Functional requirements for CD1 in development and function of invariant Natural Killer T (iNKT) cells

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Functionele benodigdheid van CD1 voor de ontwikkeling en functie van invariante NKT (iNKT) cellen
(met een samenvatting in het Nederlands)

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Contents

Summary

Activation of the adaptive immune response depends on the presentation of antigen (Ag) in a form that can be recognized by specialized T cells. This process requires Ag processing and association of Ag-derived fragments with molecules encoded by the major histocompatibility complex (MHC) or CD1 locus. Much emphasis on Ag processing and presentation in the last decades has focused on the processing and presentation of 1) extracellular peptide Ags via Class II MHC complexes and 2) cytosolic peptide Ags via Class I MHC complexes. However, some pathogens and tumor cells have evolved evasive mechanisms to escape these 'conventional routes' of Ag processing and presentation. **Chapter 2** highlights recent developments in two alternative pathways in professional Ag presenting cells (APCs), 1) those of peptide cross-presentation via Class I MHC complexes to $CD8^+$ T cells and 2) the presentation of lipid Ags in association with CD1 to natural killer T cells (NKT). The purpose of the research described in this thesis is to clarify the role of CD1d on the development and activation of NKT cells during pathogenic and autoimmune diseases.

The review in **Chapter 2** summarizes how lipid Ag degradation and presentation can be modulated via triggering of pattern recognition receptors (PRR) by pathogen-associated molecular patterns (PAMPs). The review also covers how certain pathogens, such as *Mycobacterium tuberculosis* (*Mtb*), can evade this immune recognition through inhibition of lipid-Ag presentation of phagosomal origin. In addition, we review how deviations in endosomal lipid-Ag processing and presentation affect the immune system of individuals suffering from lysosomal glycosphingolipid storage diseases.

Vα14 invariant (i)NKT cells (hereafter referred to as iNKT cells) are the key effector cells activated via the presentation of both exogenous and endogenous lipids on CD1d. Activated iNKT cells stimulate cells contributing to both innate and adaptive immune responses through the rapid release of cytokines. It is important to understand the mechanisms behind the development and activation of iNKT cells, because of the role they play in bridging the innate and adaptive immune systems.

The positive selection of iNKT cells in mice requires CD1d-mediated Ag presentation by $CD4^+CD8^+$ double positive (DP) thymocytes. Maturation of newly selected iNKT cells continues in the periphery and also involves CD1d expression. CD1d molecules acquire Ags for presentation in endosomal compartments, to which CD1d molecules have access through an intrinsic CD1d-encoded tyrosine motif and by association with the Class II MHC chaperone, invariant chain (Ii). Until recent, existing mouse models were insufficient to clarify the roles that the CD1d-intrinsic sorting motif plays in CD1d-mediated positive selection, post-selection terminal maturation and stimulation of matured iNKT cells. In **Chapter 3** we describe a new mouse model in which all CD1d is replaced by CD1d-enhanced yellow fluorescent fusion protein (EYFP). CD1d-EYFP molecules are stable, present lipid-Ags, and have near normal subcellular distribution. Yet, the internalization rate of CD1d-EYFP molecules from the cell surface to endosomes is especially reduced in T cells, which alters the development of iNKT cells. With these mice we could clarify that the intrinsic CD1d-encoded tyrosine motif influences the thymic selection of iNKT cells, while the CD1d-encoded motif is dispensable for the peripheral maturation.

Thymocytes do not express Ii, and CD1d therefore strictly relies on its own CD1d-encoded tyrosine-based sorting motif for localization in lysosomes. In professional APCs, however, endosomal sorting of CD1d could be mediated by the CD1d-encoded tyrosine-based

sorting motif, or via association of CD1d with Ii. In **Chapter 3** and partly in **Chapter 4** we studied whether in professional APCs, the CD1d-encoded tyrosine-based sorting motif and Ii-encoded sorting motif can supplement for each other in mediating lysosomal localization, Ag acquisition and presentation by CD1d. To this end we used CD1d-EYFP/EYFP mice in combination with mice deficient for Ii or the Ii-processing enzyme cathepsin S (CatS). Experiments with these mice demonstrate that both the CD1d-encoded and Ii-encoded endosomal sorting motifs are necessary to induce the activation of iNKT cells by professional APCs in the periphery. In addition, we demonstrate that thymic selection of iNKT cells not only occurs on DP thymocytes but also involves CD1d-Ag presentation by Ii and CatS-expressing professional APCs.

Invariant NKT cells play a role in the early immune response against *Mycobacterium tuberculosis* (*Mtb*) by suppressing intracellular bacterial growth when activated by antigenic CD1d/lipid complexes on *Mtb*-infected cells. Tuberculosis is one of the most prevalent deadly diseases and the Bacille Calmette–Guérin (BCG) vaccine against tuberculosis unfortunately affords insufficient protection against *Mtb*. Therefore, it is necessary to develop better prophylactic strategies and to understand the mechanisms that are critical in the protective immunity to *Mtb*. *Mtb* persists in phagosomes of myeloid cells by preventing fusion of phagosomes with lysosomal compartments, thereby avoiding acidic pH and exposure to lysosomal hydrolases. It is not understood how *Mtb* presence in phagosomes can yield antigenic CD1d/lipid complexes, which are thought to be generated in lysosomes. The identity of the endosomal compartment that facilitates pathogen replication is also unknown. To address these questions, we focused in **Chapter 4** on Ii and CatS. Ii regulates endosome fusion and architecture, and is required for endosomal transport. Cells lacking CatS exhibit enlarged endosomal compartments without multivesicular structures. Our results demonstrate that *Mtb* survival benefits from obstruction of endosomal transport in Ii-deficient, but not in CatS-deficient macrophages. *Mtb* replication does require Ii-mediated phagosomal fusion to lysosomes. Lysosomes but not phagosomes possess endosomal architecture conducive for *Mtb* replication, a process that benefits the pathogen. Lysosomal presence of *Mtb* is, however, equally pivotal for induction of CD1d-dependent iNKT cell responses in the host. We thus highlight the lysosome as a potential therapeutic target in the development of therapeutic strategies against *Mtb* infections.

Invariant NKT cells also regulate immune tolerance as during autoimmunity and inflammation. In **Chapter 5** we describe a role for iNKT cells in modulating 'conventional' $CD8^+$ T cell responses to Ag expressed in liver, using transferrin-ovalbumin (Tf-mOVA) mice. The presence of Ag on Tf-mOVA hepatocytes leads to the retention of Ag-specific $CD8^+$ T cells in the liver, which induce hepatitis upon restimulation. This makes the Tf-mOVA mice a useful mouse model for autoimmune hepatitis. We tested the function and effects of naïve mOVA-specific class I MHC-restricted T cells (OTI), which were adoptively transferred into Tf-mOVA or $CD1d^{-/-}$ Tf-mOVA mice in the presence or absence of iNKT-cell agonist α-galactosylceramide (αGalCer). Our findings demonstrate how rapid cytokine production by activated CD1d-restricted iNKT cells stimulates intrahepatic $CD8^+$ T cell effector responses to Ag expressed in liver. We thus highlight the rapid cytokine production by iNKT cells as a potential intervention point for targeted immunotherapy to autoimmune and possibly (viral) infectious liver diseases.

The studies in this thesis highlight the importance and mechanisms behind CD1d-restricted iNKT cell development, and function during infectious disease and autoimmunity. In specific, we elucidate the distinct requirements for CD1d intracellular transport for development and function of iNKT cells. Furthermore, we demonstrate the importance of lysosomal localization of *Mtb* for both pathogen replication and induction of CD1d-restricted iNKT cell response against *Mtb* infection. In addition, we show a critical role for CD1d-restricted iNKT cells in stimulating intrahepatic $CD8^+$ T cell responses to liver-expressed self-Ag. Finally, our findings elucidate previously unknown targets for therapeutic strategies against autoimmune and infectious disease.

Samenvatting

Activatie van het adaptieve immuunsysteem berust op de presentatie van antigeen (Ag), in een vorm die herkend kan worden door gespecialiseerde T cellen. Dit proces vereist de verwerking van Ag en de associatie van Ag-fragmenten met moleculen die gecodeerd zijn door het 'major histocompatibility complex' (MHC) of het CD1 locus. De afgelopen decennia heeft het focus binnen het Ag-presentatie- en verwerkingsonderzoek gelegen bij de verwerking en presentatie van 1) extracellulair peptide-Ag via Klasse II MHC complexen en 2) intracellulair peptide-Ag via Klasse I MHC complexen. Echter, sommige pathogenen en tumor cellen hebben ontsnappingsstrategieën ontwikkeld om aan deze 'conventionele' Ag-verwerking en presentatie te ontsnappen. Hoofdstuk 2 belicht de recente ontwikkelingen in het onderzoek naar twee alternatieve routes voor antigeen-presentatie in professionele Ag-presenterende cellen (APCs), namelijk: 1) kruispresentatie van peptide via Klasse I MHC complexen aan $CD8^+$ T cellen en 2) presentatie van lipiden via CD1 moleculen aan Natural Killer T cellen (NKT). Het onderzoek beschreven in dit proefschrift heeft als doel om de rol van CD1d in de ontwikkeling en functie van NKT cellen tijdens pathogene infecties en auto-immuun ziekten te verduidelijken.

Hoofdstuk 2 vat samen hoe lipide-afbraak en -presentatie gemoduleerd wordt door stimulatie van 'patroon-herkennende receptoren (PRR) met pathogeen-geassocieerde moleculaire patronen (PAMPs). Dit hoofdstuk beschrijft tevens hoe bepaalde pathogenen, zoals *Mycobacterium tuberculosis* (*Mtb*) deze immuun-herkenning kunnen ontwijken door de presentatie van lipiden vanuit fagosomen te blokkeren. Daarnaast wordt bediscussieerd hoe afwijkingen aan de endosomale lipide Ag-verwerking en -presentatie het immuunsysteem beïnvloeden bij patiënten met lysosomale glycosfingolipiden opslagziekten.

Vα14 invariante NKT cellen (hierna genoemd iNKT cellen) zijn de belangrijkste effector cellen die geactiveerd worden via de presentatie van zowel exogene als endogene lipiden via CD1d. Middels snelle uitscheiding van cytokines stimuleren geactiveerde iNKT cellen andere cellen die bijdragen aan de aangeboren en adaptieve immuun respons. Het is van belang om de mechanismen achter het ontwikkelen en activeren van iNKT cellen te begrijpen. iNKT cellen spelen namelijk een belangrijke rol in de koppeling van het aangeboren en adaptieve immuun systeem.

Positieve selectie van iNKT cellen in muizen vereist presentatie van lipide-Ag op CD1d door $CD4^+CD8^+$ dubbel positieve (DP) thymocyten. De ontwikkeling van nieuwgevormde iNKT cellen wordt voortgezet in de periferie en berust op de expressie van CD1d. CD1d moleculen verkrijgen Ag in endosomale compartimenten, die ze bereiken met behulp van een intrinsiek CD1d-gecodeerd tyrosine motief en door associatie met de Klasse II MHC chaperonne, 'invariant chain' (Ii). Tot voorkort, waren de bestaande muizenmodellen ongeschikt om de rol van het intrinsiek CD1d-gecodeerd tyrosine motief tijdens positieve selectie, post-selectieve terminale ontwikkeling en stimulatie van iNKT cellen, te verklaren. In Hoofdstuk 3 beschrijven we een nieuw muizenmodel waarin alle CD1d vervangen is door CD1d gefuseerd met 'versterkt geelfluorescerend fusie eiwit' (EYFP). CD1d-EYFP moleculen zijn stabiel, presenteren lipide-Ag en vertonen een vrijwel normale subcellulaire distributie. Echter, in T-cellen hebben de CD1d-EYFP moleculen een vertraagd beloop van internalisering van het cel oppervlak naar de endosomen, wat de ontwikkeling van iNKT cellen beïnvloed. Met deze muizen hebben we kunnen verklaren dat het intrinsiek CD1d-gecodeerde tyrosine motief de selectie van iNKT cellen in de

thymus beïnvloedt, terwijl het CD1d-gecodeerde motief voor de perifere ontwikkeling niet noodzakelijk is.

Thymocyten brengen geen Ii tot expressie, waardoor CD1d uitsluitend op zijn eigen CD1d-gecodeerde tyrosine motief is aangewezen voor lokalisering in de lysosomen. Echter, in professionele APCs wordt de endosomale sortering van CD1d beïnvloed door zowel het intrinsiek CD1d-gecodeerde tyrosine motief als de associatie van CD1d met Ii. In Hoofdstuk 3 en gedeeltelijk in Hoofdstuk 4 bestuderen we of het CD1d-gecodeerde tyrosine motief en het Ii-gecodeerde sorteringsmotief elkaar aanvullen tijdens lokalisering in lysosomen, en acquisitie en presentatie van Ag door CD1d. Hiervoor is gebruik gemaakt van CD1d-EYFP/EYFP muizen in combinatie met muizen die deficiënt zijn voor Ii of het Ii-verwerkend enzym, cathepsin S (CatS). Experimenten met deze muizen wijzen uit dat zowel het CD1d-gecodeerde als het Ii-gecodeerde sorteringsmotief nodig zijn voor de activering van iNKT cellen door professionele APCs in de periferie. Tot slot blijkt dat selectie van iNKT cellen in de thymus niet alleen gebeurt op DP thymocytes, maar ook wordt beïnvloed door presentatie van lipiden/CD1d complexen door professionele APCs die wel Ii en CatS tot expressie brengen.

Invariante NKT cellen spelen een rol in de vroege immuunrespons tegen *Mycobacterium tuberculosis* (*Mtb*). De iNKT cellen, geactiveerd door antigene CD1d/lipiden complexen op *Mtb*-geïnfecteerde cellen, onderdrukken de intracellulaire bacteriële groei. Tuberculose is een van de meest voorkomende dodelijke ziekten en het Bacille Calmette–Guérin (BCG) vaccin biedt helaas onvoldoende bescherming tegen *Mtb*. Daarom is de ontwikkeling van betere profylactische strategieën en het begrip van de mechanismen achter de beschermende immuunrespons tegen *Mtb* noodzakelijk. *Mtb* overleeft in fagosomen van myeloïde cellen door fusie van deze fagosomen met lysosomale compartimenten te voorkomen. Hierdoor wordt zure pH en blootstelling aan lysosomale hydrolases vermeden. Het is onbekend hoe de aanwezigheid van *Mtb* in de fagosomen kan leiden tot presentatie van antigene CD1d/lipiden complexen, waarvan de samenstelling vermoedelijk in de lysosomen plaats vindt. De identiteit van de endosomale compartimenten waar pathogeen replicatie plaats vindt is tevens onbekend. Om deze vragen te kunnen beantwoorden hebben we ons in Hoofdstuk 4 gefocust op Ii en CatS. Ii reguleert de fusie van fagosomen met lysosomale compartimenten en is tevens van belang voor het endosomaal transport. In CatS-deficiënte cellen zijn de endosomale compartimenten vergroot en ontbreken multivesiculaire structuren. Onze resultaten wijzen uit dat *Mtb* profiteert van obstructie van het endosomale transport in Ii-deficiente, maar niet in CatS-deficiente macrofagen. Ii-gemedieerde fusie van fagosomen met lysosomen is noodzakelijk voor replicatie van *Mtb*. Lysosomen, maar niet fagosomen, bezitten de endosomale architectuur die nodig is voor de replicatie van *Mtb*. De lysosomale lokalisering van *Mtb* blijkt echter even zo cruciaal voor de inductie van de CD1d-afhankelijke iNKT cel respons in de gastheer. Derhalve zou bij de ontwikkeling van nieuwe therapeutische strategieën tegen *Mtb* infecties het lysosoom als een potentiëel therapeutisch doelwit kunnen dienen.

Invariante NKT cellen spelen ook een rol bij het reguleren van immunologische tolerantie bijvoorbeeld in de context van auto-immuun ziekten en ontstekingen. Met behulp van transferrine-ovalbumine (Tf-mOVA) muizen, beschrijven we in Hoofdstuk 5 een rol voor iNKT cellen tijdens de modulatie van de 'conventionele' $CD8^+$ T cel respons tegen lever-Ag. De aanwezigheid van Ag op Tf-mOVA hepatocyten leidt tot de retentie van OVA-specifieke $CD8^+$T cellen in de lever. Door restimulatie wordt hepatitis opgewekt, en

derhalve vormen deze muizen een model voor auto-immuun hepatitis. We testen de functie en effecten van naïeve mOVA-specifieke Klasse I MHC-beperkte T cellen (OTI) die in de aan- of afwezigheid van α-galactosylceramide (αGalCer), een potente iNKT-cel activator, in Tf-mOVA of $CD1d^{-/-}$Tf-mOVA muizen werden geïnjecteerd. Onze resultaten tonen aan hoe cytokine-productie door geactiveerde CD1d-specifieke iNKT cellen de intrahepatische $CD8^+$ T cel respons tegen lever-Ag stimuleert. Deze bevindingen vormen mogelijk een interventie punt voor immuno-therapie tegen auto-immuniteit en (virale) lever infecties.

Tezamen benadrukken de studies besproken in dit proefschrift het belang en de mechanismen achter de CD1d-specifieke iNKT cel ontwikkeling en functie gedurende infecties en auto-immuun ziekten. In het specifiek, verhelderen we de benodigdheden van CD1d intracellulair transport tijdens de ontwikkeling en functie van iNKT cellen. Daarnaast, tonen we aan dat de lysosomale lokalisering van *Mtb* van belang is voor zowel de pathogeen replicatie als de inductie van de CD1d-gemedieerde iNKT cel response tegen de *Mtb* infectie. Bovendien, tonen we aan dat CD1d-specifieke iNKT cellen een cruciale rol spelen in de stimulatie van $CD8^+$ T cellen tegen zelf-Ag, welke tot expressie wordt gebracht in de lever. Al met al belichten we met onze resultaten tot voorkort ongekende mikpunten voor de therapeutische strategieën tegen auto-immuun en infectie ziekten.

Chapter 1

General introduction

CD1 and immunity

The immune system is a versatile defense system against invading pathogenic microorganisms and cancers. It consists of humoral and cellular components that play a role during the innate or adaptive immune responses. It has long been the paradigm that the adaptive immune system relies on peptide antigens presented by major histocompatibility complex (MHC) molecules and recognized by T cells. The discovery that unlike MHC molecules, CD1 molecules present lipids instead of peptides that function as T cell antigens revealed a yet unappreciated complexity to the adaptive immune system [1,2]. The review in **Chapter 2** covers how several signals instigate the processing and CD1-mediated presentation of antigenic lipids in antigen presenting cells (APCs). The majority of CD1-restricted T cells are natural killer T (NKT) cells that express invariant TCRα chains, NK cell markers and depend on the expression of CD1 for their selection, development and maturation [3-10]. The data in **Chapter 3** shows that invariant (i)NKT cells depend on endosomal trafficking of CD1 for their development and function. The iNKT cells that specifically respond to CD1-lipid antigen presentation by the rapid release of cytokines, thereby amplify the innate response [3], activate and regulate cells from the adaptive immune system and thus function as a bridge between the innate and adaptive immune responses [4,5]. In **Chapter 4** it is discussed how intracellular pathogen trafficking is necessary for pathogen replication and induction of CD1-mediated immune defense during *Mycobacterium tuberculosis* infection. Since most iNKT cells display self-lipid reactivity [6-10], they play an important role in tolerance maintenance and autoimmunity. **Chapter 5** highlights the role of CD1-restricted iNKT cells in an autoimmune hepatitis model.

The origins of CD1

CD1 molecules got their name "Cluster of differentiation 1" (CD1) at the First International Workshop on Human Leukocyte Differentiation antigens, since they were the first human leukocyte differentiation antigens that were defined by monoclonal antibodies [11]. CD1 genes have since been discovered in many other mammals [12-23] and chickens [24].

In all studied animals except chickens [24-26] the CD1 genes are found in regions outside the MHC locus [14,27]. The CD1 and Class I and II MHC genes probably all arose in the primordial MHC and may have diverged quite early in their history, at least around the same time as the bird-mammal divergence, 250-300 million years ago (mya) [28,29], or even earlier at the time of the appearance of the jawed vertebrates, ~600 mya [24].

CD1 genes

Compared to Class I and II MHC genes, allelic variation of CD1 genes is extremely limited. Five linked and active CD1 genes, CD1A to CD1E have been identified in humans [27,30-34]. Based on sequence divergence the five known CD1 genes can be ordered into three groups: (1) CD1A, CD1B and CD1C, (2) CD1D and an intermediate group (3) CD1E [28,30,35]. Chicken CD1 genes could not be classified according to the existing isoforms, and are therefore named *ch*CD1.1 and *ch*CD1.2 [24-26]. Different evolutionary pressure might have led to different copy numbers or deletion of separate CD1 isoforms in other animals. Muroid rodents such as mice [14] most likely lost the group 1 CD1 genes due to a chromosomal break [36-38] and thus only express CD1D (group 2) homologs. Mice have

duplicate CD1D genes located in tail-to-tail orientation on chromosome 3 [14,36,39,40], the functional gene *m*CD1D1 and the pseudogene *m*CD1D2 [41]. Since CD1d genes are expressed in most mammalian species studied, mice can be used to study the properties and functions of group 2 CD1, which is the focus in this thesis.

CD1 from genes to proteins

CD1 genes are organized in a similar way to Class I MHC genes, with separate exons encoding for a leader peptide, three immunoglobulin-like extracellular domains (two membrane-distal domains α1 and α2 and a membrane proximal domain α3) a transmembrane region and a generally short intracellular carboxy-terminus [31,34]. Overall mouse CD1d molecules have a 36 kDa polypeptide backbone and depending on the amount of glycosylation at the 5 *N*-linked glycosylation sites, a molecular weight between 49 and 55 kDa [39,42]. **Figure 1** displays the molecular weight of CD1d molecules compared to CD1d linked to enhanced yellow fluorescent protein (CD1d-EYFP) on a Western blot (see also Figure 1 in **Chapter 3**).

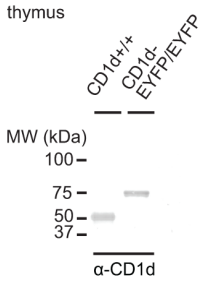

FIGURE 1. Molecular weight of mouse CD1d. Whole thymocyte lysates were separated on a 4-20% Tris-HCl gel and transferred to PVDF membrane. Membranes were blotted using polyclonal anti-CD1d Ab. The CD1d-EYFP fusion protein is positioned at 76 kDa (CD1d heavy chain: 49 kDa; EYFP: 27 kDa) compared to WT-CD1d heavy chain at 49 kDa.

Crystal structures of mouse CD1d revealed an overall fold similar to Class I MHC molecules [43], see **Figure 2** for a cartoon of the structure similarities of CD1d and Class I and II MHC molecules. The α-chain folds into three domains of which the immunoglobulin-like α3 domain non-covalently associates with β2m [44-47]. Although murine CD1d associates with β2m [39,48,49], there is indirect evidence from β2m-deficient mice that β2m-free CD1d molecules may exist as well [50-53].

The α1 and α2 domains form the binding groove superdomain [43]. This antigen binding groove however, is deeper, narrower and much more hydrophobic compared to related Class I and II MHC molecules [43]. The binding groove consists of two deep major pockets, designated A' and F', which are lined with hydrophobic residues optimal for binding long hydrophobic chains such as lipid tails [43]. Significant differences in the binding grooves, contribute to distinctive ligand binding characteristics for the different CD1 isoforms found in humans [54].

The discovery and use of the synthetic form of marine sponge-derived α-galactosyl ceramide (αGalCer) proved that CD1d molecules indeed present (glyco-)lipid antigens to NKT cells [1,7,55-58]. Since the discovery of αGalCer, several naturally occurring endogenous and exogenous antigens, potentially recognized by NKT cells have been identified (see the later paragraph on "CD1 antigens" for a selected overview).

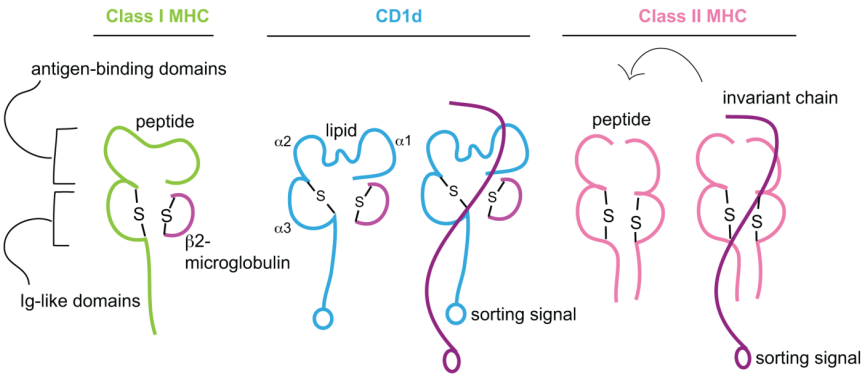

FIGURE 2. Graphic comparison between CD1d and Class I and II MHC molecules. X-ray crystallographic studies of mouse CD1d revealed a significant overall homology with Class I MHC molecules [43]. The α-chain folds into three domains of which the immunoglobulin-like α3 domain non-covalently associates with β2m [44-47]. The α1 and α2 domains form the binding groove superdomain which is deeper, narrower and much more hydrophobic compared to related Class I and II MHC molecules [43]. The binding groove consists of two deep major pockets, which are lined with hydrophobic residues optimal for binding long hydrophobic chains such as lipid tails [43]. The trafficking of mouse CD1d is not only mediated by the tyrosine-based sorting motif present in the cytoplasmic tail of CD1d, but also by association with the invariant chain (Ii) or Class II MHC molecules/Ii complexes [59-61].

CD1 assembly and intracellular trafficking

CD1d is constitutively expressed by many cells, in particular antigen presenting cells (APCs) including dendritic cells (DCs) and macrophages (Mϕ) [51] and other cells including, keratinocytes in the skin and epithelial cells in hair follicles [62], Kupffer cells and endothelial cells lining liver sinusoids [63], hepatocytes in mice and upon disease induction in humans [64] cortical thymocytes [65] B cells, particularly marginal zone B cells [66] and T cells in liver and to a lesser degree in the spleen and lung [51]. Intracellular, CD1 molecules are found in different compartments depending on their trafficking patterns.

CD1 heavy chains are assembled in the endoplasmic reticulum (ER), where they are glycosylated, by the addition of N-linked oligosaccharides, which enables the binding to the chaperones calnexin, calreticulin and the thiol reductase ERp57 [67-69]. Eventually CD1 molecules associate with β2m, although this is not strictly necessary to exit the ER [39,48,49]. In the ER CD1d molecules are loaded with self-lipids such as GPI with a chaperone-like function as described below [70,71]. This lipid-loading process is mediated by microsomal triglyceride transfer protein (MTP) [72,73].

After assembly in the ER, CD1 molecules follow the secretory route through the Golgi apparatus to the plasma membrane, where the ER-derived self-lipids can be presented to a specific subset of NKT cells [8]. CD1 molecules are then internalized in clathrin-coated pits, a process which for human CD1b, CD1c and CD1d and most likely mouse CD1d, is mediated through the interaction of the adaptor protein complex-2 (AP-2) with the CD1

tyrosine-based sorting motif [74-78]. After internalization the different CD1 isoforms follow different routes. CD1b and mouse CD1d traffic through late endosomal and lysosomal compartments via the interaction of AP-3 with the tyrosine-based sorting motif, before recycling back to the plasma membrane [75,79-81]. It has been suggested that MTP, although it is an ER-resident lipid transfer protein, is involved in the recycling of CD1d from the lysosomes to the plasma membrane [82]. In contrast, human CD1c and CD1d contain tyrosine-based motifs that do not bind to AP-3, while CD1a lacks the tyrosine-based motif entirely, leading to their main intracellular distribution within early endosomes and recycling endosomes respectively [77,78,81]. The trafficking of mouse CD1d is not only mediated by tyrosine-based sorting motif present in the cytoplasmic tail of CD1d, but also by the invariant chain (Ii) [59,60]. Mouse CD1d molecules can associate with Ii or Class II MHC molecules/Ii complexes [60,61], which direct CD1d complexes directly from the trans-Golgi network to the endosomal compartments without first reaching the plasma membrane [60].

