]. This would project the LRP to have a more prominent role in lipoprotein metabolism by orchestrating the hydrolysis of TGs and

their delivery to non-hepatic tissues. In addition to its contribution to CM remnant clearance and TG hydrolysis, it has been shown that inhibition of LRP binding *in vivo* can lead to elevated levels of β-VLDL (VLDL remnants) in the circulation [128]. Altogether these reports show that LRP, in concert with LDLr, can affect critical pathways of lipoprotein metabolism and their inability to function in the liver disrupt the ability to clear cholesterol rich CM from the circulation leading to dysregulation of lipoprotein content.

LRP as a modulator of adaptive immunity

The expression of LRP in APCs modulates function of the adaptive immune system by promoting activation of T cells. One of the ways in which LRP does this is by increasing the content of extracellular components that can be internalized by APCs and presented to T cells. An early example of this was shown by studying the way in which α2M can serve as a vessel of transport for the model antigen hen egg lysozyme (HEL) [129]. In this study, it was determined that α2M can form complexes with HEL only while α2M is undergoing activation by the nucleophile action of methylamine. Activation of α2M induces a conformational change hypothesized to "trap" and inactivate proteins in the serum. This is followed by endocytosis of α2M-HEL complexes, which is known to be the main mechanism by which α2M promotes inhibition of serum proteinases. In this report it was shown that the ability of HEL to induce secretion of IL-2 by HEL-specific T cell hybridomas was increased 200-fold when incubated with MΦs that had been pulsed with α2M-HEL complexes. These results showed that antigenic proteins in complex with LRP ligands can undergo

endocytosis at a much faster rate than non-bound antigenic proteins and can also lead to enhanced activation of T cells.

LRP ligands manipulated *in vitro* can be used *in vivo* to elicit targeted T cell responses. An example showing this is the adjuvant effect of heat shock proteins (HSPs). HSPs are molecular chaperones whose expression is upregulated when cells are exposed to temperature increase and in response to other stressors such as glucose and oxygen deprivation [130]. It was initially thought that the main function of HSPs is to prevent proteins from aggregating or mis-folding when cells are exposed to extracellular stressors. However it has been shown that basal levels of HSPs are present under normal cellular conditions and they promote folding of nascent proteins [131]. Early studies by Udono *et al.* showed that an HSP measured at 70 kilodaltons (hsp70) can be isolated from methylcholanthrene (Meth A) induced sarcoma in BALB/cJ mice and elicit a protective immune response against tumors [132]. In this work, mice immunized with hsp70 isolated from Meth A tumors showed a dramatic reduction in tumor size when a high dose of hsp70 (9 μg/mice) was used. However, tumor protection was lost when hsp70 isolated from healthy tissues was used as an immunization agent. These results showed that hsp70 from healthy tissues lack tumor protection, yet the hsp70 derived from tumors contains critical components capable of fending off tumor formation. Later research by Blachere *et al.* showed that hsp70 can be a carrier of small peptides that elicit cytotoxic T cell responses [133]. For example, the authors showed that peptides derived from ovalbumin and vesicular stomatitis virus led to a robust generation of $CD8^+$ T cell responses with antigenic specificity when immunized with hsp70:peptide complex,

while hsp70 or peptide alone failed to generate antigen-specific $CD8^+$ T cells. In this manner hsp70 functions as an adjuvant which can lead to T cell responses restricted by MHC class I. Interestingly, LRP has been shown to be the main receptor capable of binding hsp70 [134] and its expression in APCs is the major contributor to T cell responses [135]. Additionally, the LRP endogenous inhibitor RAP can inhibit HSP binding on the surface of APCs [136] and also diminish the ability of HSP:peptide complexes to elicit adaptive immune responses [137]. In this system, LRP binding to its ligand is a major contributor to adaptive immunity by enhancing the adjuvant effect by HSPs.

LRP expression and its association to human pathologies

LRP and Cancer

The role of LRP in cancer has been studied extensively with mixed results and conclusions [138,139]. Several reports have shown that LRP loss/downregulation can lead to aggressive cancer phenotypes and unfavorable clinical outcomes [140–145]. However other studies have shown that increased expression of LRP can have a similar effect [121,146–149]. While a clear role for LRP in cancer has not been defined, the most likely explanation is that LRP function is dependent on the tumor microenvironment and the molecules available for LRP binding that can modulate tumor growth.

LRP and Alzheimers Disease (AD)

LRP can bind ligands involved in the pathogenesis of AD such as amyloid precursor protein (APP), apoE and α2M [150,151]. All of these have been shown to be independent contributors to the progression of AD. Additionally, multiple genome wide association studies have shown that LRP polymorphism C667T has a genetic link to the progression of AD [152–154] and APP uptake rate in the temporo-parietal cortext of the brain is affected in patients with this genetic mutation [155]. Surprisingly, the C to T change in exon 3 of the LRP gene does not lead to an alteration in amino acid sequence and the resulting protein in patients with the C667T mutation is identical to those without it. While this discounts a possible mechanistic link or change in LRP function, it raises the possibility that LRP protein levels are affected over time because of this mutation. Coincidentally, LRP levels in humans have been shown to decrease over time as part of the brain aging process, but at a much faster rate in AD patients [151]. Mutations that decrease LRP expression in the brain could have severe defects in the degradation of APP protein which is a major contributor of AD pathology.

LRP and multiple sclerosis (MS)

Loss of myelin in neurons is the key pathologic event that leads to MS. LRP has been shown *in vitro* to be involved in the binding and uptake of myelin [156]. Due to this, LRP had been designated as a potential contributor to MS, but little clinical evidence exists that supports this notion. However, a recent report in which frozen brain tissue from 13 MS patients was analyzed post mortem showed a significant upregulation of

LRP mRNA levels (p=0.008) in MS brains when compared to healthy human controls of similar age groups [157]. While it is doubtful that LRP function is a causative agent of MS, one could speculate that once disease is initiated and myelin begins to be degraded, increased levels of LRP could accelerate the rate at which neurons are stripped of this essential protein. Therefore, inhibition of LRP function could substantially decrease the rate at which myelin degradation occurs in MS.

LRP and proliferative retinopathy (PR)

Given the ubiquitous nature of LRP expression and its diverse ligands, in some cases its role in human pathologies has only been investigated briefly. For example, increased expression of LRP has been reported in PR where it was found to be localized in the inner limiting membrane, astrocytes and inner photoreceptor matrix of diabetic patients [158]. In addition, the same report shows an increase of α2M presence in the neo-vascularized tissue measured by immunohistochemistry. In a separate study, α2M prevalence was found in a proteomic analysis of plasma from diabetic patients and suggested that it could be used as a pathological marker to predict diabetic patients who will suffer from PR [159]. All of these features were absent in healthy controls and strongly suggests that LRP ligand binding and LRP signaling could be involved in the pathology of PR.

LRP and ischemic cardiomyopathy (ICM)

Expression of LRP can lead to lipid accumulation in the form of intracellular cholesteryl esters (CE) in human ischemic myocardium. One of the main features

leading to ICM is lipid overload experienced by cardiomyocytes that lead to contractile dysfunction and arrhythmias [160]. Researchers have speculated that overexpression of LRP and other lipoprotein receptors in ischemic hearts can modulate uptake and accumulation of CEs. In fact, quantitative mRNA and Western blotting in hearts from 18 ICM patients showed significant upregulation in ischemic hearts and that only LRP had a direct correlation with the levels of CE in cardiomyocytes [161]. The possibility exists that inhibition of LRP binding in the ischemic heart could help decrease the intracellular accumulation of CEs.

Summary

INKT cells have been determined by multiple research groups to play a role in a myriad of chronic inflammatory and autoimmune disorders. In some such as EAE, iNKT cell activation ameliorates disease scores, while in others such as atherosclerosis it exacerbates lesion areas. In fact, the immunoregulatory potential of iNKT cells makes them attractive therapeutic targets to modulate progression of human disease. The activation of iNKT cells is mediated by the presence of exogenous and endogenous lipids and their access to the presentation machinery of APCs.Further research is required to understand the manner in which modification of glycolipids can affect the quality of the iNKT cell response and how the presence of lipoprotein receptors influences iNKT cell secretion of pro- and anti-inflammatory cytokines.

Critical components of the iNKT cell presentation machinery include proteins involved in lipoprotein metabolism that can have direct or indirect effects in the ability

of APCs to prepare and load glycolipids onto CD1d. This step has been shown to specifically involve receptors that share homology and function with LRP such as LDLr. Due to its wide expression in leukocytes, in particular APCs, and ability to survey endocytic compartments, we hypothesize that LRP can play a role in the intracellular processes that lead to iNKT cell activation. The work presented in this dissertation details a new mechanism by which a previously known lipoprotein receptor modulates iNKT cell activation. Only with the acquisition of this knowledge, will scientists and clinicians be able to devise strategies that harness the ability of iNKT cells for the benefit of human health.

CHAPTER II

SPECIFIC DELETION OF LDL RECEPTOR-RELATED PROTEIN ON MACROPHAGES SKEWS CYTOKINE PRODUCTION BY INVARIANT NATURAL KILLER T CELLS

Abstract

Expression of molecules involved in lipid homeostasis such as the low density lipoprotein receptor (LDLr) on antigen presenting cells (APCs) has been shown to enhance invariant natural killer T (iNKT) cell function. However, the contribution to iNKT cell activation by other lipoprotein receptors with shared structural and ligand binding properties to the LDLr has not been described. In this study, we investigated whether a structurally related receptor to the LDLr, known as LDL receptor-related protein (LRP), plays a role in iNKT cell activation. We found that, unlike the LDLr which is highly expressed on all immune cells, the LRP was preferentially expressed at high levels on $F4/80^+$ macrophages (MΦs). We also show that $CD169^+$ MΦs, known to present antigen to iNKT cells, exhibited increased expression of LRP compared to $CD169^-$ MΦs. To test the contribution of MΦ LRP to iNKT cell activation we used a mouse model of MΦ LRP conditional knockout (LRP-cKO). LRP-cKO MΦs pulsed with glycolipid alpha-galactosylceramide (αGC) elicited normal IL-2 secretion by iNKT hybridoma and *in vivo* challenge of LRP-cKO mice led to normal IFN-γ, but blunted IL-4 response in both serum and intracellular expression by iNKT cells. Flow cytometric analyses show similar levels of MHC class-I like molecule CD1d on LRP-cKO MΦs and normal glycolipid

uptake. Survey of the iNKT cell compartment in LRP-cKO mice revealed intact numbers and percentages and no homeostatic disruption as evidenced by the absence of programmed death-1 and Ly-49 surface receptors. Mixed bone marrow chimeras showed that the inability iNKT cells to make IL-4 is cell extrinsic and can be rescued in the presence of wild type APCs. Collectively, these data demonstrate that, although MΦ LRP may not be necessary for IFN-γ responses, it can contribute to iNKT cell activation by enhancing early IL-4 secretion.

Introduction

Although research has vastly increased our knowledge of iNKT cell function, less is known about the cellular components in antigen presenting cells (APCs) that present glycolipid ligands to activate iNKT cells. Studies focused on understanding iNKT cell immunity have shown that proteins classically involved in lipoprotein metabolism, such as the microsomal triglyceride transfer protein (MTP) [55], low density lipoprotein receptor (LDLr) [62], scavenger receptors (SRs) [68], and cholesterol membrane transporters [162] can modulate iNKT cell homeostasis and activation. The mechanisms by which MTP, LDLr and SRs on APCs modulate iNKT cell activation are unknown, but data suggest a critical role in glycolipid uptake and presentation. MTP has been shown to be essential in the loading of endogenous lipids into the hydrophobic pocket of CD1d [163] serving to stabilize this molecule for appropriate expression. MTP-deficient APCs lack CD1d expression on the cell surface and iNKT cells fail to develop in fetal thymus organ culture when treated with MTP inhibitors [57]. On the other hand the LDLr binds to apoE present on the surface of many lipoproteins, which can function as vessels for

glycolipid transport. Targeting glycolipid uptake via the LDLr in APCs enhances CD1d-mediated antigen presentation and leads to increased iNKT cell activation [62,63]. This shows that molecular pathways related to lipoprotein homeostasis and metabolism are closely linked to the modulation of iNKT cell function.

A similar protein to LDLr initially described in lipoprotein metabolism, but later found to bind approximately 30 different ligands, is the LDL receptor-related protein (LRP, also referred to as CD91) [164]. In mice, LRP was initially discovered in hepatocytes [165], but is currently thought to be expressed in most cells of the body, including many immune cells [88,134,166–168]. The expression of LRP on APCs has been shown to enhance the adaptive immune response by facilitating antigen uptake [169–171]. LRP can modulate innate immune responses by binding pseudomonas exotoxin A [172], rhinovirus particles [173] and collectins [174]. For iNKT cell activation, the LRP may be important by binding apoE-containing lipoproteins [175], facilitating processing of the lipid transport proteins called saposins (necessary for CD1d loading) [176] and/or by interacting with the ER chaperone protein calreticulin on the cell's membrane leading to phagocytosis of opsonized pathogens and apoptotic bodies [177]. Given these characteristics of LRP and the fact that it can actively recycle through endocytic compartments [178], we hypothesized that this surface receptor plays an active role in glycolipid antigen presentation and subsequent activation of iNKT cells. In this study, we demonstrate that LRP is highly expressed in specialized macrophages (MΦs) capable of iNKT cell activation. We also show that MΦ LRP can modulate iNKT activation *in vivo* and is necessary for IL-4, but not IFN-γ secretion.

Results

LRP is differentially expressed on immune cells

We measured LRP expression on immune cells isolated from tissues in which iNKT cells are known to reside and encounter antigen. We collected spleen, liver and lymph nodes from C57Bl/6 (WT) mice and stained with a fluorescently labeled antibody (5A6) specific for the 85 KDa subunit of LRP [84]. Flow cytometric analysis demonstrated that F4/80$^+$ splenic MΦs express the highest levels of LRP (ΔMFI = 44.7 ± 2.4) compared to T cells (ΔMFI = 1.6 ± 0.4), B cells (ΔMFI = 10.4 ± .35) and DCs (ΔMFI = 15.1 ± 0.7) (Figure 5A, B, top row). Similar LRP expression profiles were observed on immune cells isolated from the liver and lymph nodes (Figure 5 A, B middle and bottom rows).

CD169$^+$ MΦs express high levels of LRP

Recent reports have shown that MΦs expressing sialic acid binding immunoglobulin-like lectin 1 (Siglec-1, also known as CD169) can mediate potent activation of iNKT cells in secondary lymphoid tissues [179]. CD169$^+$ MΦs can cross-present tumor antigens [180] and take up liposomal particles conjugated to CD169 ligands [181]. One such study demonstrated that αGC conjugated to CD169 ligands elicited an iNKT cell response two orders of magnitude higher that administration of free

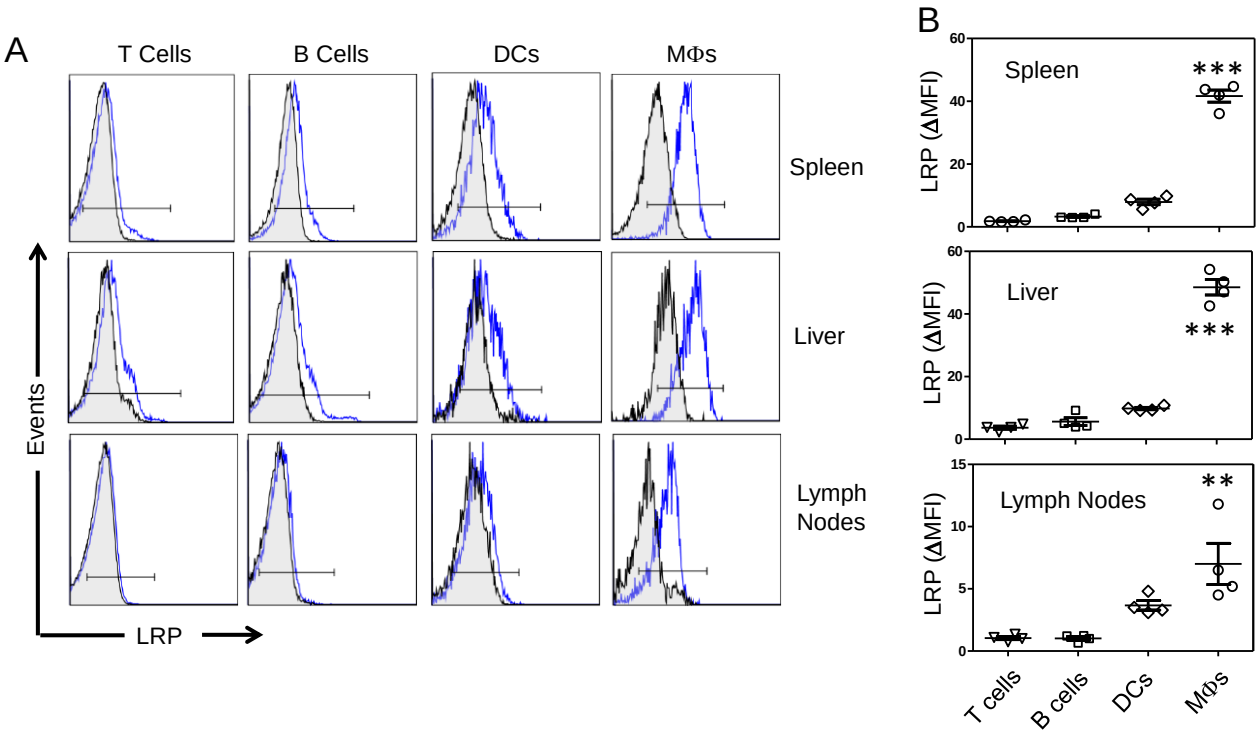


Figure 5: LRP expression in T cells, B cells, DCs and MΦs. (A) Representative histograms for LRP (blue line) versus isotype control (black line) staining in spleen (top row) liver (middle row) and lymph nodes (bottom row). Mononuclear lymphocytes from from 8-to-12 week old WT mice (n=4) were stained with fluorochrome-conjugated antibodies for T cells (TCRβ), B cells (B220), DCs (CD11c) and MΦs (F4/80). **(B)** Graphs showing quantification of mean fluorescent intensity (MFI =LRP Antibody- isotype antibody) of LRP on lineage-specific populations. Symbols represent individual mice. Bars represent mean and standard error. *** denotes $p < 0.0001$ compared to T cells, B cells and DCs. ** denotes $p < 0.001$ compared to T cells and B cells. *P* value was determined by a one-way ANOVA with a Bonferroni post-test. Histograms are representative of three separate experiments with 4 mice each. Scatter plots show individual mice from one representative experiment of three separate experiments.