The exchange of ER-derived lipids for other self-lipids or pathogen-derived lipids can occur at the plasma membrane, but mainly occurs in the endocytic compartments, where glycolipid antigen processing takes place ([8,83-85], additionally see **Chapter 2** for a review on endosomal processing for CD1d-mediated antigen presentation). Accessory molecules such as saposins [86-89], apolipoprotein E [90] and for the human isoforms, CD1e [35,91,92] assist in the loading of lipids in the endocytic compartments. It has been suggested that the cysteine proteases cathepsin S and L (CatS and CatL) also have a role in CD1d-mediated antigen presentation, although the mechanisms by which they affect CD1d function have not yet fully been delineated [93,94]. See **Figure 3** for a representation of the trafficking of CD1d.

Given that lipids are sorted into different compartments and traffic according to their biophysical properties [95,96], it has been proposed that the trafficking of the different CD1 isoforms evolved to sample all the compartments to which lipid antigens are differentially delivered [37,97]. Much of what is known to date about CD1-mediated antigen presentation is obtained from mouse-based experiments. Until recent, those models were not sufficient to study whether CD1d in mice also loads different lipids depending on the endosomal trafficking and what the effect would be on thymic iNKT cell selection and peripheral maturation.

We generated a new mouse model in which all CD1d is fused to enhanced yellow fluorescent protein (EYFP) on the cytosolic tail. In these mice the internalization rate of CD1d-EYFP molecules from the plasma membrane into the endosomal pathway is delayed, in for example $CD4^{+}CD8^{+}$ double positive (DP) thymocytes. Invariant NKT cells are positively selected on DP thymocytes ([6,98-100] see also the later paragraph on “Selection and development of CD1-specific T cells”). This delay in CD1d endocytosis most likely altered the loading and presentation of endogenous lipids, thereby skewing the iNKT cell repertoire during positive selection ([101], **Chapter 3**). In addition, the use of CD1d-EYFP mice deficient in Ii, demonstrated that the presentation of lysosomal antigen on professional APCs to activate of iNKT cells, requires both the CD1d tyrosine-based sorting motif and Ii ([101], **Chapter 3**). Thus, although mice have only CD1d, its tyrosine-based sorting motif and the interaction with molecules, such as AP-3 and Ii, allows it to cover the entire endocytic pathway, representing the different trafficking pathways of several different CD1 isoforms.

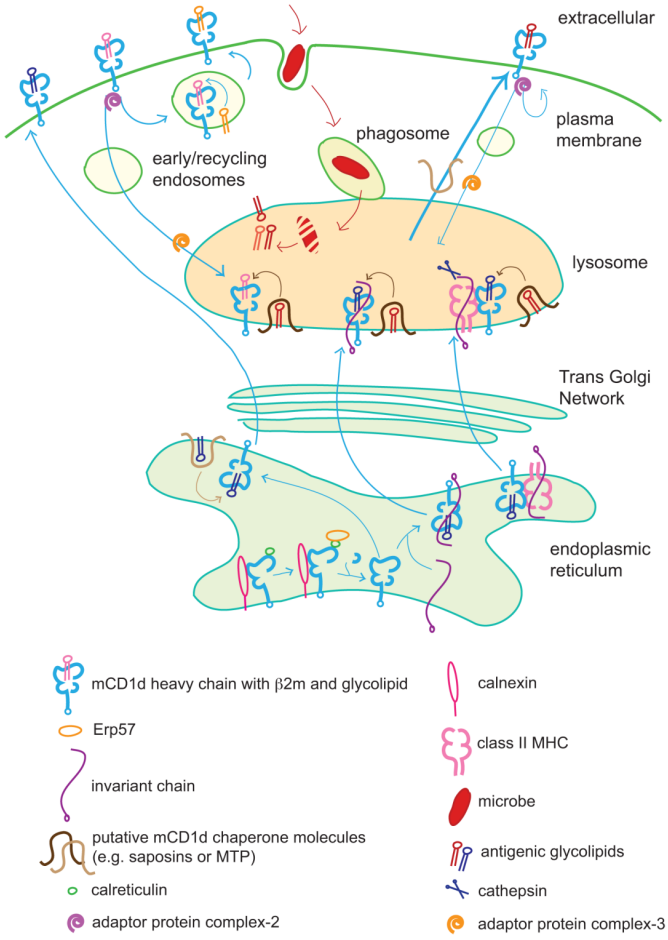

FIGURE 3. Intracellular distribution and trafficking of mouse CD1d. Complexes are assembled in the endoplasmic reticulum (ER), where they are glycosylated and bound to the chaperones calnexin, calreticulin and thiol reductase ERp57 [67-69]. Eventually CD1 molecules associate with β2m, although this is not strictly necessary to exit the ER [39,48,49]. In the ER CD1d molecules are loaded with self-lipids with a chaperone-like function [70,71]. This lipid-loading process is mediated by microsomal triglyceride tranfer protein (MTP) [72,73]. After assembly in the ER, CD1 molecules follow the secretory route through the Golgi apparatus to the plasma membrane, where the ER-derived self-lipids can be presented to a specific subset of NKT cells [8]. Mediated by the adaptor protein complex-2 (AP-2), CD1 molecules are then internalized in clathrin-coated pits [74-78]. After internalization mouse CD1d traffics through late endosomal and lysosomal compartments via the interaction of AP-3 with the tyrosine-based sorting motif, before recycling back to the plasma membrane [75,79-81]. MTP might be involved in the recycling of CD1d from the lysosomes to the plasma membrane [82]. Mouse CD1d molecules can associate with Ii or Class II MHC molecules/Ii complexes [60,61], which direct CD1d complexes directly from the trans-Golgi network to the endosomal compartments without first reaching the plasma membrane [60]. Most likely, cysteine proteases, such as the Ii-degrading cathepsin S also have a role in CD1d-mediated antigen presentation [93,94]. The exchange of ER-derived lipids for other self-lipids or pathogen-derived

lipids can occur at the plasma membrane, but mainly occurs in the endocytic compartments (especially lysosomes), where glycolipid antigen processing takes place [8,83-85]. Pathogenic content is delivered to the lysosomes for degradation via phagolysomal fusion. Accessory molecules such as saposins [86-89] assist in the loading of lipids in these endocytic compartments.

Triggering of CD1-mediated antigen presentation

Dendritic cells (DCs) function as primary phagocytic sentinels, which upon ligation of their inflammatory cytokine- and pathogen-associated molecular pattern- (PAMP) receptors, can turn into potent antigen presenting cells (APCs) capable of (NK)T cell stimulation [[102,103] and reviewed in [104]]. Exogenous lipid antigens can be taken up via receptors, such as C-type lectins, the mannose receptor and low-density lipoprotein receptors (LDL-R) [83,90,105,106], or via phagocytosis of apoptotic blebs [107,108]. Triggering of pathogen-recognition receptors on DCs promotes CD1d-mediated lipid presentation [5,109,110]. During microbial infections the CD1d-mediated antigen-presentation pathway is rearranged, sometimes in combination with increased synthesis of endogenous lipids [111], to accommodate the stimulatory effects of CD1d-restricted iNKT cell responses [5,109,110,112]. In **Chapter 2**, we summarize how several signals instigate the rearrangement of the antigen processing and presentation machinery in endosomes of antigen presenting cells for induced cell-surface display of antigenic peptide-Class I MHC and lipid-CD1d complexes [113].

CD1d–restricted NKT cells

Selection and development of CD1-specific NKT cells

CD1d-restricted NKT cells are strictly thymus-derived $CD4^+$ or $CD4^-CD8^-$ double-negative (DN), or $CD8^+$ (the latter not present in mice) T cells which can be divided into a predominant subset (group I) that expresses a relatively invariant (i) TCR alpha chain (Vα14-Jα18 in mice; Vα24-Jα18 in humans) coupled with a restricted subset of TCR beta chains (Vβ8.2, Vβ7 and Vβ2 in mice, and Vβ11 in humans), and a less prevalent subset that express diverse TCRs (group II) [114-117]. The αGalCer–reactive Vα14 iNKT cells constitute most of the $NK1.1^+$ T cell population in the spleen and represent >80% of the $NK1.1^+$ T cells in the thymus and liver [114,115].

The development of NKT cell precursors diverges from that of mainstream thymocytes at the $CD4^+CD8^+$ double-positive (DP) stage [118,119] After random rearrangement and expression of the canonical Vα14 chain coupled with a restricted TCR β receptor, these iNKT cell precursors are positively selected on neighboring CD1d-expressing DP cortical thymocytes [6,98-100]. Positively selected Vα14 iNKT cell precursors then mature by gradually expressing CD44, CD69, NK1.1 and other NK lineage receptors (such as Ly49G2, Ly49C, Ly49I, CD94, NKG2D, Ly6C and 2B4) [120-124]. The transition from immature $NK1.1^-$ to mature $NK1.1^+$ Vα14 iNKT cells is an important step during the terminal differentiation of iNKT cells, which occurs mainly outside the thymus and requires the presence of CD1d [100,125,126].
The trafficking of CD1d is mediated by its intrinsic tyrosine-based sorting motif and the interaction with invariant chain/Class II MHC complexes [59,60] and most likely influences the sampling of lipid antigens. It is generally accepted that Vα14 iNKT cell development occurs on DP thymocytes, since the expression on only DP thymocytes, using

Lck promotor driven CD1d was sufficient to drive normal Vα14 iNKT cell selection [100,127,128]. Studies using CD1d tail-deleted mutants have demonstrated that an intact CD1d tyrosine-based sorting motif is essential for the positive thymic selection of Vα14 iNKT cells [8,59]. Using the CD1d-EYFP mice, in which all CD1d is expressed as CD1d-EYFP fusion proteins as described above, we confirmed that endosomal sorting mediated by the CD1d-intrinsic tyrosine motif is critical for thymic selection of Vα14 iNKT cells ([101], **Chapter 3**). DP thymocytes do not express the invariant chain. However, we found a significant delay in Vα14 iNKT cell maturation and a decrease in $V\beta7^+$ Vα14 iNKT cells in the thymus of $Ii^{-/-}$ mice, suggesting a role for Ii-expressing professional APCs during the selection and maturation of Vα14 iNKT cells in the thymus (Manuscript in submission, **Chapter 4**). These Ii-expressing APCs, may well be thymic dendritic cells, which previously have been suggested to mediate negative selection of Vα14 iNKT cells [127,129], or immigrant DCs, such as for the selection of regulatory NKT cells during anterior chamber of the eye associated immune deviation (ACAID) [130].

In the periphery, Vα14 iNKT cells are exposed to both professional APCs, which express invariant chain and non-professional APCs, such as peripheral T cells, that do not express invariant chain. The homeostasis of Vα14 iNKT cells in the periphery is independent of the presence of CD1d in the periphery: CD1d does not affect the survival or expansion rate of iNKT cells in the periphery [125,131]. However, the terminal differentiation of iNKT cells in the periphery does require the presence of CD1d [100,125,126]. With the use of the CD1d-EYFP mouse model, we were able to demonstrate that the intrinsic CD1d sorting motif is dispensable for the terminal differentiation of Vα14 iNKT cells in the periphery [101], **Chapter 3**). In contrast, we found that the activation of differentiated Vα14 iNKT cells in the periphery, requires both the CD1d-tail encoded sorting motif and Ii [101], **Chapter 3**).

Activation and effector functions of CD1-specific NKT cells

The total NKT cell pool in mice constitutes only 0.4-1% of the total lymphocyte population in the thymus, bone marrow, spleen, lymph nodes and intraepithelial lymphocytes, while they are most frequent (20-30%) in the liver [115,132,133]. The main functional difference between Vα14 invariant NKT cells and non-Vα14 NKT cells is that the natural Vα14 iNKT cell ligands require endosomal trafficking of CD1d and lysosomal processing for their presentation at the cell surface, whereas non-Vα14 NKT cell ligands are normally presented by tail-truncated CD1d, which is defective in endosomal trafficking and likely presents antigens loaded in the secretory pathway or at the cell surface [8,59].

Vα14 iNKT cells secrete large amounts of T helper type 1 (Th1) (e.g. IFN-γ, TNF-α and IL-2) and/ or Th2 (IL-4, IL-13, IL-5 and IL-10) cytokines within minutes after TCR stimulation [134-137]. The cytokine response of iNKT cells is differentially regulated by costimulatory pathways including CD28 and CD40 [137-140] and downregulatory mechanisms involving inhibitory NK receptors such as Ly49A, C/I and Ly49G2 [141,142].

The production of cytokines by iNKT cells, sometimes in combination with IL-12 from DCs can lead to the activation of NK cells [143-145], (memory-) $CD4^+$ and $CD8^+$ T cells [146-150], macrophages [151], and B cells [146,152-155]. Finally, activated iNKT cells also regulate the suppressive activity of T regulatory cells (Tregs) [156,157] [156].

Invariant NKT cells thus function as a bridge between innate and adaptive immune responses, by activating and regulating cells from both branches of the immune system.

In addition to prompt cytokine secretion, iNKT cells are also cytolytic and release perforin, granzymes and express apoptosis-inducing ligands, such as FasL and TNF-related-apoptosis-inducing ligand (TRAIL) [134,158,159]. The cytotoxicity of iNKT cells is enhanced by IL-2 and IL-12 and is important during antimicrobial and antitumor immunity [134,160].

CD1 antigens

Using the marine sponge-derived αGalCer proved that CD1d molecules present (glyco-)lipid antigens to NKT cells [1,7,55-58]. To date αGalCer is the most extensively studied ligand for CD1d molecules. It is a broad-range stimulant for NKT cells and has been tested for therapeutic purposes against tumors [158,161], autoimmune diseases [162-165] and infections [166-170].

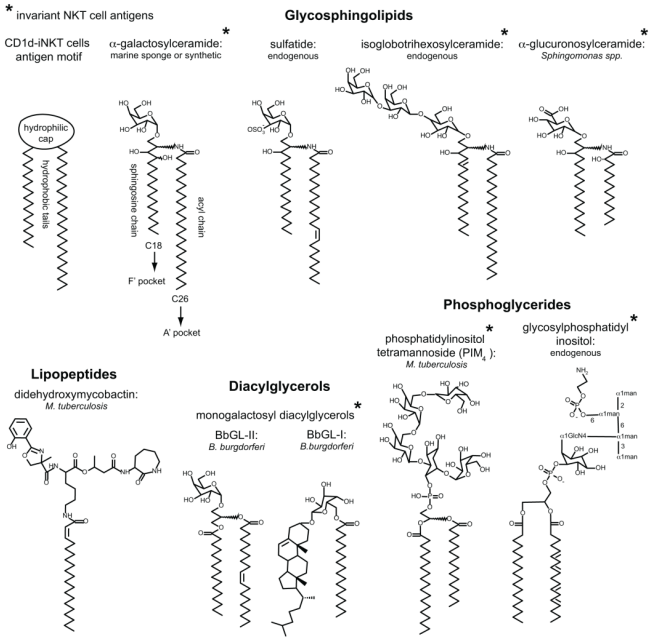

FIGURE 4. Structures of microbial and self lipids presented by CD1 molecules. Structural examples of antigens presented by CD1 molecules, grouped according to lipid classes. Typically CD1d-presented antigens have two aliphatic hydrocarbon chains bound to a hydrophilic cap, as represented by the simple schematic of the CD1d antigen motif. Crystal structures of CD1d in complex with a short-chain synthetic variant of αGalCer revealed that both lipid components fit snugly into the A' and F' pockets. The sphingosine chain is inserted in the F' pocket whereas the acyl chain is anchored within the A' pocket [171]. Other CD1 isoform-presented antigens, such as lipopeptides can have a single alkyl tail. All structures coded with an asterisk (*) are mCD1d antigens known to stimulate invariant (i) NKT cells.

Glycolipids are typically found in the plasma membranes of animal, bacterial and plant cells and consist of a hydrophilic oligosaccharide with a linkage to the hydrophobic lipid component. Glycosphingolipids, such as αGalCer are complex glycolipids, which contain an extra ceramide (sphingosine) lipid component. See **Figure 4** for a structural overview of CD1d-binding lipids. Crystal structures of CD1d in complex with a short-chain synthetic variant of αGalCer revealed that both lipid components fit snugly into the A' and F' pockets. The sphingosine chain is inserted in the F' pocket whereas the acyl chain is anchored within the A' pocket [171]. The exposed hydrophilic sugar moiety, possibly interacting with the α1 and α2 domains of CD1d, is potentially involved with the T cell receptor (TCR) recognition [172,173]. See **Figure 5** for a graphic interpretation of the interaction of the TCR with the lipid/CD1d complex.

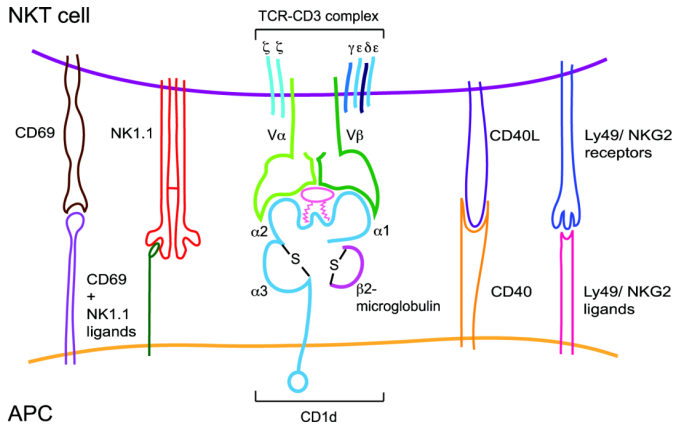

FIGURE 5. Graphic model of the interactions between the T cell receptor and the CD1d/lipid complex. This graphic represents the proposed model of glycolipid antigen recognition. The aliphatic hydrocarbons of the glycolipid are sequestered from aqueous solvent within the hydrophobic CD1d antigen-binding groove. The hydrophilic cap protrudes from the groove so that it is positioned adjacent to the exposed α-helices of the CD1d protein. The exposed hydrophilic sugar moiety, possibly interacting with the α1 and α2 domains of CD1d, is potentially involved with the T cell receptor (TCR) recognition [172,173]. Multiple signaling pathways are likely to operate during the interaction between NKT cell and CD1d-expressing cell. TCR-CD3 complex stimulation triggers both Th1 and Th2 cytokine secretion, whereas NK1.1 or IL12R may selectively promote Th1 functions. By analogy with CD4 and CD8 on conventional T cells, NK1.1 could signal as a coreceptor or theoretically cross-link TCR-CD1 complexes [175]. Activation of iNKT cells is further differentially regulated by costimulatory pathways including CD28 and CD40 [137-140] and downregulatory mechanisms involving inhibitory Ly49 receptors [141,142].

Since the discovery of αGalCer, several naturally occurring endogenous and exogenous antigens, potentially recognized by NKT cells have been identified. However, the physiological significance for most of these antigens remains elusive until they can be extracted and identified from isolated native CD1d and their presence proves essential during *in vivo* NKT cell selection and activation. For example, *Mycobacterium tuberculosis* (*Mtb*) express CD1d ligands [174]. However, it is yet to be shown whether *Mtb*-derived antigens or endogenous antigens induce CD1d-restricted NKT cell-mediated immune

protection **(Chapter 4**, manuscript in submission). Therefore, most experimental work on mechanisms involved with CD1d Ag loading and presentation to date still rely on αGalCer and few identified antigens.

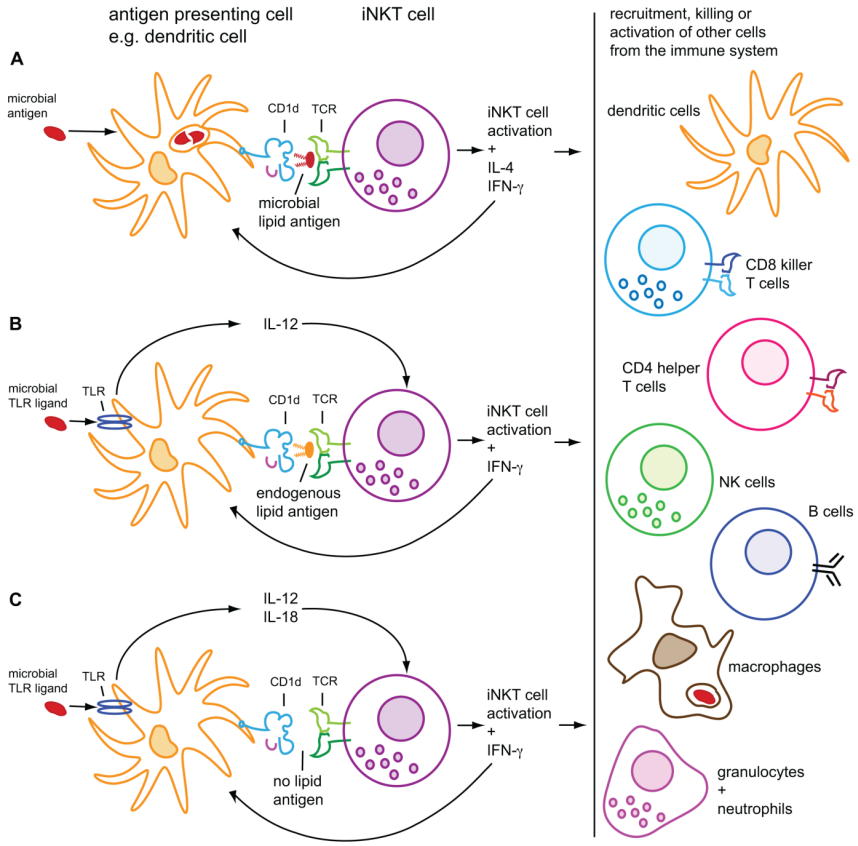

FIGURE 6. Different pathways leading to the activation of invariant (i)NKT cells during microbial infections. A. Direct activation via recognition of microbial antigen. Microbial antigens such as glycosphingolipids from *Sphingomonas* spp. and galactosyldiacylglycerols from *Borrelia burgdorferi* can induce iNKT cell activation by engaging the invariant TCRs, without Toll-like receptor (TLR) or interleukin-12 (IL-12) signaling [109,191,224]. Activated iNKT cells in turn regulate/recruit/kill the dendritic cells (DCs) and other immune cells via cytokine, chemokine and granzyme/perforin production and expression of apoptosis-inducing ligands. **B**. Cytokine- and endogenous antigen-mediated activation. Lipopolysaccharide (LPS) from for example *Salmonella typhimurium* can stimulate TLRs on DCs and induce IL-12 release. IL-12 produced by LPS-stimulated DCs combined with the recognition of endogenous lipid antigen presented by CD1d can activate iNKT cells [5,109]. **C**. Cytokine-mediated activation. Certain stimuli are potent enough to activate iNKT cells via cytokines, without necessary presentation of lipid antigen on CD1d. For example, *Escherichia coli* LPS-stimulated DCs secrete IL-12 and IL-18, which are sufficient for potent IFN-γ production by iNKT cells [3].

Endogenous antigens

Self-lipid antigens play an important role during the thymic selection of NKT cells [176,177], maintenance of tolerance [178,179], autoimmunity [162-164] and it is suggested that they are involved in the indirect stimulation of NKT cells during certain microbial infections ([5,177], see the later paragraph on "iNKT cells and pathogenic diseases"). Isoglobotrihexosylceramide (iGB3) is a weak mammalian self-glycosphingolipid that can stimulate both mouse and human NKT cells [109,177] and is implicated in the indirect NKT cell activation during *salmonella typhimurium* infections [177]. The finding that iGb3-synthase-deficient mice, which lack the entire family of isoglobo-series glycosphingolipids, have normal numbers and development of NKT cells [180], suggests that there may be several self-lipids playing a role in the thymic selection of NKT cells and the activation of NKT cells. Cell-free studies showed that purified small mammalian phospholipids such as phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) can stimulate various NKT cell populations, albeit weakly compared to αGalCer [10]. Glycosylphosphatidylinositol (GPI) extracted from isolated mouse CD1d, was also identified as a natural CD1d-ligand [181], and like PI is derived from the ER [70,84]. It is proposed that these phospholipids have a chaperone-like function, occluding the hydrophobic binding groove of CD1 during assembly until they are exchanged for glycolipid antigens within the endocytic pathway for display on the plasma membrane [70,84]. Other mammalian self-lipids such as sulfatide, an immunodominant species in myelin [182], monosialoganglioside GM1 [183,184], and disialoganlioside (GD3) [185] have been implicated in experimental autoimmune encephalomyelitis, multiple sclerosis and anti-tumor responses, respectively.

Pathogen-derived antigens

A number of pathogen-derived lipids recognized by NKT cells have been characterized and since CD1d-restricted NKT cells play a role in a broad spectrum of infectious diseases, many more are expected to be identified in the future. The α-galacturonosylceramides from *Sphingomonas* [186-190] and *Ehrlichia muris* bacteria were the first bacterial antigens described that could activate the majority of mouse and human CD1d-restricted NKT cells [109,191-194]. However, it is unclear whether these α-proteobacteria are actually pathogenic. CD1d was shown to be required for the control of infection with the Lyme disease spirochete *Borrelia burgdorferi* [195,196] and instead of α-linked sphingolipids, another class of lipids, diacylglycerols [197,198] were found to stimulate NKT cells [199]. Phosphoglycolipids from different protozoan parasites (*Trypanosoma cruzi*, *Leishmania donovani*, *Plasmodium falciparum* and *Trypanosoma brucei*) have been identified [200-202]. However, most protozoan parasites did not evoke significant NKT cell-mediated immune responses [201,203,204] and so far only lipophosphoglycan from *L. donovani* could stimulate NKT cells [200]. Yet, NKT cell-deficient mice showed increased susceptibility to *L. donovani* and *B. burgdorferi* [199,200], emphasizing the role of NKT cells during antimicrobial responses.

Mycobacterium tuberculosis (*Mtb*) is the causing agent for one of the deadliest infections worldwide [205,206]. Importantly, several lipid antigens from mycobacteria have been identified [2,174,207-211] and it was shown that CD1d-restricted NKT cells are stimulated during mycobacterial infections [212,213]. Most of the unique lipids found in *Mtb* can be presented by the human group 1 CD1 molecules to activate clonally diverse T cells. These

antigens include CD1a-binding mycopeptides, such as didehydroxymycobactin (DDM) [211], CD1b-binding glucomonomycolate (GMM) [207,209], diacylated sulpholipids [208] and lipoarabinomannan (LAM) [2] and the CD1c-binding lipopeptides mannosylphosphomycoketides [209,210]. So far only phosphatidylinositol mannoside (PIM) has been described to activate both human and mouse CD1d-restricted NKT cells [174] and induce NKT cell-rich granuloma lesion formations [214]. However, it is unclear if PIM or any other *Mtb*-derived lipid is responsible for CD1d-restricted iNKT cell activation during an actual *Mtb* infection. Although our study was not designed to identify the antigenic lipid presented during *Mtb* infection, our observation that *Mtb* does not optimally localize to lysosomal compartments in infected Ii-deficient Mϕ, in combination with the finding that those infected Ii-deficient Mϕ are unable to activate iNKT cells that suppress bacterial growth, suggests that a lysosomal *Mtb*-derived antigen or *Mtb*-induced self antigen is involved in CD1d-restricted iNKT cell activation during infection (Manuscript in submission, **Chapter 4**).

iNKT cells and pathogenic diseases

Invariant NKT cells have been implicated in protective immune responses against several pathogens, such as viruses [215-218], parasites [219,220], fungi [221] and bacteria [151,196,212,222]. Most examples suggest that iNKT cells are beneficial during pathogenic infections. However, they can also exacerbate the disease outcome, as demonstrated by the findings that anti-CD1d mAb blocking during *Listeria monocytogenes* infection improved survival and the pathogen-specific Th1 response [213], and iNKT-cell deficient mice had less severe damage during *Salmonella choleraesuis* infection compared to controls [223].

There are three main ways iNKT cells can be activated during microbial infections (see **Figure 6**). First, iNKT cells can be activated directly upon recognition of microbial lipid antigens presented by CD1d molecules on the surface of infected cells. For example the recognition *Sphingomonas*- and *Borrelia*-derived lipids is entirely dependent on CD1d and independent of Toll-like receptor (TLR)-mediated activation of APCs or IL-12 [109,191,224]. Second, iNKT cells can be activated indirectly when weak reactivity to self-glycolipids is amplified by costimulatory cytokines, such as IL-12 released from DCs triggered via TLR signaling. This mode of iNKT cell activation occurs during *Salmonella typhimurium* infections [5,109]. Finally, upon TLR signaling DCs secrete cytokines that can activate iNKT cells without TCR engagement, which in turn amplify the innate immune response [3]. The iNKT cell response against viruses, which by default do not express glycolipid compounds, involves TLR9 signaling and consequent production of IL-12, while the necessity of CD1d-mediated presentation may not be absolute [225].

CD1d and iNKT cells during mycobacteria infections

Mtb characteristics spread and clinical implications

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). *Mtb* are slow replicating obligate aerobe bacteria that are classified as acid-fast gram-positive bacteria (summarized in [226]). More than 100 years after the discovery of the tubercle bacillus by Robert Koch [227], TB is still one of the most prevalent diseases in developing countries [206] and a leading cause of morbidity and mortality worldwide

[205,228]. More than 9 million people are infected each year with *Mtb* [205]. Only about 10% of those infected develop TB. The majority of otherwise healthy patients experience a mild self-limiting disease (primary TB) after their first infection, which clinically heals [229,230]. Yet, due to co-infection with Human immunodeficiency virus (HIV) and the appearance of multidrug-resistant tuberculosis, around two million people die annually [205,228].

Therapeutic strategies against Mtb

The current *M. bovis* Bacille Calmette–Guérin (BCG) vaccine against tuberculosis affords insufficient protection against *Mtb* [summarized in [206,231,232]], causing the development of better prophylactic strategies to be an international research priority. New vaccine research and development includes recombinant BCG, attenuated *Mtb*, monoclonal antibodies [233,234], DNA vaccines [235] and recombinant antigen-based subunit vaccines [236], summarized in [232]. In order to improve these therapeutic strategies it is important to understand the immunological mechanisms that are critical in the protective immunity to *Mtb*.

Immune response against Mtb

Humoral and in particular cellular immunity are important in the protection of *Mtb*. *Mtb* replicates primarily within host Mϕ and Mϕ activation induced by the cytokines IFN-γ [237] and tumor necrosis factor-α [238] is important in the control of *Mtb*. Antibodies may play a role in host defense against *Mtb* [239,240]. However, mouse studies have shown that $CD4^+$ T cells, especially of the Th1 phenotype, are the predominant factor in the protection against *Mtb* [241], in part by producing cytokines such as IFN-γ [242]. The role of $CD4^+$ T cells in the protection against *Mtb* has also been confirmed in humans [243]. In addition, both mouse and human studies have suggested a role for $CD8^+$ T cells in the protection against *Mtb* [244-246], partially dependent on perforin [247].

Role of CD1 and CD1-restricted T cells during Mtb infection

Until recently evidence for the essence of CD1 antigen presentation and CD1-restricted NKT cells in the protection against *Mtb* has been contradictory. Studies that used $CD1d^{-/-}$ [244,246,248] or NKT cell knockout ($J\alpha281^{-/-}$) mice [169,249] could show at most marginal, if any, role for CD1 or NKT cells in the protection against *Mtb*. Yet, upregulation of CD1 surface expression has been observed upon *Mtb* [110,112] and BCG [250] infection. CD1d mediated antigen presentation by DCs occurs rapidly upon *Mtb* infection [251] and even CD1 expression on uninfected bystander cells appeared crucial for the indirect activation of CD1-restricted $CD8^+$ T cells during *Mtb* infection [107]. Furthermore, it has been shown that CD1d-restricted NKT cells rapidly produce large amounts of IFN-γ when activated by *Mtb*-infected cells [208], that they play a role in granuloma formation under certain conditions [252]. Although the activation of NKT cells with *Mtb*-specific antigens [208,252,253] or αGalCer [166] proved beneficial, there was little evidence to support their requirement for optimum immunity against *Mtb* infection. Using the mouse model for tuberculosis, it has finally been clarified that both CD1d-mediated antigen presentation and CD1d-restricted iNKT cell activation contribute to immunity against *Mtb* infection [212].

We established a role for endosomal trafficking in the CD1d-restricted iNKT cell response during *Mtb* infection (Manuscript in submission, **Chapter 4**). In invariant chain (Ii)-deficient mice, CD1d molecules strictly rely on their intrinsic tyrosine-based sorting motif for intracellular trafficking [60]. Furthermore, in the absence of Ii endosomal fusion is inhibited, resulting in smaller lysosomes and altered endosomal trafficking of membrane proteins and endosomal content [254-256]. The absence of Ii, prevented CD1d surface upregulation in Mϕ upon *Mtb* infection and inhibited the activation of iNKT cells (Manuscript in submission, **Chapter 4**), suggesting that Ii-mediated trafficking of CD1d through the endosomal pathway is necessary for the acquisition of antigenic lipids that can activate iNKT cells during *Mtb* infection.

Mtb evasion of the host immune system

Many organisms try to evade the host immune system, (summarized in [113], **Chapter 2**). *Mtb* tries to evade the microcidal capabilities of APC through the modulation of host signaling pathways resulting in long-term persistence in infected hosts. As one method of escape *Mtb* evades Class II MHC peptide antigen presentation by the precocious induction of DC maturation, but this did not lead to its evasion of CD1d lipid antigen presentation [251]. It is generally thought that in Mϕ *Mtb* persists and replicates within the phagosomes, where it survives by preventing lysosomal fusion (phagosome maturation), presumably mediated by its expression of phosphatidylinositol lipoarabinomannan (LAM) [257,258] and maintains extensive communication with early endosomal traffic, mediated by phosphatidylinositol mannoside (PIM) [259], to provide access to nutrients for survival and growth [260-262]. However, eventually *Mtb* has to escape from phagolysosomes to replicate in the cytosol of non-apoptotic cells, causing significant cell death within a week [263]. Our results with Mϕ that lack Ii imply that *Mtb* intracellular growth requires pathogen presence in lysosomes to facilitate *Mtb* translocation from the endosomal pathway to the cytosol. Localization of *Mtb* in the lysosomes is inhibited in Mϕ that lack Ii and prevents optimal bacterial growth in the cytosol, but eventually benefits the bacteria by preventing lysosome-acquired antigen presentation by CD1d and subsequent iNKT cell activation (Manuscript in submission, **Chapter 4**).

iNKT cells, tolerance, tumors and autoimmunity

Many iNKT cells are autoreactive in that they respond functionally to presentation of self-lipids by CD1d molecules [6-10]. This iNKT cell autoreactivity may be important for their ability to constitutively promote self immunological tolerance and also forms the basis for the indirect activation of iNKT cells during microbial infections (as described above).

Invariant NKT cells can have either beneficial or deteriorating functions, often depending on their secretion of either Th1 or Th2-type cytokines. For example, the activation of iNKT cells and their production of Th1-type cytokines, is important in the initiation of anti-tumor immune responses in a variety of tumor-metastasis models [58,158], whereas they can also downregulate anti-tumor immune responses through the production of the Th2-type cytokine IL-13 [264].

Invariant NKT cells also play an important role in tolerance-regulation in some models of allogeneic and xenogeneic transplantation tolerance [265-267], systemic tolerance

[178,179] and for the regulation of maternal-fetal interactions [268]. In addition, several animal models indicate that iNKT cells prevent autoimmunity and inflammation, either when activated naturally or when using αGalCer or related compounds. The stimulation of iNKT cells is beneficial to collagen-induced arthritis [269] and diminishes colitis in a dextran sodium sulfate-induced colitis model [270]. In addition, iNKT cell activation ameliorates autoimmune diabetes in non-obese diabetic (NOD) mice [162,164,271,272] and protects against experimental autoimmune encephalomyelitis (EAE, a mouse model for multiple sclerosis) [163,165], by shifting pathogenic Th1-type immune responses to nonpathogenic Th2-type responses [162-164].

Yet, sometimes iNKT cells rather promote autoimmunity, for example in the spontaneous model of systemic lupus erythematosus that arises in (NZB x NZW)F_1 mice, where iNKT cells augment Th1-type immune responses and autoantibody secretion that contribute to lupus development [273]. In addition, airway hypersensitivity experiments indicate that Th2-type cytokine production by iNKT cells is required for susceptibility, while mice lacking iNKT cells are resistant to developing hypersensitivity [274,275]. In line with studies using concanavalin A-induced hepatitis, a mouse model for human autoimmune hepatitis [276-278], we found that iNKT cells are required for hepatitis induction ([279], **Chapter 5**). Using Tf-mOVA mice that express ovalbumin as a self-antigen under the transferrin promoter, we demonstrated a critical role of CD1d-restricted iNKT cells in $CD8^+$ T cell-mediated hepatitis. The administration of a single low dose of αGalCer stimulated Th1-type cytokine production by iNKT cells, which enhanced the cytolytic activity and IFN-γ production by OVA-specific $CD8^+$ T cells, leading to hepatitis and subsequent liver damage ([279], **Chapter 5**).

Clinical implications

As outlined, iNKT cells can either exacerbate disease, or prevent overt autoimmunity via mechanisms involving the production of Th2-type cytokines, but they can also initiate powerful antitumor and antimicrobial immune responses through the production of Th1-type cytokines, resulting in the activation of both innate and adaptive immune cells. All together, this makes the iNKT cell population a potential target for therapeutic strategies. Current strategies are focused on the selective modulation of iNKT cell responses, for example through the administration of αGalCer and other glycolipids that can act as adjuvants [58,151,158,166-168,170,216,218,221,280-282], sometimes in combination with cytokines [164,283], or possibly in combination of TLR ligands [284,285] or protein antigens to enhance $CD4^+$ and $CD8^+$ T cell responses [149,150,286], and finally through the administration of αGalCer pulsed DCs [287-289]. Other strategies include the adoptive transfer of adequate numbers of appropriately polarized iNKT cells [287,290]. The studies covered in this thesis eventually contribute to the knowledge on how CD1d-restricted iNKT cells can be targeted for therapeutic strategies against autoimmunity and infectious disease (see discussion, **Chapter 6**).

Scope of the thesis

Chapter 2 includes a review of how several signals instigate lipid antigen processing and CD1d-mediated antigen presentation in antigen presenting cells. In addition, the review covers mechanisms pathogens employ to escape immune recognition. The requirement of

the endosomal trafficking for CD1d-restricted invariant NKT cell development and function is addressed in **Chapter 3** and partly in **Chapter 4**. **Chapter 4** further covers the necessity of *Mycobacterium tuberculosis* to traffic from phagosomes to lysosome for pathogen replication and induction of CD1d-mediated immune defense. In **Chapter 5**, the role of CD1d-restricted invariant NKT cells in stimulating intrahepatic $CD8^+$ T-cell responses to liver antigen was assessed. **Chapter 6** contains a summarizing discussion and presents a perspective for targeting CD1d-mediated antigen presentation and CD1d-restricted invariant NKT cells in therapeutic strategies against autoimmunity and infectious disease.

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Chapter 2

Endosomal processing for antigen presentation mediated by CD1 and Class I major histocompatibility complex: roads to display or destruction

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Abstract

The presentation of antigen in a form that can be recognized by T lymphocytes of the immune system requires antigen processing and association of antigen-derived fragments with molecules encoded by the major histocompatibility complex (MHC) locus or by the CD1 locus. Much emphasis on antigen processing and presentation in the last decades has focused on what we consider 'conventional routes' of antigen processing and presentation, whereby extracellular antigens are processed for presentation via Class II MHC complexes and cytosolic antigens are presented as peptide–Class I MHC complexes. We here highlight two other pathways in myeloid dendritic cells, those of lipid antigen presentation in association with CD1 and of peptide cross-presentation via Class I MHC complexes. Some pathogens evade immune recognition through inhibition of antigen presentation of phagosomal origin. Deviations in endosomal antigen processing and presentation are also seen in individuals suffering from glycosphingolipid lysosomal lipid storage diseases. We summarize recent developments in the endosomal antigen processing and presentation pathway, for display as lipid–CD1 complexes to natural killer T cells and as peptide–Class I MHC complexes to CD8 T cells.

Introduction

The initiation of strong adaptive immune responses that yield memory requires the processing and presentation of antigen as peptide–major histocompatibility complex (MHC) complexes or as lipid–CD1 complexes to T lymphocytes (1). Class I MHC is expressed on most nucleated cells, whereas CD1 expression and Class II MHC expression are more restricted, being constitutive on professional antigen-presenting cells (APCs) [i.e. on dendritic cells (DCs), B cells and macrophages]. Thymic selection of MHC-restricted T lymphocytes occurs by presentation of peptide–MHC complexes on thymic stroma cells, while selection of CD1-restricted natural killer T cells (NKT cells) occurs on CD1-expressing cortical thymocytes, as shown in mice (2,3). MHC-restricted T cells require clonal expansion to execute key roles in adaptive immune responses, whereas CD1-restricted NKT cells act within hours of stimulation by secreting polarizing cytokines (4). Class I MHC and CD1 molecules both contain one heavy chain [of approximately 43,000–49,000 molecular weight (MW)] that assembles with β2-microglobulin (12 000 MW) and endogenous peptide or lipid in the endoplasmic reticulum, respectively. Exogenous antigens provide alternative sources of peptides for Class I MHC, for loading in phagosomes (5). The number of CD1 isoforms expressed varies among species: humans express several antigen-presenting isoforms of CD1 – CD1a, CD1b, CD1c and CD1d – that complement one another in the sampling of antigen from various endosomal compartments (6), whereas mice only express the CD1d isoform (7). Humans additionally express CD1e, which during DC maturation translocates from the Golgi to lysosomes, where CD1e is thought to facilitate the selection of antigenic lipids for surface display via other members of the CD1 family (8).

Much of what is known to date about CD1-mediated antigen presentation and T-cell selection is obtained from mouse-based experiments. Semi-invariant NKT cells represent approximately 80% of all CD1d-restricted T cells in mice and express a T-cell receptor (TCR) that contains a Vα14-Jα18 assembled with Vβ8, Vβ7 and Vβ2-containing TCR β chains (9). Antigens recognized by semi-invariant NKT cells are derived from lipid membranes in endosomes and lysosomes from foreign or host origin (9) As can be

expected from the cargo loaded into the antigen-binding grooves, the hydrophobic CD1d-binding groove contains two large cavities that facilitate the binding of non-polar alkyl chains and exposes the polar glycolipid headgroup (10), whereas MHC molecules harbor a groove that is lined with charged residues to facilitate the binding of peptide anchor residues (11,12). Some peptides can bind CD1 and these are overall hydrophobic in character. The MHC locus is polygenic (> 200 genes in human) and exceptionally polymorphic, whereas CD1 gene products are monogenic or have a very limited range of alleles. The single CD1d protein that is expressed in mice, however, can adopt different conformations that facilitate the binding of related ceramides from endogenous and acquired sources (i.e. iGb3 and alpha-Galactosyl ceramide) (10), thereby allowing the presentation of multiple antigens. One can imagine that other CD1 isoforms may take on multiple conformations as mouse CD1d does: human CD1b can present glucose monomycolates that vary significantly in their lipid tail fine structures and overall lengths (13), a capability that may be attributable to conformational flexibility. Variability in CD1 conformations may therefore increase the antigen-presentation repertoire presented to CD1-restricted T-cell clones.

T-cell stimulation requires instructive signals that supplement signals incited by triggering of the TCR. DCs in particular are proficient at T-cell stimulation to mount the adaptive immune responses most appropriate for the pathogen at hand. DCs can rapidly and effectively form peptide–MHC complexes or lipid–CD1 complexes derived from encountered pathogens, and rapidly up-regulate the surface expression of costimulatory molecules and chemokine receptors (14). The possession of an elaborate endosomal pathway, where antigens are degraded and loaded onto MHC or CD1, is likely to contribute to the success of DCs in stimulating antigen-specific T-cell responses. Foreign-derived antigens are most readily acquired at barrier surfaces with the outside environment; only when pathogens succeed at breaching the physical and chemical barriers of skin and mucosa are they exposed to cells of the immune system. It is becoming increasingly clear that DCs form a network of cell types that have specialized characteristics: DCs found in barrier tissues acquire mobility when pathogen-recognition receptors exposed on their plasma membrane are triggered, whereas other DC subtypes found in secondary lymphoid tissues (i.e. murine $CD8\alpha^+$ DCs) do not have significant migratory capacities but are instead optimized for antigen cross-presentation (15). DCs also have a potent ability to process and present antigenic lipids in association with CD1. Triggering of pathogen-recognition receptors often coincides with the ligation of receptors to inflammatory cytokines, for example interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), on DCs. Signals integrated from these receptors instigate in DCs the rearrangement of the antigen processing and presentation machinery in endosomes for induced cell-surface display of antigenic peptide–MHC and lipid–CD1 complexes.

Pathogen recognition receptors as modulators of endosomal antigen processing

Ligation of receptors specific for pathogen-associated molecular patterns (PAMPs) expressed by DCs deliver signals that can stimulate the differentiation of DCs from being primarily phagocytic sentinels into potent APCs capable of T-cell stimulation. These receptors are collectively called pattern recognition receptors (PRRs) and include three major families: Toll-like receptors (TLRs); intracytoplasmic nucleotide oligomerization

domain (NOD)-like receptors; and cell-surface C-type lectin receptors. DCs can also be activated indirectly by capture of fragments of cells that died in response to infection or tissue injury. DCs express selected sets of PRRs belonging to these receptor families, the composition of which helps focus the type of adaptive response that can be raised by DC subtypes. Langerhans' cells (LCs), for example, do not express TLR4 and TLR5, whereas dermal interstitial DCs do express TLR4 and TLR5 (16). There is, furthermore, integration of signals downstream of PRRs: the simultaneous ligation of TLRs 3, 4 and 8 yields a 20–50-fold increase in interleukin (IL)-12 p70 production compared with the single ligation of either TLR (17,18): and TLR4 is located at the cell surface, whereas TLRs 3 and 8 are found intracellularly, in endosomal compartments, thereby illuminating the fact that signal integration occurs distal from TLR cytosolic domains. Use of divergent sets of adaptor molecules for signal transduction (i.e. TIRAP-MyD88 and TRAM-TRIF) can direct the TLR-induced production of selective pro-inflammatory cytokines.19 Cytosolic triggering of PRRs, through ligation of NOD-like receptors, can, moreover, activate a caspase-1-activating multiprotein complex called inflammasome (20) NACHT, LRR, and pyrin domain-containing proteins (NALPs), which constitute the largest subfamily of the NOD-like receptors, process pro-inflammatory caspases and the cytokines IL-1β and IL-18 into their active forms (21). If combined triggering of PRRs potentiates DC activation, what is the effect of the simultaneous ligation of multiple TLRs on the antigen processing and endosomal transport of peptide–MHC or lipid–CD1 complexes?

The loading of Class II MHC and of CD1d with antigenic cargo occurs in specializations of late endosomal and lysosomal compartments (22) Endosomal tubulation is an early measure of DC maturation and is thought to facilitate the transport of peptide-loaded Class II MHC complexes to the cell surface (23,24). In maturing DCs, CD1 molecules are not mobilized to endosomal tubules and do not exhibit rapid surface display as seen for Class II MHC (25,26). The loading of Class I MHC for cross-presentation can occur in early endosomes/phagosomes (5,27) as well as in the endoplasmic reticulum (28) and perhaps also in a hybrid form of these two combined (29,30). The assortment of Class I MHC molecules that are found in early endosomal compartments are recycling Class I MHC molecules from the surface (31,32). The origin of Class I MHC complexes that localize to the cell surface during DC maturation, however, is new synthesis rather than transfer of existing Class I MHC present in endosomal/lysosomal compartments (33) and is therefore not expected to be strongly represented in endosomal tubulations. The DC maturation-associated disposition of Class I MHC, Class II MHC and mouse CD1d complexes in various subcellular compartments is summarized in Fig. 1. Antigen degradation in endosomal compartments is also regulated in DCs (34,35): DCs exhibit less lysosomal proteolysis than macrophages (36–38). In DCs, a low level production of reactive oxygen species produced by NADPH oxidase consumes protons in the phagosomal lumen, supporting a near-neutral pH environment and favouring peptide retention for presentation to T cells.

This delay in antigen degradation apparently promotes antigen cross-presentation via Class I MHC (38). Control of antigen degradation may additionally involve regulation in spatially confined antigen-containing compartments rather than regulation at a cellular level, for example by local ligation of PRRs (39). TLRs can be triggered inside endosomal compartments by phagocytosed microbes (40). Triggering of PRRs can induce maturation of selective PAMP-containing phagosomes and thereby promotes the generation of peptide–MHC complexes in PAMP-containing phagosomes (41). PRR triggering promotes

CD1d-mediated lipid presentation (42–44). It is not clear, however, whether NADPH oxidase activity modulates the generation of lipid–CD1 complexes in DCs.

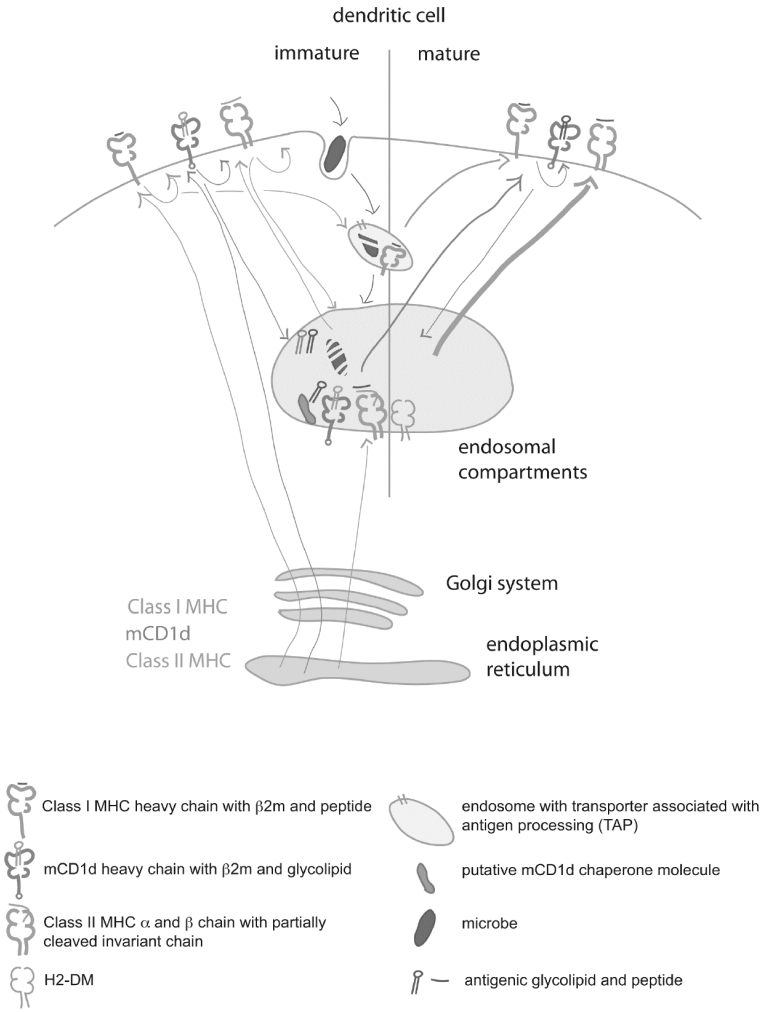

FIGURE 1. Intracellular distribution and trafficking of Class I major histocompatibility complex (MHC), Class II MHC and mouse CD1d (mCD1d) in maturing dendritic cells. Complexes are assembled in the endoplasmic reticulum (ER). To allow them to fold into functional complexes in the ER, chaperone molecules are co-assembled: antigenic peptide–Class I MHC; invariant chain–Class II MHC; and endogenous lipid–CD1d (i.e. phosphatidylinositol and glycosyl-phosphatidylinositol) (96,97). After arrival at the cell surface by routing through the secretory pathway, Class I MHC display and CD1d molecules recycle through early endocytic compartments, from where CD1d molecules are sorted to late endocytic/lysosomal compartments for exchange of ER-derived lipid for antigenic lipid cargo. Most nascent invariant chain–Class II MHC complexes arrive in the endosomal pathway directly from the Golgi system for acquisition of antigenic peptide. Chaperone proteins can facilitate antigen loading in the ER (Class I loading complex for Class I MHC

(98) and some processes of CD1d assembly (99), and lipid transfer proteins for lipid loading) (100) and in the endosomal pathway (H2-DM for Class II MHC (101,102) and lipid transfer proteins for mCD1d) (92,95,103). Maturation of DCs rearranges intracellular trafficking to promote the presentation of newly acquired antigen from ER or endosomes at the cell surface, for inspection by appropriately restricted T cells. Arrow thickness represents the relative rate of translocation of Class I MHC, Class II MHC and mCD1d complexes. β2m, beta-2-microglobulin.

Pattern recognition receptors as modulators of endosomal composition

The ligation of TLRs induces the transcription of genes that collectively facilitate the differentiation process in DCs called maturation. The integration of TLR-induced signals makes use of adaptor proteins, the most studied of which is MyD88, which is involved in signal transduction downstream of TLRs 1, 2, 4, 5, 6, 7 and 9 in mice (45). MyD88-deficient APCs exhibit deficiencies in antigen cross-presentation and are defective in eliciting functional cytotoxic T lymphocytes (CTL) *in vivo* (46).

Cross-presentation of phagosomal antigens by myeloid DCs requires processing by the proteasome in most experimental systems, thereby inferring the necessity to transfer protein fragments from the endosomal pathway into the cytosol (47). It was suggested that the cross-presentation of soluble antigens which contain PAMPs is especially dependent on the loading of Class I MHC in early endosomes/phagosomes: antigen cross-presentation required TLR4/MyD88-mediated signals for phagosomal recruitment of transporter associated with antigen processing (TAP) heterodimers (TAP1/2) (5). Triggering of TLR4 via treatment with lipopolysaccharide (LPS) also stimulates, in DCs, the rearrangement of endosomal compartments to exhibit tubular structures (23,24,48,49). Class I MHC molecules are also concentrated in multivesicular bodies (MVB) of the endosomal pathway (50), which represent the structures from which tubular endosomes are formed (48). MyD88-deficient DCs do not form tubular endosomes when triggered with LPS, yet do form tubular endosomes when triggered with Cryptococcus neoformans (51). C. neoformans can ligate TLR2 in addition to TLR4, whose signaling cascades both involve MyD88 as well as TIRAP and TRIF, respectively (45). The ligation of TLR3 (which is expressed on the endosomal membranes of $CD8\alpha^+$ DCs) with double-stranded RNA (dsRNA) present in the cell-associated form of phagocytosed virus-infected cells promotes cross-priming and induction of antigen-specific CTLs (52). Co-ligation of multiple TLRs also potentiates CTL responses *in vivo* (53). What is known about endosomal mechanisms that link TLR ligation with endosomal remodeling and possibly with antigen presentation via Class I MHC or CD1?

Endosomal sorting of Class I MHC and CD1 for antigen acquisition

DC maturation induced by TLR ligation stimulates new synthesis of Class I MHC molecules and induces antigen loading of small stores of phagosomal Class I MHC (5). A fraction of the cellular content of Class I MHC is found in late endosomal compartments (50,54). Surface display of endosomal Class I MHC via exosome release, however, is down-regulated when DCs undergo maturation (55). Upon exposure to inflammatory stimuli, Class II MHC molecules acquire antigen in phagosomes/lysosomes, then accumulate on the plasma membrane to display peptides to CD4 T cells as part of DC maturation (56,57). Maturation-induced relocation of antigen-loaded Class I MHC and

CD1 from the endosomal pathway to the cell surface is relatively modest when compared with the maturation-induced relocation of antigen-loaded Class II MHC. Stimulation of antigen-specific CD8 T-cell clones to acquire cytotoxic killing of target cells requires as few as three peptide–Class I MHC complexes (58), which is one to two orders of magnitude less than required to stimulate naïve CD4 T cells (59,60). Unlike Class II MHC, the trafficking of CD1 molecules and the presentation of lipid antigens is comparable between both immature and mature DCs (25,61,62) TLR-mediated signals stimulate specific antigen loading of Class I MHC and CD1 complexes in the endosomal pathway, with less consequence to the ongoing surface-directed traffic.