αGC [182]. Interestingly, we found significantly higher LRP expression levels on F4/80^{+}CD169^{+} MΦs compared to F4/80^{+}CD169^{-} MΦs in spleen (Figure 6A) and liver of WT mice (Figure 6B). These data demonstrate that LRP is highly expressed on MΦs known to elicit potent iNKT cell responses.

Conditional knockout leads to deletion of LRP on MΦs

In order to test MΦ LRP function in iNKT cell activation, we used mice harboring a MΦ knockout of LRP [183]. In this mouse model, LRP$^{flox/flox}$ mice were crossed with LyzM-Cre mice, which have cre recombinase under the control of the lysozyme M promoter. Flow cytometry staining of splenic and liver MΦs (Figure 7A and 7B) shows that, compared to WT, LRP-cKO mice have decreased LRP expression on splenic and hepatic MΦs (71% and 68%, respectively). As expected, other immune cells in LRP-cKO mice such as T cells, B cells and DCs had no reduction of LRP expression, verifying MΦ specificity. Further analysis of LRP-cKO MΦs subpopulations showed that CD169^{+} MΦs have a 52% reduction in LRP expression (Figure 8). The staining observed in LRP-cKO MΦs could be attributed to non-specific antibody binding, however the isotype control produces a negative signal, showing that 5A6 antibody staining is because of residual LRP expression. Taken together, these data show that LRP-cKO mice have a deletion of LRP on MΦ, including specialized CD169^{+} MΦs that are capable of presenting antigens to iNKT cells.

LRP deficiency on MΦ does not alter CD1d expression or iNKT cell homeostasis

We asked whether LRP deletion in MΦs leads to changes in CD1d expression or iNKT cell homeostasis. LRP deletion had no effect on CD1d expression either on spleen

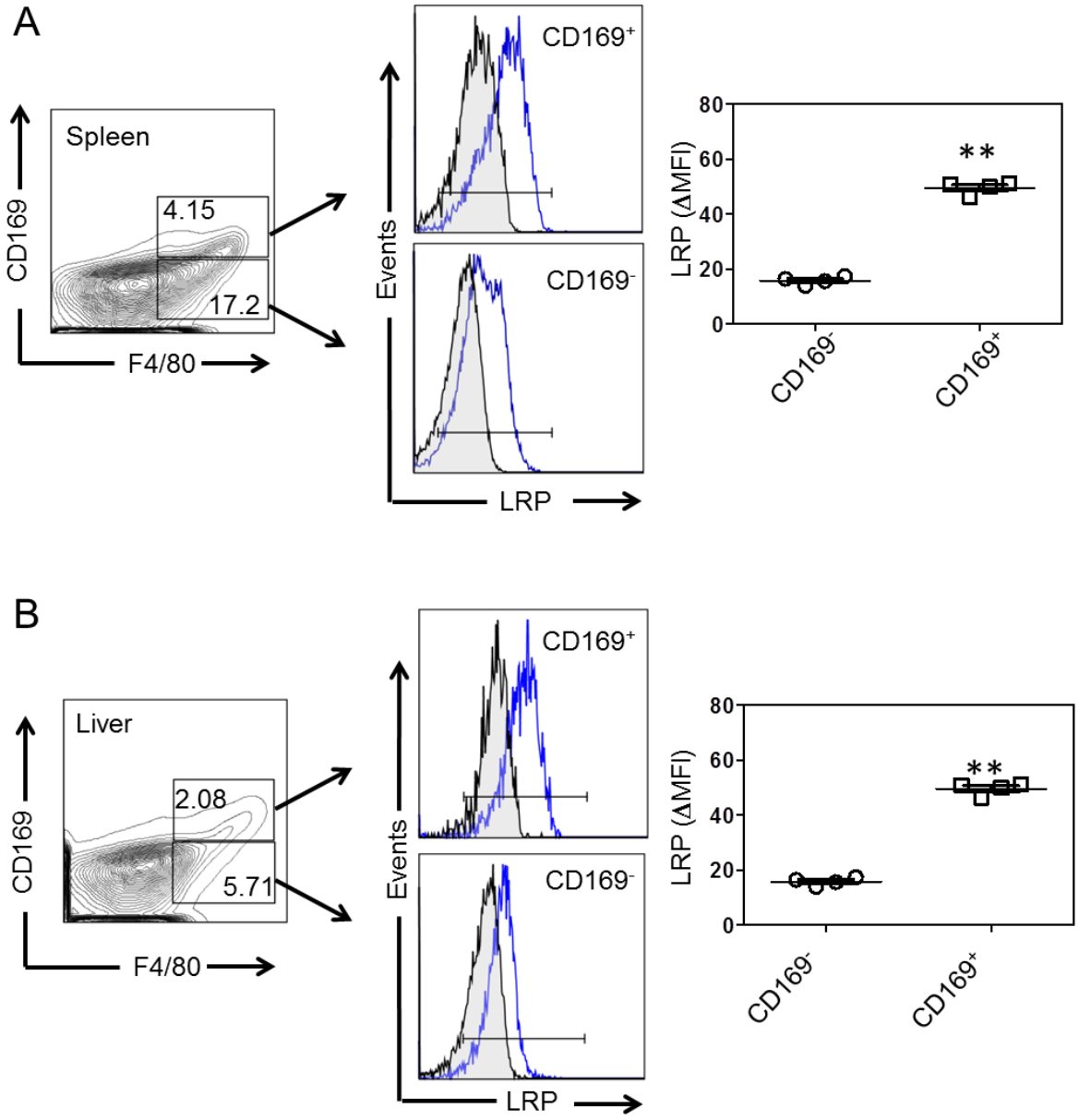


Figure 6: LRP expression in CD169$^+$ MΦs. (A) Spleen and **(B)** liver mononuclear cells from 8-to-12 week old WT mice (n=4) were stained with fluorochrome-conjugated antibodies for MΦs (F4/80) and CD169. Left panels show dot plots for F4/80 and CD169 and middle panels show representative histograms of LRP expression (blue line) versus isotype control (black line). Graphs show quantification of mean fluorescent intensity (MFI =LRP antibody- isotype antibody). Bars represent mean and standard error. **denotes $p < 0.001$. *P*-value determined by Student's *t*-test. Histograms are from one representative experiment of three separate experiments. Scatter plots show individual animals from one representative experiment of three.

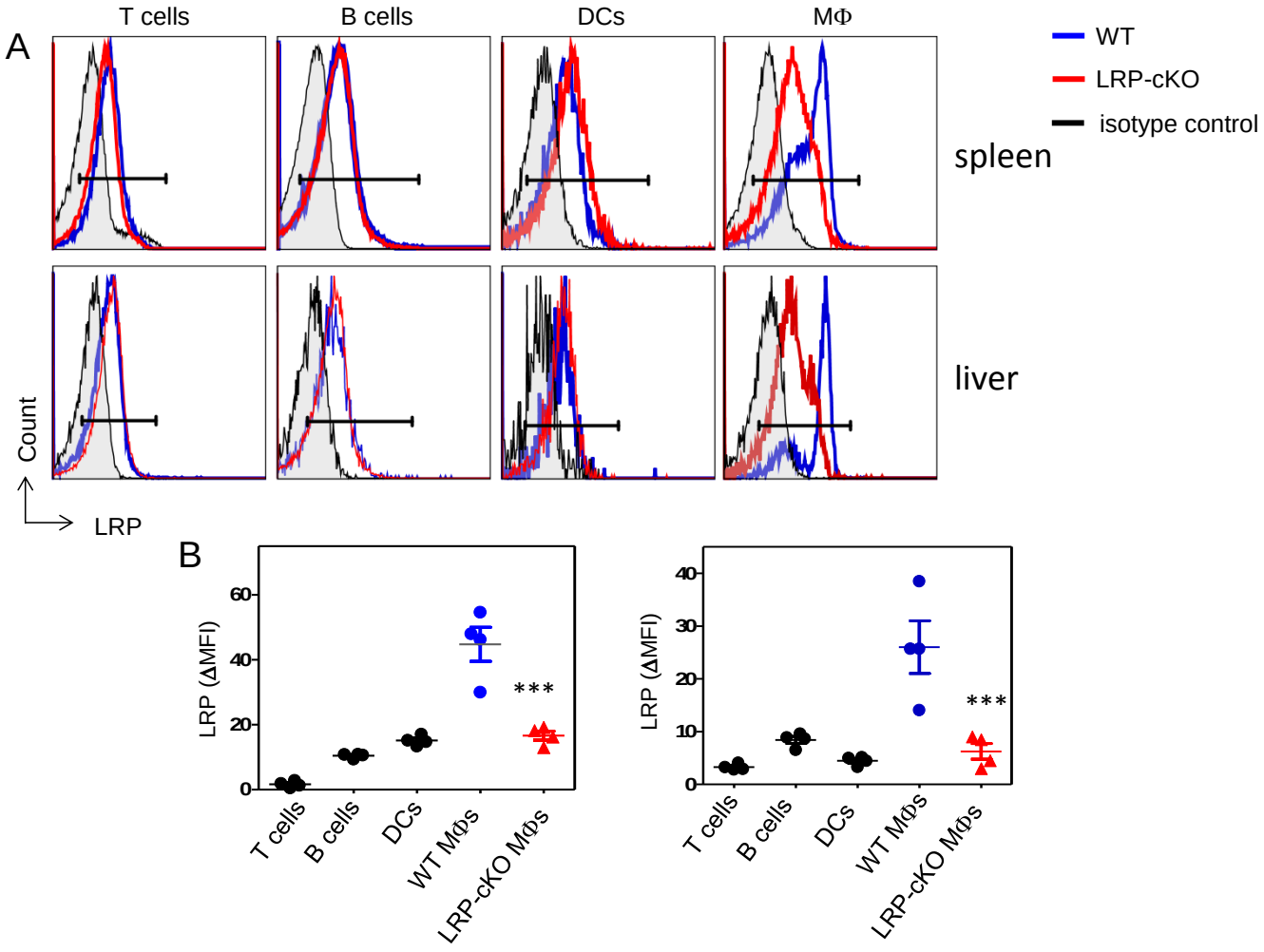


Figure 7: LRP-cKO mice exhibit knockout of LRP in MΦs. (A) Spleen and liver mononuclear cells from 8-to-12 week old WT and LRP-cKO (n=4) were stained with fluorochrome-conjugated antibodies for T cells (TCRβ), B cells (B220), DCs (CD11c) and MΦs (F4/80). **(B)** Graphs show quantification of mean fluorescent intensity (MFI =LRP antibody MFI- isotype antibody MFI) of LRP. Each symbol represents an individual mouse from one representative experiment of three separate experiments. Bars represent mean and standard error. *** denotes $p < 0.0001$ and ** denotes $p < 0.001$ when comparing WT MΦs to LRP-cKO MΦs, p value was determined by Student's t test.

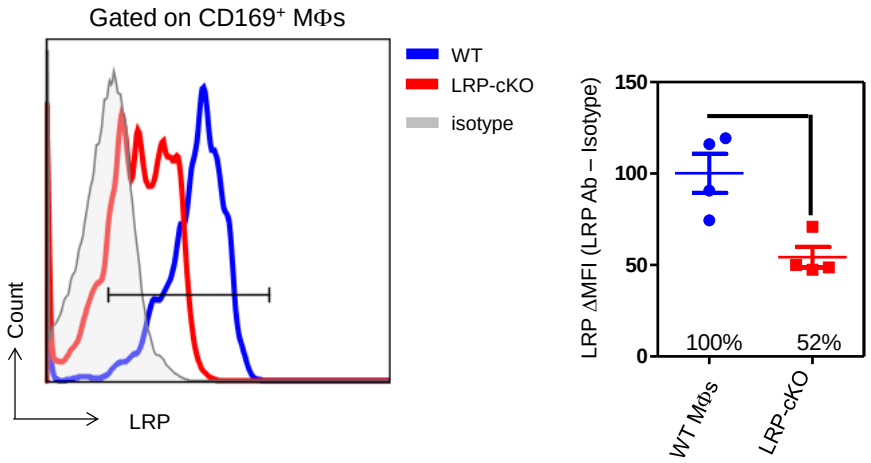


Figure 8: LRP-cKO mice exhibit knockout of LRP in CD169^{+} MΦs. Splenocytes from 8-to-12 week old WT (n=4) and LRP-cKO (n=4) were stained with fluorochrome-conjugated antibodies MΦs (F4/80) and CD169. Graph shows quantification of mean fluorescent intensity (MFI =LRP antibody MFI- isotype antibody MFI) of LRP. Each symbol represents an individual mouse from one representative experiment of three separate experiments. Bars represent mean and standard error.

MΦs, liver MΦs, or double positive ($CD4^+CD8^+$) thymocytes (Figure 9A). Co-cultures of WT or LRP-cKO thymocytes with iNKT hybridoma (DN32.D3) led to similar levels of IL-2 secretion (Figure 9B), showing that LRP-cKO thymocytes have no defects in presentation of endogenous glycolipid ligands. Analysis of thymic cells demonstrates normal expression of development markers in LRP-cKO iNKT cells when compared to WT (Figure 10). Additionally, iNKT cells, isolated from spleen and liver were present at normal frequencies (Figure 11A-C) and expressed normal levels of the prototypic markers of homeostasis and activation (Figure 11D and E). Finally, *in vitro* uptake of BODIPY-labeled αGC was not affected by LRP deficiency on MΦs as measured by flow cytometry (Figure 12A and B). However, a pulse-chase experiment where unlabeled αGC was used to pulse pMΦs prior to chase with BODIPY-αGC showed a faster turnover of LRP-cKO pMΦs as BODIPY-αGC was higher than WT pMΦs (Figure 12 C and D). This difference is observed as early as 4 hours, but reaches statistical significance only at 8 and 16 hours. These data suggests that LRP deletion in MΦs does not affect uptake of glycolipids but can alter cellular glycolipid turnover.

LRP deficiency on MΦs does not affect iNKT activation in vitro

Although we did not see changes in uptake of αGC by LRP-deficient MΦs, LRP is also important for cell signaling. Therefore, to determine whether decreased LRP expression modulates iNKT cell activation, we first harvested thioglycollate-elicited peritoneal MΦs (pMΦs) from WT and LRP-cKO mice and determined their LRP expression. Similar to previous reports [183], we found a 53% decrease in LRP expression on LRP-cKO pMΦs when compared to WT (Figure 13A and B). To measure

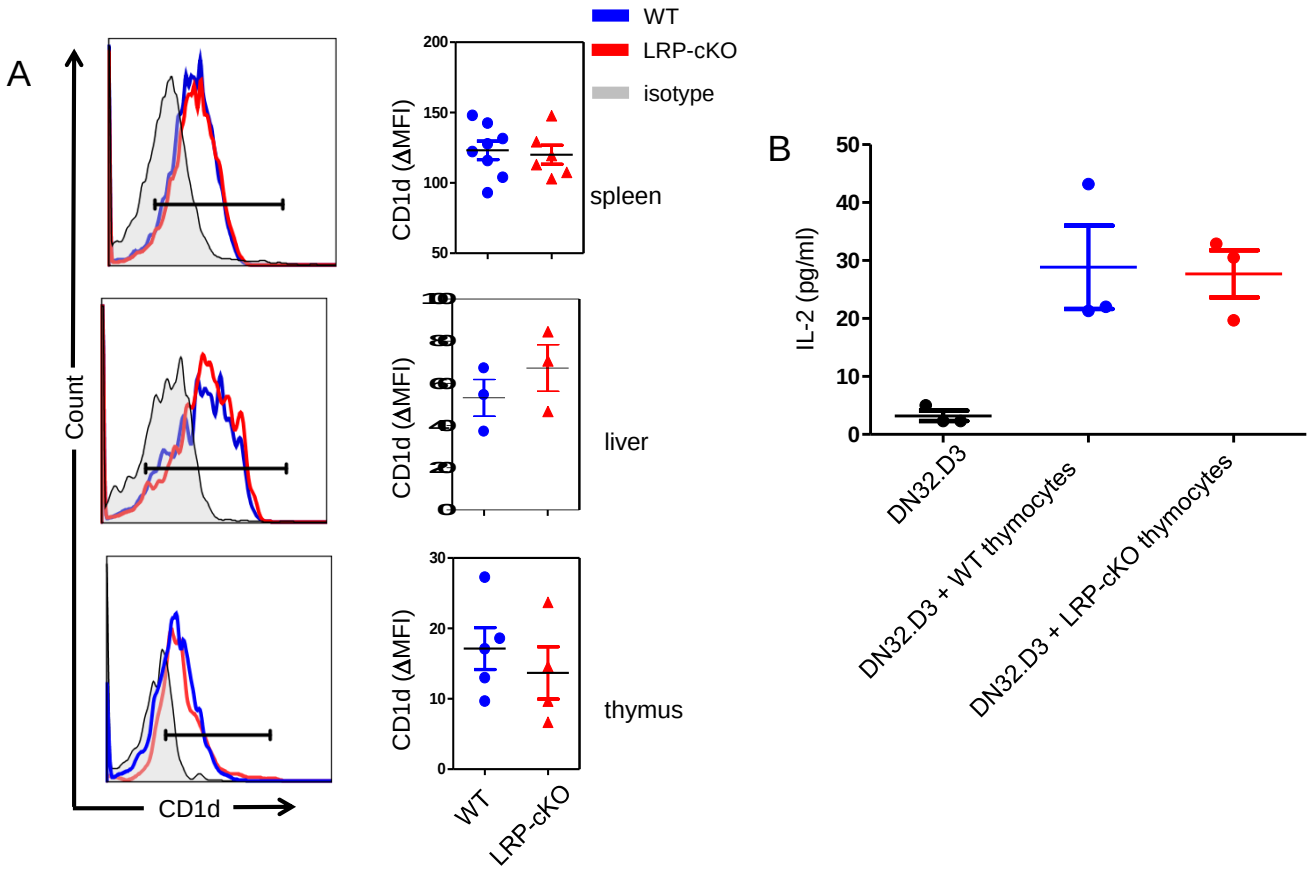


Figure 9: CD1d expression in spleen MΦs , liver MΦs and double positive ($CD4^+$ $CD8^+$) thymocytes. (A) Dot plots (left panels) of pMΦs stained with fluorochrome-conjugated antibodies B cells (B220), and pMΦs (CD11b). Representative histogram (middle) shows LRP expression gated on $CD11b^+$ cells. Mean fluorescent intensity (LRP antibody – isotype antibody). **(B)** ELISA measurement of IL-2 production by DN32.D3 hybridomas cocultured with WT or LRP-cKO thymocytes. Data points show standard error and mean. Data is representative of three independent experiments.