Receptor-mediated uptake effectively introduces antigen into the cross-presentation pathway, whereas antigen internalized by way of fluid-phase uptake is mostly excluded from cross-presentation (63). Some receptors are more effective than others at targeting of antigen for cross-presentation: Gram-negative bacteria (whose uptake by DCs is facilitated by binding to complement receptor 3) are fully degraded, whereas peptide antigen expressed by bacteria internalized via binding to receptors to the Fc portion of immunoglobulins (Fc-receptors) are effectively cross-presented (63). Recruitment of the Rab27a small GTPase to phagosomal membranes is involved in this antigen cross-presentation process in DCs, which also includes NADPH oxidase recruitment (64). CD1-mediated antigen presentation of exogenous antigen also benefits from receptor-mediated uptake, for example via the C-type lectins Langerin and DC-SIGN, mannose receptor and low-density lipoprotein receptors (LDL-R) (65–68), although details that link receptor-mediated uptake with endosomal processing of lipid antigens are scarce (68). Uptake of apoptotic blebs (for example through binding to β3 integrins or to CD14) can also introduce antigenic cargo to Class I MHC and CD1 molecules into uninfected bystander DCs, as was shown using apoptotic macrophages that harbored Salmonella typhimurium or *Mycobacterium tuberculosis* (69,70).

Ceramides, lipids that are composed of a sphingosine and fatty acid chain, are crucial for sorting lipids and proteins into intravesicular membranes of exosomes and MVBs (71). The endosomal-sorting mechanisms for peptide loading of Class II MHC in MVBs are relatively well established (22). In unstimulated cells, both glycosphingolipid (GSL) ligands and CD1d are also found in MVBs (72). Microbial infection of susceptible hosts, however, rearranges the CD1-mediated antigen-presentation pathway to accommodate the stimulatory effects of CD1-restricted NKT-cell responses (43,44). Microbial infections yield presentation of acquired lipids or mobilized self-derived lipids by APCs. Infection with *Ehrlichia muris* and *Sphingomonas capsulata*, for example, causes CD1d-mediated NKT-cell activation primarily through direct recognition of microbial lipids (43). By contrast, *S. typhimurium* infection causes stimulation of autoreactive NKT cells through a combination of endogenous GSL presentation via mouse CD1d and PRR-induced IL-12 production (43,44). The outer membranes of *M. tuberculosis* bacilli contain lipid ligands for CD1-mediated presentation and lipid adjuvants that induce CD1 antigen-processing pathways through TLRs (26,42,70). Finally, infection of host cells can increase the synthesis of endogenous GSL and stimulate GSL-specific T cells in a CD1- and TCR-dependent manner, as shown using *Escherichia coli*, *Bacilus subtilis*, *Staphylococcus aureus* and *Mycobacterium bovis*-bacille Calmette Guérin (BCG) (73). Induction of invariant NKT (iNKT)-cell responses upon viral infection in mice involves TLR9 signaling and consequent production of IL-12, while the necessity of CD1d-mediated presentation may not be absolute (74).

Virus-infection interference with endosomal antigen presentation mediated by CD1 and Class I MHC

DC activation accomplished through TLR ligation, for example, is pivotal for effective antigen cross-presentation as discussed above. Infection with certain large viruses that harbor ligands for TLRs can, however, also interfere with antigen cross-presentation. The β-herpes virus, mouse cytomegalovirus (MCMV), contains immune-evasion genes that may affect some aspects of antigen cross-presentation as it does for the direct pathway of Class I MHC presentation (75). Herpes simplex virus-1 (HSV-1) and herpes simplex virus-2 (HSV-2), for example, express a protein called ICP47, which blocks peptide transport via the TAP1/2 transporter (76). Human cytomegalovirus (HCMV) contains another TAP1/2 inhibitor, US6 (77). The use of a US6-transferrin chimeric molecule was used to infer the role of TAP1/2 in transporting antigenic peptide from the cytosol into phagosomes in the process of cross-presentation of phagosomal antigen (5).

Mouse DCs that are infected with MCMV activate CD1d-restricted iNKT cells in co-culture systems and *in vivo* (74). Human DCs that are infected with the human cytomegalovirus (HCMV) or HSV-1 also up-regulate antigen presentation via CD1d, which is the only group 2 CD1 molecule (78). However, HCMV infection of human DCs inhibits NKT-cell activation by CD1a, b and c (the so-called group 1 CD1 molecules), as characterized by accumulation of CD1 in endosomal/lysosomal compartments and by the direct measurement of CD1b-restricted NKT-cell activation presented by infected DCs (79). One possible explanation for differential responses to viral insult of group 1 and group 2 CD1 molecules in humans lies in regulatory elements in the 5' untranslated region, which is conserved for group 1 CD1 and distinct for CD1d (80). At high multiplicity of infection (MOI) of HSV-1 (MOI of 5–10), however, CD1d-mediated presentation is also blocked, possibly through inhibition of CD1d recycling to the cell surface (72,81).

Bacterial infection interference with endosomal antigen presentation mediated by CD1 and Class I MHC

Intracellular bacteria, including mycobacteria, also have escape mechanisms for antigen presentation mediated by Class I MHC or CD1. Bacteria can evade Class II MHC antigen presentation by infected cells through alteration of phagosome–lysosome fusion or biosynthesis, intracellular trafficking and surface expression of Class II MHC, as recently reviewed (82). Peptide–Class I MHC complex presentation can be obstructed through interference with the surface expression of Class I MHC (e.g. as in infections with *Chlamydia pneumoniae*, *Salmonella enteritidis*, *Yersinia enterocolitica* and *Klebsiella pneumoniae*) (83–86). Bacterial products can inhibit the cross-presentation of particulate antigen via Class I MHC in a manner that requires PAMP binding to TLRs and MyD88. Inhibition of cross-presentation through this route occurs through interference with phagosome maturation and antigen proteolysis, as shown for the M. tuberculosis 19 000 MW lipoprotein, CpG DNA and LPS (87). Moreover, cell wall-associated alpha-glucan from *M. tuberculosis* can induce monocytes to differentiate into DCs that lack CD1 expression, fail to up-regulate CD80 and produce IL10, rendering them unable to prime effector T cells or present lipid antigens to CD1-restricted T cells (88).

Interference with CD1-mediated antigen loading in inherited disease

Class II MHC molecules acquire their peptide cargo in specialized compartments of the late endosomal pathway, including MVBs, where the peptide-exchange factor HLA-DM (H2-DM in mice) localizes (89,90). In analogy, late endosomal compartments are enriched for lipid-binding proteins, some of which may function in the exchange of endoplasmic reticulum-derived lipids for antigenic lipids onto CD1 molecules (i.e. saposins A, B, C and D) and Niemann-pick type C2 protein (91,92). Saposins function in a non-enzymatic manner, the exact mechanisms of which are not yet fully understood. Lack of saposin function causes lipid storage diseases, such as a form of Krabbe disease, which develops as a result of saposin A deficiency (93). Other known sphingolipid activator proteins are GM2 and saposins B, C and D, which are all post-translational products of the prosaposin gene (94). Mice that are deficient in prosaposin lack Vα14 iNKT cells, yet have a normal ability to present peptide–Class II MHC complexes (95). Deficiencies that involve only proteins for antigen cross-presentation, while excluding those concerned with the direct pathway of Class I MHC-mediated presentation, are to our knowledge yet to be described. Candidates should be found amongst proteins involved with the phagosomal milieu, especially those related to antigen processing, peptide transport past the phagosomal membrane and assembly into peptide–Class I MHC complexes.

Concluding remarks

The mechanisms that support the endosomal processing machinery in professional APCs for presentation of exogenous antigen via Class I MHC or CD1 complexes are currently being uncovered. It is our hope that new accessory proteins and pivotal mechanisms are found that facilitate selectively, in the endosomal microenviroment, the sorting of antigen, proteolysis and loading of Class I MHC and CD1. Elucidation of the endosomal processes that underlie the display of antigen-loaded Class I MHC and CD1 complexes should yield insights for future therapies aimed at potentiation of cytotoxic T-cell responses to phagocytosed antigens of viral or microbial origin.

Disclosures

All authors declare no conflicts of interest.

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Chapter 3

Distinct requirements for CD1d intracellular transport for development of Vα14 iNKT cells[1]

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Abstract

The positive selection of Vα14 invariant (i)NKT cells in mice requires CD1d-mediated Ag presentation by $CD4^+CD8^+$ thymocytes. Maturation of newly selected iNKT cells continues in the periphery and also involves CD1d expression. CD1d molecules acquire Ags for presentation in endosomal compartments, to which CD1d molecules have access through an intrinsic CD1d-encoded tyrosine motif and by association with the Class II MHC chaperone, invariant chain. In this study, we report the generation of mice in which all CD1d is replaced by CD1d-enhanced yellow fluorescent fusion protein (EYFP). CD1d-EYFP molecules are stable, present lipid Ags, and have near normal subcellular distribution. CD1d-EYFP molecules mediated positive selection of Vα14 iNKT cell precursors at decreased efficiency, caused a delay in their terminal maturation, and did not invoke Vα14 iNKT cell effector function as wild-type CD1d could. Using these mice, we show that the intrinsic CD1d-encoded sorting motif mediates thymic selection and activation of Vα14 iNKT cells by professional APCs, while for peripheral terminal differentiation the intrinsic CD1d sorting motif is dispensable.

Introduction

Ag presentation via CD1 molecules is important in the protection against viral and bacterial pathogens, against autoimmune diseases and for effective development of antitumor responses (1, 2, 3). CD1 molecules are assembled in the endoplasmic reticulum from CD1 H chain, β2-microglobulin and endogenous lipids. Incorporated lipids are thought to stabilize the CD1/β2-microglobulin complexes until exchange for antigenic lipids is accomplished, analogous to the role of invariant chain $(Ii)^3$-derived peptide in Class II MHC assembly (4, 5, 6). Lipid-loaded CD1 arrives at the plasma membrane within 30 min after assembly and is rapidly introduced into the endocytic pathway, mediated by a tyrosine-based sorting motif present in the CD1 cytosolic tail or through interaction with invariant chain/Class II MHC complexes (7, 8, 9). Recycling through the endocytic pathway from the cell surface results in exchange of lipid Ags, as CD1 molecules transit back and forth between the plasma membrane and lysosomes.

CD1d controls the development and function of a subset of strictly thymus-derived lymphocytes called NKT cells. Among the CD1d-restricted NKT cells are those that express a relatively invariant (i) TCR α-chain (Vα14-Jα18 in mice; Vα24-Jα18 in humans) coupled with a restricted subset of TCR β-chains (Vβ8.2, Vβ7, and Vβ2 in mice, and Vβ11 in humans), and those that express diverse TCRs. These α-galactosylceramide αGalCer)-reactive Vα14 iNKT cells constitute most of the $NK1.1^+$ T cell population in the spleen and represent >80% of the $NK1.1^+$ T cells in the thymus and liver (10, 11). The development of NKT cell precursors diverges from that of mainstream thymocytes at the $CD4^+CD8^+$ double-positive (DP) stage (12, 13, 14, 15, 16). Upon random rearrangement and expression of the canonical Vα14 chain coupled with a restricted TCR β receptor, these DP thymocytes can be positively selected on neighboring CD1d-expressing $CD4^+CD8^+$ cortical thymocytes (17, 18, 19, 20). Such positively selected Vα14 iNKT cell precursors are prompted to mature following a sequence from $CD24^+CD44lowNK1.1^-$ via $CD24^-$ $CD44lowNK1.1^-$ and $CD24^-CD44^+NK1.1^-$ to $CD24^-CD44^+NK1.1^+$ (12, 15, 16, 21, 22). Studies have shown that the periphery is primarily seeded with immature $CD44^+NK1.1^-$ Vα14 iNKT cells, which eventually mature outside the thymus (12, 16). At the final maturation step NK1.1 and other NK lineage receptors (such as Ly49G2, Ly49C, Ly49I,

CD94, NKG2D, Ly6C, and 2B4) are expressed (12, 15, 16, 21, 22). The transition from $NK1.1^-$ to $NK1.1^+$ Vα14 iNKT cells is important for terminal differentiation of iNKT cells and requires the presence of CD1d in the periphery (20, 23). A developmental block at this stage is linked to major deficiencies in Vα14 iNKT cell function, as for example, in diabetes-prone NOD mice in which selective deficiency of $NK1.1^+$ Vα14 iNKT cells predisposes to onset of autoimmune diabetes (24, 25).
Peripheral iNKT cell maturation involves professional APCs, in which CD1d endocytosis may occur via the CD1d intrinsic tyrosine motif or via association with Ii. Although Vα14 iNKT cells fully depend on an intact CD1d cytosolic tail for self-Ag recognition during thymic selection (9, 26), in the periphery, Vα14 iNKT cells are exposed to CD1d-positive cells that express invariant chain (i.e., professional APCs, including dendritic cells (DCs)), and cells that do not express invariant chain, in nonprofessional APCs such as peripheral T cells. Strong reactivity of iNKT cells for CD1d/self-glycolipid complexes results in their removal from the iNKT cell repertoire, as was shown using transgenic mice that overexpress CD1d and in fetal thymic organ cultures containing the high affinity ligand αGalCer (27, 28). In those experiments, the CD1d endosomal trafficking requirements for peripheral maturation could not be judged because postselection thymocytes did not survive. Mutant mice in which CD1d is expressed only on cortical thymocytes (driven by the Lck promotor) and professional APCs (driven by the I-Eα promotor) develop Vα14 iNKT cells that undergo normal selection and terminal differentiation (20). In those mice, postselection maturation of iNKT cells in the periphery could be contributed by either CD1d-tail-mediated endocytosis or Ii-assisted endocytosis. We generated a new mouse model in which all CD1d is expressed as CD1d-enhanced yellow fluorescent fusion protein (EYFP) fusion proteins. CD1d-EYFP molecules exhibit normal subcellular distribution and CD1d-EYFP molecules can support development of Vα14 iNKT cells, albeit at a reduced rate. Using these mice, we clarified the roles that the CD1d-intrinsic sorting motif plays in CD1d-mediated positive selection, postselection terminal maturation, and stimulation of matured Vα14 iNKT cells.

Materials and Methods

Mice

Six- to eight-week-old mice on C57 Bl/6 background were used throughout the study. Wild-type (WT) mice were purchased from The Jackson Laboratory. CD1d-deficient mice were described previously (29). CD1d-EYFP/EYFP mice were generated using homologous recombination in embryonic stem cells (Bruce 4 line). The genomic sequence encoding exons 1-6 and part of the 3' untranslated region were used as homologous regions. A linker sequence between exon 6 and EYFP was inserted using the following primer: ACC GGT CCT CCT CCA GCA GGA CTC CTG GGA CAG CCG GTC GCC ACC.

For mixed bone-marrow chimeras, 6-wk-old CD1d-/- hosts were subjected to two consecutive doses of 600 Rads irradiation with 2 h in between. Within 8 h after the second irradiation, irradiated mice were injected retro-orbitally with 1 x 107 bone marrow cells isolated from femurs of donor mice (1:1 mix of CD45.1 CD1d-/- : CD45.2 CD1d+/+ or CD45.1 CD1d-/- : CD45.2 CD1d-EYFP/EYFP). All chimeras showed a ratio of CD45.1:CD45.2 expressing thymocytes and splenocytes between 0.3 and 0.7, demonstrating the comparable capacity of CD1d-/- , CD1d+/+ and CD1d-EYFP/EYFP bone marrow cells to reconstitute general lymphocyte population. After 6 wk of grafting,

the mice were analyzed for the presence and development of Vα14 iNKT cells. All mice were bred and maintained in a barrier facility and studies were performed according to institutional guidelines for animal use and care.

Abs and reagents

CD1d:PBS-57 (αGalCer) tetramers were provided by the National Institute of Allergy and Infectious Disease MHC Tetramer Core facility, Atlanta, GA. Abs were acquired from eBiosciences, BD Biosciences, BioLegend, and Invitrogen. Annexin V was acquired from BD Biosciences. Dr. A. Bendelac (Howard Hughes Medical Institute, University of Chicago, Chicago, IL) provided cells excreting rat-anti-mouse CD1d (19G11), Dr. G. Besra (School of Biosciences, University of Birmingham, Birmingham, U.K.) provided αGalCer and Gal(α1→2)GalCer and Dr. H. Ploegh provided polyclonal rabbit anti-EGFP Ab.

Immunoblot

Thymocytes were lysed with NP40 lysis buffer (2% NP40, 150 mM NaCL, 1 mM EDTA, 0.02% NaN3, 0.5 mM Trisbase (pH 10.4)) and protease inhibitor (Roche). Lysates were boiled, separated on a 4-20% Tris-HCL gel, and transferred to a polyvinylidene fluoride membrane (Bio-Rad). Membranes were blotted using a polyclonal anti-mCD1d Ab (19G11) or a polyclonal anti-EGFP Ab, which cross-reacts with EYFP.

Vα14 iNKT cell enrichment from the liver

Mouse liver was perfused with PBS and homogenized trough a stainless steel mesh. After 5 min, centrifugation at 2000 rpm, the liver cells were taken up in 9 ml of PBS and mixed with 5 ml of 80% Percoll. This suspension was overlayed on 3 ml 80% Percoll and the gradient was spun for 20 min at 2000 rpm. The interface containing the liver lymphocytes was collected, washed with PBS, and RBC were lysed using ammoniumchloride.

Flow cytometry and CD1d endocytosis assay

Cells were stained with the relevant mAb on ice in the presence of Fc-block (2.4G2). Vα14 iNKT cells and their different developmental stages were detected by staining with αGalCer-loaded CD1d-tetramers and Abs against TCRβ, CD44, and NK1.1, unless mentioned otherwise. Incubating thymic Vα14 iNKT cells at 37°C for 30 min induced apoptosis. The amount of apoptosis was determined by staining with Annexin V following the manufacturer's suggestions. For the endocytosis assay, thymocytes were stained for 1 h on ice in DMEM with PE-conjugated CD1d mAb in the presence of 10 μg/ml cycloheximide to block de novo synthesis. Cells were washed and subsequently incubated for various times at 37°C. Surface-bound mAb was stripped using 300 mM glycine/1% FCS solution (pH 2, 3 min) before neutralization (300 mM glycine/1% FCS solution, pH 7), staining for surface markers, and fixation (10 min, 4% paraformaldehyde). CD1d endocytosis was determined by the relative intensity of internalized anti-CD1d mAb. Normalized residual surface CD1d was calculated by setting surface CD1d-PE fluorescence at 100% and subtracting the percentage of internalized CD1d-PE.

Vα14 iNKT stimulation assays

$CD11c^+$ MACS-purified spleen DCs were stimulated for 4 h with 100 ng/ml αGalCer or Gal(α1→2)GalCer. After two washes, freshly isolated liver Vα14 iNKT cells were added to

the culture for 24 (for IL-4) or 48 (for IFN-γ) h. Vα14 iNKT cells in enriched leukocytes from CD1d-EYFP/EYFP liver were 2-fold reduced compared with WT and were corrected for by addition of twice the number of Vα14 iNKT cell-enriched leukocytes to the Ag-laden DC cultures. IFN-γ and IL-4 secretion was measured by ELISA following the manufacturer's suggestions (eBiosciences).

Statistical analyses

Data are shown as mean ± SEM. Unpaired two-tailed t test was used to compare two groups. A p-value of at least 0.05 was considered statistically significant. Analysis was performed using Prism 4.0 for Mac software (GraphPad Software).

Results

Mice expressing CD1d-EYFP fusion proteins

Mice have only two CD1 genes, CD1d1 and CD1d2. Expression of CD1d2 protein in mice is restricted to thymocytes and is considered nonfunctional (30, 31). We focused on CD1d1, hereafter referred to as CD1d, and generated knock-in mice in which the CD1d locus was replaced by a version encoding CD1d-EYFP fusion protein by homologous recombination (Fig. 1A). The phosphoglycerate kinase promoter-driven neomycin resistance gene in the targeting vector, flanked by loxP sites, was deleted by breeding with cre-deleter mice (Fig. 1A). Development of CD4 and CD8 T cells, B cells, and DCs was unaffected by the knock-in mutation (data not shown). Thymocytes from CD1d-EYFP/EYFP and WT mice show similar protein levels of CD1d (Fig. 1B, left; polyclonal anti-CD1d blotting Ab). The presence and abundance of CD1d-EYFP polypeptide in whole thymus lysate was also comparable in amount to MHC Class II-EGFP β-chain polypeptide (32) (Fig. 1B, right; polyclonal anti-EGFP Ab, which also recognizes EYFP protein). Thymocytes showed the expected 49 kDa CD1d product in WT mice, and a 76 kDa fusion protein product in CD1d-EYFP/EYFP mice (composed of the 49 kDa CD1d together with 27 kDa EYFP polypeptide) (Fig. 1B). No free EYFP was detected. Therefore, EYFP detected by flow cytometry (Fig. 1C) or visualized by microscopy (Fig. 1D) represents CD1d molecules labeled with EYFP (CD1d-EYFP), and CD1d-EYFP molecules are stable in the cellular environment. Using flow cytometry on fresh peripheral blood B lymphocytes, we showed that CD1d-EYFP/EYFP mice express double the amount of EYFP fluorescence compared with CD1d-EYFP/+ mice that harbor one CD1d-EYFP and one untagged CD1d allele (Fig. 1C).

Direct visualization of intracellular localization of CD1d-EYFP fusion proteins

CD1d molecules acquire their antigenic cargo in the lysosomal pathway. CD1d molecules therefore predominantly localize to the cell surface and the lysosomal pathway (26). We purified DCs from bone marrow and determined the subcellular distribution of CD1d-EYFP molecules by confocal microscopy. DCs were analyzed immediately after magnetic cell sorting based on CD11c-expression to prevent maturation and DC attachment to coverslips. Using Z-stack analysis, CD1d-EYFP fluorescence was found in intracellular vesicular structures of the endo/lysosomal pathway, as well as on the cell surface in DCs (Fig. 1D).

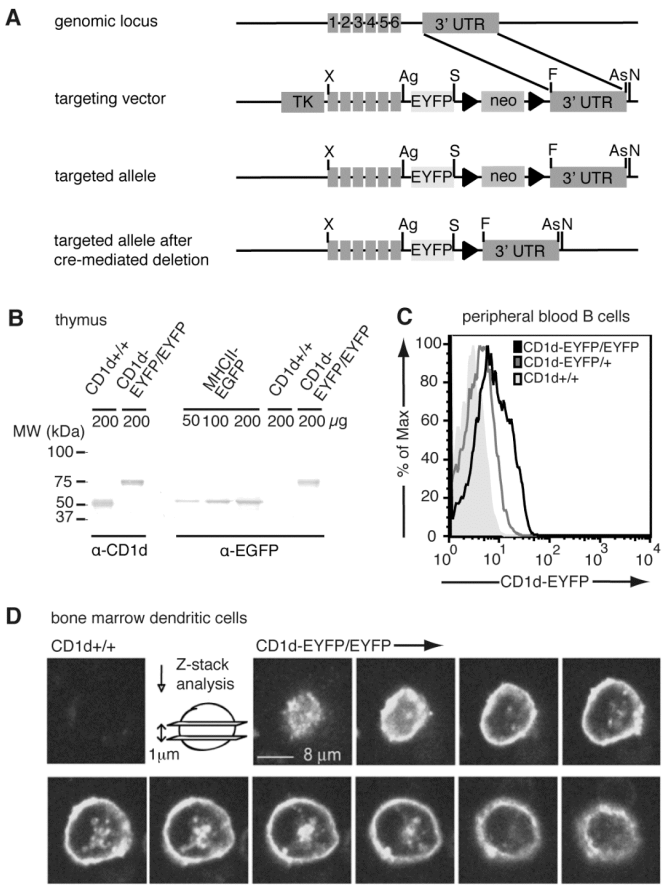

FIGURE 1. Generation and characterization of CD1d-EYFP knock-in mice. ***A***, Schematic representation of the targeting construct consisting of a floxed (triangles) phosphoglycerate kinase promoter-driven neomycin (neo) resistance sequence, flanked by a 2683 bp XhoI/AgeI homologous fragment upstream to which we fused EYFP (SalI) and by a 538 bp FseI/AscI homologous fragment downstream. NotI digestion was used for plasmid linearization. The thymidine kinase (tk) sequence was used for counter selection. ***B***, Whole thymocyte lysates were separated on a 4-20% Tris-HCl gel and transferred to polyvinylidene fluoride membrane. Membranes were blotted using polyclonal anti-CD1d or anti-EGFP Ab. The CD1d-EYFP fusion protein is positioned at 76 kDa (CD1d H chain: 49 kDa; EYFP: 27 kDa) compared with WT-CD1d H chain at 49 kDa or I-Ab-EGFP at 56 kDa. ***C***, EYFP signal was detected using flow cytometry on peripheral blood B cells. Whole blood from CD1d+/+, CD1d-EYFP/+, and CD1d-EYFP/EYFP mice was stained with fluorophore-conjugated Abs against B220 (CD45RA). EYFP fluorescence representing CD1d-EYFP on $B220^{+}$ peripheral blood B cells was detected in the FL-1 channel on a FACSCanto flow cytometer. ***D***, Fresh bone marrow-derived DCs from CD1d+/+ and CD1d-EYFP/EYFP mice were enriched by magnetic cell sorting, based on CD11c expression and visualized by spinning disc confocal microscopy at x100 original magnification. Images shown are from one representative cell per mouse model. For the CD1d-EYFP/EYFP bone marrow-derived DC, one cell was visualized as 10 cross-sections on the Z-axis, using a 1-μm separation interval.

Vα14 iNKT cell development in CD1d-EYFP/EYFP mice

The positive selection and terminal differentiation of Vα14 iNKT cells requires CD1d-mediated Ag presentation by both cortical thymocytes and professional APCs (20, 23). To determine whether CD1d-EYFP can fully substitute for its unlabeled counterpart, we asked whether CD1d-EYFP molecules can support Vα14 iNKT cell development *in vivo*. Vα14 iNKT cells were characterized by staining with anti-TCRβ and CD1d-tetramers loaded with the pan-iNKT cell ligand αGalCer and analyzed by flow cytometry. CD1d-EYFP/EYFP mice showed a ~60% reduction in tetramer-positive Vα14 iNKT cells in the thymus, and ~80% reduction in spleen and liver compared with WT (Fig. 2). No Vα14 iNKT cells were detected in CD1d-/- mice, as previously described (29). Thus, CD1d-EYFP molecules *in vivo* can support Vα14 iNKT cell development, albeit with reduced efficiency compared with WT CD1d molecules.

Thymic selection of Vα14 iNKT cells expressing one CD1d or CD1d-EYFP allele

The reduced Vα14 NKT cell number in CD1d-EYFP/EYFP mice indicates a defect in the functioning of CD1d-EYFP. Alternatively, this observation could be caused by a reduced expression of CD1d-EYFP compared with WT, as CD1d-EYFP/EYFP thymocytes display less CD1d at the cell surface than do CD1d+/+ thymocytes ($p < 0.05$) (Fig. 3A). However, CD1d-EYFP/+ heterozygous mice exhibited normal Vα14 iNKT cell numbers, as do CD1d+/- heterozygous mice that express 50% less CD1d at the cell surface (Fig. 3, A and B). Thus, the expression of a single WT CD1d allele suffices to support normal thymic Vα14 iNKT cell selection, and the decreased Vα14 iNKT cell development in CD1d-EYFP/EYFP mice was unlikely to result from the ~25% decrease in CD1d surface expression. CD1d-EYFP/EYFP mice moreover exhibited normal percentages of DP thymocytes (Fig. S1).

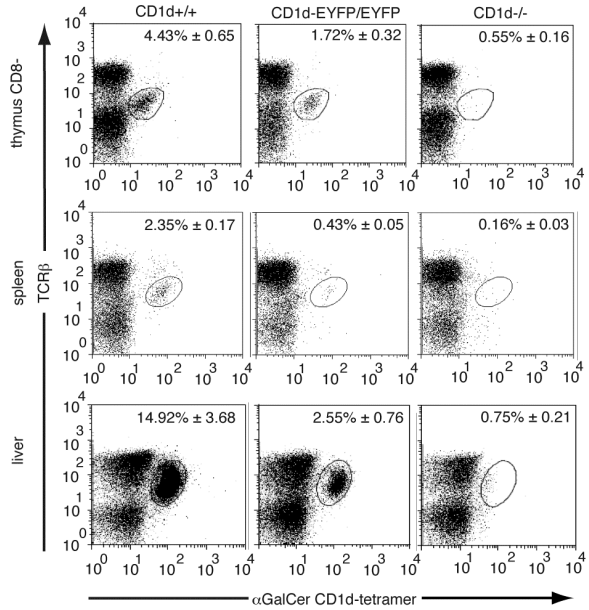

FIGURE 2. Impaired thymic selection of Vα14 iNKT cells in CD1d-EYFP/EYFP mice. Thymocytes, splenocytes, and liver leukocytes were stained with mAbs against TCRβ, CD19, and CD8α and αGalCer-loaded CD1d-tetramers, and analyzed by flow cytometry. B cells ($CD19^+$) and $CD8^+$ cells were electronically removed during analysis. Data shown are representative of at least five mice per group.

Thymic selection of Vα14 iNKT cells expressing one CD1d or CD1d-EYFP allele

The reduced Vα14 NKT cell number in CD1d-EYFP/EYFP mice indicates a defect in the functioning of CD1d-EYFP. Alternatively, this observation could be caused by a reduced expression of CD1d-EYFP compared with WT, as CD1d-EYFP/EYFP thymocytes display less CD1d at the cell surface than do CD1d+/+ thymocytes ($p < 0.05$) (Fig. 3A). However, CD1d-EYFP/+ heterozygous mice exhibited normal Vα14 iNKT cell numbers, as do CD1d+/- heterozygous mice that express 50% less CD1d at the cell surface (Fig. 3, A and B). Thus, the expression of a single WT CD1d allele suffices to support normal thymic Vα14 iNKT cell selection, and the decreased Vα14 iNKT cell development in CD1d-EYFP/EYFP mice was unlikely to result from the ~25% decrease in CD1d surface expression. CD1d-EYFP/EYFP mice moreover exhibited normal percentages of DP thymocytes (Fig. S1).

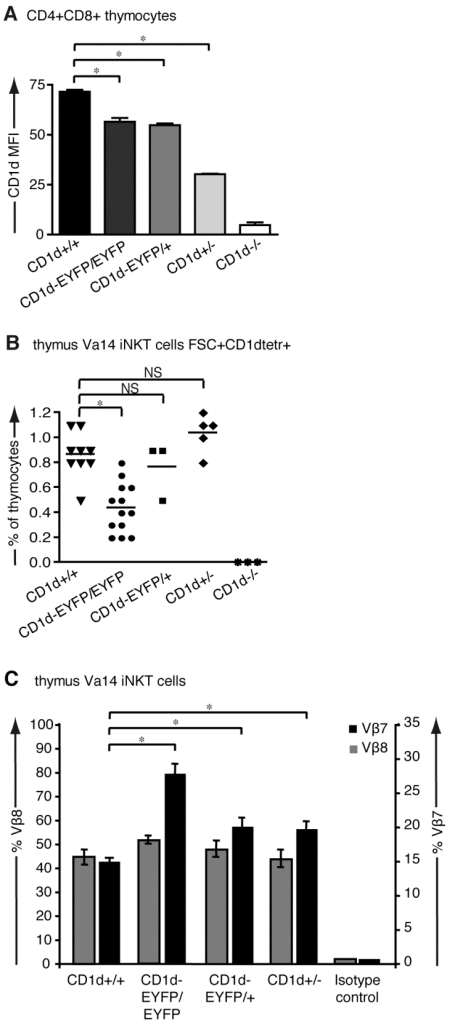

FIGURE 3. CD1d surface display and Vα14 iNKT cell development in heterozygous and homozygous CD1d mutant mice. ***A***, DP thymocytes from 6- to 9-wk-old mice were stained with PE-conjugated CD1d mAb and surface expression was analyzed by flow cytometry. Surface CD1d expression between CD1d+/+ and CD1d-EYFP/EYFP or CD1d-EYFP/+ is significantly different ($p < 0.05$). ***B***, Percentage of Vα14 iNKT cells. Thymocytes from 6- to 9-wk-old mice were stained with αGalCer-loaded CD1d-tetramers. CD1d-EYFP/EYFP mice contain significantly less Vα14 iNKT cells compared with CD1d+/+ mice (*, $p < 0.0001$). CD1d-EYFP/+ mice and CD1d+/- mice contain comparable numbers of Vα14 iNKT cells to CD1d+/+ mice (NS, $p > 0.1$). ***C***, Skewing of Vβ repertoire in CD1d-EYFP/EYFP mice. Thymocytes from different mice were stained with mAbs against TCRβ, Vβ7, Vβ8, and αGalCer-loaded CD1d-tetramers and analyzed by flow cytometry. Percentage of Vβ7 (black, right axis) and Vβ8 (gray, left axis) positive Vα14 iNKT cells. CD1d-EYFP/EYFP mice display significantly more Vβ7 positive Vα14 iNKT cells compared with CD1d+/- or CD1d-EYFP/+ mice, which display more Vβ7 positive Vα14 iNKT cells compared with CD1d+/+ (*, $p < 0.001$). Bar graphs and dot plots are representative example of three experiments with at least three mice per group; the bars represent the mean of duplicate values and the brackets indicate SEM.

The thymic selection of Vβ7$^+$, but not Vβ8.2$^+$, Vα14 iNKT cells is favored in situations where endogenous CD1d ligand concentration are suboptimal, as was shown in CD1d+/- mice (33), while Vβ7$^+$ Vα14 iNKT cells are relatively diminished upon CD1d overexpression (28). We asked whether endogenous Ag presentation by CD1d-EYFP molecules is suboptimal, as an explanation for decreased Vα14 iNKT cell development. To this end, we examined the TCR Vβ repertoire of Vα14 iNKT cells in CD1d-EYFP knock-in mice. We found that thymic Vα14 iNKT cells from CD1d-EYFP/EYFP mice displayed a bias in their Vβ repertoire, with an overrepresentation of Vβ7$^+$ usage that was more pronounced than was earlier shown in CD1d+/- mice ($p < 0.001$) (Fig. 3C). Use of the TCR Vβ domain shapes the selection of Vα14 iNKT cells by endogenous ligand displayed by CD1d (33). Therefore, our data suggests that presentation of endogenous lipid Ags supporting thymic selection of Vα14 iNKT cells is suboptimal in CD1d-EYFP/EYFP mice when compared with WT mice.

Incomplete Vα14 iNKT cell selection and maturation in CD1d-EYFP thymus

We next asked how suboptimal endogenous Ag presentation during positive selection affects thymic development of Vα14 iNKT cells. To this end, we generated mixed bone marrow chimeras in which we tracked CD1d-/- bone marrow precursors for their ability to give rise to Vα14 iNKT cells when transferred into CD1d-expressing recipient hosts (15), and measured the expression of the maturation markers CD44 and NK1.1 on Vα14 iNKT cells. In these chimeras, CD45.1 CD1d-/- Vα14 iNKT cell precursors were selected on DP thymocytes expressing either WT CD45.2 CD1d (CD1d-/- : CD1d+/+ chimeras) or CD45.2 CD1d-EYFP (CD1d-/- : CD1d-EYFP/EYFP chimeras). CD1d-/- hosts were lethally irradiated and reconstituted with a 1:1 mix of bone marrow cells, distinguishable by expression of separate CD45 isoforms. After 6 wk of engraftment, the mice were analyzed for the presence and development of Vα14 iNKT cells. CD1d-EYFP/EYFP chimeras supported thymic development of fewer Vα14 iNKT cells when compared with CD1d+/+ chimeras ($p < 0.0001$), causing a reduction of ~80% in Vα14 iNKT cells (Fig. 4A). We next analyzed three successive Vα14 iNKT cell thymic developmental stages, CD44$^-$ NK1.1$^-$, CD44$^+$NK1.1$^-$, and CD44$^+$NK1.1$^+$ by flow cytometry, as described in previous studies (12, 15, 16, 22). The majority of thymic Vα14 iNKT cells extracted from CD1d-EYFP/EYFP chimeras were present in the earlier stages (predominantly CD44$^+$NK1.1$^-$) of development, with fewer than 15% of cells exhibiting the mature CD44$^+$NK1.1$^+$ stage. In CD1d+/+ chimeras, Vα14 iNKT cells were more mature overall, with 40% of cells being found in the CD44$^+$NK1.1$^+$ stage ($p < 0.05$) (Fig. 4B). Thus, CD1d-EYFP molecules are defective at supporting thymic selection and thymic maturation of Vα14 iNKT cells.

Delay in thymic Vα14 iNKT cell maturation selected on CD1d-EYFP molecules

After birth, the Vα14 iNKT cell compartment continues to expand and mature, with a cellular increase with age up to 50 days (12, 15, 16, 34) after which thymic Vα14 iNKT cell numbers drop (35). In newborn mice, the majority of thymic Vα14 iNKT cells are CD44$^-$ NK1.1$^-$, which gradually acquire CD44 and NK1.1 expression (12, 15). Also in the periphery, CD44$^+$NK1.1$^-$ thymic emigrants progressively develop into CD44$^+$NK1.1$^+$ Vα14 iNKT cells (35). We analyzed thymic Vα14 iNKT cells from three age cohorts of CD1d-EYFP/EYFP and control mice accordingly. Throughout time, thymic Vα14 iNKT cells from CD1d-EYFP/EYFP mice significantly remain in the earlier stages of development (predominantly CD44$^-$NK1.1$^-$) compared with CD1d+/+ mice ($p < 0.05$) (Fig. 5A). To

establish further that Vα14 iNKT cells are inhibited in their thymic maturation when selected on CD1d-EYFP molecules, we analyzed for down-regulation of the developmental markers Slamf1 (CD150) and CD69. Developing iNKT cells temporarily display high levels of Slamf1 expression on DP Vα14 iNKT cell precursors ($CD24^+CD44^-NK1.1^-$) (36). CD69 is also temporarily expressed following positive selection of MHC-restricted T cells (37) and after positive selection of Vα14 iNKT cells (12, 15, 16, 21, 22). In 6 -to 9-wk-old mice, we found that Vα14 iNKT cells that were selected on CD1d-EYFP molecules retained significantly high surface levels of Slamf1 ($p < 0.006$) (Fig. 5B) and CD69 ($p < 0.0001$) (Fig. 5C) up until the last stage of development compared with CD1d+/+ mice. Similar data was found in mice 2-5 wk and 10-13 wk of age (data not shown).

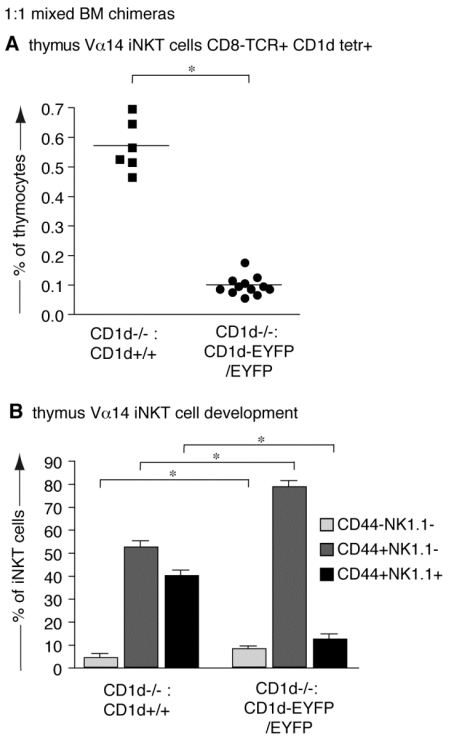

FIGURE 4. Incomplete differentiation and maturation of Vα14 iNKT cells selected on CD1d-EYFP in mixed bone marrow chimeras. ***A***, Percentage of total Vα14 iNKT cells in thymus of mixed bone marrow chimeras. Six weeks postengraftment, thymocytes from mixed bone-marrow chimeras were stained with αGalCer-loaded CD1d-tetramers and analyzed by flow cytometry. Mixed bone marrow chimeras reconstituted with CD1d-EYFP/EYFP exhibited significantly fewer Vα14 iNKT cells than mixed bone marrow chimeras reconstituted with CD1d+/+ marrow (*, $p < 0.05$). ***B***, Thymocytes from ***A*** were stained with mAbs against CD44, NK1.1 and analyzed by flow cytometry. αGalCer-loaded CD1d-tetramer positive Vα14 iNKT cells were divided into three successive development stages, from $CD44^-NK1.1^-$ via $CD44^+NK1.1^-$ to $CD44^+NK1.1^+$. Results visualized are from the CD45.1 CD1d-/- compartment and displayed in bar graphs are representative of two experiments with five mice per group. Thymic Vα14 iNKT cells from CD1d-/- origin, selected on CD1d-EYFP/EYFP thymocytes, significantly remain in the earlier stages of development compared with CD1d-/- Vα14 iNKT cells selected on CD1d+/+ thymocytes (*, $p < 0.05$).

Furthermore, thymic Vα14 iNKT cells from CD1d-EYFP/EYFP mice exhibited increased levels of apoptosis, as measured by flow cytometric analysis of Annexin V staining, which could partially account for the reduction of Vα14 iNKT cells in CD1d-EYFP/EYFP mice (Fig. S2, A and B). This increase in apoptosis was found in each age group and in each of the three stages of development ($p < 0.05$). Taken together, our data shows that suboptimal endogenous Ag presentation by CD1d-EYFP molecules results in decreased thymic maturation of developing Vα14 iNKT cells.

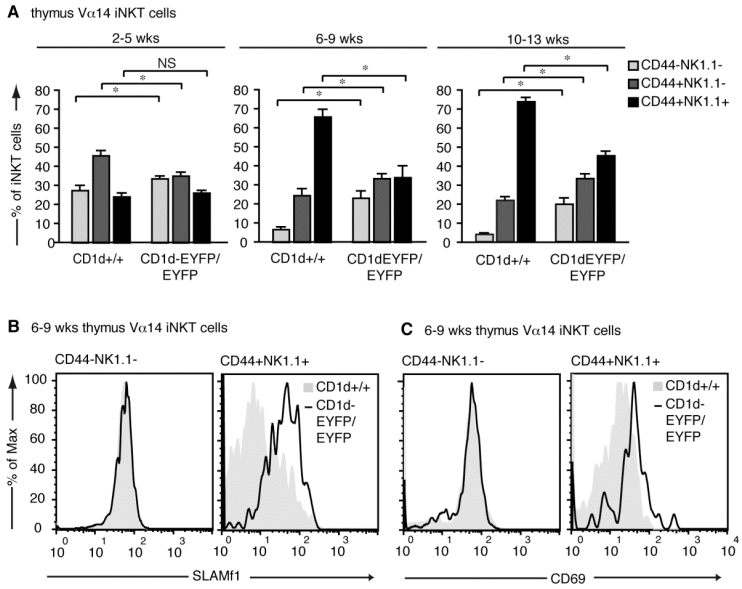

FIGURE 5. Age-dependent delay in differentiation and maturation of Vα14 iNKT cells selected on CD1d-EYFP molecules. ***A***, Thymic Vα14 iNKT differentiation and maturation. Thymic Vα14 iNKT were subdivided into three stages, ranging from early and immature Vα14 iNKT cells ($CD44^-NK1.1^-$) via $CD44^+NK1.1^-$ to mature Vα14 iNKT cells ($CD44^+NK1.1^+$). At least four mice in each group were analyzed independently and results were displayed in a bar graph with SEM. In all age-groups analyzed, thymic Vα14 iNKT cells from CD1d-EYFP/EYFP mice remain in the earlier stages of development compared with CD1d+/+ mice (*, $p < 0.04$). ***B***, Thymocytes from A were stained with mAbs against Slamf1 (CD150) or CD69 (C) and analyzed by flow cytometry. Histograms shown are from representative 6- to 9-wk-old mice of three experiments each with at least four mice per group. For all age groups, significantly more Vα14 iNKT cells from CD1d-EYFP/EYFP mice remain high for Slamf1 ($p < 0.006$) and CD69 ($p < 0.0001$) after the first stage of development ($CD44^-NK1.1^-$) compared with CD1d+/+ mice.

CD1d-EYFP molecules support normal peripheral maturation of Vα14 iNKT cells

The majority of Vα14 iNKT cells exits the thymus in immature stage ($CD44^+NK1.1^-$) and matures in the periphery (12, 16). Acquisition of NK1.1 by these immature Vα14 iNKT cells and acquisition of full effector function requires interaction with CD1d in the periphery (23) by professional MHC Class II-expressing APCs (20). We next asked whether CD1d-EYFP molecules can mediate postselection maturation of Vα14 iNKT cells. Vα14 iNKT cells from spleens of CD1d-EYFP/EYFP and CD1d+/+ mice showed comparable $CD44^+NK1.1^+$ profiles, suggesting that CD1d-EYFP molecules support peripheral maturation of Vα14 iNKT cells (Fig. 6A). Do phenotypically mature Vα14 iNKT cells selected on CD1d-EYFP molecules produce normal amounts of cytokine upon activation? WT spleen DCs were allowed to stimulate Vα14 iNKT cells extracted from either WT or CD1d-EYFP/EYFP livers. In addition to αGalCer glycolipid Ag, we used Gal(α1→2)GalCer, which requires internalization and removal of the terminal sugars by

the lysosomal enzyme, α-galactosidase A, for CD1d-mediated presentation to occur (38). CD1d-EYFP/EYFP Vα14 iNKT cells produced normal levels of IFN-γ upon αGalCer and Gal(α1→2)GalCer presentation by WT spleen DCs (Fig. 6, B and D). However, IL-4 production by CD1d-EYFP/EYFP Vα14 iNKT cells was nearly absent compared with their WT counterparts (Fig. 6, C and E). In addition, IL-2 production by CD1d-EYFP/EYFP Vα14 iNKT cells was reduced upon αGalCer and Gal(α1→2)GalCer presentation by WT spleen DCs ($p < 0.05$) (Fig. S3, A and B). Thus, endogenous Ag presentation by CD1d-EYFP molecules during thymic selection results in Vα14 iNKT cells that produce normal levels of IFN-γ, decreased levels of IL-2, and no IL-4.

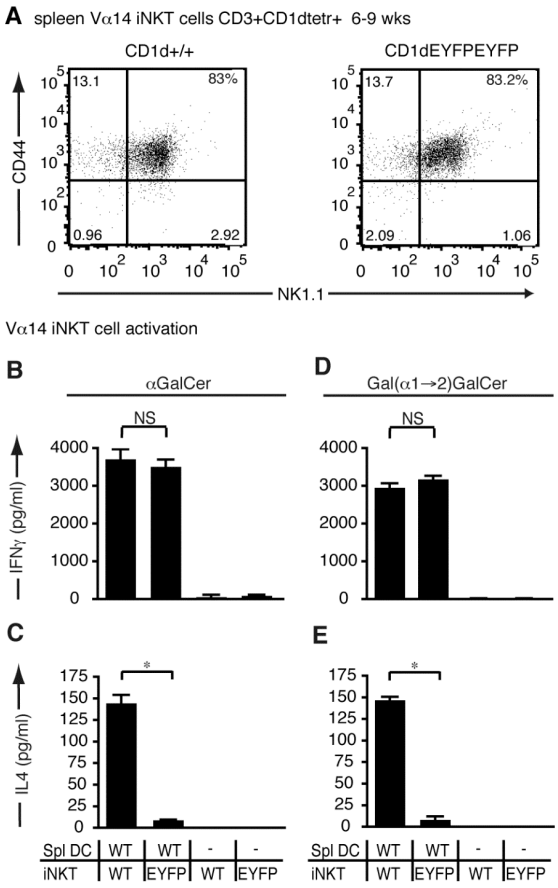

FIGURE 6. Normal peripheral maturation, but lack of Ag-induced IL-4 production by Vα14 iNKT cells selected on CD1d-EYFP molecules. ***A***, Maturation of Vα14 iNKT cells in the periphery. Spleen Vα14 iNKT were subdivided into immature ($CD44^+NK1.1^-$) and mature ($CD44^+NK1.1^+$) Vα14 iNKT cells. Histograms shown are representative from 6- to 9-wk-old mice of three experiments each with at least four mice per group. In all age groups, equal fractions of CD1d-EYFP/EYFP Vα14 iNKT cells and CD1d+/+ Vα14 iNKT cells expressed NK1.1 (NS, $p > 0.05$). Activation of Vα14 iNKT cells *in vitro*. Spleen DCs and liver Vα14 iNKT cells from WT (CD1d+/+) or CD1d-EYFP/EYFP mice were cocultured in the presence of 100 ng/ml αGalCer (***B*** and ***C***) or Gal(α1→2)GalCer (***D*** and ***E***). Results displayed in bar graphs are representative of two experiments. Shown are the mean of triplicate values with SEM. Production of IFN-γ was comparable (NS, $p > 0.05$), while IL-4 production by CD1d-EYFP/EYFP Vα14 iNKT cells was significantly reduced compared with CD1d+/+ counterparts (*, $p < 0.001$).

Endocytosis of CD1d-EYFP molecules in thymocytes, $CD4^+$ T cells, and DCs

We had noticed that in DP thymocytes and spleen $CD4^+$ T cells of CD1d-EYFP/EYFP mice, the surface expression of CD1d measured by flow cytometry appears as narrower mean fluorescence intensity histogram peaks compared with WT (Fig. S4A). In the DP

thymocytes, this tighter surface CD1d distribution resulted in a significant decrease in mean fluorescence intensity compared with WT ($p < 0.05$), while in spleen, $CD4^+$ T cells from homozygous CD1d-EYFP mice, the number of surface-expressed CD1d molecules was increased (reflected in a narrower histogram peak that is shifted more to the right, $p < 0.05$). On professional APCs (spleen DCs) from CD1d-EYFP/EYFP mice, however, CD1d surface expression was comparable to their WT counterparts (Fig. S4A).

To determine whether the increase in surface-expressed CD1d related to decreased endocytosis of CD1d-EYFP, we next analyzed the rate of Ab-binding-induced endocytosis in thymocytes, $CD4^+$ T cells, and DCs from CD1d+/+, CD1d-EYFP/EYFP, and CD1d-/- mice. Cells were labeled on ice using PE-conjugated anti-CD1d in the presence of cycloheximide to block protein synthesis. Aliquots of cells were transferred to 37°C, and collected at sequential times thereafter. Ab bound to surface CD1d was stripped by acid treatment, and the extent of internalized CD1d bound by PE-conjugated anti-CD1d Ab was visualized by flow cytometry (39). The internalization rate of CD1d-EYFP molecules in DP thymocytes was significantly reduced compared with WT ($p < 0.05$) (Fig. 7, A and B). Control thymocytes from CD1d-deficient mice show no CD1d staining at any of the time points (Fig. S4B). Similar to their thymic counterparts, endocytosis of CD1d-EYFP molecules in $CD4^+$ T cells was significantly reduced compared with WT CD1d molecules ($p < 0.05$) (Fig. 7A). Compared with DP thymocytes, Ab-binding-induced CD1d endocytosis in spleen-derived DCs is much reduced and without significant difference between CD1d-EYFP/EYFP and WT DCs ($p > 0.05$) (Fig. 7, A and B).

Effector function of end-differentiated Vα14 iNKT cells from CD1d-EYFP/EYFP and Ii-/- mice

Thymocytes do not express Ii, and in thymocytes CD1d therefore strictly relies on its own CD1d-encoded tyrosine-based sorting motif for localization in lysosomes. In professional APCs, however, endosomal sorting of CD1d could be mediated by the CD1d-encoded tyrosine-based sorting motif, or via association of CD1d with the Class II MHC-chaperone Ii (7, 8, 9). We asked whether in professional APCs, the CD1d-encoded tyrosine-based sorting motif and Ii-encoded sorting motif can supplement for each other in mediating lysosomal localization, Ag acquisition, and presentation by CD1d. To this end, we tested whether Gal(α1→2)GalCer can be processed and presented by spleen-derived DCs, when either CD1d-EYFP is expressed, or Ii is absent, or in DCs from mice that harbor both mutations (Ii-/- CD1d-EYFP/EYFP mice).

DCs from indicated mouse strains were loaded with graded amounts of Gal(α1→2)GalCer for 4 h, washed, and allowed to stimulate WT Vα14 iNKT cells. WT DCs presenting 100 pg/ml αGalCer to WT Vα14 iNKT cells were included as a control to determine the response potential of the Vα14 iNKT cells in this assay. WT DCs strongly induced IFN-γ production and IL-4 production in Vα14 iNKT cells (Fig. 8, A and B). DCs from CD1d-EYFP/EYFP mice, Ii-/- mice, and the combined Ii-/- CD1d-EYFP/EYFP mice did not induce IFN- γ production by WT Vα14 iNKT cells ($p < 0.001$), while also IL-4 production was significantly reduced ($p < 0.05$). DCs from CD1d-/- mice did not induce cytokine production in WT Vα14 iNKT cells. Similarly, presentation of Gal(α1→2)GalCer by CD1d-EYFP/EYFP spleen DCs resulted in significantly less IL-2 production by WT Vα14 iNKT cells ($p < 0.05$) (third bar in Fig. S3B). Taken together, endosomal sorting of CD1d

relies on both its self-encoded tyrosine-based motif, and on association with Ii in professional APCs in the periphery, to stimulate Vα14 iNKT cells. Thus, both the CD1d-encoded and Ii-encoded endosomal sorting motifs are necessary to induce activation of Vα14 iNKT cells by professional APCs in spleen.

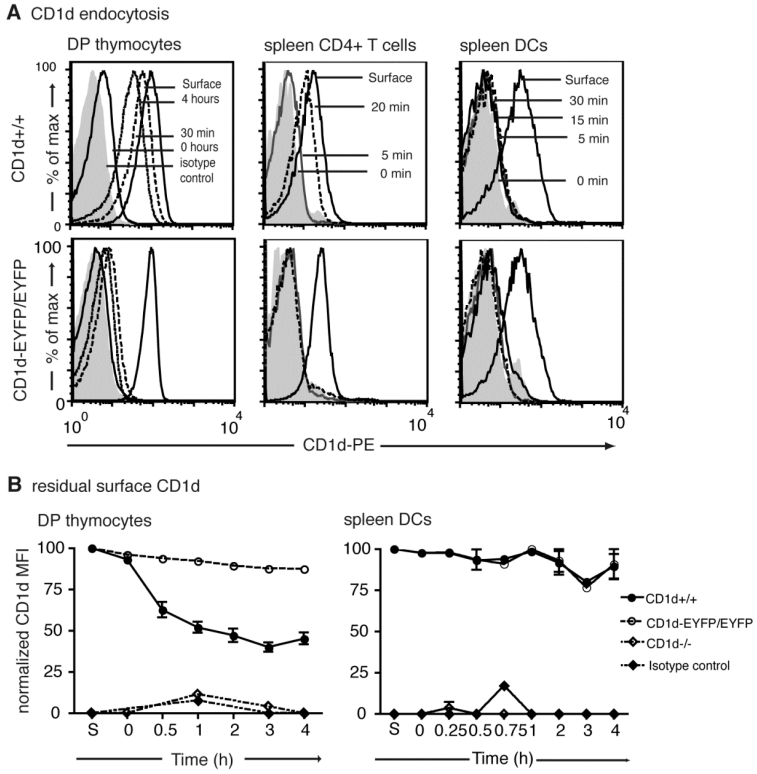

FIGURE 7. Delay in endocytosis of CD1d-EYFP fusion proteins in T cells, but not in professional APCs. ***A*** and ***B***, The extent of internalized CD1d bound by PE-conjugated anti-CD1d Ab in thymocytes, $CD4^+$ T cells and DCs from CD1d+/+ and CD1d-EYFP/EYFP mice was visualized by flow cytometry. CD1d-EYFP molecules on DP thymocytes exhibit a significantly longer surface retention time than WT CD1d molecules, demonstrated within 0.5 h of endocytosis induction ($p < 0.005$). CD1d-EYFP molecules on $CD4^+$ T cells in the periphery exhibit a significantly longer surface retention time than WT CD1d molecules ($p < 0.05$). Professional APCs in the periphery display less CD1d endocytosis activity than T cells. No difference was found between endocytosis of CD1d-EYFP molecules and CD1d (NS, $p > 0.05$). Histograms shown are representative of three independent experiments. Data points in the graph represent the mean of quadruplet values and brackets indicate SEM.