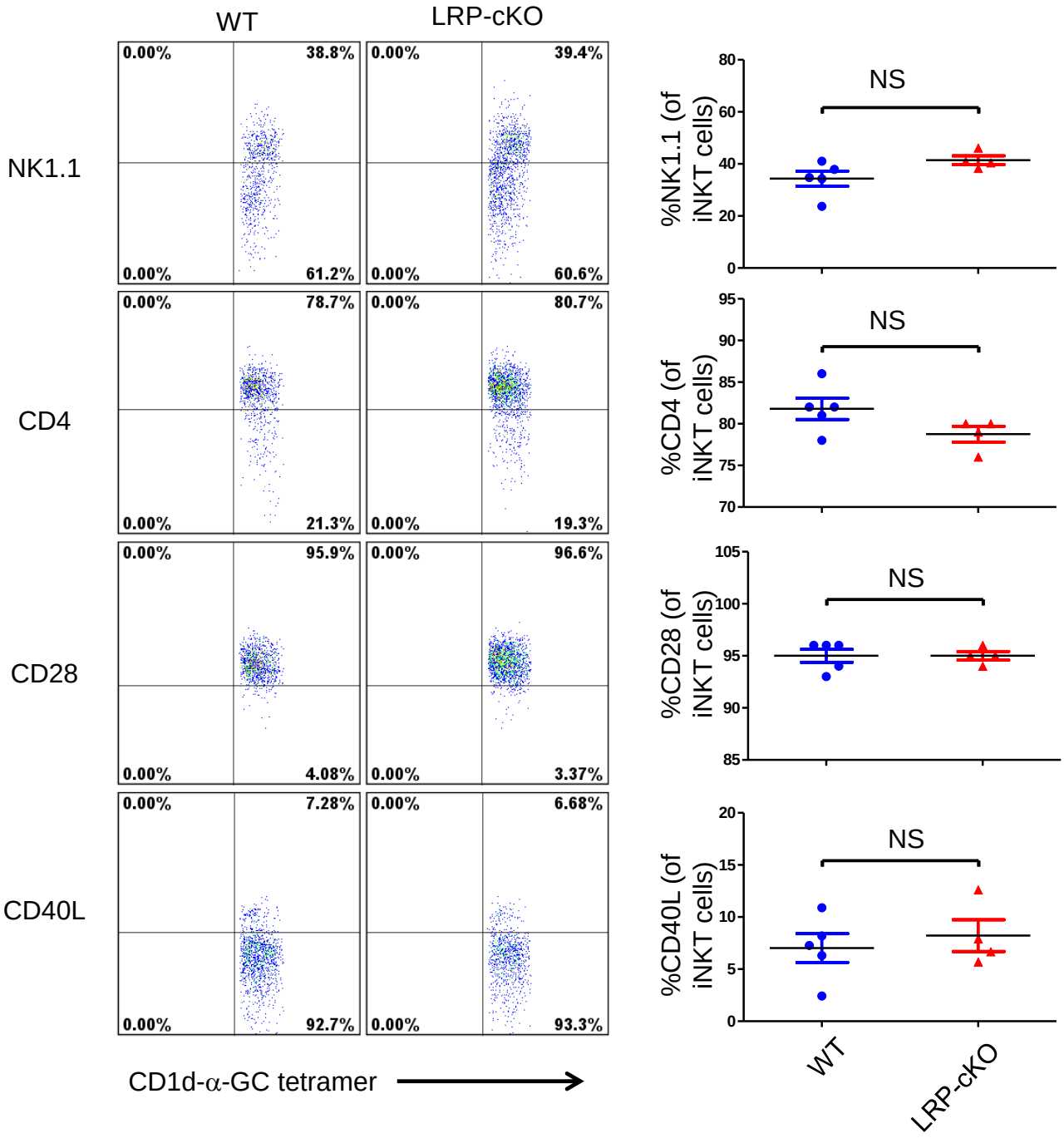


Figure 10: Thymic analysis of iNKT cells. Representative dot plots (left panels) of thymic cells and quantification (scatter plots). Cells were stained with fluorochrome conjugated antibodies for B cells (B220), T cells (TCRβ), αGC-CD1d-tetramer, and homeostatic markers of iNKT cells: NK1.1, CD4, CD28 and CD40L.

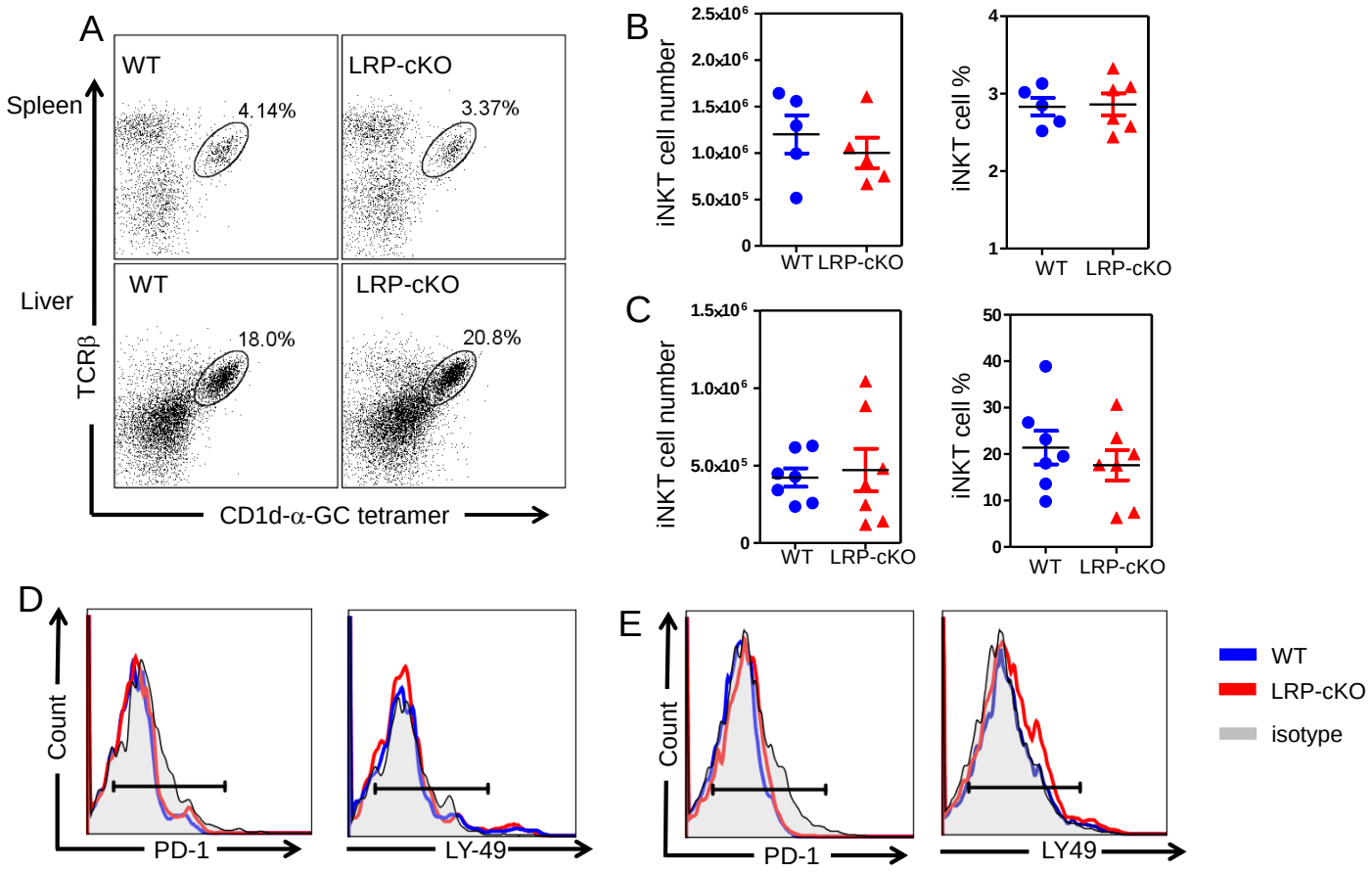


Figure 11: LRP-cKO deficiency in MΦs leads to normal development of iNKT cells. (A) Spleen and **(B)** liver mononuclear cells from 8-to-12 week old WT and LRP-cKO were stained with fluorochrome-conjugated antibodies for T cells (TCRβ), B cells (B220) and CD1d-αGC tetramer. iNKT gate ($TCR\beta^{int}Tet^{+}B220^{-}$) shows frequency of iNKT cells. Graphs show quantification of total iNKT cell number and frequency in **(B)** spleen and **(C)** liver, bars represent mean and standard error. Spleen (left dot plot) and liver (right dot plot) iNKT cells stained with **(D)** PD-1 and **(E)** Ly-49 fluorochrome conjugated antibodies. Shown are representative histograms gated on iNKT cells. Results are representative of one experiment from three independent experiments.

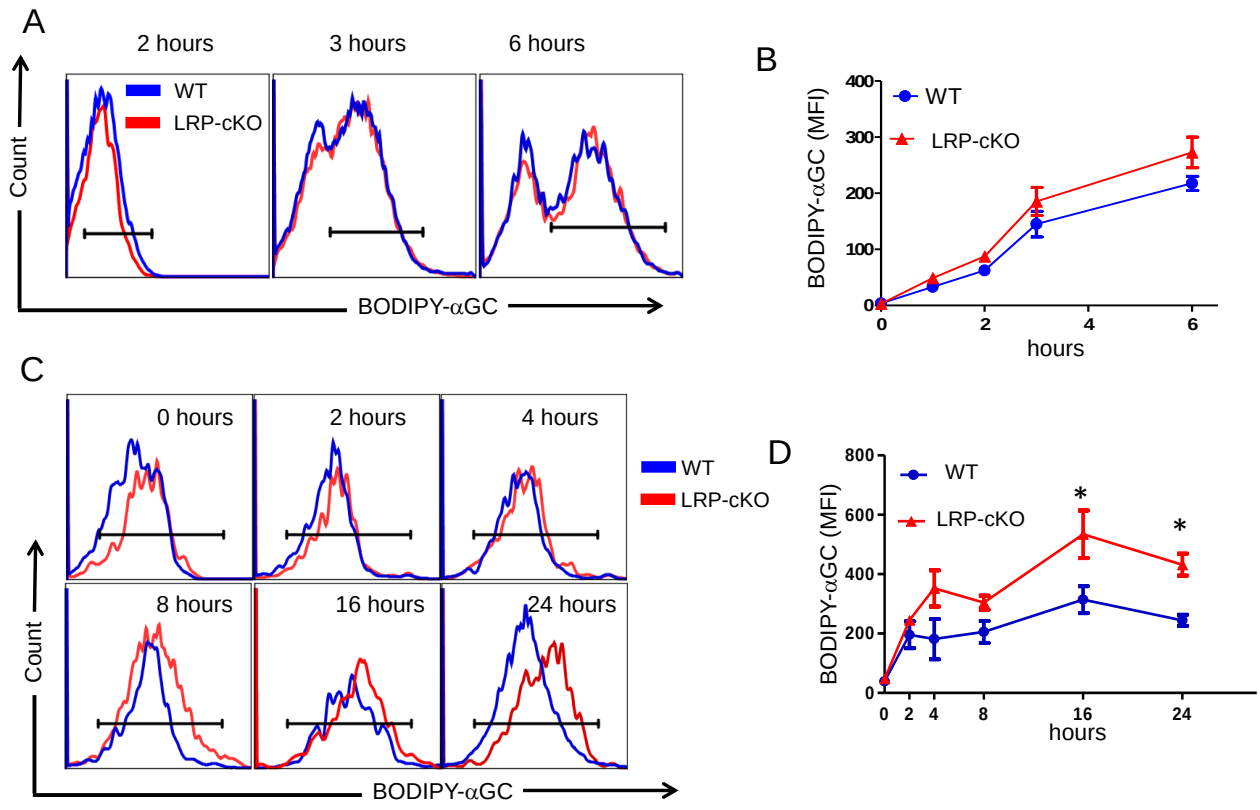


Figure 12: Peritoneal MΦ uptake of fluorescently labeled αGC (BODIPY-αGC). pMΦs from WT (n=3) and LRP-cKO (n=4) were incubated with 1mg/ml of BODIPY-αGC and harvested at the indicated times. This was followed by incubation with fluorochrome conjugated antibodies for CD11b,CD11c and B220. Shown are **(A)** representative histograms and **(B)** MFI quantification in $CD11b^{+}CD11c^{-}B220^{-}$ pMΦs pulsed with unlabeled αGC for four hours and chased with labeled BODIPY-αGC at the indicated hours. BODIPY-αGC (MFI) was measured by flow cytometry MΦs. **(C)** Representative histograms of WT (n=3) and LRP-cKO (n=3) and **(D)** MFI quantification in $CD11b^{+}CD11c^{-}B220^{-}$ pMΦs . Results shown are representative of an experiment from three independent experiments. Bars represent mean and standard error and * denotes p <0.05.

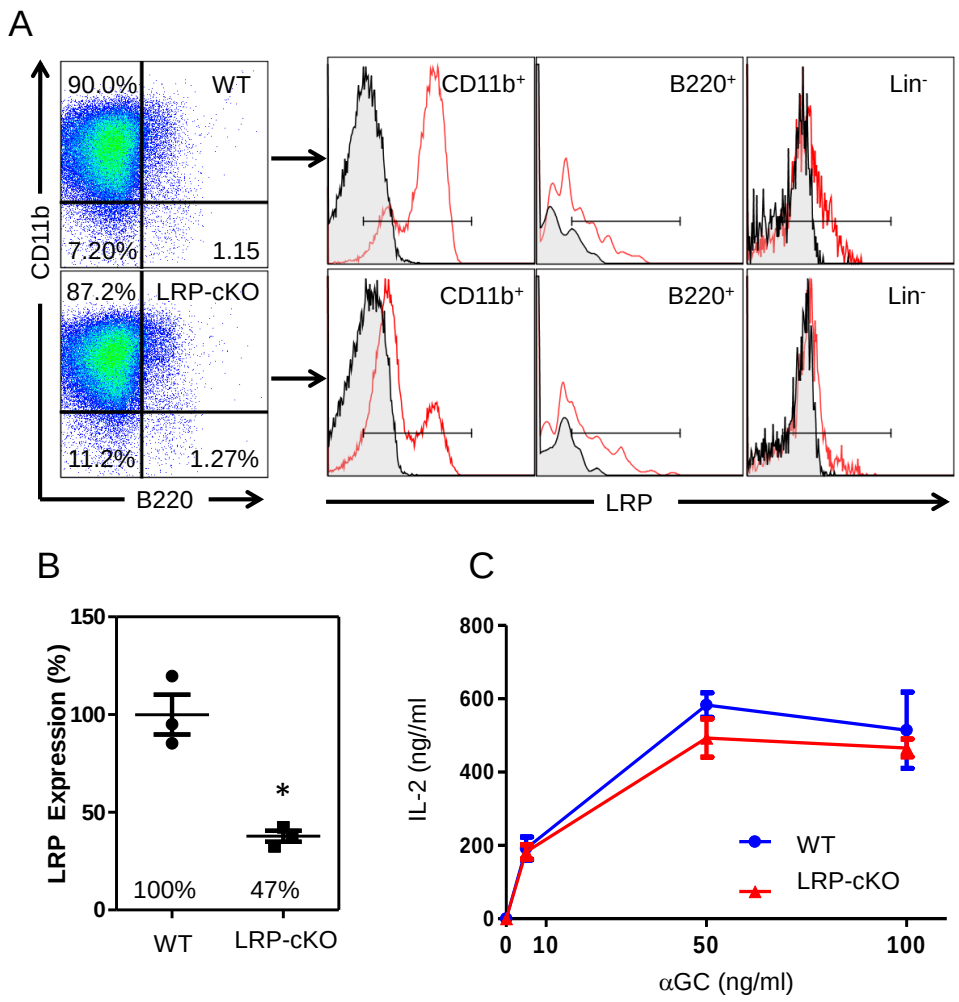


Figure 13: LRP deletion in pMΦs does not affect IL-2 secretion by iNKT cell hybridomas. (A) Left panel shows representative flow cytometry dotplots of WT and LRP-cKO MΦs along with histograms showing LRP expression in macrophages ($CD11b^+$) B cells ($B220^+$) and lineage negative cells from the peritoneal cavity. **(B)** Quantification of LRP expression in pMΦs from WT (n=3) and LRP-cKO (n=3) mice. Each data point represents an individual mouse and results are from one representative experiment of three separate experiments **(C)** WT (n=3) and LRP-cKO (n=3) pMΦs incubated with iNKT cell hybridomas for 48 hours. Each sample well was done in triplicates and IL-2 levels were determined by ELISA. Results are from one representative experiment of three separate experiments. Shown bars represent mean and standard error.

iNKT cell activation, pMΦs were pulsed with αGC and incubated with iNKT cell hybridoma, DN32.D3, and secreted IL-2 was measured in 48 hour culture supernatants by ELISA. We observed a slight but reproducible decrease in IL-2 when iNKT cells were incubated with LRP-cKO pMΦs (Figure 13C). However, this did not reach statistical significance. *In vitro* incubation of freshly isolated splenocytes from WT or LRP-cKO mice with αGC for 24, 48, 72 hours likewise showed no statistically significant difference in IFN-γ or IL-4 levels in culture supernatants (Figure 14), but a slight reduction in IL-4 at 48 hours of culture. These data suggest that *in vitro* activation of iNKT cells by MΦs is not dependent on LRP expression.