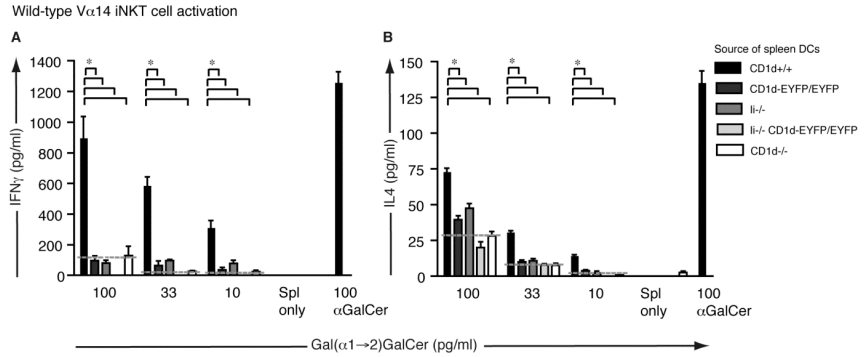

FIGURE 8. Endosomal Ag loading and stimulation of Vα14 iNKT cells by professional APCs requires endosomal sorting motifs of Ii and CD1d-cytosolic tail. Activation of Vα14 iNKT cells *in vitro*. CD11c$^+$ MACs-sorted spleen DCs from WT (CD1d+/+), CD1d-EYFP/EYFP, Ii-/-, Ii-/- CD1d-EYFP/EYFP or CD1d-/- mice were cocultured with WT liver Vα14 iNKT cells in the presence of different amounts of Gal(α1→2)GalCer (***A*** and ***B***). WT DCs cultured in the presence of 100 pg/ml αGalCer to WT Vα14 iNKT cells were included as a control to determine the response potential of the Vα14 iNKT cells in this assay. Results displayed in bar graphs are representative of two experiments. Shown are the mean of triplicate values with SEM. WT DCs induced IFN-γ and IL-4 production in WT Vα14 iNKT cells, while DCs from CD1d-EYFP/EYFP mice, Ii-/- mice and the combined Ii-/- CD1d-EYFP/EYFP mice did not induce IFN-γ nor IL-4 production in WT Vα14 iNKT cells.

Discussion

A remaining question in the field of CD1d-mediated Ag presentation and Vα14 iNKT cell development concerns the intracellular endosomal trafficking routes of CD1d during Vα14 iNKT cell development. In contrast to conventional, MHC-selected CD4 and CD8 T cells, Vα14 iNKT cells require Ag presentation by both nonprofessional and professional APCs to obtain terminal maturation. Although thymic selection occurs on DP thymocytes presenting CD1d, final CD1d-mediated maturation events after their thymic emigration, by interaction with professional APCs (17, 18, 19, 20, 23). Endosomal tyrosine-based sorting motifs in the CD1d-tail and in Ii mediate endosomal localization of CD1d (7, 8, 9), which therefore could result in exposure to nonoverlapping Ags due to differential localization in non-Ii-expressing cells (i.e., DP cortical thymocytes) and Ii-expressing cells (i.e., professional APCs). In this study, we investigated the role of the CD1d-encoded endosomal sorting motif in thymic development, peripheral maturation, and activation of mature Vα14 iNKT cells.

A comparative study for the role of professional APCs and other CD1d-expressing cells in peripheral maturation of Vα14 iNKT cells is complicated, because in most mutant mice in which CD1d-mediated presentation is affected, both thymic development and peripheral maturation of Vα14 iNKT cells is hampered, resulting in thymic deletion of Vα14 iNKT cells during thymic development and inability for their study during peripheral maturation. The importance of peripheral maturation events for Vα14 iNKT cell function, however, is clear. In NOD mice, for example, Vα14 iNKT cells do develop but are unable to fully

mature, which results in a predisposition to insulitis development (24, 25). We generated a new mouse strain in which all CD1d is expressed as CD1d-EYFP fusion proteins, in which we investigated the role of CD1d endosomal sorting and Vα14 iNKT cell development and maturation.

In the homozygous CD1d-EYFP mice, a sizable fraction of Vα14 iNKT cells of 40% pass successfully through positive selection. This is in contrast with CD1d tail-deleted mice, in which Vα14 iNKT cells were decreased to ~10% (9). In CD1d-EYFP/EYFP mice, the thymic selection defect probably differs from the defect in CD1d tail-deleted mice in which CD1d access to endosomal compartments is blocked. In CD1d-EYFP/EYFP mice, CD1d-EYFP molecules do localize to endosomal compartments, albeit at a different kinetic rate. Using CD1d+/- mice, it was shown that in situations where endogenous CD1d ligand concentration are suboptimal, thymic selection of Vβ7$^+$, but not Vβ8.2$^+$, Vα14 iNKT cells is favored (33), while Vβ7$^+$ Vα14 iNKT cells are relatively diminished upon CD1d overexpression (28). The hierarchy of Vβ-chain usage (Vβ8, Vβ7, and Vβ2) is already established during positive selection and is not altered thereafter through preferential cellular expansion (21, 33). CD1d-EYFP/EYFP mice also show an overrepresentation of Vβ7 usage by Vα14 iNKT cells, suggesting that endogenous Ag presentation by CD1d-EYFP molecules is suboptimal. Endogenous Ag presentation during thymic selection, however, is not absent as in CD1d-tail deleted mice, and CD1d-EYFP/EYFP mice are still able to support development of 40% of Vα14 iNKT cells.

Maturation of Vα14 iNKT cells in both the thymus and the periphery can be monitored by acquisition of NK1.1 and CD44 (12, 16) and is CD1d-dependent (20, 23). In thymus of CD1d-EYFP/EYFP mice, acquisition of NK1.1 and CD44 was delayed, but was compensated for in full during peripheral maturation of Vα14 iNKT cells capable of seeding in the periphery. NK1.1 expression signals a maturation of the cytokine response toward high IFN-γ production (12, 16, 22). We confirm this data, as terminally matured Vα14 iNKT cells in CD1-EYFP/EYFP mice were fully capable of producing IFN-γ, but not IL-4. In humans, invariant NKT cells (characterized by Vα24/Vβ11 TCR junctions) are readily distinguished based on expression of CD4: CD4$^+$ Vα24 iNKT cells are the exclusive producers IL-4 and IL-13 upon primary stimulation, but could also produce Th1 cytokines, whereas the double negative (DN) Vα24 iNKT cells had a strict Th1 profile producing IFN-γ and TNF-α (40, 41). It is yet unclear whether in mice a similar divide of CD4$^+$ and DN Vα14 iNKT cells in production of Th1 and Th2 responses exists (37, 42, 43, 44, 45). In CD1d-EYFP/EYFP mice, it is a possibility that IL-4-producing Vα14 iNKT cells are specifically deleted during thymic selection on CD1d-EYFP molecules. In mice, CD4$^+$Vα14 iNKT cells constitute the majority of the Vα14 iNKT cells in the periphery. Both CD4$^+$ and DN Vα14 iNKT cell subsets showed comparable decreases in numbers present in CD1d-EYFP/EYFP spleen. There was no selective depletion of DN Vα14 iNKT cell subsets that could explain the absence of IL-4 production (data not shown).

CD1d-EYFP/EYFP mice exhibit a narrower mean fluorescence peak in CD1d surface expression in DP thymocytes and CD4$^+$ T cells, representing a more homogenous population of cells expressing similar surface levels of CD1d. This tighter distribution of surface CD1d-EYFP could be caused by the delay in internalization rate. Tail-deleted CD1d molecules exhibit a slower internalization rate than WT CD1d, but recycling (reappearance from intracellular compartments) is undisturbed (8). For CD1d-EYFP molecules, a slower endocytosis rate resulted in accumulation of CD1d at the cell surface in

peripheral $CD4^+$ T cells, but not for $CD4^+CD8^+$ thymocytes. Recycling assays using primary cells were unsuccessful in our hands. The rate of CD1d-EYFP recycling could possibly be faster in $CD4^+$ T cells than thymocytes, which would explain the accumulation on the surface in $CD4^+$ T cells and not in $CD4^+CD8^+$ thymocytes.

The homeostasis of Vα14 iNKT cells in the periphery is independent of the presence of CD1d in the periphery: CD1d does not affect the survival or expansion rate of iNKT cells in the periphery (23, 46). CD1d-EYFP/EYFP mice exhibit a decrease in Vα14 NKT cell numbers in spleen and liver. Because thymic output of postselection Vα14 iNKT cells is decreased in CD1d-EYFP/EYFP mice, fewer recent thymic emigrants are seeded in the periphery, which is a likely cause for the observed lower set-point for Vα14 iNKT cell homeostasis in the periphery.

Stimulation of terminally differentiated Vα14 iNKT cells by presentation of lysosomal Ag on professional APCs required unmodified CD1d and presence of Ii. Thus, while CD1d-EYFP molecules were capable of mediating peripheral maturation of Vα14 iNKT cells, they did not succeed in provoking IFN-γ and IL-4 production in Vα14 iNKT cells. Taken together, in this study, we describe a new mouse model, in which all CD1d is expressed as CD1d-EYFP fusion proteins. An unexpected phenotype of these mice clarified the role of CD1d endosomal sorting motifs in Vα14 iNKT cell selection and peripheral iNKT cell maturation, during thymic selection and by peripheral Ii-expressing professional APCs. Endosomal sorting mediated by the CD1d-intrinsic tyrosine motif is critical for thymic positive selection. In the periphery, CD1d endosomal sorting does not require the intrinsic CD1d-encoded sorting motif for terminal maturation of Vα14 iNKT cells. Activation of differentiated Vα14 iNKT cells, however, does require both the CD1d-tail encoded sorting motif, and cannot be compensated for by the Class II MHC-associated Ii.

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Disclosures

The authors have no financial conflict of interest.

Footnotes

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F.S. and M.B. designed research; F.S., M.B., and D.S. performed research; N.V. and G.B. contributed new reagents; F.S. and M.B. analyzed data; and F.S. and M.B. wrote the paper.

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[3] Abbreviations used in this paper: Ii, invariant chain; i, invariant; αGalCer, α-galactosylceramide; DP, double-positive; DC, dendritic cell; EYFP, enhanced yellow fluorescent fusion protein; Gal(α1→2)GalCer, galactosyl(α1→2) galactosylceramide; DN, double negative; WT, wild type.

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Supplemental data

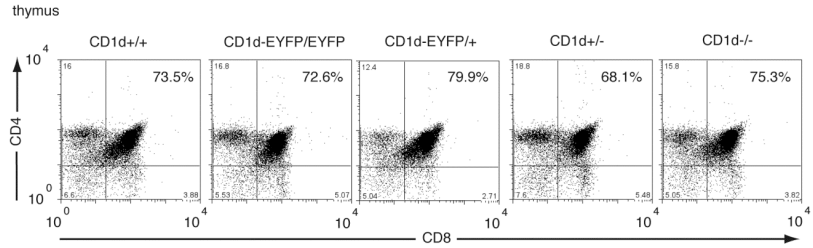

FIGURE S1. Normal development of DP thymocytes in CD1d-EYFP expressing mice. Thymocytes from 6-9 week old mice were stained with CD4 and CD8 mAbs and percentage of DP thymocytes was assessed by flow cytometry. Surface CD1d expression between CD1d+/+ and CD1d-EYFP/EYFP or CD1d-EYFP/+ is significantly different ($p<0.05$). Comparable development of DP thymocytes was found in CD1d+/+, CD1d-EYFP/EYFP, CD1d-EYFP/+, CD1d+/- and CD1d-/- mice (NS, $p>0.1$).

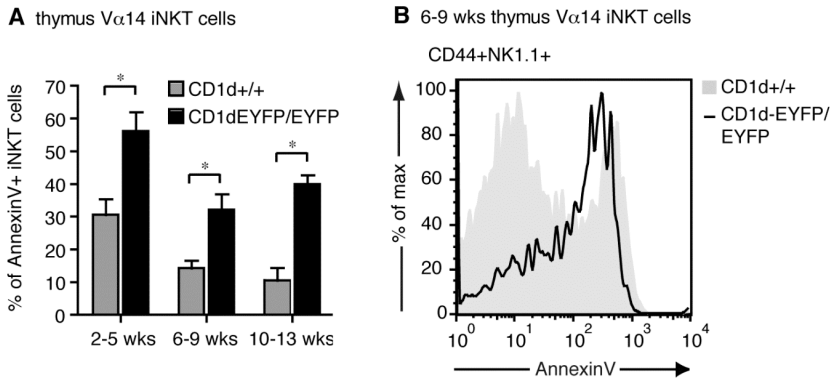

FIGURE S2. Increased apoptosis among Vα14 iNKT cells from CD1d-EYFP/EYFP mice. ***A***, Thymocytes from CD1d+/+ or CD1d-EYFP/EYFP mice were incubated for 30 minutes at 37°C to induce apoptosis. After incubation cells were incubated with Annexin V, mAbs against TCRβ, CD44, NK1.1, ‹GalCer-loaded CD1d-tetramers and analyzed by flow cytometry. Data shown are representative of at least 3 experiments. The bars represent the mean of at least triplicate values and the brackets indicate SEM. Vα14 iNKT cells from CD1d-EYFP/EYFP mice undergo significantly more apoptosis than CD1d+/+ Vα14 iNKT cells at any age (* $p<0.01$). ***B***, Representative histogram of AnnexinV staining on mature ($CD44^+NK1.1^+$) Vα14 iNKT cells from 6-9 week old mice. Mature CD1d-EYFP/EYFP Vα14 iNKT cells exhibit significantly more Annexin V staining than CD1d+/+ Vα14 iNKT cells ($p<0.05$).

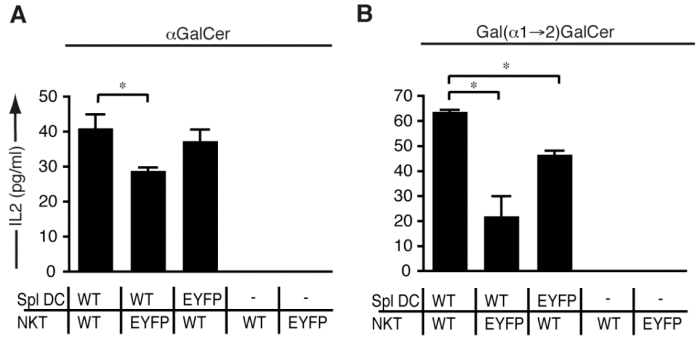

FIGURE S3. Decreased production of antigen-induced IL-2 by Vα14 iNKT cells selected on CD1d-EYFP molecules. Activation of Vα14 iNKT cells *in vitro*. Spleen DCs and liver Vα14 iNKT cells from WT (CD1d+/+) or CD1d-EYFP/EYFP mice were co-cultured in the presence of 100 ng/ml αGalCer (***A***) or 100 ng/ml Gal(α1→2)GalCer (***B***). Results displayed in bar graphs are representative of 2 experiments. Shown are the mean of triplicate values with SEM. Production of IL-2 by CD1d-EYFP/EYFP Vα14 iNKT cells was significantly reduced compared to their CD1d+/+ counterparts (* $p<0.001$). Presentation of Gal(α1→2)GalCer by CD1d-EYFP/EYFP spleen DCs resulted in significantly less IL-2 production by wild-type Vα14 iNKT cells (* $p<0.05$).

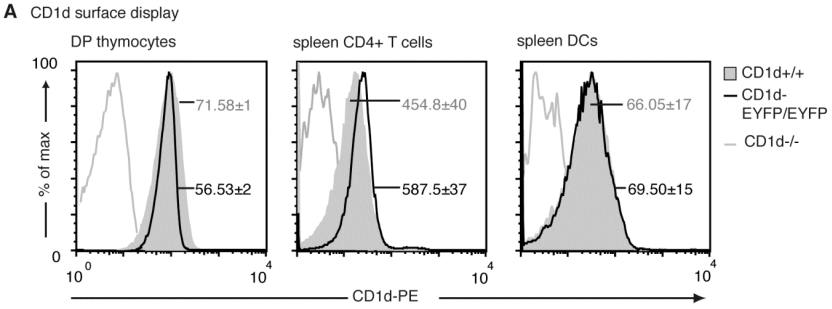

FIGURE S4A. Increased surface expression and delay in endocytosis of CD1d-EYFP fusion proteins in T cells, but not in professional APCs. ***A***, Surface expression of CD1d on DP thymocytes, spleen $CD4^+$ T cells and spleen DCs. DP thymocytes and spleen $CD4^+$ T cells, but not spleen DCs from CD1d-EYFP/EYFP mice express increased surface levels of CD1d compared to wild-type (T cells: $p<0.05$; DCs: NS, $p>0.05$). Histograms shown are representative of 3 independent experiments. Data points in the graph represent the mean of quadruplet values and brackets indicate SEM.

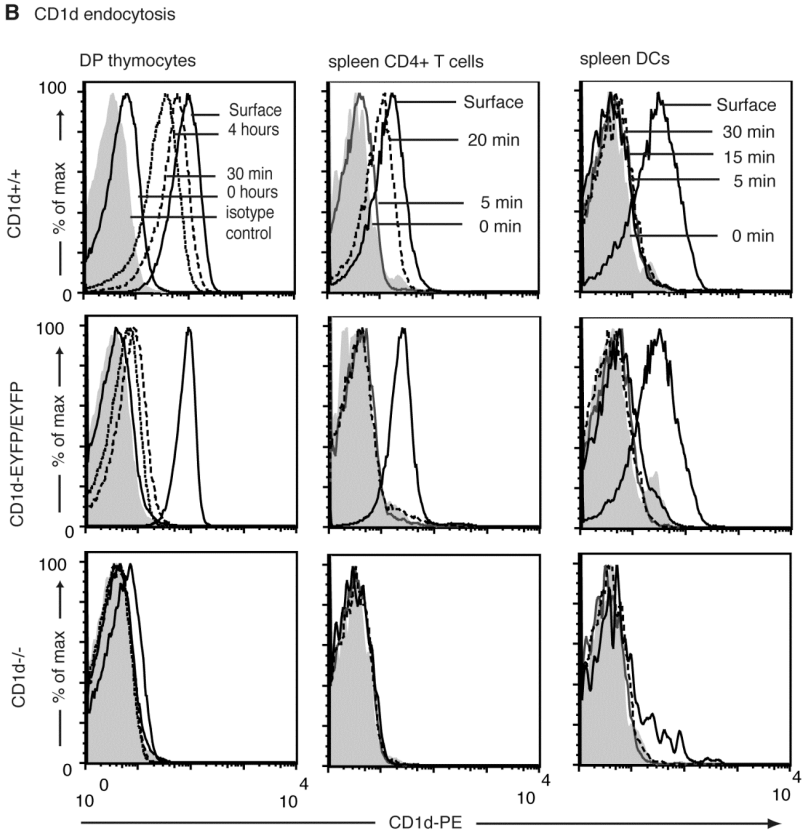

FIGURE S4B. Increased surface expression and delay in endocytosis of CD1d-EYFP fusion proteins in T cells, but not in professional APCs. ***B***, The extent of internalized CD1d bound by PE-conjugated anti-CD1d antibody in thymocytes, CD4+ T cells and DCs from CD1d+/+, CD1d-EYFP/EYFP and CD1d-/- mice was visualized by flow cytometry. CD1d-EYFP molecules on DP thymocytes exhibit a significantly longer surface retention time than wild-type CD1d molecules, demonstrated within 0.5 hours of endocytosis induction ($p<0.005$). CD1d-EYFP molecules on $CD4^+$ T cells in the periphery exhibit a significantly longer surface retention time than wild-type CD1d molecules ($p<0.05$). Professional APCs in the periphery display less CD1d endocytosis activity than T cells. No difference was found between endocytosis of CD1d-EYFP molecules and CD1d (NS, $p>0.05$). Control thymocytes from CD1d deficient mice show no CD1d staining at any of the time points. Histograms shown are representative of 3 independent experiments. Data points in the graph represent the mean of quadruplet values and brackets indicate SEM.

Chapter 4

Requirement for *M. tuberculosis* trafficking from phagosomes to lysosomes for pathogen replication and induction of immune defense.

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Abstract

Mycobacterium tuberculosis (*Mtb*) are intracellular pathogens that persist in phagosomes of myeloid cells. Pathogen survival is supported by *Mtb*-encoded factors that prevent fusion of phagosomes with lysosomal compartments, thereby avoiding acidic pH and exposure to lysosomal hydrolases and proteases. NKT cells of the immune system can be stimulated through recognition of antigenic CD1d/lipid complexes on *Mtb*-infected cells, and when activated can ameliorate disease. It is not understood how *Mtb* presence in phagosomes can yield antigenic CD1d/lipid complexes, which are thought to be generated in lysosomes. The identity of the endosomal compartment that facilitates pathogen replication is also unknown. To address these questions, we focused on the invariant chain (Ii) Class II MHC chaperone, which regulates endosome fusion and architecture, and is required for endosomal transport. *Mtb* have normal ability to infect Ii-deficient macrophages, but exhibit a significant defect in pathogen replication. Infected Ii-deficient macrophages selectively retain *Mtb* in $EEA1^+$ phagosomes and do not elicit CD1d-mediated NKT cell activation. Thus, while *Mtb* growth benefits from obstruction of endosomal transport, *Mtb* replication does require phagosomal fusion to lysosomes. Lysosomes but not phagosomes possess endosomal architecture conducive for *Mtb* replication, a process that benefits the pathogen. Lysosomal presence of *Mtb* is, however, equally pivotal for induction of CD1d-dependent NKT cell responses in the host.

Author summary

Invariant natural killer T cells (iNKT) play an important role in the early immune response against *Mycobacterium tuberculosis* (*Mtb*) by suppressing intracellular bacterial growth. Both iNKT cell development and activation involves presentation of CD1d/lipid complexes at the cell membrane of infected cells. The lipid antigens are retrieved from vesicles of the endosomal pathway called lysosomes. CD1d endosomal trafficking involves association with the invariant chain (Ii) Class II MHC chaperone. Ii also controls endosomal fusion and function. Ii-deficient mice develop iNKT cells that exhibit deficiencies in maturation and do not yield protection against *Mtb* infected macrophages. In $Ii^{-/-}$ macrophages *Mtb* are retained in EEA^+ phagosomes, inhibiting *Mtb* growth, yet, *Mtb* escape iNKT cell-mediated killing of $Ii^{-/-}$ macrophages. Ii is thus required for *Mtb* localization to a lysosomal compartment with architecture conducive for facilitation of *Mtb* replication and also for the generation of antigenic CD1d/lipid complexes. New therapeutic strategies against *Mtb* infection include adjuvant therapies targeted to infected myeloid cells and potentiation of iNKT cell responses. This study clarifies the necessity for lysosomal localization of *Mtb* for both pathogen growth and for CD1-restricted iNKT cell response against *Mtb*.

Introduction

Mycobacterium tuberculosis (*Mtb*) causes chronic tuberculosis in at least nine million people and more than 1,7 million deaths each year [1]. The long-established *M. bovis* Bacille Calmette–Guérin (BCG) vaccine against tuberculosis affords insufficient protection against *Mtb*, summarized in [2]. Thus, development of better prophylactic strategies and the need to understand the mechanisms that are critical in the protective immunity to *Mtb* have great international research priority. *Mtb* replicates primarily within host myeloid cells including macrophages (Mϕ) and the resistance to *Mtb* infection involves Mϕ activation

mediated by the immune mediators IFN-γ [3] and tumor necrosis factor-α [4]. A readily mobilized and potent source of IFN-γ and tumor necrosis factor- α is found in stimulated iNKT cells.

Mtb survive in the endosomal pathway of infected cells [5]. The endosomal requirements that support the replication of *Mtb* remain incompletely defined. Phagosomal presence of Mtb ensures continued access of *Mtb* to nutrients, although *Mtb* have also been shown to persist in lysosomes [6]. The endosomal pathway also encompasses compartments where CD1d molecules acquire their antigenic cargo. Lysosomes in particular accommodate the generation of antigenic CD1d/lipid complexes. When isolated cells are exposed to *Mtb* or *Mtb*-specific antigens, their surface CD1d expression is upregulated [7,8] and CD1d-mediated antigen presentation is induced [9,10]. *Mtb*-induced antigen presentation can activate CD1d-restricted iNKT cells, which in turn can have a protective role against *Mtb* infection [11,12]. In addition to suppressing intracellular *Mtb* growth in infected Mϕ in vitro, CD1d-restricted iNKT cells contribute to protection in whole animals, which indicates that they are physiologically activated during infection [11-13].

The intracellular trafficking of lipid-loaded CD1d molecules from the secretory pathway to the endocytic pathway involves a tyrosine-based sorting motif in the cytosolic tail of CD1d, and in professional antigen presenting cells (APCs) additionally includes interaction of CD1d with invariant chain (Ii)/ Class II MHC complexes [14-16]. In the endosomal pathway, ER-derived antigen is replaced by antigenic lipid antigen, after which antigenic CD1d/lipid complexes traffic to the cell surface [17,18]. The requirements of endosomal transport for presentation of *Mtb*-induced antigens are not fully understood.

Amongst professional APCs, *Mtb* particularly infects Mϕ and DCs. We investigated whether *Mtb* endosomal trafficking is required for intracellular *Mtb* growth and for its immunological answer being *Mtb*-induced iNKT cell responsiveness. Endosomal trafficking involves the fusion of endosomal vesicles, a process in which Ii plays a critical role [19-22]. The role of Ii in CD1d-mediated presentation is therefore two-fold: facilitating directed transport of CD1d/lipid complexes towards the endosomal pathway, and for fusion of endosomal vesicles within this pathway. We earlier established the endosomal trafficking mutant phenotypes of Mϕ deficient in Ii and in Ii-processing enzyme CatS [19]. In the absence of Ii endosomal fusion is inhibited, resulting in smaller lysosomes and altered endosomal trafficking of membrane proteins and endosomal content [20-22]. Lack of CatS causes enlarged endosomal compartments that lack multivesicular morphology [19,23]. We investigated the possible requirement for *M. tuberculosis* to traffic from phagosomes to lysosomes and for endosomal multivesicular morphology, to support pathogen replication and induce CD1d-mediated immune defense.

Results

In order to address the structural requirements of the endosomal pathway for *Mtb* survival and induction of CD1d-driven immune defense, we made use of mouse models known to exhibit either defects in endosomal fusion and resulting small endosomal compartments (Ii knock-out) or defects in endosomal degradation of Ii remnants resulting in enlarged endosomal compartments that lack multivesicular structures (CatS knockout). We first

characterized these mouse models for their ability to express CD1d and CD1d-restricted Vα14 invariant (i)NKT cells (hereafter referred to as iNKT cells).

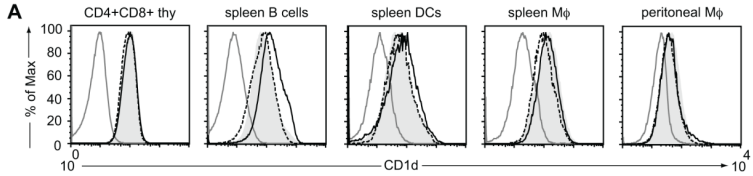
Modification of endosomal structures inhibits the maturation of iNKT cells.

We first assessed the cell surface CD1d expression level under steady-state conditions in our mutant mice. To this end, we performed flow cytometry analyses using anti-CD1d-mAb on Ii-expressing cells (i.e., B cells, macrophages, Mϕ and dendritic cells, DCs) and Ii-non-expressing cells (i.e., thymocytes). CD1d surface expression on double positive ($CD4^+CD8^+$) thymocytes of $Ii^{-/-}$ mice is similar to WT control mice, whereas CD1d surface expression on B cells in the spleen of $Ii^{-/-}$ mice is increased ($p<0.01$) (Figure 1A). Our data showing increased CD1d expression on $Ii^{-/-}$ B cells confirms published data [15], and likely reflects expansion of $CD1d^{high}$ marginal zone B cell numbers in $Ii^{-/-}$ mice [24]. CD1d surface expression on spleen DCs, steady-state spleen macrophages (Mϕ) and elicited peritoneal Mϕ of $Ii^{-/-}$ mice is similar to WT mice. The absence of cathepsin S (CatS) did not alter the surface expression of CD1d in the cell types analyzed (Figure 1A).

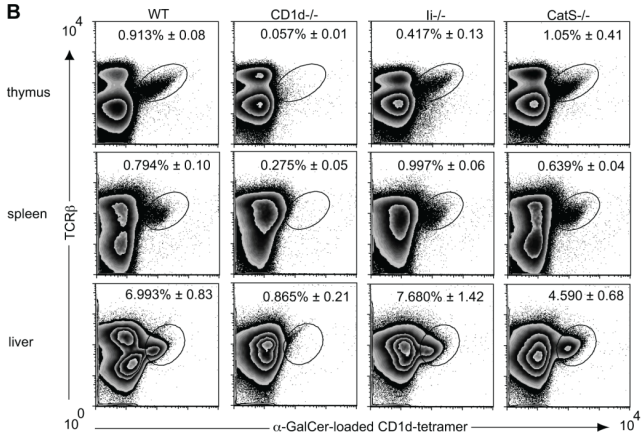
CD1d endosomal localization is required for the loading of antigenic lipids onto CD1d, and modulation of the endosomal pathway may therefore affect CD1d-driven iNKT cell development. We established the number of iNKT cells in unstimulated wild-type, Ii-/- and $CatS^{-/-}$ mice using αGalCer-loaded CD1d-tetramers. We found that lack of Ii or CatS does not alter the percentage of iNKT cells in spleen and liver (Figure 1B). However, the percentage of thymic iNKT cells in $Ii^{-/-}$ mice was reduced compared to WT (* $p<0.05$), confirming earlier published data [25,26].

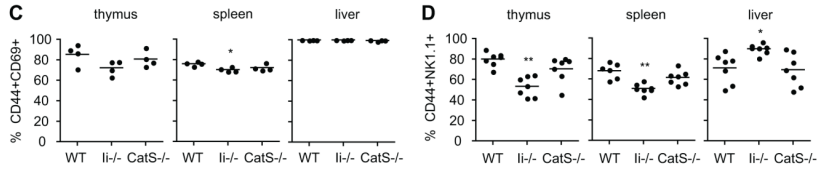
How does modification of the endosomal pathway affect maturation of CD1d-restricted iNKT cells? We stained cells from thymus, spleen and liver with αGalCer-loaded CD1d-tetramers and antibodies against TCRβ, CD44, CD69 and NK1.1 and analyzed by flow cytometry. The maturation of iNKT cells from $CatS^{-/-}$ mice, measured by expression of CD69 and NK1.1 is similar to WT (Figures 1C and 1D, respectively). iNKT cells from $Ii^{-/-}$ mice are delayed in maturation with significantly lower percentages expressing CD69 in the spleen (* $p<0.05$) and NK1.1 in the thymus and spleen (** $p<0.01$). In $Ii^{-/-}$ liver, more iNKT cells than in WT liver express a $CD44^+NK1.1^+$ phenotype, possibly reflecting local activation of liver-resident iNKT cells.

The selection of Vβ TCR domains assembled with the invariant Vα14 chain shapes the avidity of TCRs on iNKT cells for endogenous ligand/CD1d complexes, to which developing iNKT cells are exposed during thymic selection [27]. A change in Vβ usage would suggest a role for Ii and CatS-expressing professional APCs in thymic selection. We found that both $Ii^{-/-}$ and $CatS^{-/-}$ mice display significantly less Vβ7 positive iNKT cells in the thymus compared to WT mice ($p<0.01$ and $p=0.045$, respectively), but normal contribution of Vβ7 iNKT cells in spleen and liver (Figure 1E). Thus, thymic selection of iNKT cells involves the presentation of lipid/CD1d complexes by Ii and CatS-expressing professional APCs.



WT	104.8 ± 4.6	113.3 ± 4.5	139.9 ± 22.7	220.3 ± 12.59	50.1 ± 2.5
Ii-/-	99.2 ± 6.3	200.3 ± 10.2 **	152.4 ± 19.3	239.5 ± 34.2	47.2 ± 5.3
CatS-/-	105.8 ± 2.3	97.19 ± 20.3	119.8 ± 10.7	198.5 ± 23.6	44.4 ± 1.7





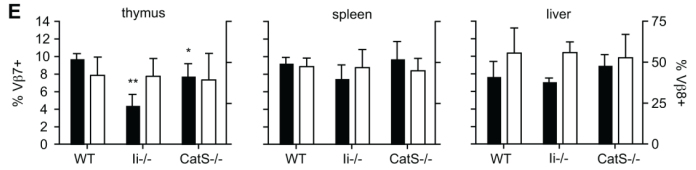


Figure 1. Defective maturation of iNKT cells in invariant chain knockout mice.
A. Normal surface levels of CD1d in $CD4^+CD8^+$ thymocytes and Mϕ of invariant chain knockout ($Ii^{-/-}$) or cathepsin S knockout ($CatS^{-/-}$) mice. Representative flow cytometry histograms of $CD4^+CD8^+$ thymocytes, $B220^+$ spleen B cells, $CD11c^+$ DCs and $F4/80^+$ Mϕ and $F4/80^+$ peritoneal Mϕ are shown for wild-type (WT, solid gray), $CD1d^{-/-}$ (gray), $Ii^{-/-}$ (black) and $CatS^{-/-}$ mice (dotted). Averages of CD1d mean fluorescent intensities (MFI) ± standard error of the mean (SEM) are summarized below corresponding histogram overlays ($4 \leq n \leq 7$). **B.** Normal percentages of iNKT cells in $CatS^{-/-}$ and $Ii^{-/-}$ mice. Representative flow cytometry dot-plots of thymus, spleen and liver are shown for different mice. iNKT cells are detected by using anti-TCRb antibody and α-galactosylceramide (αGalCer)-loaded CD1d-tetramers. Average percentages ± SEM are summarized in each dot-plot (n=6-7). **C and**

D. Biased maturation of iNKT in $Ii^{-/-}$ mice. Cells from thymus, spleen and liver from different mice were stained with αGalCer-loaded CD1d-tetramers and antibodies against TCRβ, CD44, CD69 (**C**) and NK1.1 (**D**) and analyzed by flow cytometry. Dots in the scatter-plots represent individual mice. iNKT cells from $Ii^{-/-}$ mice are delayed in maturation with significantly lower percentages expressing CD69 in the spleen (* $p<0.05$) and NK1.1 in the thymus and spleen (** $p<0.01$), but not in liver. **E.** Skewing of Vβ repertoire in $Ii^{-/-}$ mice. Cells from thymus, spleen and liver, from different mice were stained with αGalCer-loaded CD1d-tetramers and antibodies against TCRβ, Vβ7, Vβ8 and analyzed by flow cytometry. Bars represent mean percentages ± standard deviation of Vβ7 (black, left axis) and Vβ8 (white, right axis) positive iNKT cells ($5\leq n\leq 7$). $Ii^{-/-}$ (** $p<0.01$) and $CatS^{-/-}$ (* $p=0.045$) mice display significantly less Vβ7 positive iNKT cells in the thymus, but not in the periphery compared to WT mice. Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT.

Unperturbed endosomal trafficking is required for upregulation of CD1d upon M. tuberculosis infection.

We recently showed that CD1d surface expression on peritoneal Mϕ is upregulated upon infection with *Mtb* [8]. Does CD1d upregulation require unperturbed endosomal trafficking and morphology? To address this question, we investigated thioglycolate-elicited peritoneal Mϕ from WT, $Ii^{-/-}$ and $CatS^{-/-}$ mice, which we infected *in vitro* with virulent *Mtb* (H37Rv) at an MOI of 10:1 for 2 hrs. After 1 or 4 days of culture, cells were stained with a mAb against CD1d and analyzed by flow cytometry. CD1d surface expression was significantly upregulated upon infection by day 4 in WT ($p<0.01$) and less notably in $CatS^{-/-}$ ($p<0.05$) but not in $Ii^{-/-}$ Mϕ (Figure 2).

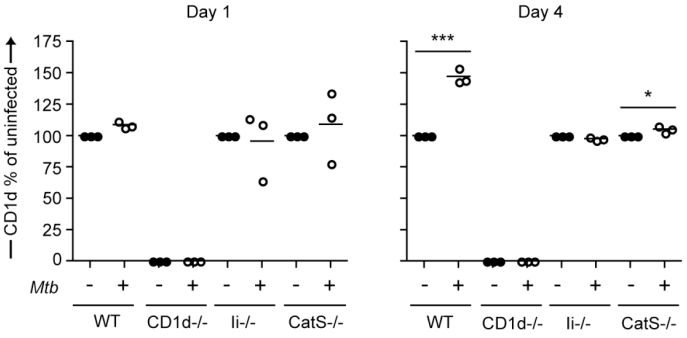


Figure 2. Ii knockout macrophages fail to upregulate CD1d upon *M. tuberculosis* infection. Uninfected or *M. tuberculosis* (*Mtb*)-infected (MOI 10:1) peritoneal Mϕ from wild-type (WT), $CD1d^{-/-}$, invariant chain knockout ($Ii^{-/-}$) and Cathepsin S knockout ($CatS^{-/-}$) mice were stained with monoclonal antibodies against F4/80 and CD1d on day 1 and day 4 post infection and analyzed by flow cytometry. Dots in the scatter-plots represent CD1d MFI of individual samples normalized to their uninfected state. Relative CD1d surface expression was significantly upregulated upon infection by day 4 in WT ($p<0.01$) and $CatS^{-/-}$ ($p<0.05$) but not in $Ii^{-/-}$ $F4/80^{+}$ Mϕ. Statistical testing was done using a one-way ANOVA with Bonferroni's post-test comparing each *Mtb*-infected experimental group to the corresponding uninfected group.

Reduced pathogen growth and absence of iNKT cell activation by M. tuberculosis infected Ii-deficient macrophages.

As CD1d upregulation is absent in Ii-deficient Mϕ, other endosomal processes that occur upon *Mtb* infection may be affected as well in these cells. We next investigated the requirement of an intact endosomal pathway for bacterial replication and induction of CD1d-dependent iNKT cell activation. We purified thioglycolate elicited peritoneal Mϕ from WT, CD1d$^{-/-}$, Ii$^{-/-}$ and CatS$^{-/-}$ mice and infected these with *Mtb* (H37Rv). Bacterial growth in the *Mtb*-infected Mϕ was assessed by determining colony-forming units (CFU) on day 1 and day 4 post-infection. We retrieved fewer live *Mtb* from Ii$^{-/-}$ Mϕ, suggesting that Ii-/- Mϕ have a reduced ability to support *Mtb* replication or can better control *Mtb* growth by day 4 compared to WT (*** $p<0.001$) (Figure 3A).

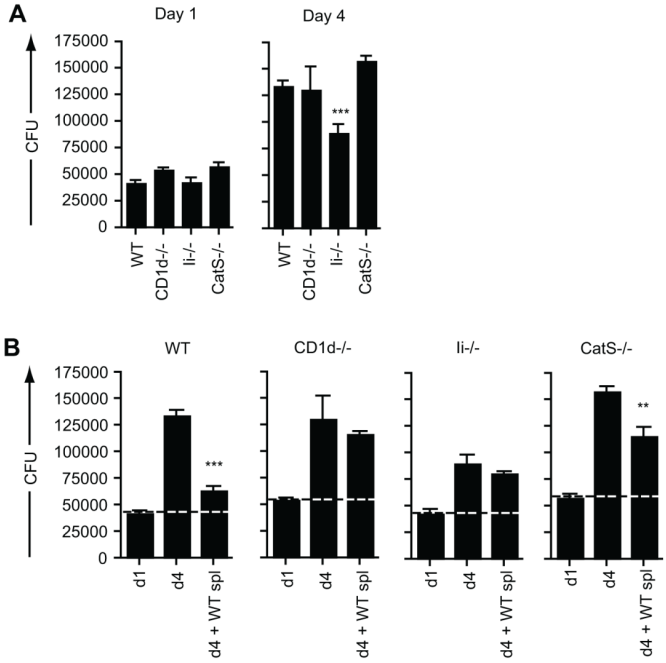


Figure 3. Naïve WT splenocytes fail to suppress bacterial replication when co-cultured with *M. tuberculosis*-infected invariant chain knockout macrophages.
Mϕ from WT, CD1d$^{-/-}$, Ii$^{-/-}$ and cathepsin S knockout (CatS$^{-/-}$) mice were infected with H37Rv and cultured in the absence (A) or presence (B) of splenocytes (spl) from uninfected WT mice. Bacterial growth in the *Mtb*-infected Mϕ was assessed by determining colony-forming units (CFU) on day 1 and day 4 post-infection. On day 1, uninfected WT splenocytes were added to the *Mtb*-infected Mϕ, and CFU were determined 72 hrs later on day 4. **A.** Expansion of *Mtb* in Ii$^{-/-}$ Mϕ by day 4 is significantly reduced compared to WT (*** $p<0.001$). Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT. Shown is one representative experiment out of three performed. Bars represent the mean ± SEM of replicate cultures (n = 3-6, ** $p<0.01$, *** $p<0.001$). **B.** CD1d and invariant chain (Ii) expression by macrophages (Mϕ) infected with *M. tuberculosis* (*Mtb*) is required for the suppression of bacterial

replication. Co-culture of WT splenocytes with *Mtb*-infected WT (*** $p<0.001$) or $CatS^{-/-}$ (** $p<0.01$) Mϕ, but not with $CD1d^{-/-}$ or $Ii^{-/-}$ Mϕ led to a significant CFU reduction. Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to the corresponding *Mtb*-infected Mϕ cultured alone for 4 days (d4). Shown is one representative experiment out of three. Bars represent the mean ± SEM of replicate cultures (n = 3-6, ** $p<0.01$, *** $p<0.001$).

To assess the induction of iNKT cell activation by infected Mϕ, we added naïve WT splenocytes as a source of iNKT cells at 24 hours post-infection, as described [11]. CFU were determined 72 hrs later on day 4. We first confirmed that co-culture of WT splenocytes with *Mtb*-infected WT Mϕ results in a significant CFU reduction (*** $p<0.001$), which was CD1d-dependent as WT splenocytes did not restrict pathogen replication in infected $CD1d^{-/-}$ Mϕ (Figure 3B) [11]. WT splenocytes were able to suppress intracellular growth of *Mtb* in $CatS^{-/-}$ Mϕ, indicating that CatS is not required for activation of CD1d-restricted iNKT cell-mediated control (*** $p<0.001$) (Figure 3B). In contrast, and in line with their failure to up-regulate cell surface CD1d expression upon *Mtb* infection (Figure 2), *Mtb* growth in infected $Ii^{-/-}$ Mϕ could not be suppressed by WT splenocytes (Figure 3B).

Unperturbed endosomal trafficking is required for development of iNKT cells that suppress M. tuberculosis replication.

CD1d-restricted iNKT cells are able to suppress intracellular *Mtb* replication (figure 3B and [11]). We asked whether iNKT cells that developed in the absence of Ii or CatS can suppress the replication of *Mtb* in Mϕ. Confirming previous findings [11] we found that splenocytes from $CD1d^{-/-}$ mice, which lack iNKT cells, are unable to restrict intracellular *Mtb* replication compared to WT splenocytes (Figure 4). Addition of $CatS^{-/-}$ splenocytes to *Mtb*-infected WT Mϕ also led to a significant reduction in bacterial CFU after 4 days (*** $p<0.001$), similar to the addition of WT splenocytes (*** $p<0.001$) (Figure 4). However, $Ii^{-/-}$ splenocytes did not suppress intracellular *Mtb* replication in infected WT Mϕ, since significantly more *Mtb* CFU were retrieved (Figure 4).

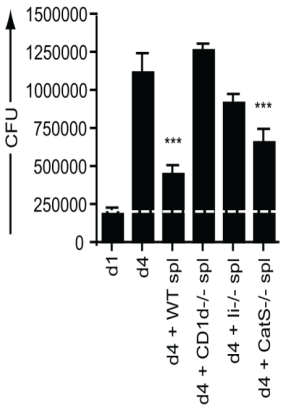


Figure 4. Naïve splenocytes from invariant chain knockout mice are unable to suppress *M. tuberculosis* replication in wild-type macrophages.
Splenocytes (spl) from $CD1d^{-/-}$ or invariant chain knockout ($Ii^{-/-}$) mice are unable to limit *M. tuberculosis* (*Mtb*) replication. *Mtb*-infected WT Mϕ were cultured alone or in the presence of splenocytes from uninfected WT, $CD1d^{-/-}$, $Ii^{-/-}$ or $CatS^{-/-}$ mice. Splenocytes were added to the *Mtb*-infected WT Mϕ 1 day post-infection. Growth of *Mtb* in the infected Mϕ was assessed by determining CFU on day 1 and day 4 post-infection. Addition of WT or $CatS^{-/-}$ splenocytes (*** $p<0.001$) but not $CD1d^{-/-}$ or $Ii^{-/-}$ splenocytes led to a significant reduction in bacterial CFU after 4 days. Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT *Mtb*-infected Mϕ cultured alone for 4 days (d4). Shown is one representative experiment out of three. Bars represent the mean ± SEM of replicate cultures (n = 3-6, *** $p<0.001$).

Phagosomal retention of M. tuberculosis in Ii-deficient macrophages.

In $Ii^{-/-}$ Mϕ, the replication of *Mtb* bacteria was significantly inhibited (figure 3A) and CD1d-mediated antigen presentation was also blocked (figure 3B). We propose that in Mϕ where endosomal trafficking is perturbed, such as in the absence of Ii, *Mtb*growth is inhibited, suggesting that endosomal trafficking is required to support *Mtb* replication. To test this hypothesis, we made use of mCherry-*Mtb* and immunostaining of Mϕ using antibody to LAMP1, which visualizes late endosomal/lysosomal compartments. In WT Mϕ most mCherry-*Mtb* is found in late endosomal/lysosomal compartments within 24 hours, characterized by expression of LAMP1 (Figure 5A). However, in $Ii^{-/-}$ Mϕ, we found that many mCherry-*Mtb* are still present in LAMP1-negative compartments at 24 hours. What is the identity of the endosomal compartment where *Mtb* accumulate in $Ii^{-/-}$ Mϕ? To this end, we performed stainings for EEA1 (early endosomal autoantigen 1) as an early endosome marker as well as a marker for phagosome maturation [28,29] and scored for presence of mCherry-*Mtb* in EEA1-positive compartments. There was no significant difference in the localization of mCherry-*Mtb* in $CD1d^{-/-}$ or $CatS^{-/-}$ Mϕ compared to WT. However, in $Ii^{-/-}$ Mϕ significantly more mCherry-*Mtb* are present in $EEA1^{+}$ phagosomes (** $p<0.01$) (Figure 5B). Thus, we show that the trafficking of *Mtb* to late endosomal/lysosomal compartments requires an unperturbed endosomal pathway that involves Ii. We also establish a requirement for normal endosomal trafficking to $LAMP^{+}$ compartments for *Mtb* replication and the ability to stimulate CD1d-restricted iNKT cells.

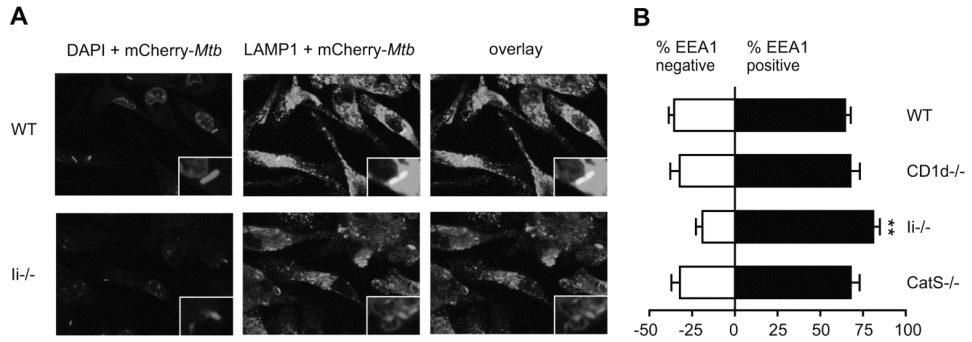


Figure 5. Altered intracellular localization of *M. tuberculosis* in invariant chain knockout macrophages.
A. *M. tuberculosis* (*Mtb*) in invariant chain knockout ($Ii^{-/-}$) Mϕ is mainly localized outside LAMP1 positive compartments. Mϕ from wild-type (WT) or $Ii^{-/-}$ mice were infected with mCherry-*Mtb* (red) and 1 day post-infection were stained with DAPI (blue) to mark the nucleus and an antibody against the lysosomal marker LAMP1 (green). The insets are 300% magnifications of representative mCherry-*Mtb* from the larger images. **B.** *Mtb* in $Ii^{-/-}$ Mϕ is retained in phagosomes/early endosomes. Mϕ from WT, $CD1d^{-/-}$, $Ii^{-/-}$ and cathepsin S knockout ($CatS^{-/-}$) mice were infected with mCherry-*Mtb*. 1 day post-infection the infected Mϕ were stained with antibodies against the early endosome marker EEA1. Sections ($39\leq n\leq 173$ in at least 3 separate scoring sets) were scored for overlap of mCherry-*Mtb* with EEA1 positive compartments. Bars represent average scoring percentages ± standard error of the mean (SEM) of mCherry-*Mtb* found in EEA1 positive (black bars) or EEA1 negative compartments (white bars). Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT.

Discussion

Mtb survive in Mϕ intracellular vesicles by evading lysosomal killing. This is accomplished by preventing the fusion of *Mtb*-harboring phagosomes with lysosomes possessing a proteolytic milieu damaging to live *Mtb* [5,28,30-35]. Both invariant chain (Ii), the Class II major histocompatibility complex (MHC) chaperone and the lysosomal cysteine protease cathepsin S (CatS) are known to control endosomal architecture and function [19-23]. Our results with Mϕ that lack Ii imply that *Mtb* intracellular growth requires unperturbed endosomal trafficking. *Mtb* replication does not require multivesicular structures within endosomal compartments, as CatS-deficient Mϕ support normal *Mtb* replication. Localization of *Mtb* to lysosomes is inhibited in Mϕ that lack Ii and results in deficiencies in the induction of CD1d-restricted iNKT cell responses from the host.

One of the events occurring during *Mtb* infection is the upregulation of CD1d at the surface of APC [7,8]. *Mtb*-induced upregulation of CD1d on Mϕ requires the presence of Ii and to a lesser extent CatS (Figure 2). In addition, *Mtb*-infected $Ii^{-/-}$ Mϕ are unable to activate iNKT cells, a crucial step in the suppression of bacterial growth (Figure 3B). Ii is necessary for sorting of CD1d from the ER towards the endo-lysosomal pathway, and plays a critical role in the presentation of endosome-derived antigen by CD1d molecules *in vivo* [15]. Together our results suggest that CD1d molecules that are upregulated during *Mtb* infection contain antigenic lipids that are derived from endosomal compartments rather than from the ER/TGN/secretory pathway. While host iNKT cells are responsive to *Mtb*-induced lipid-CD1d complexes generated in Ii-proficient Mϕ, it remains elusive whether the lipid cargo is from self or *Mtb* origin.
CD1d-restricted iNKT cell activation is important to immunity against *Mtb* infection [11]. Despite the presence of normal percentages of iNKT cells in $Ii^{-/-}$ spleen (Figure 1), splenocytes from $Ii^{-/-}$ mice were unable to suppress *Mtb* growth in wild-type Mϕ (Figure 4), which may involve a difference in iNKT cell specificity (supported by a demonstrated bias against Vβ7 usage) and/or arrested maturation (Figures 1C-E). CatS seemed dispensable for the education of *Mtb*-responsive iNKT cells since $CatS^{-/-}$ iNKT cells were still capable to control *Mtb* growth in infected wild-type Mf (Figure 4). CD1d molecules interact with Ii for trafficking towards and within the endosomal pathway [15,16]. Incompletely cleaved Class II MHC-Ii complexes in APCs of $CatS^{-/-}$ mice accumulate in enlarged lysosomes thereby inhibiting the Class II MHC-mediated presentation of certain peptide antigens [23,36-38]. A corresponding defect in CD1d-mediated presentation of lipid antigens may exist in $CatS^{-/-}$ APCs. However, we did not find any evidence for this possibility.

Although *Mtb* evolved multiple strategies to survive in phagosomes [5,28,30-35] eventually the mycobacterium needs to replicate and spread to other cells after the host-cell has succumbed [6]. Earlier studies suggested that *Mycobacterium* species may translocate from the endosomal pathway into the cytosol [39-43]. We focused our studies on the endosomal pathway. Our new data shows that *Mtb* growth requires endosomal trafficking, which we propose may involve transport of *Mtb* growth factors (i.e., nutrients), or localization of *Mtb* to compartments harboring co-factors or an membrane architecture that facilitate *Mtb* replication. Ii molecules on different vesicles can promote endosome docking and fusion and thereby control endosomal traffic of membrane proteins, endosomal content and lysosomal size [19-22]. Reduced *Mtb* growth and increased co-localization of *Mtb* in EEA1 positive compartments therefore imply that in $Ii^{-/-}$ Mϕ *Mtb* is subject to phagosome

maturation, although endosomal trafficking is blocked. We propose that the absence of Ii prevents fusion of matured (EEA1 positive) phagosomes with lysosomes (LAMP1 positive), thereby denying *Mtb* access to late endosomal/lysosomal compartments for optimal replication. Preventing *Mtb* access to the late endosomes/lysosomes [9] might have promoted host-cell survival. However we did not find any difference in cell survival at day 4 of *Mtb* infection between any of the mutant and wild-type Mϕ (our unpublished data). Taken all this together, the lack of CD1d-antigen presentation in *Mtb*-infected $Ii^{-/-}$ Mϕ (Figure 3B) could be the result from altered CD1d intracellular trafficking and/or *Mtb*-antigen not localizing to the lysosomes where CD1d antigen loading occurs.

We did not find a major effect of the absence of CatS, and corresponding lack of multivesicular membrane structures and enlarged structures on endosomal trafficking of *Mtb*: intracellular localization of *Mtb* in $CatS^{-/-}$ Mϕ in EEA1- and LAMP- compartments was comparable to WT (our unpublished data). We interpret this as normal *Mtb* replication, corroborated by normal bacteria replication in $CatS^{-/-}$ Mϕ.

Taken together, our findings elucidate the dependence of *Mtb* on endosomal fusion and lysosomal presence, for optimal pathogen survival. We believe our data shows that the lysosome is pivotal for induction of *Mtb*-directed CD1-restricted iNKT cell responses. New mycobacteria vaccine strategies may therefore aim at targeting the lysosomal presence of *Mtb*.

Materials and Methods

Mice

Six- to eight- week old mice, all on C57Bl/6 background were used throughout the study. Wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). CD1d-knockout ($CD1d^{-/-}$) mice [44], Ii-knockout ($Ii^{-/-}$) mice [45] and Cathepsin S knockout ($CatS^{-/-}$) mice [38] were described previously. All mice were bred and maintained in a barrier facility and studies were performed according to institutional guidelines for animal use and care.

Antibodies and reagents

CD1d:PBS-57 (αGalCer analog) tetramers were kindly provided by the National Institute of Allergy and Infectious Disease MHC Tetramer Core facility, Atlanta. Antibodies used were rat anti-mouse CD1d (1B1, Biolegend), rat anti-mouse CD4 (RM4-5, BD Pharmingen), rat anti-mouse CD8 (53-6.7, BD Pharmingen), Armenian Hamster anti-mouse CD11c (HL3, BD Pharmingen), rat anti-mouse CD44 (IM7, eBioscience), Armenian hamster anti-mouse CD69 (H1.2F3, Biolegend), rat anti-mouse B220 (RA3-6B2, Biolegend), rat anti-mouse F4/80, NK1.1 (PK136, Biolegend), rat anti-mouse Vβ7 (TR310, BD Pharmingen), rat anti-mouse Vβ8 (KJ16, Serotec), Armenian Hamster anti-mouse TCRβ (H57-597, Biolegend), rat anti-mouse LAMP1 (eBioscience), polyclonal rabbit anti-mouse EEA1 (CalBiochem). Secondary antibodies used for immunofluorescence were Alexa 647 Fluor-conjugated goat anti-rabbit IgG and Alexa 488 Fluor-conjugated chicken anti-rat IgG (Molecular Probes).