LRP deficiency alters iNKT cell secretion of IL-4 but not IFN-γ in vivo

Although we did not see an effect of MΦ-specific deletion of LRP on *in vitro* iNKT cell responses to αGC, we were interested in understanding whether the magnitude and/or kinetics of the *in vivo* iNKT cell response to αGC might be altered in LRP-cKO mice where the spatial integrity of the secondary lymphoid tissues would remain intact. To do this, we challenged WT and LRP-cKO mice with αGC (4μg/mouse i.p.) and measured serum IFN-γ and IL-4 at 2, 12 and 24 hours. ELISA results from this experiment show the near absence of IL-4 production by iNKT cells in LRP-cKO mice (Figure 15A). In addition to this we measured a statistically significant reduction in IL-4^+ iNKT cells (Figure 15B). In WT mice, the kinetics and levels of IFN-γ were not affected by LRP deficiency on MΦs. Lower doses of αGC (1μg and 0.5μg per mouse) did not recapitulate a similar difference in IL-4 secretion between WT and LRP-cKO mice

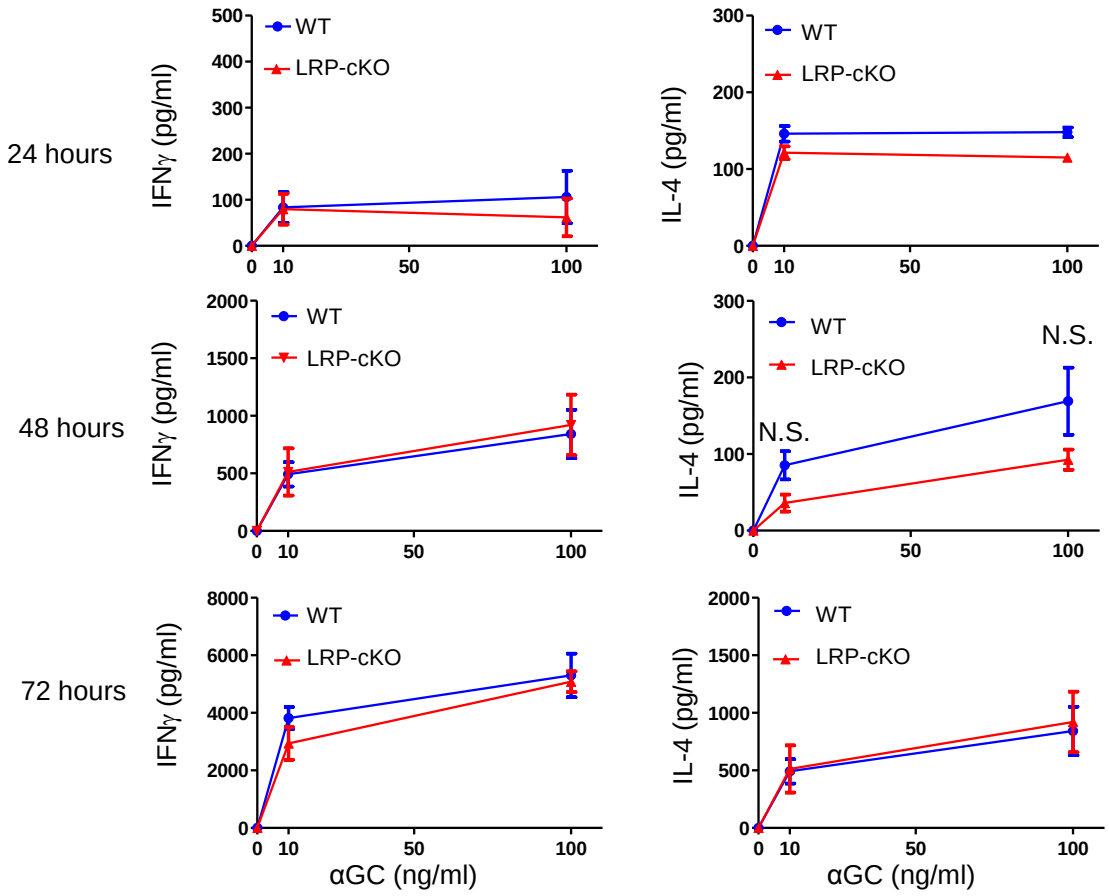


Figure 14: WT and LRP-cKO splenocytes challenged with αGC. Splenocytes from WT and LRP-cKO mice were stimulated with indicated concentration of αGC for 24, 48 and 72 hours. Supernatants were assayed for IFNγ and IL-4 by ELISA. Results are from one representative experiment of three independent experiments. Data points show standard error and mean of 3 mice in each group. N.S. stands for data sets that are statistically not significant.

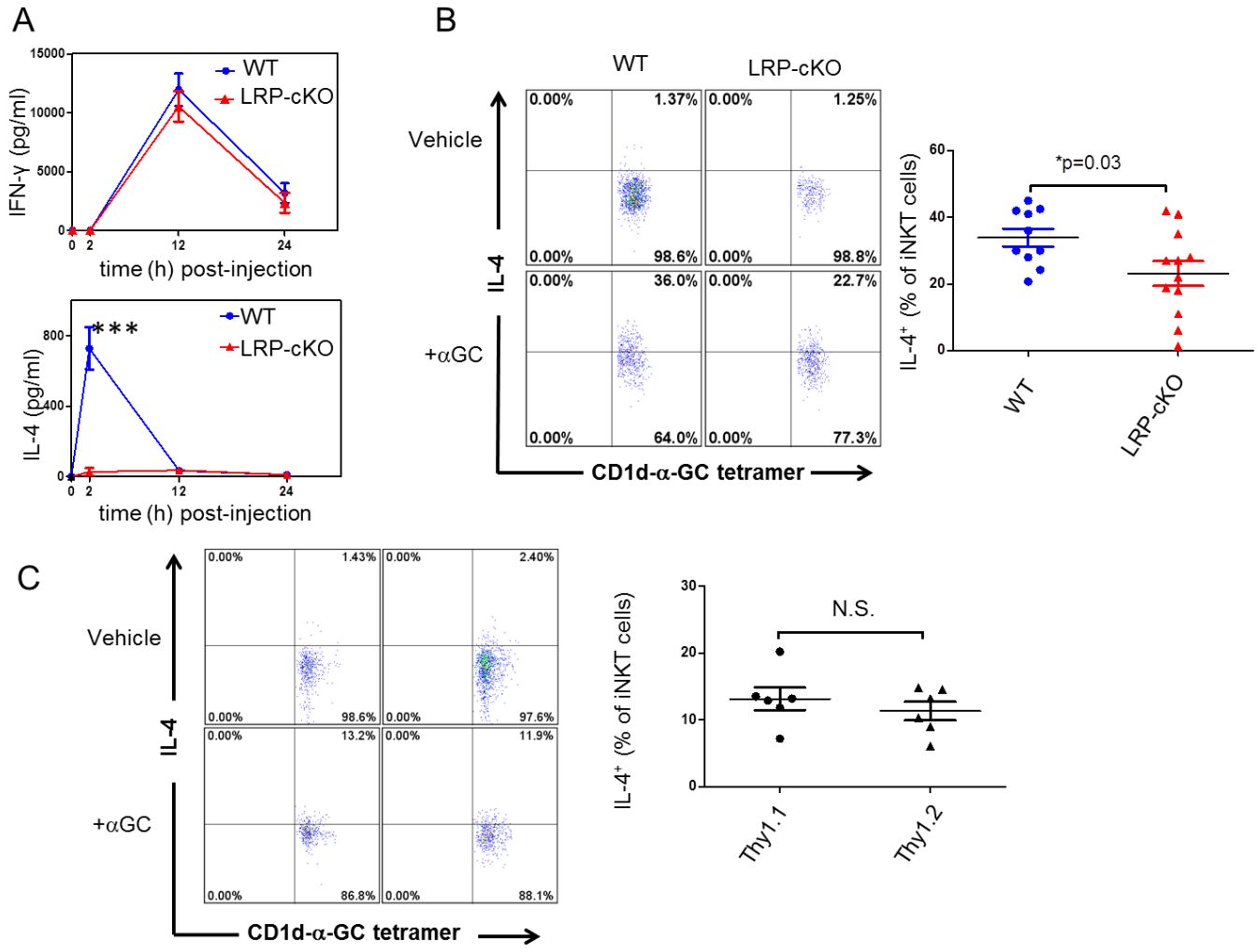


Figure 15: LRP deficiency in MΦ's results in normal IFN-γ response but decreased IL-4. (A) Mice were challenged with 4 μg αGC/mouse and serum assayed for IFN-γ and IL-4 by ELISA. Data points show standard error and mean. WT (n = 4 and 6 mice for 12 and 24 hour, respectively) and LRP-cKO (n = 5 and 4 for 12 and 24 hours, respectively), each point represents an individual mouse from a representative experiment of three separate experiments **(B)** WT (n=7) and LRP-cKO mice (n=8) challenged with 4 μg αGC/mouse or vehicle control. Flow cytometry stain to detect intracellular molecules was performed for IL-4 and antibodies for TCRβ, B220, and CD1d-αGC-tetramer to select iNKT cells. iNKT cells were defined as $TCR\beta^{intermediate}$, $B220^{-}$, $CD1d\text{-}\alpha GC\text{-}tetramer^{+}$. Each point represents an individual mouse from a representative experiment of three separate experiments. **(C)** Chimeric mice were generated by transplanting Thy1.1 and LRP-cKO (Thy1.2) at a 1:1 ratio into $LysMCre^{-/-}$ $LRP^{flox/flox}$ (Thy1.2) recipient mice. Isolation of splenic iNKT cells and intracellular cytokine stain of IL-4 was performed 4 weeks after transplantation. Dot plots on the left show percentage of $Thy1.1^{+}$ or $Thy1.2^{+}$ iNKT cells expressing IL-4. Graph on the right shows quantification of $IL\text{-}4^{+}$ iNKT cells. Data presented from this experiment is the compilation of two separate experiments where each data point represents a single mouse. Bars show standard error and mean. *** denotes $p < 0.0001$. *P*-value determined by Student's *t*-test.

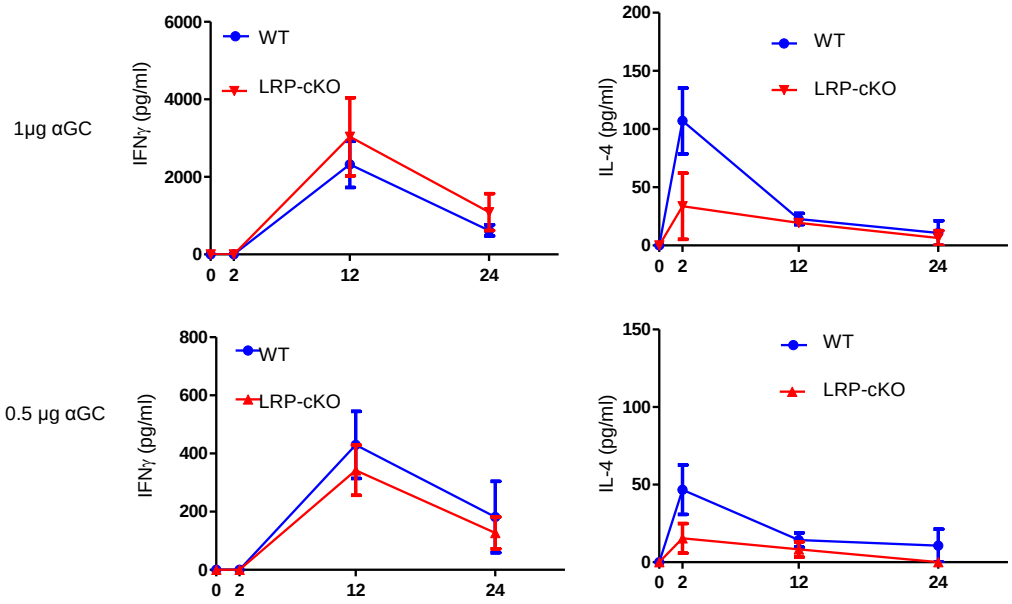


Figure 16: WT and LRP-cKO mice challenged with αGC. Mice were challenged with 1 µg or 0.5 µg αGC/mouse and blood collected at the indicated time points. Serum was assayed for IFN-γ and IL-4 by ELISA. Data points show standard error and mean. WT (n = 3 for 2, 12 and 24 hours, respectively) and LRP-cKO (n = 3 for 12 and 24 hours). Results shown are representative of 3 independent experiments.

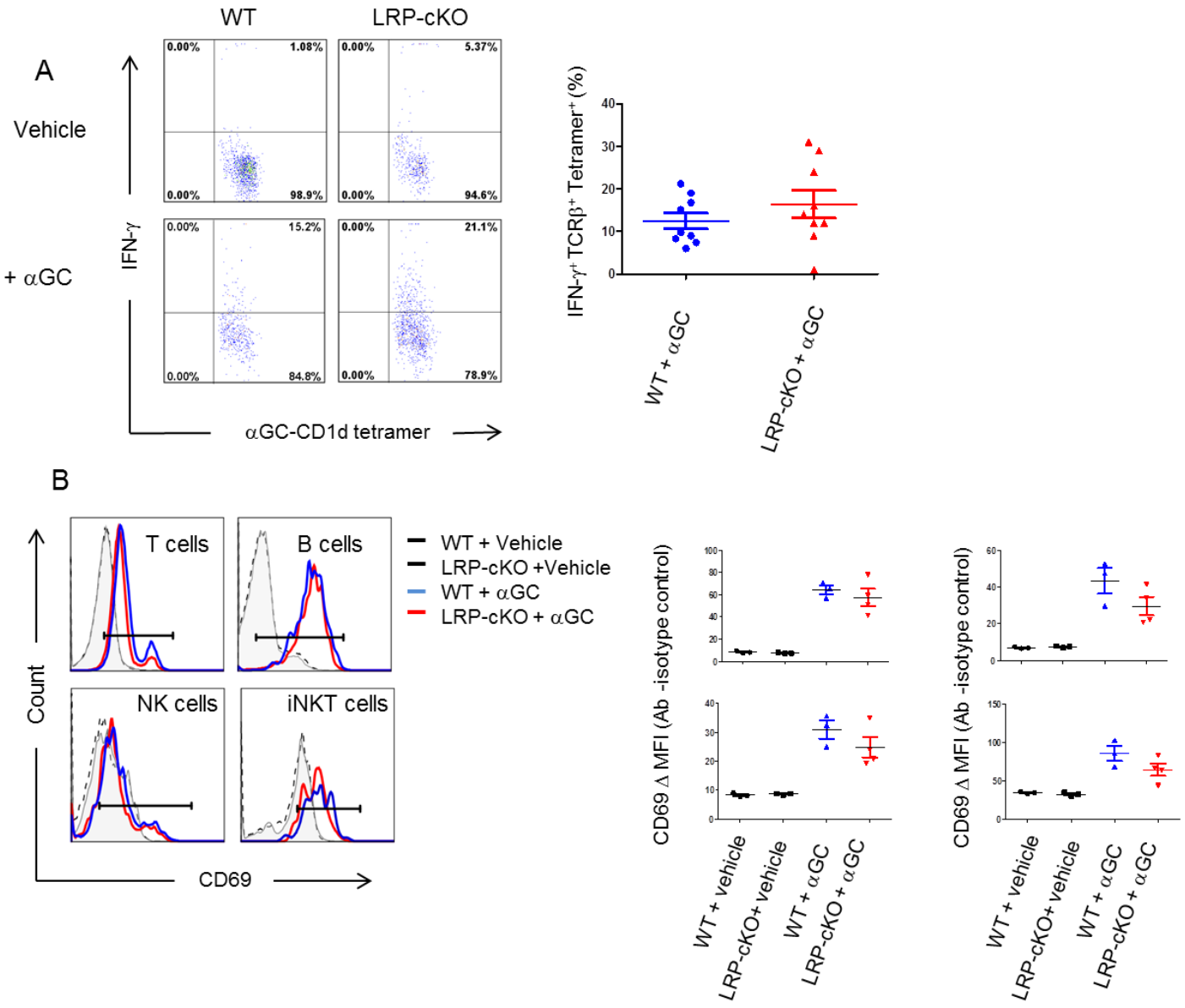


Figure 17: αGC leads to normal iNKT cell IFN-γ production and normal transactivation of the immune system of LRP-cKO mice. (A) WT (n=7) and LRP-cKO mice (n=8) challenged with vehicle or 4 μg αGC/mouse. Flow cytometry stain to detect intracellular molecules was performed for IFN-γ and antibodies for TCRβ^{int}, B220^{-}, CD1d-αGC-tetramer. Each point represents an individual mouse. Graph represents quantification of IFN^{+} TCRβ^{int}Tetramer^{+} cells **(B)** Representative histograms of WT and LRP-cKO splenocytes from mice injected with αGC. Splenocytes were isolated from mice 24 hours after injection and stained with fluorochrome-conjugated antibodies for T cells (TCRβ), B cells (B220), NK cells (NK1.1) and αGC-CD1d tetramer (iNKT cells). Graphs represent quantification of CD69 mean fluorescent intensity (ΔMFI = MFI – isotype control). Symbols represent individual mice. Bars represent mean and standard error. Each data point represents individual mice. Bars represent mean and standard error.

(Figure 16). The presence of normal IFN-γ (Figure 17A) responses is supported by similar levels of transactivation of T cells and NK cells as measured by CD69 upregulation (Figure 17B). These results suggest that, *in vivo,* MΦ LRP is not required for IFN-γ secretion, but can impact production of IL-4 by iNKT cells.

Decreased IL-4 expression by iNKT cells in LRP-cKO mice is cell extrinsic

Although iNKT cells in LRP-cKO mice should not be directly affected we wanted to confirm that decreased IL-4 production in response to αGC was not due to an iNKT cell intrinsic deficiency. To do this we generated bone marrow chimeras using a 1:1 ratio of bone marrow from Thy1.1 and LRP-cKO mice (Thy1.2) transplanted into recipient WT mice at a 1:1 ratio. Four weeks after transplant, mice were injected with αGC and splenocytes analyzed for intracellular IL-4 cytokine stain. The results from this experiment (Figure 15C) show the production of IL-4 by $Thy1.2^+$ iNKT cells (LRP-cKO) is rescued to $Thy1.1^+$ iNKT cell levels in the 1:1 mixed bone marrow chimeras. This result suggests that the decrease in IL-4 measured in LRP-cKO mice is cell extrinsic and that iNKT cells from LRP-cKO mice can respond normally in the presence of LRP-sufficient macrophages.

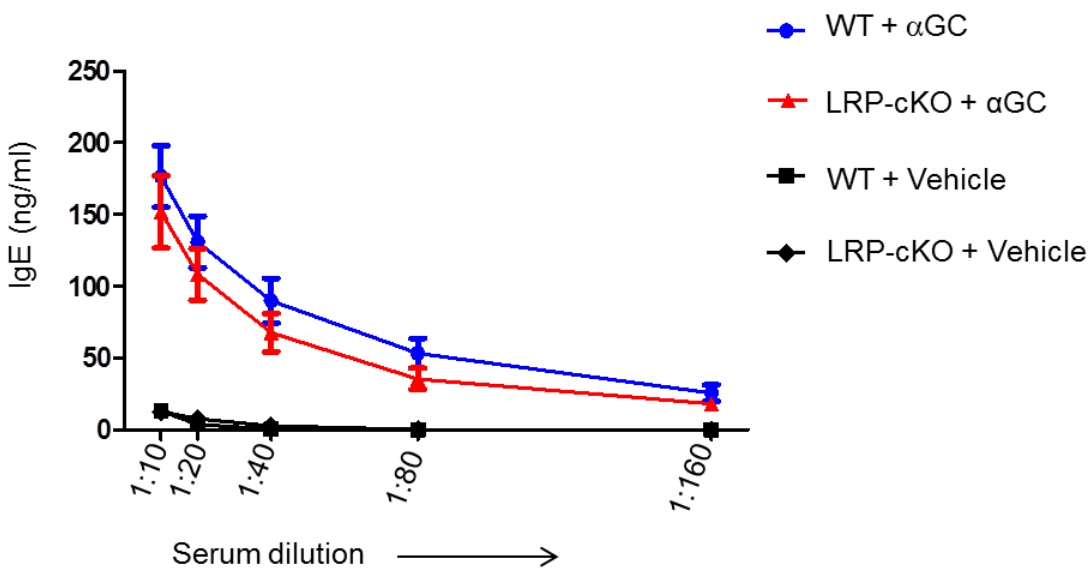
LRP-cKO mice have a normal IgE response when challenged with αGC

To determine whether lack of IL-4 response in LRP-cKO mice to αGC could lead to a deficiency in the adaptive immune response, we measured production of IgE. It has been established that a single dose of αGC can lead to a robust increase in the presence of serum IgE 6 days after αGC injection [184]. To test this, we injected WT

and LRP-cKO mice and obtained serum at day 6 and measured IgE levels by ELISA (Figure 18). Titration curves of IgE in this experiment show that, despite a lack of early burst of IL-4, LRP-cKO mice are still able to produce a normal, although reduced, IgE response. This result suggests that, while LRP deficiency in MΦs decreases iNKT cell IL-4 production, the main cytokine involved in IgE production, it is not necessary for increased IgE.

Discussion

Several studies have reported that activation of the immune system can reciprocally regulate the expression of lipoprotein receptors in APCs. For example, exposure of MΦs to IFN-γ can exert dramatic changes of LRP [185,186], VLSL [187], and (SRs) [188]. However, far less is known about how these changes in lipid-binding receptors might influence immune responses. LRP's ubiquitous nature, ligand diversity and involvement in intracellular signaling cascades allow it to be a receptor capable of affecting a multitude of cellular processes. Unfortunately, the examination of LRP's expression and function has been largely confined to human cell lines [189,190], mouse cell lines [191,192] and primary mouse cells [134,183]. The $LRP^{flox/flox}$ mouse model [193] has allowed a wide number of conditional knockouts which verify its multicellular distribution and function [171,194,195]. By crossing it with LyzM-Cre mice, we observed close to a 70% reduction of LRP in primary cells (Figure 4), which is reminiscent to other $Cre^{+/-}$ models such as the adipocyte specific aP2-Cre [196] and neuron specific synapsin-Cre [197]. In all these cases a full deletion was not observed but rather a near



Covarrubias *et al.* Fig 6

Figure 18: Induction of IgE in LRP-cKO mice is normal when compared to WT. Both WT (n=8) and LRP-cKO (n=6) mice were challenged with αGC (4μg/mouse) or vehicle (WT, n=3, LRP-cKO n=2). Blood was collected at 0 and 6 days and total serum IgE levels measured by ELISA. Bars represent mean and standard error. These results are from one experiment.

complete reduction of LRP. In this study, we show that in the steady state, T cells express low levels of LRP followed by B cells. DCs express a small amount of LRP, but MΦs express higher levels of LRP (Figure 4). This demonstrates that on immune cells, LRP expression is largely restricted to phagocytes and professional APCs supporting its role in antigen presentation and modulation of immunity.

Using a conditional mouse model of MΦ LRP knockout, we show that reduction of LRP expression in MΦs does not influence iNKT activation *in vitro* (Figure 13C and 14), but abolishes IL-4 production *in vivo* (Figure 15A). This lack of IL-4 response was not the result of altered development and/or homeostasis of iNKT cells (Figure 10 and 11). Because we did not observe changes in iNKT cell activation using single cell suspensions *in vitro*, but did see a near absence of early iNKT cell IL-4 production following *in vivo* challenge, we hypothesized that spatial orientation of the LRP expressing MΦs may be important to early iNKT cell responses. We focused our studies on spleen because 1) marginal zone MΦs express receptors that allow them to capture blood borne antigens [198], 2) spleen contains an elaborate distribution network which disseminates molecules present in the blood to white pulp where APCs are located [199], and 3) iNKT cells that reside in the spleen are exposed to blood borne antigen within minutes after they enter the bloodstream [179]. Our attempts to measure iNKT cell activation in liver and LN shortly after αGC injection were not successful. Therefore, whether iNKT cells in liver and lymph nodes undergo a similar exposure to blood borne antigens is an issue that needs to be addressed in future studies.

Indeed, previous studies have demonstrated that MΦs in the splenic marginal zone (MZ) are the first APCs to activate iNKT cells in response to blood-borne

glycolipids [179]. Using time-lapse multiphoton microscopy the authors showed that, following antigen challenge, splenic iNKT cells quickly localize to the MZ and come into contact with MZ $CD169^+$ MΦs. Interestingly, when the MZ integrity was compromised following injection of clodronate liposomes, a method of MΦ-specific depletion, there was a dramatic decrease in iNKT cell cytokine production in response to *in vivo* challenge with particulate αGC [41]. *In vitro* comparison of antigen presenting efficiency of DCs and MZ MΦs to iNKT cells showed that, although MZ MΦs were able to activate iNKT cells *in vitro*, DCs were clearly superior. Therefore, the data suggest that the spatial interaction of iNKT cells with the MZ MΦs soon after injection of αGC is the most plausible explanation for the observed *in vivo* decreases in iNKT cell activation.

More specifically, it is possible that at 2 hours the localization of LRP-expressing MΦs in the MZ induces iNKT cells to secrete IL-4. However, at later times other APCs (such as DCs) are primarily responsible for iNKT cell activation and the resulting IFN-γ response. Because *in vitro* disruption of the spleen to make single cell suspensions would destroy this spatial integrity allowing for iNKT cells to be activated by DCs, this decrease in IL-4 production in response to αGC would not be observed. In support of this hypothesis, our study demonstrates that splenic $CD169^+$ MZ MΦs express higher levels of LRP than the $CD169^-$ MΦs (Figure 5). Therefore LRP expression in these $CD169^+$ MΦs may influence the early activation of iNKT cells and consequently lead to decreased IL-4 at 2 hours.

The expression of surface receptors that can direct glycolipid uptake and subsequent iNKT cell activation suggests multiple pathways are responsible for delivery to APCs. To date, no specific receptor has been described that carries out this function.

In fact, the hydrophobic nature of glycolipids prevents their individual presence in the serum and would require their association to serum-borne particles. Van Den Elzen *et al.* found that apoE-containing lipoproteins, specifically VLDL could associate with iNKT cell activating glycolipids such as αGC's close family member galactosyl(α1-2) galactosylceramide [62]. The investigators went on to demonstrate that apoE-associated glycolipid was efficiently taken up by the LDLr and enhanced iNKT cell activation. More recent studies demonstrate that, like the LDLr, other lipid receptors such as SRA can bind and facilitate uptake of glycolipid antigens by DCs to activate iNKT cells [68]. Preference for receptor/glycolipid interaction was determined to be more dependent on chemical structure of the lipid. Interestingly, both SRA- and LDLr-deficient DCs showed decreased ability to activate the iNKT cell hybridoma DN32.D3 in response to αGC *in vitro*. However, *in vivo* challenge of SRA and LDLr-deficient mice with soluble αGC led to the same blunted IL-4 response we observed in LRP-cKO mice (Figure 15A). How these receptors contribute to such different cytokine responses remains to be clarified. One possibility is that all three receptors (LRP, SRA and LDLr) are required for an optimal IL-4 response *in vivo*. Another may be that loss of any one of these receptors leads to changes in intracellular lipid compositions and that the iNKT cell IL-4 response is sensitive to these changes. We did not observe changes in uptake of BODIPY-αGC in LRP-cKO MΦs in our studies (Figure 12A and B), However, it is possible that this is the result of the limitation of our *in vitro* system. Surprisingly, in a pulse chase experiment, we did see increased appearance of BODIPY-αGC in LRP-cKO MΦ indicating that lipid exchange may be accelerated in the absence of LRP (Figure 12C and D). Therefore, one might hypothesize that more rapid replacement of

αGC with endogenous lipids in LRP-cKO MΦs results in a blunted, early IL-4 response *in vivo*. Further investigation is warranted to determine whether there are *in vivo* changes in antigen-uptake or whether loss of these receptors affects lipid rafts, or if other lipid pools important for cell activation are required.

Collectively, this study demonstrates that LRP expression in MΦs does not alter total activation of iNKT cells *in vivo* but, like the LDLr and SRA, LRP plays a role in IL-4 secretion by iNKT cells. This result is remarkable as it suggests a scenario where LRP can be manipulated to modulate iNKT cell responses. Activation of iNKT cells has been associated with altered progression of cancer, autoimmune disorders and atherosclerosis. Understanding how lipoprotein receptors affect their function can lead to targeted therapies to harness the immunoregulatory potential of iNKT cells.

CHAPTER III

ACTIVATION OF INVARIANT NATURAL KILLER T CELLS LEADS TO LOSS OF SPLENIC MACROPHAGES AND MODULATES EXPRESSION OF LDL RECEPTOR-RELATED PROTEIN

Abstract

Challenge of mice with αGC is known to induce phenotypical changes in iNKT cells in secondary lymphoid organs. INKT cells downregulate surface TCR and expand robustly 3 days after αGC injection. However, the changes that APCs undergo in response to αGC challenge and subsequent iNKT cell activation have not been well-documented. In this study, we investigated whether changes in MΦ content was altered during the course of αGC challenge. We found that 12 hours after αGC injection there is a 50% loss of $F4/80^+$ MΦs in the spleen. We determined that this loss is not due to downregulation of the $F4/80^+$ surface marker, as flow cytometry to detect intracellular molecules showed a similar loss in MΦ percentage. We also found that in the peritoneal cavity, where αGC is administered, experiences a similar loss of resident peritoneal MΦs. In contrast to these observations we measured that lymph node cellular content remains normal throughout the course of αGC challenge. Previous reports have shown that loss of LRP can play a role in cellular migration and metastatic properties of cells [121,138]. In fact, we also found out that LRP expression in remaining splenic MΦs was decreased by about 50% 12 hours after αGC injection. Using $CD1d^{-/-}$ mice that lack

iNKT cell responses, we show that loss of MΦs and MΦ LRP is dependent upon iNKT cell activation. Since previous reports have shown that exogenous IFN-γ can induce loss of LRP in cell culture [185], we determined the role iNKT cell derived IFN-γ has in MΦ and MΦ LRP loss. We found that treatment with IFN-γ neutralizing antibody prior and during iNKT cell activation prevented the loss of splenic MΦ and MΦ expression of LRP. Thus, αGC activation of iNKT cells promotes loss of splenic MΦ and splenic expression of LRP through secretion of IFN-γ.

Introduction

The dynamics of mouse iNKT cells in response to glycolipid challenge have been well documented and it has been shown that αGC can induce long-term phenotypic changes in iNKT cells. For example, a report by Parekh *et al.* showed that following a single injection of αGC splenocytes proliferate less and secrete less cytokines *in vitro* when restimulated with αGC [26]. *In vivo,* iNKT cells become undetectable by conventional methods of flow cytometry in as little 8 hours after αGC injection. This was initially thought to be due to massive amounts of iNKT cell death [200]. However, the mechanism was later determined to be an immediate internalization of iNKT cell surface receptors followed by a 10-fold expansion of iNKT cells at day 3 [201]. A single αGC injection can render iNKT cells unresponsive *in vitro* for as long as a month after αGC challenge. While iNKT cell dynamics have been studied extensively, the concomitant effect of αGC injection on APCs in the spleen has not been documented. Previous work by Barral *et al.* showed that iNKT cells must localize to the MZ in the spleen where they can interact with MΦs that can

process glycolipids to activate iNKT cells [179]. Barral et al. showed that these MΦs were present in the spleen at 2 hours after particulate αGC injection, however at longer time frame more representatives of iNKT cell activation were not presented. In a previous study, it was determined that DCs present in the lymph nodes can migrate outside of tissue in response to a single 4μg/mouse dose of αGC [202]. This led us to hypothesize that MΦs in the spleen can also be affected by αGC and undergo similar phenotypical changes.

Results

F480$^+$ MΦs become undetectable in the spleen 12 hours after αGC injection

To determine whether MΦs undergo changes in response to αGC injection, we treated WT mice i.p. with αGC and tracked F4/80$^+$ MΦs at 12, 24, 48 and 72 hours after injection in spleen (Figure 19A-B) and LNs (Figure 20A-B). We found that in spleen, the number and percentage of MΦs dropped at 12 hours and recovered to normal levels at 72 hours. This was not evident in the LNs where the MΦs remain constant throughout duration of the experiment. The surface antigen F4/80 is a glycoprotein used to identify MΦs in the spleen, brain (microglia), liver (Kupffer's cells) and skin (Langerhan cells) [203,204]. Previous work has shown that activation of MΦs can lead to the downregulation and internalization of surface F4/80 when MΦs are challenged with Bacillus Calmette-Guerin [205] and in response to T cell

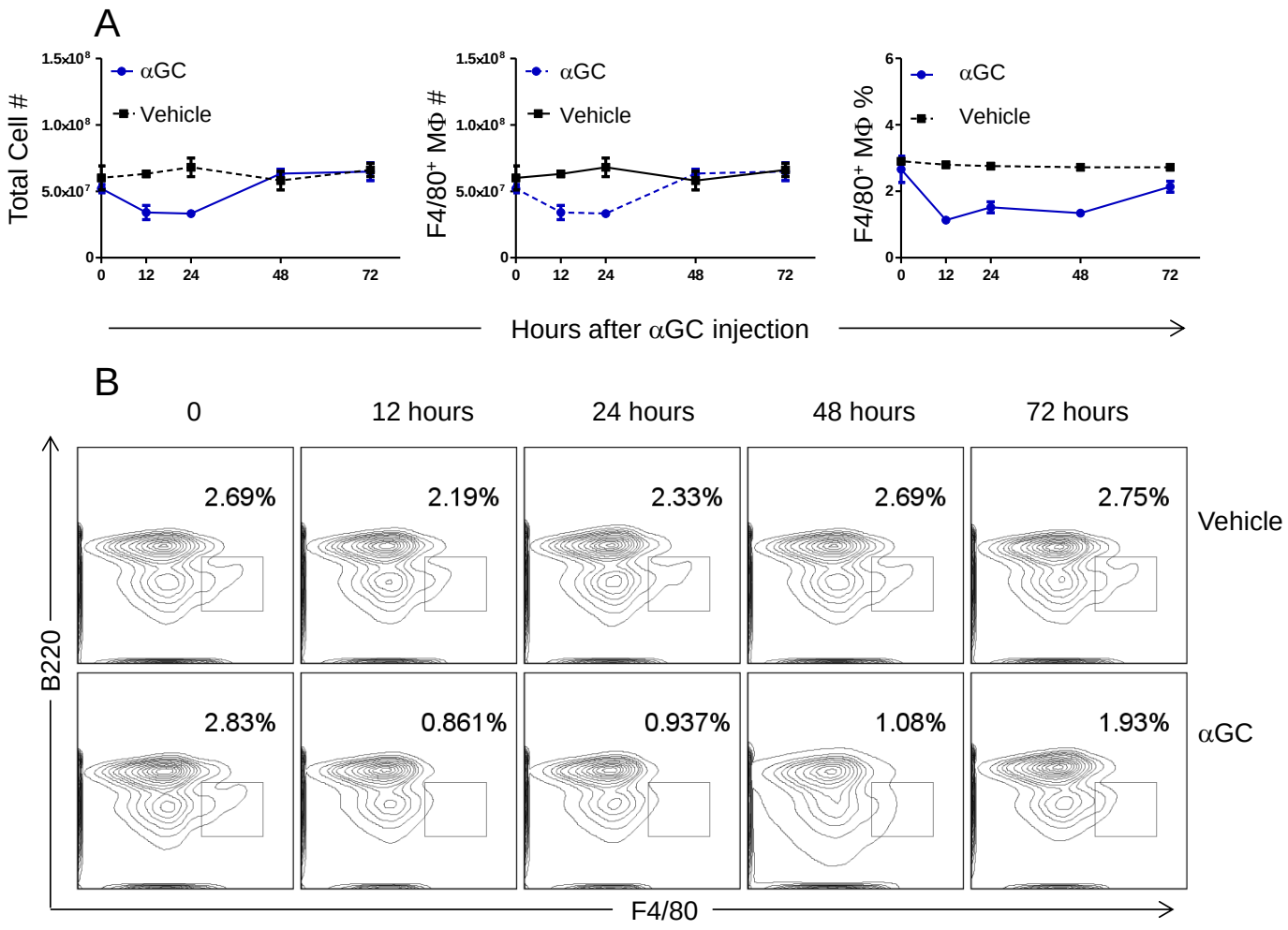


Figure 19: αGC challenge leads to loss of F4/80⁺ MΦs in the spleen. WT mice were injected i.p. with 4 μg/mouse of αGC and splenocytes collected at the indicated time points. **(A)** From left to right, quantification of total cell number, F4/80⁺ MΦs number and F4/80⁺ percentages of WT αGC (n=3) and WT (n=2) mice. Data points show standard error and mean **(B)** Representative flow cytometry dot plots of splenic MΦs.

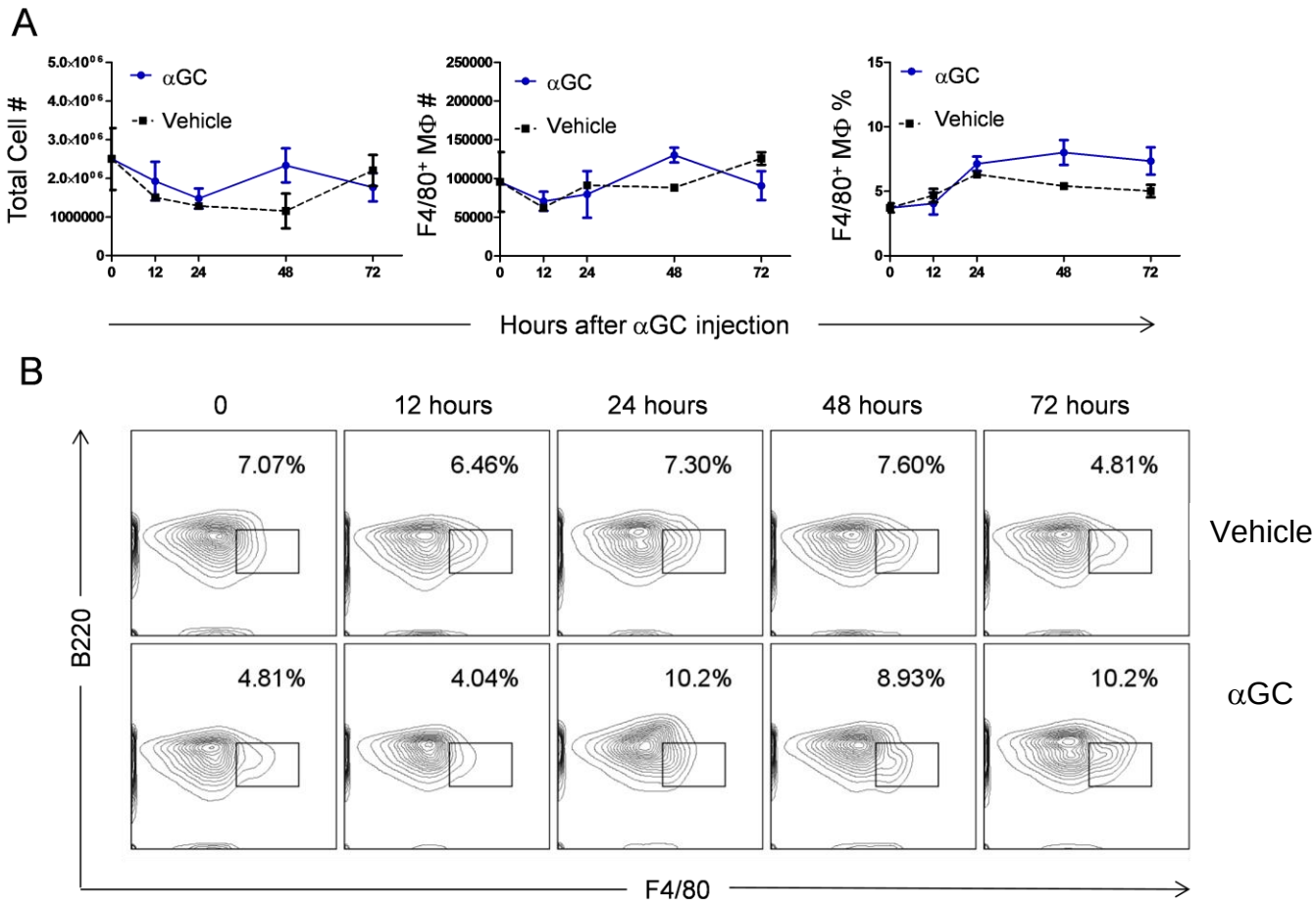


Figure 20: αGC challenge does not lead to loss of F4/80$^+$ MΦs in the lymph nodes. WT mice were injected i.p. with 4μg/mouse of αGC and brachial lymph nodes collected at the indicated time points. **(A)** From left to right, quantification of total cell number, F4/80$^+$ MΦs number and F4/80$^+$ percentages of WT αGC (n=3) and WT (n=2) mice. Data points show standard error and mean **(B)** Representative flow cytometry dot plots of splenic MΦs.

activation [206]. We questioned whether F4/80$^+$ MΦs in the spleen would also internalize this receptor, which would exclude their detection by conventional flow cytometry methods. To answer this, we performed flow cytometry for intracellular molecules to detect internalization of F4/80 after αGC injection. Intracellular staining presented in Figure 21 shows a higher percentage of F4/80 expression of MΦs than the surface stain presented in Figure 19. This suggests that portions of F4/80 can be found in intracellular compartments. However, an overall decrease in F4/80$^+$ MΦ percentage was measured in both experiments. Together, these data show that the decrease in number of MΦs is not due to downregulation of F4/80 from the surface but a different mechanism which causes loss of the MΦs from the spleen.

F480$^+$ MΦs do not migrate to the site of αGC injection

The migration of lymphocytes from secondary lymphoid organs to peripheral tissues is mainly controlled by chemotaxis and chemokines associated in the site of inflammation. The classical mechanism of lymphocyte migration has been described to be due to the presence of bacterial components, leukotrienes, chemokines and a large family of motility inducing molecules widely known as chemoattractants [207]. All of these molecules can act in concert to orchestrate the mobilization of lymphocytes to sites that require immunologic attention. We questioned whether injection of αGC in the peritoneal cavity would induce MΦs from the spleen to migrate to this site in an effort to take up remaining glycolipid that was unable to enter the circulation. To test this, we injected αGC and collected resident pMΦs at

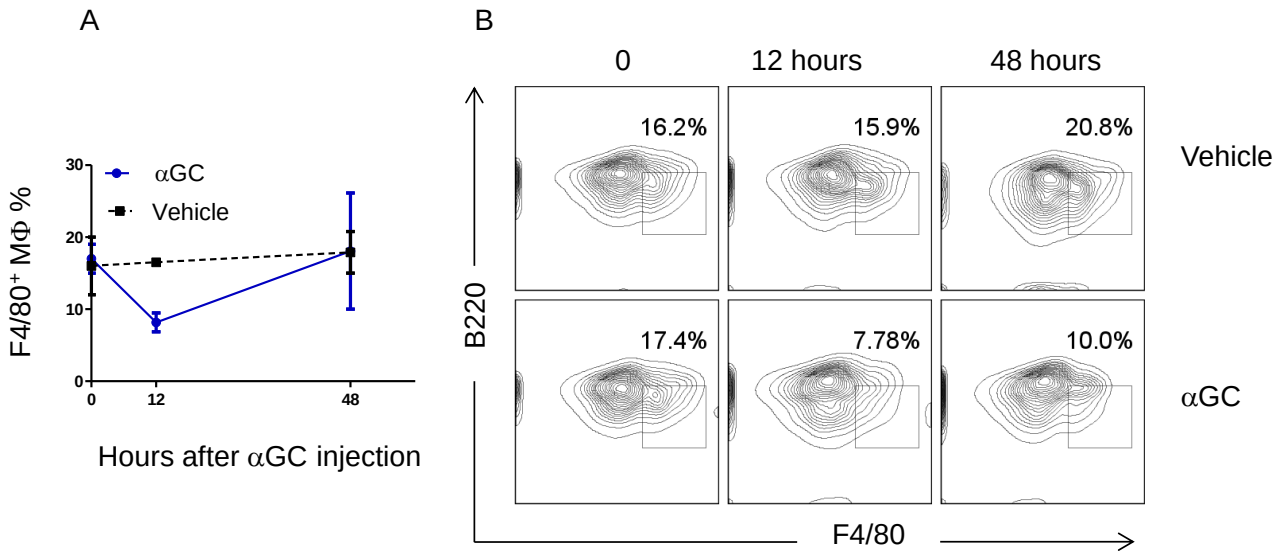


Figure 21: Splenic MΦs do not downregulate F4/80 in response to αGC challenge. WT mice were injected i.p. with 4μg/mouse of αGC and splenocytes collected at the indicated time points. **(A)** Quantification of flow cytometry to detect intracellular molecules to stain F4/80^{+} in of WT αGC (n=3) and WT vehicle (n=3) injected mice. Data points show standard error and mean. **(B)** Representative flow cytometry dot plots of splenic MΦs.

12 and 48 hours after injection. Figure 22 shows a sharp decline of pMΦs at 12 hours and unlike the spleen, the percentage of pMΦs in the peritoneal cavity remained low 48 hours after injection. These data show that the loss of splenic MΦs and pMΦs is not the result of a localized immune response that causes migration of MΦs to the peritoneal cavity.

Injection of αGC causes downregulation of LRP in MΦs

The expression of LRP has been shown to play a role in vascular cell proliferation [208], implantation of the embryo [209] and induction of cellular adhesion that prevents metastasis [210]. In fact, a recent report by Llorente-Cortes et al. showed that inhibition of LRP in human hepatic stellate cells (HHSCs) by siRNA or LRP neutralizing antibody led to higher proliferative rates and higher migration rates than LRP competent cells. This knockdown of LRP was associated with increased levels of TGF-β secretion and phosphorylation of ERK 1,2, a protein involved in the scaffolding signaling necessary for migration. Interestingly, a recent report showed that LRP can modulate Gi-dependent sphingosine-1-phosphate signaling which is responsible for restricting lymphocyte migration and egress from secondary lymphoid tissues [211]. Therefore, we questioned whether LRP could play a role in the MΦ loss observed in the spleen. We injected WT mice i.p. with αGC and measured LRP expression in the MΦs remaining in the spleen. The histogram in Figure 23A shows that MΦs isolated from mice injected with αGC have decreased LRP expression quantified by MFI in F4/80$^+$ cells (Figure 23B). The largest drop in LRP expression is measured at 12 and 24 hours and becomes

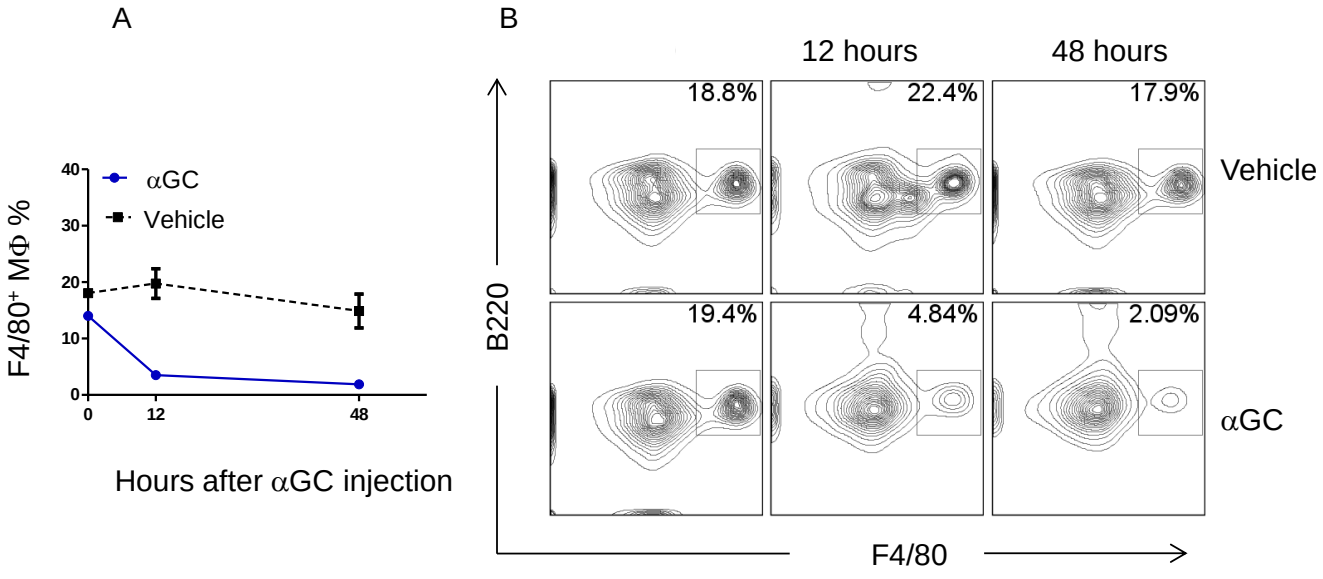


Figure 22: αGC challenge leads to loss of F4/80 MΦs in the peritoneal cavity. WT mice were injected i.p. with 4μg/mouse of αGC and pMΦs collected at the indicated time points. **(A)** Quantification flow cytometry stain showing F4/80$^+$ percentages of WT αGC (n=3) and WT vehicle (n=3) injected mice. Data points show standard error and mean **(B)** Representative flow cytometry dot plots of splenic MΦs.

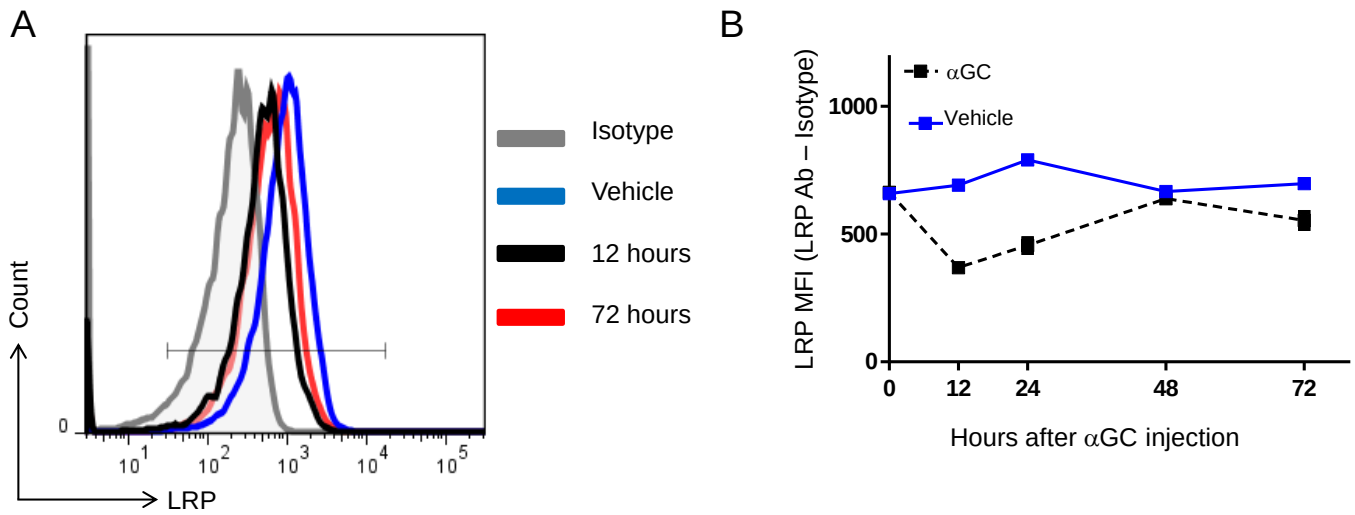


Figure 23: αGC challenge leads to downregulation of LRP in splenic F4/80$^+$ MΦs. WT mice were injected i.p. with 4μg/mouse of αGC and splenocytes collected at the indicated time points. **(A)** Flow cytometry representative histogram showing LRP expression in mice injected with the indicated conditions. **(B)** Quantification of LRP expression in WT F4/80$^+$ MΦs αGC (n=3) and vehicle (n=2). Data points show standard error and mean.

normalized at 48 hours. Interestingly, this normalization of LRP expression occurs at the same time point (72 hours) shown in Figure 19 where MΦs reappear on the spleen. This suggests a scenario where splenic MΦs require expression of LRP when present in this organ. Altogether these data show that MΦs that remain in secondary lymphoid organs following αGC challenge exhibit a drop in LRP expression.

Challenge of αGC in iNKT cell deficient mice prevents MΦ loss and LRP downregulation

We determined that MΦs from the spleen do not migrate to the site of injection in the peritoneal cavity (Figure 22), thus, we hypothesized that this MΦ loss and concomitant LRP downregulation was in response to iNKT cell activation mediated by αGC. To test this, we injected WT and iNKT cell-deficient $CD1d^{-/-}$ mice with αGC. Twelve hours following injection $CD1d^{-/-}$ mice maintain normal levels of MΦs comparable to vehicle treated controls (Figure 24A). Additionally, a representative histogram (Figure 24B) and quantification of LRP MFI (Figure 24C) show that $CD1d^{-/-}$ mice do not downregulate LRP expression in response to αGC injection. Altogether, these results show that iNKT cell activation is responsible for the loss of MΦs in the spleen and also for the downregulation of LRP in response to αGC.

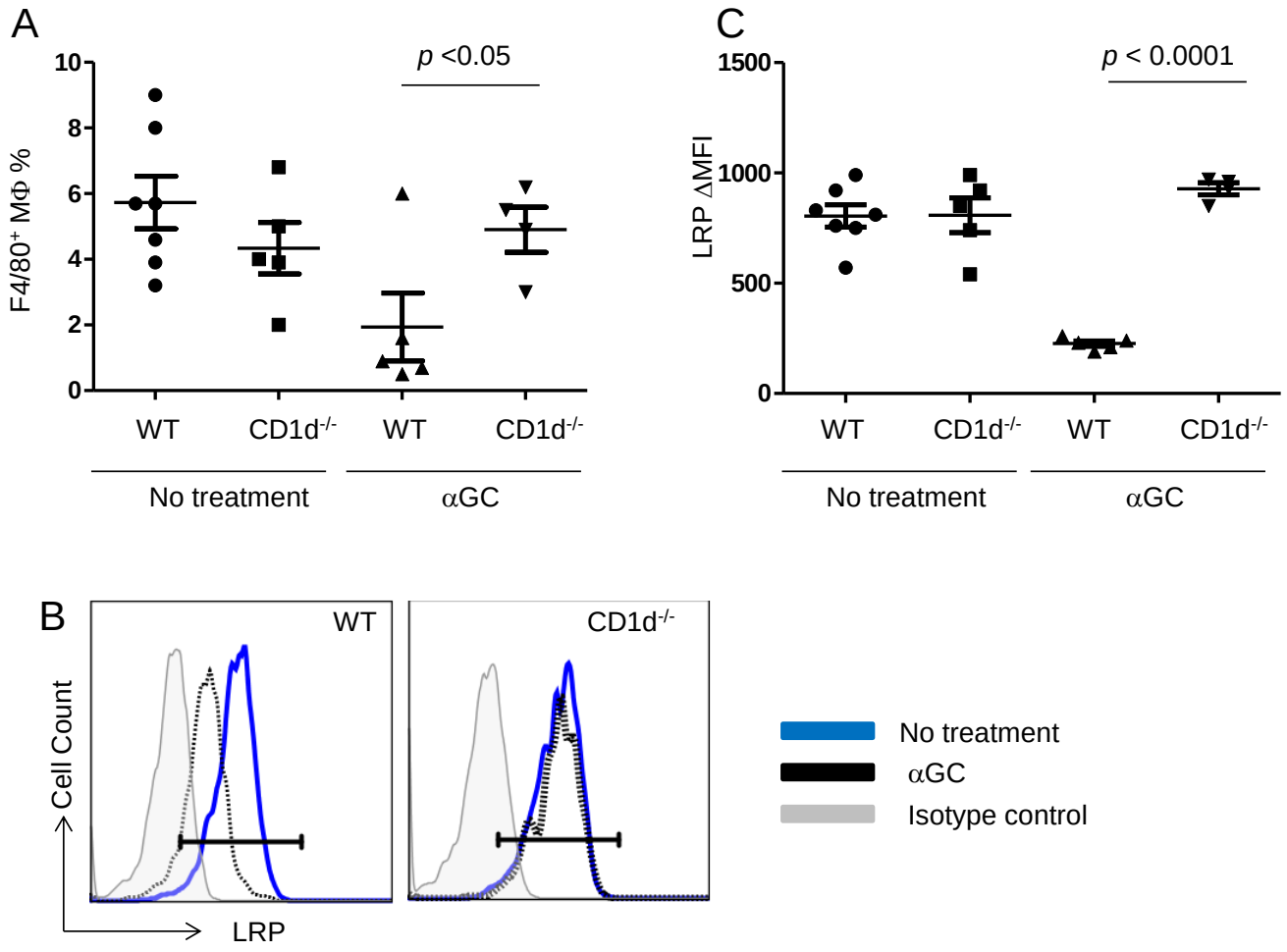


Figure 24: F4/80^{+} MΦs from CD1d$^{-/-}$ do not downregulate LRP expression in response to αGC. WT and CD1d$^{-/-}$ mice were injected i.p. with 4μg/mouse of αGC and splenocytes collected at the indicated time points. **(A)** Quantification of F4/80 MΦs percentages in mice that received vehicle or αGC. **(B)** Representative flow cytometry histograms of LRP gated on F4/80^{+} MΦs and **(C)** Quantification of ΔMFI (LRP antibody – isotype control) LRP expression in WT F4/80^{+} MΦs αGC and vehicle. Data points show standard error and mean.

MΦ loss in the spleen and LRP downregulation in response to αGC challenge is IFNγ-dependent

IFN-γ is secreted by iNKT cells in response to αGC and reaches its peak in the circulation between 12 and 24 hours. Assays for intracellular cytokines can detect IFN-γ presence in iNKT cells in a little as 4 hours following αGC challenge, suggesting that *in situ* IFN-γ may promote physiological effects earlier than the time required to peak in the circulation. Previous studies have shown that recombinant murine IFN-γ treatment of murine MΦ cell line RAW264.7 leads to a significant decrease of LRP expression and ability to take up activated α2-M. This IFN-γ-mediated decrease in LPR occurred at the transcriptional level and resulted in a 70% reduction in LRP mRNA [212]. A later report showed that treatment with TGF-β prior to IFN-γ was sufficient to antagonize the decrease in LRP expression and that other molecules secreted in response to inflammation by the immune system such as nitric oxide synthase (NOS) and tumor necrosis factor did not have a similar effect on LRP expression as IFN-γ. Due to this, we hypothesized that the IFN-γ secreted by iNKT cells could be responsible for the loss of MΦs in the spleen and downregulation of LRP expression. To test this, we treated WT mice with αGC and used neutralizing antibody for IFN-γ to inhibit its function during iNKT cell activation. We found that effective neutralization of IFN-γ (Figure 25A) prevents the loss of LRP levels (Figure 25B) and maintains normal levels of $F4/80^{+}$ MΦs when compared to mice that received isotype control. This result shows that IFN-γ secrete by iNKT cells can modulate MΦ in the spleen as well as LRP their loss of LRP expression.

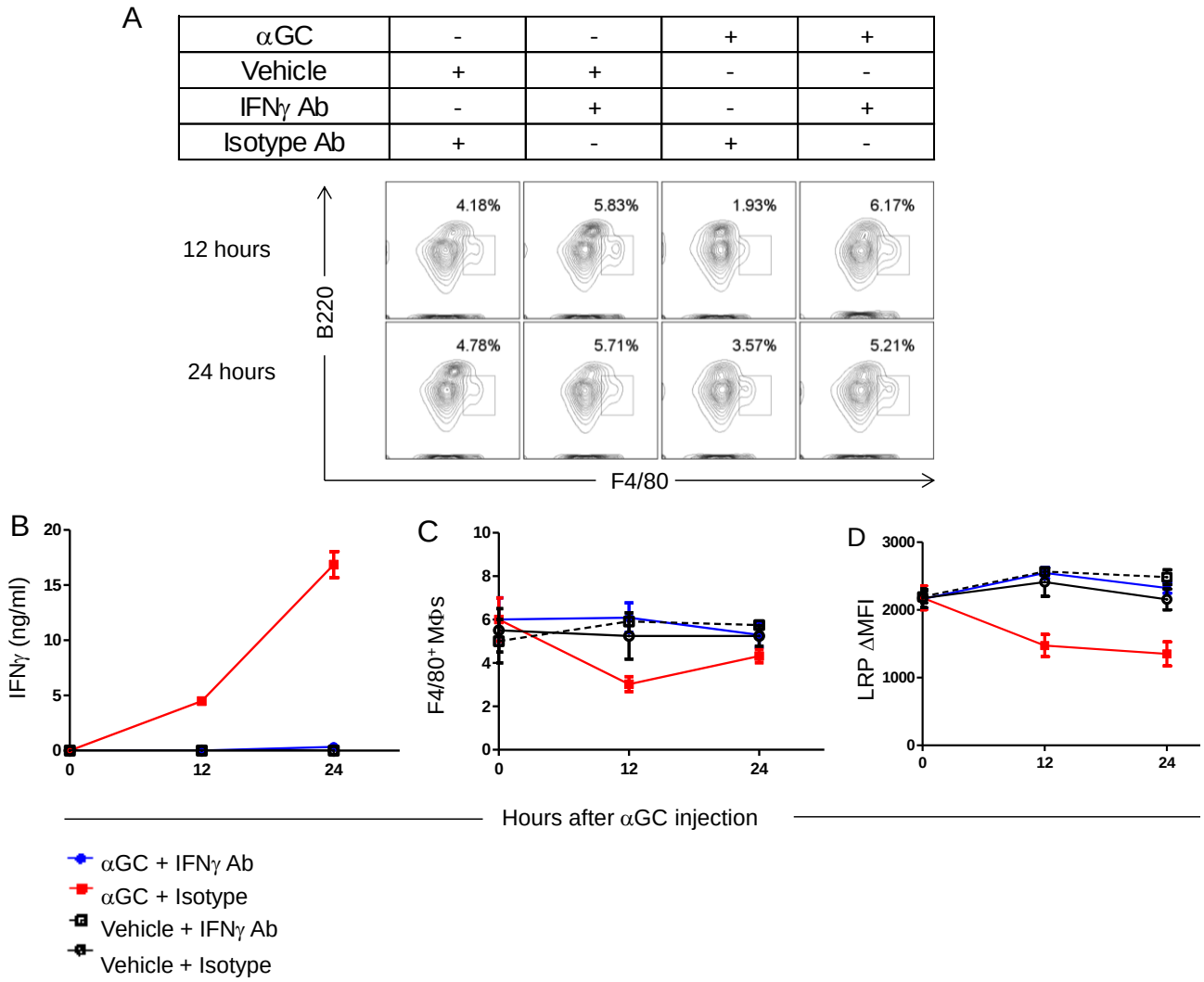


Figure 25: Neutralization of IFNγ before and during αGC challenge prevents loss of splenic F4/80$^+$ MΦs and downregulation of LRP expression. WT mice were injected i.p. with 200μg/ mouse of neutralizing IFN-γ hours prior to αGC challenge. This was followed by αGC injection and 100μg/mouse of neutralizing IFNγ antibody every 4 hours leading to collection of splenocytes. Distribution of mice sacrificed at 12 hours is as followed: WT mice injected with αGC and isotype (n=5), WT mice injected with αGC and IFN-γ neutralizing Ab (n=5), WT mice injected with vehicle + IFNγ Ab (n=3), WT mice injected with vehicle and isotype controls (n=3). Distribution of mice sacrificed at 24 hours is as follow WT mice injected with αGC and isotype (n=5), WT mice injected with αGC and IFNγ neutralizing Ab (n=3), WT mice injected with vehicle + IFNγ Ab (n=2), WT mice injected with vehicle and isotype controls (n=2) **(A)** Representative flow cytometry dot plots of F4/80$^+$ MΦs injected with indicated conditions. **(B)** Results from IFNγ ELISA derived from serum **(C)** Quantification of F4/80$^+$ MΦs percentages in the spleen and **(D)** Quantification of ΔMFI (LRP antibody – isotype control) LRP expression in WT F4/80$^+$ MΦs αGC and vehicle. Data points show standard error and mean.

Discussion

In this study, we showed that challenging WT mice with αGC leads to loss of MΦs in the spleen (Figure 19) and peritoneal cavity (Figure 22) and is accompanied by decreased LRP expression (Figure 23). We also demonstrated that intracellular staining for F4/80 (Figure 22) was unable to account for the loss of MΦs in the spleen suggesting that internalization of this receptor is not responsible for decreased numbers of MΦs in the spleen. Additionally, $CD1d^{-/-}$ mice were protected from the loss of splenic MΦs and decrease in LRP expression (Figure 24). Moreover, LRP expression and MΦ content remained intact when IFN-γ was neutralized after αGC challenge (Figure 25). These data suggest that IFN-γ secretion by activated iNKT cells causes loss of MΦs and LRP expression.

We hypothesized that loss of MΦs in the spleen is associated with LRP expression because previous reports have shown that inhibition of LRP leads to an increase in migration and proliferation of human hepatic stellate cells (HHSCs) and fibroblasts [213,214]. However, other studies of LRP's role in cell migration show the opposite effect. For example, *Song et al.* showed in the human glioblastoma cell line, U87, that LRP knockdown by siRNA had a 60% decrease in migratory activity in two and three dimensional migration assays [215]. In this work, a number of human lung cancer cell lines (H292, H441, H520 and SK-LU-1) were used to corroborate the results found in U87 cells and, across the board, siRNA knockdown had a striking correlation with cell migration *in vitro.* The decreased in cell migration was attributed to a concomitant decrease in expression and secretion of metallic metalloproteinase (MMP)-2 and MMP-9. Both of these MMPs have been studied

extensively in the cancer field for their contribution to extracellular matrix (EM) breakdown and neovascularization of gliomas [216]. This is an interesting study because MMP-2 and MMP-9 have not been officially reported as LRP ligands, yet their expression is affected at the transcriptional level when LRP is knocked down. The authors attribute this effect to changes in intracellular signaling events under LRP deficiency, yet provide no direct mechanism. The most important piece of data supporting this hypothesis is that transfection of U87 cells with an LRP mini receptor (LRPmr) unable to bind ligands, but with a functional intracellular signaling domain, restores expression of MMP-2 and MMP-9 and also cellular migratory capacity. Collectively, these data show the complexity of LRP function as it can play ligand independent roles such as glioblastoma cell migration. Therefore, study of LRP physiology is a complex issue as it binds a large collection of ligands and has intracellular signaling function. Every study performed to increase experimental knowledge about LRP has to take into account the cellular and signaling context in which they are presented.

Another possibility is that iNKT cell-mediated downregulation of MΦ LRP can be a protective mechanism via decreased uptake of LRP ligands that can be toxic. The most well-studied LRP ligand shown to promote cellular apoptosis is a molecule released by pathogen *Pseudomonas aeruginosa* called Exotoxin A (PEA) [172]. This toxic molecule is responsible for the inhibition of ADP-ribosylation and consequently leads to eukaryotic cell death. Previous work demonstrated that MΦs can gain protection against PEA when LRP is inhibited [217]. Using a macrophage-like cell line HS-P. The authors showed that these cells express functional LRP and exhibit

high sensitivity to PEA. However, exogenous addition of LRP inhibitor RAP prevented PEA induced cell death. Interestingly, in the same report, the authors show that challenge of MΦs with LPS can also induce rapid downregulation of LRP (80% decrease in 6 hours). The results from these reports suggest that LRP downregulation can be a general mechanism that protects MΦs from toxic molecules even those that are not able to bind LRP directly such as LPS. Interestingly, it has been shown that the main immune cell involved in protection against *P. aeruginosa* is the iNKT cell . In a mouse model of *P. Aeruginosa* mediated corneal perforation, it was shown that 5 hours post infection, the first cell type present in this tissue was iNKT cells. As infection persisted, NK and $CD4^+$ T cells became more important for diminishing bacterial load and delaying the time to corneal perforation. Depletion of NK and NKT cells by asialo-GM-1 antibody is sufficient to accelerate cornea perforation by 4 days [218]. While the role of MΦs or dendritic cells in containment of *P. aeruginosa* was not studied in this report, one could hypothesize that iNKT cells provide protection to the cornea and surrounding tissues through activation by bacterial glycolipids which results in IFN-γ production, downregulation of LRP and subsequently decreased binding and toxicity of PEA. The data presented in this chapter is the first available evidence that iNKT cell activation can modulate expression of lipoprotein receptors.

CHAPTER IV

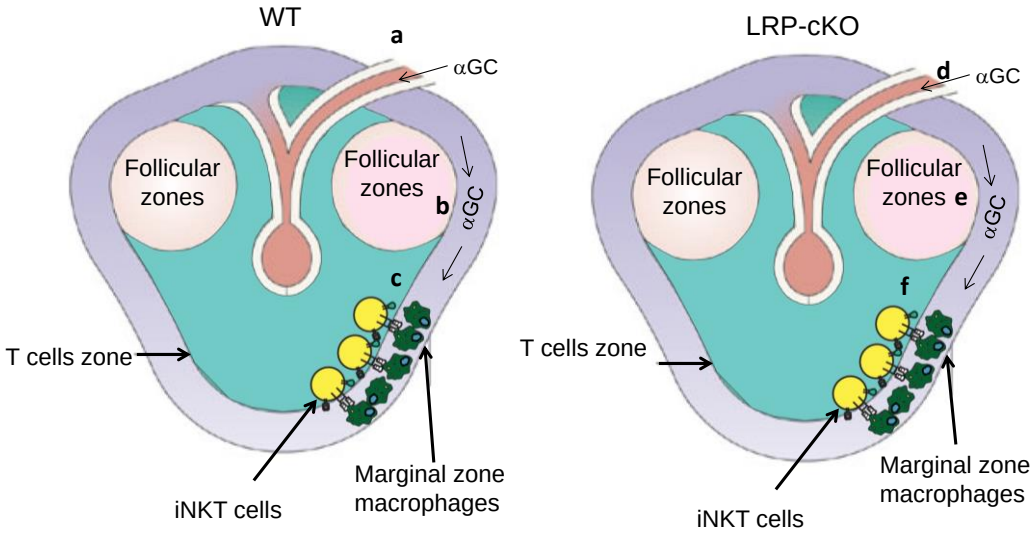
GENERAL DISCUSSION

Collectively, the data presented in this dissertation support the hypothesis that expression of LRP in MΦs is critical for a normal iNKT cell response to glycolipid antigen αGC. Using LRP-cKO mice, an LRP specific knockout in MΦs, we have shown that iNKT cells develop normally in primary and secondary lymphoid organs and LRP-cKO MΦs have normal levels of MHC-class I-like molecule CD1d that directs activation of iNKT cells. Moreover, we did not observe changes in homeostatic surface markers that could indicate spontaneous activation of iNKT cells. Our *in vitro* data demonstrated that activation *in vitro* of iNKT with αGC yields normal secretion of IFN-γ and IL-4 (Figure 14), the main cytokines associated with a normal iNKT cell response. However, *in vivo* challenge of LRP-cKO mice led to a dramatic reduction of IL-4 while secretion of IFN-γ was normal (Figure 15A). Additionally, bone marrow transplants demonstrated that the decrease in IL-4 secretion in LRP-cKO mice is iNKT cell extrinsic, because IL-4 secretion is restored when 50% of LRP competent bone marrow is transplanted into WT mice (Figure 15C).

Our studies demonstrated that glycolipid turnover is affected in LRP-deficient MΦs. Using a fluorescently labeled glycolipid (BODIPY-αGC) we show that in LRP-cKO MΦs take up glycolipids in a normal fashion (Figure 12A), however these MΦs have a faster turnover rate of lipids. Additionally, we tested whether the decrease in

serum IL-4 would prevent the conversion of IgM to IgE and found that although there was an overall trend to decreased IL-4 at 7 days, these results were not statistically significant and that IgE conversion was normal. Furthermore, a single injection of αGC is sufficient to elicit loss of MΦs and LRP expression in the spleen (Figure 19). As determined by the *in vivo* challenge of αGC and IFN-γ neutralization (Figure 25), we demonstrated that iNKT cell derived cytokine production is directly responsible for LRP downregulation. We hypothesize that iNKT cells mediate downregulation of LRP as an immunoprotective mechanism which prevents uptake of toxic LRP ligands, such as PEA and propose further experiments to test this hypothesis.

We have shown that LRP plays a role in iNKT cell activation and that cytokines secreted by iNKT cells *in vivo* can have a reciprocal effect on the expression of MΦ LRP. Figure 26 is a working model that summarizes the phenotypes observed in WT and LRP-cKO mice in response to αGC stimulation. In WT mice αGC enters the spleen through the vasculature (Figure 26a) and circulates through the white pulp of the spleen (Figure 26b) where it is taken up by $CD169^+$ MZMΦs (Figure 26c). In a normal response αGC is loaded onto CD1d and iNKT cells begin secreting IL-4 and producing IFN-γ. At this point, $CD169^+$ MZMΦs are exposed to an iNKT cell cytokine storm that includes large amounts of IFN-γ. At 12 hours, IFN-γ exposure leads to mobilization and egress of MΦs and decrease in LRP expression in remaining MΦs. At 72 hours, the majority of the IFN-γ response has resolved and the content of splenic MΦs returns to basal levels. Whether this normalization is caused by local MΦ proliferation of migration from MΦs in peripheral lymphoid organs is an issue that will have to be addressed in future studies.



Downregulation of LRP and loss of splenic macrophages mediated by IFNγ.

Absence of IL-4 response and change in glycolipid flux in macrophages

Adapted from [257]

Figure 26: Model representation of WT and LRP-cKO spleen cross section. Glycolipids such as αGC enter the spleen through the high endothelial venule (a,d). After blood reaches this tissue it is drained through the white pulp where marginal zone macrophages reside (b,e). These marginal zone macrophages are in close proximity to iNKT cells that reside in the T cell zones where both cells can come in contact and initiate an immune response (c,f).

Under LRP deficiency, αGC enters the spleen in an identical fashion as WT spleen (Figure 26d), yet once it reaches MZMΦs through the white pulp it is taken up by LRP-cKO MΦs and processed differently than in WT MΦs (Figure 26e and f). This leads to absence of an early IL-4 response and normal IFN-γ throughout duration of the iNKT cell response. In this scenario, MΦ LRP plays a role in the early processing of αGC and its ability to induce secretion of early iNKT cell cytokines such as IL-4. Consistent with this idea, others have shown that the spatio-temporal location of MΦs in the spleen plays a direct role in the activation of iNKT cells [179]. While other reports have focused on the ability of DCs to promote the bulk of iNKT cell activation [51] and B cells have been studied for their ability to provide maintenance signals to iNKT cells essential for activation and expansion [219], MΦs have been ignored as APCs that can activate iNKT cells. In fact, earlier reports have argued that MΦs play no role in the activation of iNKT cells [220]. Bezbradica *et al.* used the FVB/N-human diphteria toxin transgenic receptor transgenic ($hDTR^{tg}$) mouse model, which leads to a full body depletion of $CD11c^+$ DCs 12 hours after administration of DT and crossed it with μMT mice that are B cell deficient. This would in turn render a mouse that is DC and B cell deficient. In their studies they found that $F4/80^+$ MΦs were left intact in the spleen, yet a portion of $CD11b^+$ MΦs were depleted. Nonetheless, the authors argue that in this DC and B cell deficient mouse model, iNKT cells were unable to be activated when challenged with αGC, providing evidence that $F4/80^+$ MΦs alone are unable to mediate iNKT cell activation. While this model is appropriate to study immune activation in the absence of DCs and B cells, no studies were done to investigate the architecture of the spleen after

diphteria toxin (DT) treatment. Also, one could speculate that the absence of B cells in secondary lymphoid organs could induce changes to the cellular distribution and architecture of the spleen so that iNKT cells are not in contact with the correct cells. Additionally, the use of hDTR mice has been challenged as it has been shown to have toxic side effects in mouse models that require an inflammatory immune response [221]. In contrast to this report, Barral and colleagues showed that selective depletion of MZ F4/80$^+$ MΦs by clodronate liposomes has a disruptive effect on the distribution of particulate αGC in the spleen leading to diminished iNKT cell activation [179]. It has been proposed that MΦs in the spleen lining the MZ are the first APC cell type that is exposed to antigen in the circulation and they play an essential role in antigen retention [222] and transfer of antigens to DCs to promote cytotoxic CD8$^+$ T cell responses [223]. Interestingly, Barral et al also showed that depletion of CD11c$^+$ DCs and F4/80$^+$ MΦs in the spleen is not sufficient to prevent overall iNKT cell activation and a residual 20% of iNKT cell cytokines is secreted in response to αGC. The mystery APC that mediates this effect has not been identified, but authors suggest that even though B cells have not been shown to present glycolipid to iNKT cells *in vivo,* it is possible that in the absence of other APCs, B cells can take on this role [224]. Nonetheless, whether direct activation of iNKT cells or indirect transfer of antigens leads to iNKT cell activation, we have shown that our conditional knockout of LRP is specific to F4/80$^+$ MΦs, and that despite a low level of LRP expression in WT DCs, they have normal LRP levels in LRP-cKO mice. Therefore, the sharp decrease of IL-4 we observe in our mouse model is dependent

on the expression of LRP and the rapid access that MZ MΦs have to blood-borne lipid antigens in the spleen.

Our laboratory and others have shown that chronic activation of iNKT cells can be pro-atherogenic [16,225]. Using a classical mouse model of hyperlipidemia, apoE$^{-/-}$ mice that were injected with αGC during a 16 and 24 week period exhibited a 2-fold increase in atherosclerotic lesions at 16 weeks and statistical significant increase respectively. It was then shown that the cytokine environment in the aorta was changed with increased IFN-γ, IL-10 and IL-4 which suggested immune dysregulation as a possible mechanism for increased lesion area. In this work, we present evidence suggesting an additional process by which αGC can be involved in the augmentation of lesion area. Figure 17 and 19 show that a single injection of αGC is sufficient to cause loss of MΦs in the spleen as early as 12 hours after injection. At this point we are unable to say that this loss of MΦs is caused by cell migration egress or localized cell death. We have shown that internalization or downregulation of F4/80 and migration to site of injection (Figure 20) are not responsible for this loss of MΦs, yet the possibility exists that these MΦs are not undergoing apoptosis in response to glycolipid challenge. It is imperative to discount this possibility and then evaluate the organ where F4/80 MΦs travel after αGC injection. However, one could speculate that if this occurred under conditions of hyperlipidemia, it could increase the availability of MΦs in the periphery to localize to the aorta and become cholesterol-laden foam cells leading to increased lesion areas. An alternative explanation of what is occurring after αGC injection can be deduced from the 72 hour time point observed in Figure 17. By this time point, MΦs

in the spleen have reached levels measured pre-αGC injection. Barring local proliferation of remaining F4/80^{+} MΦs, this could hint at a scenario where activation of iNKT cells mobilizes MΦs to travel to peripheral tissues in an effort to survey for pathogens. Therefore in the absence of any real threats such as αGC injection, MΦs can return to the spleen and populate this organ once again. While no current data supports this hypothesis future studies using transgenic CD45.1 and CD45.2 mice under parabiotic conditions can test whether MΦs can migrate out of secondary peripheral lymphoid organs in response to αGC.

A main limitation in studying LRP biology is the absence of a global knockout (KO). This is not possible because LRP plays an essential role in the implantation of the embryo during early to mid-gestation [209]. Therefore, the use of lineage specific conditional knockouts has been the method of choice to study LRP function. In our work we show that our LRP-cKO mouse model represents a 70% specific knockdown of LRP in MΦs (Figure 6), but not a full deletion characteristic of global knockouts. The knockdown measured in our work is reminiscent of other conditional knockdowns where cre recombinase was used to target LRP expression [164,195,196,226]. Therefore, the phenotypes presented in our studies and cited reports need to be labeled as a reduction of LRP expression and not a full knockout. Due to this caveat, we do not know if the remaining 30% of LRP in MΦ is responsible for a degree of iNKT cell activation both *in vivo* such as the IFN-γ measured at 24 hours (Figure 14A, Figure 15) or the normal levels of both IFN-γ and IL-4 measured *in vitro* (Figure 13) . Until newer genome engineering technologies can yield more efficient levels of LRP deletion in MΦs without

compromising embryo implantation, our LRP-cKO mouse model is currently the best approach to study absence of LRP in MΦs.

Another main limitation of our study is the use of αGC as a ligand for iNKT cell activation. It was discussed in Chapter 1 that contrary to dogma, a recent report has provided sufficient evidence to entertain the idea that αGC is present in mammalian cells [41]. Even if future studies and other research groups show this to be the case, the fact is that we have used concentrations of αGC that would exceed those detected in mammalian cells (0.02% of all glycolipids). Therefore, using αGC at these doses makes it difficult to fully appreciate the physiological signifance of the role of LRP. However, this could be countered by a study that showed *Bacteroides fragilis* strain NCTC 9343, a normal component of human microbiota, can produce a near identical glycolipid to αGC called αGC_{bf} [227]. Dysregulation of the human microbiota has recently become a topic of discussion as a cause for a variety of human disorders [228–230]. Therefore, one could speculate that certain conditions in which microbiome dysregulation leads to uncontrolled growth of certain bacterial species, such as *B. fragilis*, could result in elevated levels of αGC_{bf}. In this scenario, LRP expressing MΦ could have access to αGC_{bf} and promote normal iNKT cell responses. The presence of iNKT cells in the GI tract has been described previously [231], yet LRP expressing-MΦs have not. It would be interesting to evaluate whether detection of αGC_{bf} is mediated by LRP expressing MΦs. This would suggest that LRP MΦs could also be involved in the regulation of gut microbiota, which can have wide ranging effects on human health.

A further limitation of our study is that we only addressed the role of MΦ LRP in the activation of type I iNKT cells. In mice, iNKT cells can be divided in two main categories: type I and type II. Type I are the ones described in this work, which respond to αGC and are CD1d restricted. These cells have been studied extensively due to technological advances leading to generation of αGC/CD1d tetramers. Type II iNKT cells on the other hand, although believed to also be CD1d restricted, are activated by a different class of galactosylceramides known as sulfatides [232,233]. Production of tetramers for CD1d/sulfatide has so far proven unsucessful, complicating isolation and examination of type II iNKT cells. However, type II iNKT cells have also been shown to play a role in regulation of human diseases such as graft-versus-host disease [234], ulcerative colitis [235] B-cell lymphomas [236], multiple sclerosis [237,238], hepatitis [239] and potential inhibition of HIV infection [240]. In fact, it has been proposed that activation of type II iNKT cells can have a suppressive effect in the activation of type I iNKT cells [241]. Therefore, the importance of type II iNKT cells in human health should not be ignored. Although, the exact mechanism by which LRP modulates iNKT cell activation has not been determined, we demonstrated that flux of fluorescently labeled αGC is altered in LRP-cKO MΦs (Figure 11). The fact that αGC or other glycosphigolipids have never been described as a specific ligand for LRP suggests that transport of lipids overall may be affected in the absence of LRP. In fact, previous work has shown that LRP associates intracellulary with the protein GULP and together they orchestrate transport of glycosphingilipids and free cholesterol to endosomes and lysosomes [176]. Therefore, the possibility exists that LRP deficiency can have an overall effect

in transport of lipids affecting both type I and type II iNKT cells. Until more effective methods become available to study and isolate type II iNKT cells *in vivo* we can only speculate as to what role lipoprotein receptors like LRP play in their activation. However, one report using a mouse model of LRP deficiency in the forebrain ($LRP^{flox/flox}$, $\alpha CMKIICre^{+/}$) showed a global defect of lipid metabolism with sulfatide being one of the main lipids altered [242]. Therefore, its possible that LRP can also affect availability of ligands known to activate type II iNKT cells.

Our work challenges a notion in the iNKT cell field that a single cell type is responsible for the activation of iNKT cells. For example, Arora and colleagues provide evidence that a specialized subpopulation of DCs positive for CD8α and DEC-205 are responsible for the central activation of iNKT cells [243]. This agrees with other groups that focus primarily on DCs as the main cells that induce iNKT cell activation [220,244–247]. Having a single set of APCs that directs all glycolipids to active iNKT cells might be an ideal scenario when the best route of administration is being chosen for glycolipid treatment. However, our work and others provide evidence that *in vivo* the APCs that activate iNKT cells may be a heterogenous population that also include $F4/80^{+}M\Phi s$. Arora *et al.* used a fluorescently labeled β-GalCer probe which has a structure nearly identical to αGC, yet has never been shown to have any stimulatory activity on iNKT cells. Using monoclonal antibodies capable of detecting αGC/CD1d complexes, the authors present data that shows a portion of glycolip that reaches the spleen associates with this specific DC subtype, yet they fail to provide evidence that $F4/80^{+}$ MΦs are not involved in this process. The only piece of evidence provided to discount MΦ involvement is a $CD11b^{+}$

immunomagnetic depletion of splenocytes, followed by a 16 hour *in vitro* culture showing no changes in IFN-γ and IL-4 in response to αGC. In our work we also saw no changes in IFN-γ and IL-4 when challenging splenocytes with αGC *in vitro*, yet the main differences in cytokine secretion were measured *in vivo*. While the debate over which APC subtype can activate iNKT cells continues, more evidence points to a heterogenous population made up of DCs, MΦs, and under certain circumstances, B cells.

Ultimately, we hope knowledge gained from this work can be used in the when designing iNKT cell based therapies. The complexity of this immune compartment is highlighted by the fact that the two main cytokines studied in iNKT cell activation are known to have opposite roles in immunity. IFN-γ is mainly known for its inflammatory effects [248,249] and microbicidal properties [250], while the anti-inflammatory effects of IL-4 have been documented previously [251–253]. When targetting iNKT cells as a possible immunoregulatory mediator, the debate will be centered about which type of iNKT cell response would benefit human health. For example, during a bacterial or viral infection we would primarily prefer an IFN-γ mediated Th1 response that would activate MΦs to phagocytose extracellular bacteria and cause somatic cells to upregulate MHC class I to expose viral epitopes. On the other hand, as a treatment for autoimmune disorders, we would prefer an IL-4 mediated Th2 response, which would induce an anti-inflammatory effect and potentially reduce the harm caused to self-tissues by the immune system. In this work we describe a pathway, that when deleted leads to the absence of the IL-4 Th2 response. Therefore, one could propose that blockade of LRP prior to iNKT cell

activation could in theory promote a Th1 IFN-γ response. This would lead to an an immediate burst of IFN-γ capable of altering the course of a bacterial infection. Ideally, this blockade of LRP would only be momentary and would have no long lasting effects on its ability to bind all of its diverse repertoire of ligands and signaling functions.

Overall, our work provides a new mechanism by which a receptor widely labeled as a lipoprotein receptor can regulate immunity. We have provided *in vivo* data that shows a cytokine bias in the absence of LRP. Additionally, we have shown that this receptor is modulated in response to iNKT cell activation. As the iNKT cell field moves forward designing potential therapeutics to treat human pathologies, the data presented in this dissertion illustrate that receptors such as LRP can play an important role in the quality of response generated by glycolipid antigens.

CHAPTER V

METHODS

Animals. Lysozyme M **(**LyzM)-Cre$^{+/-}$,LRP$^{flox/flox}$ (hereafter referred to as LRP-cKO) mice were a kind gift from Dr. Sergio Fazio (Department of Medicine, Vanderbilt University Medical Center) and have been described previously [183]. Briefly, mice harboring loxP sites flanking the LRP gene [193] were crossed with mice expressing cre-recombinase under the control of macrophage LyzM promoter [254]. Littermate LyzM-Cre$^{-/-}$, LRP$^{fl/fl}$ were used as wild type (hereafter referred to as WT) controls. For some experiments, C57Bl/6 mice were used as indicated. B6.PL-Thy1<a>/CyJ mice were obtained from the Jackson Laboratory. CD1d$^{-/-}$ mice were a kind gift from Dr. Luc Van Kaer (Department of Microbiology and Immunology, Vanderbilt University Medical Center). All mice were on the C57BL/6 background. Mice were maintained in the Vanderbilt University animal care facility and had access to food and water *ad libitum*. All procedures were approved by Vanderbilt University Medical Center's Institutional Animal Care and Use Committee.

Cell isolation. Isolation of leukocytes from thymus, liver, lymph nodes and spleen have been described previously [255]. In short, spleens were digested with 1mg/mL collagenase type II (Sigma) in Hank's balanced salt solution (HBSS, Mediatech). Digested spleens were passed through a 70μm cell strainer and red blood cells lysed by osmotic shock. Livers were perfused with cold PBS, digested with

collagenase type II and pressed through a 70μm cell strainer. Hepatic leukocytes were then isolated from the interface of a 40/60% Percoll gradient (GE Healthcare, Piscatway, NJ). Peritoneal macrophages were collected in PBS 3 days after peritoneal injection of 3% thioglycollate (Fluka).

Flow Cytometry. Single-cell suspensions of mononuclear leukocytes were blocked for 15 minutes at RT in a 1:50 dilution of Fc receptor block (BD Biosciences) in FACS buffer (1X HBSS, 1% BSA, 4.1 mM sodium bicarbonate, and 3 mM sodium azide). The following fluorescently labeled antibodies were diluted 1:100 in FACS buffer and incubated with cells for 45 minutes at 4^{o}C: CD11b-V450, Thy1.1-V450 (1:300 dilution), CD69-FITC, CD1d-PE, CD4-PE, TCRβ-PE, CD8-PerCP, CD45-PerCP, Thy1.2-PeCy7, CD11c-APC and CD45R-APC-cy7 (all from BD Pharmingen), NK1.1-PerCP, F4/80-PeCy7 (from eBiosciences) and Siglec-1-PE (Biolegend) LY49C/F/H/I (clone 14B11, from BD Biosciences). To stain iNKT cells, α–galactosylceramide-CD1d tetramers- APC (NIH tetramer facility) were used. iNKT cells were defined as $TCR\beta^{+}B220^{-}$ $TCR\beta^{int}$ $tetramer^{+}$. To stain LRP for flow cytometry, we fluorescently labeled 5A6 LRP clone (gift from Dr. Dudley Strickland, University of Maryland) with APEX Alexa Fluor-488 antibody labeling kit (Invitrogen) according to manufacturer's protocol. Conjugated 5A6-Alexa Fluor-488 antibody was titrated to determine optimal binding dilution. In order to stain total levels of LRP (surface and intracellular), cells were stained for surface markers prior to addition of LRP antibody. Surface labeled cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) reagents according to manufacturer's protocol.

Mouse IgG2b-Alexa Fluor 488 as the matched isotype control (BD Biosciences). Labeled cells were analyzed on a MACSquant seven-color flow cytometer (Miltenyi Biotec) and data analyzed with FlowJo software (Tree Star).

Enzyme-linked Immunosorbent Assay. Mouse IL-2, IL-4, IFN-γ and IgE were measured by standard sandwich ELISA according to the manufacturer's protocol (BD Pharmingen).

Measurement of In Vitro and In Vivo Responses to αGC. Splenocytes were plated at $2.5x10^5$ cells/well in RPMI (Hyclone) media containing 10% FBS (Sigma), penicillin-streptomycin with 50μmol/L L-glutamine (Gibco) and 50μmol/L β-mercaptoethanol (Sigma) with the indicated concentrations of αGC. Supernatants were collected after 72 hours of culture and cytokine levels determined by ELISA. For peritoneal MΦs, $1.0x10^5$ cells/well were incubated with $1.0x10^5$ of murine iNKT hybridoma DN32.D3 (from Dr. Albert Bendelac, University of Chicago, described in [256]). Supernatants were collected 48 hours after culture and IL-2 measured by sandwich ELISA. For *in vivo* measurements, 4μg, 1μg or 0.5μg αGC reconstituted in vehicle buffer (0.5% polysorbate) was i.p. injected in a total volume of 200μl. An equal volume of vehicle was injected in to control mice. At the specified times following injection, plasma was collected and splenocytes were isolated, stained and analyzed by flow cytometry.

Pulse of Peritoneal MΦs with BODIPY-αGC. Peritoneal MΦs (pMΦs) were obtained as described previously. A total of $5x10^5$ cells/tube were incubated with 1μg/mL BODIPY-αGC (gift from Dr. Paul Savage, Brigham Young University) and harvested at the time points noted in the text. For the pulse-chase experiment, pMΦs were preincubated with αGC for 4 hours. After this time, cells were washed an incubated with BODIPY-αGC.

Generation of mixed bone marrow chimeras. WT recipient mice were irradiated with 950 rads using a cesium source and bone marrow from donor mice was transplanted via retro-orbital injection with a 1:1 mixture ($2x10^6$ total cells) from $LysMcre^{+/-}$, $LRP^{flox/flox}$ (Thy1.2) and C57BL/6 (Thy1.1) mice. Recipient mice were maintained on sterile water with sulfamethaxazole-trimetroprim for 3 weeks. Chimeras were analyzed 4 weeks after transplant.

Immunization with αGC and induction of Immunoglobulin E (IgE). Mice were injected with 4μg/mouse of αGC or vehicle buffer (0.5% polysorbate). Serum was collected prior to injection and also 6 days after immunization to measure IgE levels.

Endogenous lipid presentation assay. We used DN32.D3 hybridoma ($1.0x10^5$/well) with thymocytes ($1.0x10^6$) in 200μL/well RPMI for 48 h, supernatants were collected and IL-2 levels measured by ELISA.

Neutralization of IFN-γ. In order to neutralize IFN-γ we used 100ug/ml injections of neutralizing antibody (R4-6A2) 2 hours prior to αGC injection and at 4 hour intervals until 24 hours. Serum was collected at 4, 8, 16 and 24 hours. Neutralizing antibody for IFN-γ was a kind gift from Dr. Thomas Aune (Vanderbilt University).

Statistical Analyses. Statistical analyses were conducted using PRISM V.5.0 software (GraphPad, La Jolla California, USA). For direct comparison between two groups, an unpaired Student's *t*-test was used and for comparisons made between three or more groups a one-way analysis of variance (ANOVA) was performed. Values are expressed as mean ± standard error of the mean unless otherwise noted. A p value < 0.05 was considered statistically significant.

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