Peritoneal Mϕ and in vitro culture

Four days after intraperitoneal injection of sterile 3% thioglycolate medium, elicited peritoneal cells were harvested by intraperitoneal lavage [8]. Mϕ were purified by positive selection with CD11b-microbeads (Miltenyi Biotech). The purified cells were >95% $F4/80^+$ $CD11b^+$, as determined by flow cytometry (data not shown). Purified Mϕ (5×10^5) were seeded into 24-well plates in complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (HyClone), penicillin/streptomycin, L-glutamine, sodium-pyruvate, 2-ME, nonessential amino acids, essential amino acids, and HEPES buffer (all from Gibco).

Bacteria and in vitro infections

This study made use of virulent *Mtb* (H37Rv), which for certain experiments expressed the fluorescent protein mCherry. The red fluorescent protein mCherry was expressed in *Mtb* H37Rv as follows. The gene encoding mCherry, which was used with permission from Roger Tsien, HHMI, was cloned into the episomal mycobacterial expression vector, pKA1, a derivative of pMV762. mCherry was constitutively expressed under the control of the hsp60 promoter and expression was verified by microscopy. Virulent *Mtb* (H37Rv) was grown to mid-log phase in Middlebrook 7H9 medium containing 10% albumin/dextrose/catalase enrichment (BD Biosciences). Bacteria were opsonized for 5 min using RPMI 1640 medium with 2% human serum (Gemini Bio-Products), 10% FBS, and 0.05% Tween 80 and then washed twice with complete medium without antibiotics. Bacteria were passed through a 5-µm syringe filter (Millipore), counted in a Petroff-Hausser chamber and added to enriched Mϕ at a multiplicity of infection (MOI) of 10:1. The length on infection was 2 hrs for all experiments. After infection unbound *Mtb* was washed away twice with complete RPMI medium without antibiotics. Infected Mϕ were cultured overnight before the addition of splenocytes (see below). The next day all wells were washed twice with RPMI medium without antibiotics.

Splenocytes as a source of iNKT cells

Spleens from the different mice were aseptically removed and mechanically homogenized with a 3-ml syringe plunger. Erythrocytes were lysed with RBC lysis buffer (1 mM KHCO3, 0.15 M NaCl and 0.1 mM sodium-EDTA, pH 7.3). Cells were washed and their viability was determined using trypan blue. Splenocytes were resuspended in complete RPMI 1640 medium without antibiotics and 2.5×10^6 splenocytes/well were added to cultures of infected Mϕ in 24-well plates.

CFU determination

Bacterial growth was quantified 24 hrs after Mϕ infection (day 1) and 72 hr after co-culture with or without naïve splenocytes (day 4). After removing the culture supernatant, cells were lysed by adding distilled water containing 1% Triton X-100 for 5 min. 10-fold dilutions were made in PBS containing 0.02% Tween-80 and plated on Middlebrook 7H11 agar plates. The number of colonies was counted 3 weeks after incubation at 37°C in a humidified CO_2 atmosphere.

Flow cytometry

Single-cell suspensions were resuspended in FACS buffer (2% FCS, 2 mM sodium azide in PBS) and incubated for 20 min with the relevant conjugated mAb on ice in the presence of anti-FcγRII/III Ab (2.4G2). Cells infected with *Mtb* were fixed with 1% paraformaldehyde overnight before analysis At least 100,000 cells for each panel were analyzed on a FACScanto (Becton Dickinson) using FlowJo software.

Immunofluorescence

Peritoneal Mϕ (described above) were allowed to adhere to cover slips in 24-well plates. 30 min after adherence cells were infected with mCherry-linked *Mtb* (H37Rv) at a MOI of 10:1 for 2 hours as described above. After 2 hrs unbound *Mtb* was washed away twice and cells were cultured in complete RPMI medium without phenol or antibiotics. 24 hrs after infection cells were washed twice with PBS and fixed with PBS containing 4% paraformaldehyde and 10% sucrose for 1 hr. After 4 washes with PBS cells were permeabilized in RPMI medium containing anti-FcγRII/III Ab (2.4G2), 10% goat serum (GIBCO-BRL) and 0.05% saponin (Invitrogen) for 15 min. Primary and secondary antibody solutions were prepared in the same medium. Cells were incubated overnight at 4°C with primary antibody solutions and washed twice before adding secondary antibody solutions for 30 min. After secondary antibody staining cells were washed twice with PBS and once with distilled water. Coverslips with the stained cells were mounted in ProLong® Gold antifade reagent with DAPI (Invitrogen) and analyzed with a 100x oil objective on a scanning microscope (Olympus). The merged images were analyzed for the co-localization of mCherry-*Mtb* with $LAMP1^+$ or $EEA1^+$ compartments.

Statistical analysis

One-way ANOVA was used to compare different groups to their control. A p-value of at least 0.05 was considered statistically significant. Analysis was performed using Prism 4.0 for Mac software (GraphPad Software, Inc.).

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Chapter 5

Critical role for CD1d-restricted invariant NKT Cells in stimulating intrahepatic CD8 T-cell responses to liver antigen

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Abstract

Background & Aims: Vα14 invariant natural killer T cells (iNKT) are localized in peripheral tissues such as the liver rather than lymphoid tissues. Therefore, their role in modulating the stimulation of conventional, major histocompatibility complex (MHC)-restricted T-cell responses has remained ambiguous. We here describe a role for Vα14 iNKT cells in modulating conventional T-cell responses to antigen expressed in liver, using transferrin-mOVA (Tf-mOVA) mice. *Methods:* Naïve ovalbumin-specific class I MHC-restricted T cells (OTI) were adoptively transferred into Tf-mOVA mice in the presence or absence of iNKT-cell agonist α-galactosylceramide, after which OTI T-cell priming, antigen-specific cytokine production, cytotoxic killing ability, and liver damage were analyzed. *Results:* Transfer of OTI cells resulted in robust intrahepatic, antigen-specific proliferation of T cells. OTI T cells were activated in liver, and antigen-specific effector function was stimulated by coactivation of Vα14 iNKT cells using α-galactosylceramide. This stimulation was absent in $CD1d^{-/-}$ Tf-mOVA mice, which lack Vα14 iNKT cells, and was prevented when interferon-γ and tumor necrosis factor-α production by Vα14 iNKT cells was blocked. *Conclusions:* CD1d-restricted Vα14 iNKT cells stimulate intrahepatic CD8 T-cell effector responses to antigen expressed in liver. Our findings elucidate a previously unknown intervention point for targeted immunotherapy to autoimmune and possibly infectious liver diseases.

Abbreviations

αGalCer, α-galactosylceramide; APC, antigen-presenting cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; OVA, ovalbumin; iNKT cell, invariant natural killer T cell; Tf, transferrin

Background & Aims

The initiation of adaptive immune responses generally entails the antigen-specific stimulation of naïve major histocompatibility complex (MHC)-restricted T cells by professional antigen-presenting cells (APCs), a process that requires prolonged contact between T cells and APCs. CD1d-restricted invariant natural killer T (iNKT) cells, in contrast, can secrete cytokines within only few hours of activation. In mice, the majority of CD1d-restricted iNKT cells are relatively invariant and are collectively called Vα14 iNKT cells.1 Human iNKT cells mostly express a Vα24-Jα18 rearranged T-cell receptor (TCR) α chain with a Vβ11-containing TCRβ chain [2].

Vα14 iNKT cells in mice (referred to as iNKT cells hereafter) are highly enriched in liver, in which they can represent up to 30% of lymphocytes [3,4] and are likely to play an important role in local immune responses. Their activation, through stimulation with the glycolipid α-galactosylceramide (αGalCer), can enhance T-cell responses to soluble protein antigens by directly interacting with dendritic cells (DCs) in a CD40-dependent manner [5-7] and can enhance antitumor cytotoxicity of NK cells and CD8+ T cells to inhibit metastasis to the liver [8,9]. Intrahepatic iNKT cells moreover play a protective role in the clearance of multiple pathogens such as picornavirus [10], herpes simplex virus [11], and *Pseudomonas earuginosa* [12]. In transgenic mouse models of hepatitis B virus (HBV) replication, Vα14 iNKT cells contribute to interferon (IFN)-α/β- and IFN-γ-dependent

inhibition of viral replication [13], and livers of hepatitis C virus (HCV) patients and patients with primary biliary cirrhosis contain large numbers of iNKT cells [14-16]. These findings support the hypothesis that iNKT cells contribute to the pathogenesis of these liver diseases. Still, the liver is an organ with paradoxical immunologic properties, functioning either as a site amendable to effective immune responses or to generation of tolerance, as appropriate [17]. We asked how intrahepatic Vα14 iNKT cells are involved in antigen-specific hepatitis whereby antigen-specific conventional CD8 T cells instigate liver injury. We here describe the role of iNKT cells in stimulating CD8 T-cell responses to antigen restricted to the liver, using a recently developed mouse model, transferrin (Tf)-mOVA mice.

Materials and Methods

Mice

Tf-mOVA,18 CD1d-deficient [10,19] and OTI [20] RAG1-deficient mice were maintained in a rodent barrier facility at Harvard Medical School. CD1d-deficient Tf-mOVA mice were generated by crossbreeding. All mice were on C57Bl/6J background and were used at 6–8 weeks of age. Studies were performed according to institutional guidelines for animal use and care. Wild-type control mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Adoptive Transfer of OTI T Cells

OTI T cells were extracted from lymph nodes and spleen of $RAG1^{-/-}$ OTI mice (>95% purity). OTI T cells (4×10^6) were injected via tail vein, with or without 100 ng αGalCer. αGalCer was synthesized in the laboratory of Dr Gurdyal S. Besra. For proliferation measurements (at 36 hours post-transfer), OTI T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen-Molecular Probes, Carlsbad, CA).

Blocking of Lymph Node Homing or Exit

For blocking of lymph node homing, OTI T cells were preincubated with neutralizing antibody to CD62L (clone MEL14) or isotype control IgG2a (clone RTK2758, 50 μg at 4°C, 30 minutes; both Biolegend, San Diego, CA). At the time of transfer and 24 hours later, 50 μg/mouse of anti-CD62L or isotype control antibody were injected intraperitoneally (IP) into splenectomized Tf-mOVA mice. For blocking of lymph node exit, Tf-mOVA mice received FTY 720 at the time of T-cell adoptive transfer and 24 hours posttransfer (Cayman Chemical, Ann Arbor, MI; 1 mg/kg) intraperitoneally. Littermate controls received phosphate-buffered saline (PBS).

Splenectomy

Mice were anesthetized before surgery, and hair was removed at the surgical site. A small incision was made on the left side of the mouse and through the peritoneal cavity. The spleen was ligated with 5-0 Ethilon (Ethicon, Inc, Sommerville, NJ) and excised from the opening. The abdominal wall incision was closed with staples (Precise; 3M Healthcare, St Paul, MN). Experiments were performed at least 7 days postsurgery.

Isolation of Mononuclear Cell Populations From Liver, Spleen, and Lymph Nodes

After perfusion (2–5 mL PBS), the liver was removed and pressed through 70-μm mesh (BD Pharmingen, Frankin Lakes, NJ). After washing in PBS, mononuclear cells were resuspended in 33% Percoll (GE Health Care, Piscataway, NJ), overlayed onto 80% Percoll and centrifuged (20 minutes at 900g). Mononuclear cells were collected from the interface. After erythrocyte lysis using NH4Cl, cells were washed twice in PBS and resuspended in PBS supplemented with 10% fetal calf serum (FCS) for flow cytometry or in RPMI medium supplemented with 5% FCS and Pen/Strep (Invitrogen-Gibco, Carlsbad, CA) for culture. Mononuclear cells were also isolated from spleen and from inguinal, axillary, mesenteric, and portal lymph nodes. Cells were prepared for flow cytometry or culture as described below.

In vitro OTI T-Cell Stimulation With DCs

DCs used in T-cell stimulation assays were purified from mesenteric and skin-draining lymph nodes (inguinal and axillary), from spleen and from liver based on CD11c expression using MACS (Miltenyi Biotec Inc, Auburn, CA). One × 105 DC were incubated with 1 × 105 naïve OTI T cells in round-bottom 96-wells plates (BD Pharmingen), in RPMI supplemented with 5% FCS and PenStrep, for 24 hours at 37°C. As a positive control, crystalline ovalbumin (OVA) was added (10 μg/mL; Sigma-Aldrich, St Louis, MO). As a negative control, 1 × 105 OTI T cells were incubated without DC. After 24 hours, CD25 and CD69 expression by OTI T cells was determined by flow cytometry.

Flow Cytometry and Intracellular Cytokine Staining

T cells, iNKT cells, and DC were preincubated 5 minutes on ice with Fc-block 1:200 (clone 2.4G2; BD Pharmingen). Immunostaining was performed for 15 minutes on ice with fluorophore-conjugated antibodies (Ab) against CD11c (clone HL3; BD Biosciences), CD8 (clone 53-6.7; Abcam Inc., Boston, MA), Thy1.1 (clone OX7; Antigenix America Inc., Huntington Station, NY), CD69 (clone H1.2F3; BD Pharmingen), CD25 (clone PC61; Biolegend), NK1.1 (clone PK136; eBiosciences, San Diego, CA), TCRβ (clone H57; eBiosciences), Vα2 (clone B20.1; BD Pharmingen), and PBS57-loaded CD1d-tetramers (NIH Tetramer Facility, Atlanta, GA). Flow cytometry analysis was performed on a FACS-Canto flow cytometer (BD Pharmingen).

For intracellular cytokine staining, OTI T cells were restimulated with SIINFEKL peptide in 96- or 48-well plates (10 μmol/L, 5 hours, 37°C) in the presence of brefeldin A (10 μg/mL; Sigma-Aldrich). Whole OVA was used as negative control (10 μg/mL; Sigma-Aldrich). For intracellular cytokine staining of iNKT cells, mice were injected intravenously (IV) with brefeldin A (250 ng/mouse) 15 minutes prior to αGalCer IV injections (100 ng/mouse). Liver iNKT cells were isolated at indicated times. Cells were stained for surface markers, fixed, permeabilized, and stained intracellular using a Cytofix/Cytoperm kit (BD Pharmingen) and fluorophore-conjugated Ab to IFN-γ (clone XMG1.2; eBiosciences), interleukin (IL)-4 (clone 11B11; BD Pharmingen), or TNF-α (clone MP6 XT22; eBiosciences).

To assay for apoptosis, OTI T cells were extracted at days 3 and 5 after adoptive transfer in Tf-mOVA mice, and nonparenchymal cells were isolated from the liver and stained for CD8 (clone 5H10; Invitrogen-Caltag Laboratories, Carlsbad, CA) and Vα2 (clone B20.1; BD Pharmingen). For detection of caspase 3, cells were resuspended at 1×10^6 cells/mL in

RPMI containing 10% FCS, 1% Pen/Strep, and 50 μmol/L β-mercaptoethanol, and 5 × 105 cells were incubated with 1 μL RED-DEVD-FMK (Red Caspase-3 Staining Kit, PromoKine, Heidelberg, Germany) for 45 minutes at 37°C.

Neutralization of IFN-γ and TNF-α Using Blocking Antibodies

Anti-IFN-γ (clone XMG1.2), anti-TNF-α (clone XT3.11), or rat IgG1 control antibody (200 μg/mL each) were IP injected at days −1, 0, 2, and 4 of IV αGalCer and OTI T-cell injection. At day 5, serum ALT levels were measured, liver pathology was scored, and OTI T cells were isolated from the liver. After restimulation with SIINFEKL, intracellular cytokines were measured.

Serum Alanine Aminotransferase Determination

Blood was collected from the tail of mice, centrifuged at 5000g for 10 minutes, and the serum was extracted. Serum alanine aminotransferase (ALT) activity was determined in serum using ALT (SGPT) reagent set (colorimetreic method by Teco Diagnostics, Anaheim, CA) according to manufacturer's instructions.

Histology

Liver tissue was fixed in 4% formalin (pH 7.0) for at least 24 hours and embedded in paraffin. Two-micrometer-thick paraffin sections were stained with H&E and were blindly scored by a pathologist (R. T. Bronson).

Statistical Analysis

IFN-γ production by OTI T cells, their absolute cell counts, serum ALT levels, and the frequencies of iNKT and NK cells were compared using the Mann-Whitney U test (Windows; Microsoft Corp, Redmond, WA; SPSS, Chicago, IL).

Results

CD8 T-Cell Priming in Tf-mOVA Mice

The self-antigen Tf-mOVA is expressed by hepatocytes and may be presented as peptide/class I MHC complexes to CD8 T lymphocytes in antigen-draining lymphoid tissues [21]. To determine in which anatomic location(s) OTI T-cell priming occurs in Tf-mOVA mice, we extracted DCs from different tissues and performed DC/T-cell cocultures (Figure 1A). DC from Tf-mOVA, but not wild-type mice, activated OTI T cells, as measured by CD69 and CD25 expression at 24 hours (Figure 1A). DC from Tf-mOVA mesenteric lymph nodes and spleen induced most OTI cell activation, whereas DC from liver and skin-draining lymph nodes (axillary and inguinal combined) induced more modest OTI T-cell activation.

To establish whether OTI T cells are activated in naïve Tf-mOVA mice *in vivo*, we adoptively transferred CFSE-labeled OTI T cells and analyzed their anatomic location and proliferation status at 36 hours posttransfer (Figure 1B). In the Tf-mOVA mice by far, most

proliferating OTI T cells were recovered from the liver, and some were detected in the spleen.

CD8 T-Cell Priming Occurs in the Liver When Lymph Node Homing or Exit Is Blocked

L-selectin (CD62L) expression of lymphocytes is critical for their homing to peripheral lymph nodes, through binding to endothelial sulfated carbohydrate ligands in the high endothelial venules [22]. Injection of neutralizing antibody to L-selectin, MEL14, blocks lymph node homing [22], whereas spleen-directed migration is L-selectin independent. To investigate where CD8 T-cell priming occurs, we transferred CFSE-labeled OTI T cells to splenectomized Tf-mOVA mice in which lymph node homing is blocked using MEL14. At 36 hours posttransfer, most OTI T cells (>96%) were recovered from livers regardless of antibody treatment (Figure 2A). Additionally, we made use of the immune-modulator FTY720, which induces the migration of lymphocytes into secondary lymphoid tissues and blocks their egress into efferent lymphatics [23]. Again, the majority of OTI T cells was recovered from livers of Tf-mOVA mice (Figure 2B, data are shown for 6 mice/group). A fraction of the cells (30%) had proliferated in the spleen, but only very few OTI T cells were present in lymph nodes. Thus, even though *in vitro* DC derived from lymph nodes of Tf-mOVA mice are potent OTI T-cell activators, *in vivo* OTI T cells are primarily primed in the liver.

Activation of iNKT Cells by αGalCer Does Not Potentiate Proliferation of Antigen-Specific CD8 T Cells

We hypothesized that rapid secretion of cytokines by iNKT cells may stimulate intrahepatic MHC-restricted T-cell responses. We therefore injected naïve CFSE-labeled OTI T cells into Tf-mOVA mice in the presence or absence of 1 single, low dose of αGalCer (100 ng/mouse). Vα14 iNKT-cell activation by αGalCer treatment did not significantly affect the intrahepatic proliferation rate of OTI T cells nor did it affect OTI proliferation in the lymph nodes or spleen (Figure 3)

Activation of iNKT Cells by αGalCer Enhances Effector Function of Liver-Resident OVA-Specific CD8 T Cells

Proliferation of T cells does not necessarily imply acquisition of effector cell capabilities. We therefore determined whether αGalCer injection potentiates the production of IFN-γ by liver-resident OTI T cells (Figure 4A). Coinjecting αGalCer with OTI T cells increased the frequency of liver-resident IFN-γ-producing OTI T cells from 5.1% (3.8%–13.5%) to 15.3% (6.5%–26.6%; P = .017). The potentiation of OTI T-cell function was mediated by iNKT cells because αGalCer did not promote IFN-γ production by OTI T cells in $CD1d^{-/-}$ Tf-mOVA mice that lack iNKT cells: 6.5% (2.1%–11.7%; P > .05) (Figure 4B). Restimulation of OTI T cells with whole OVA (10 μg) as a negative control induced background IFN-γ levels.

To investigate further whether iNKT-cell activation facilitates intrahepatic effector function of OTI T cells in Tf-mOVA mice, *in vivo* cytolysis assays were performed. Coinjecting αGalCer with OTI T cells caused a profound increase in specific lysis of SIINFEKL-pulsed splenocytes in the liver (Figure 4C).

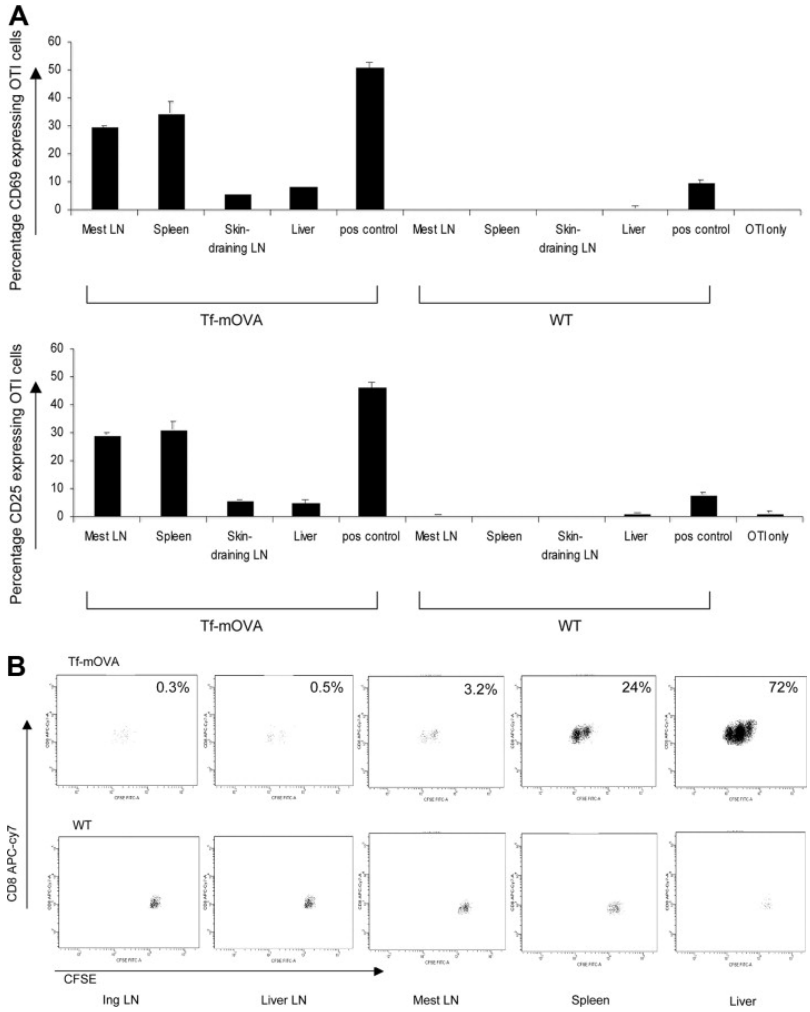

FIGURE 1. Activation of OTI T cells in a mouse expressing mOVA as liver-antigen. (***A***) Analysis of OTI T cell activation *in vitro*. OTI T cells were cultured with DCs from Tf-mOVA mice or wild-type mice and were analyzed for activation markers at 24 hours. Tf-mOVA DCs but not wild-type DCs from liver, spleen, and lymph nodes were able to activate OTI T cells in the absence of added OVA antigen, as measured by up-regulation of CD69 and CD25. Data shown of a representative experiment of 3 experiments using 4 mice per experiment. (***B***) Analysis of OTI T-cell activation *in vivo*. CFSE-labeled OTI T cells were transferred into wild-type or Tf-mOVA mice (4×10^6 T cells/mouse) and analyzed for OTI T-cell homing and proliferation in lymph nodes, spleen, and liver at 36 hours. In the Tf-mOVA mice (top panels), most proliferating OTI T cells were recovered from liver, whereas few OTI T cells were found in mesenteric (Mest LN) inguinal (Ing LN), and the liver-draining portal lymph node (Liver LN). Numbers depicted in the upper right corner of each dot plot reflect the percentage of OTI T cells present of OTI T cells recovered from all compartments combined. In WT mice (bottom panels), OTI T cells did not proliferate and were mostly recovered from the lymph nodes. Data shown are from a representative experiment of 5 experiments performed with 4 mice each.

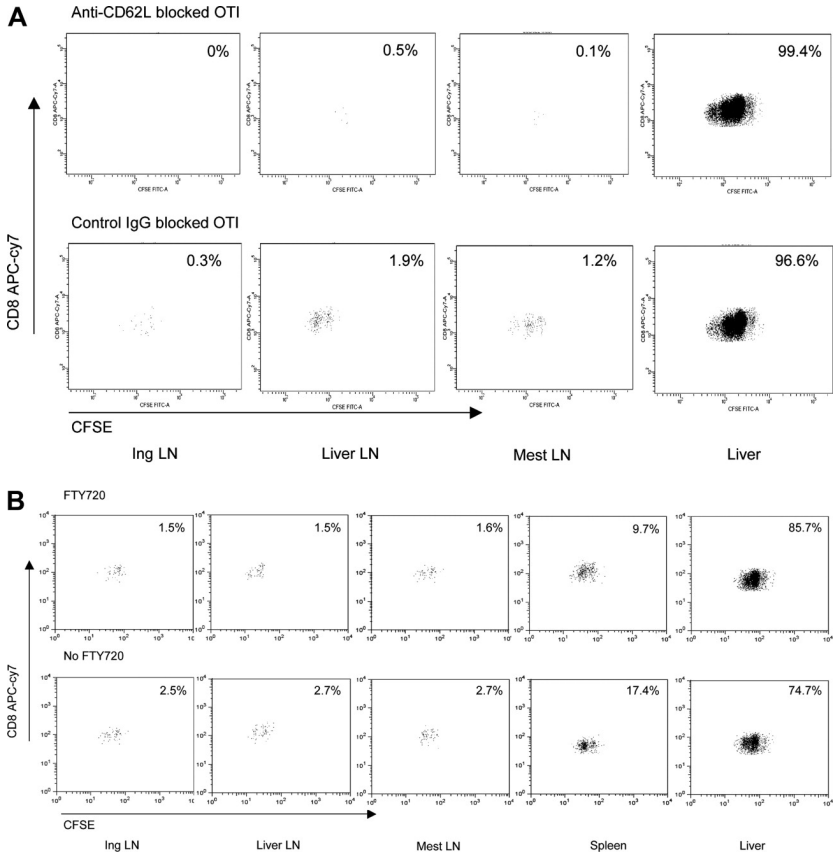

FIGURE 2. CD8 T-cell priming is unaffected when lymph node homing or exit is blocked. (***A***) To block lymph node homing of T cells, splenectomized Tf-mOVA mice were treated with MEL14 antibody (anti-CD62L) or isotype control antibody (control IgG) and injected with 4×10^6 CFSE-labeled OTI T cells that were preincubated with MEL14 or control antibody. The percentage in the upper right corner of each dot plot reflects the fraction of OTI T cells present in liver, inguinal (Ing LN) and mesenteric (Mest LN) lymph nodes, and the liver-draining portal lymph node (Liver LN) of the total OTI T cells recovered. Data shown are from a representative experiment of 3 experiments with 4 mice each. (***B***) To block lymph node exit of T cells, Tf-mOVA mice were treated with FTY720 and injected with 4×10^6 CFSE-labeled OTI T cells. The percentage in the upper right corner of each dot plot reflects the fraction of OTI T cells present in each organ, as described above. Data shown are from a representative experiment of 3 experiments with 4 mice each. A control experiment showing the functionality of the FTY720 reagent is shown in Supplemental Data ***S1***.

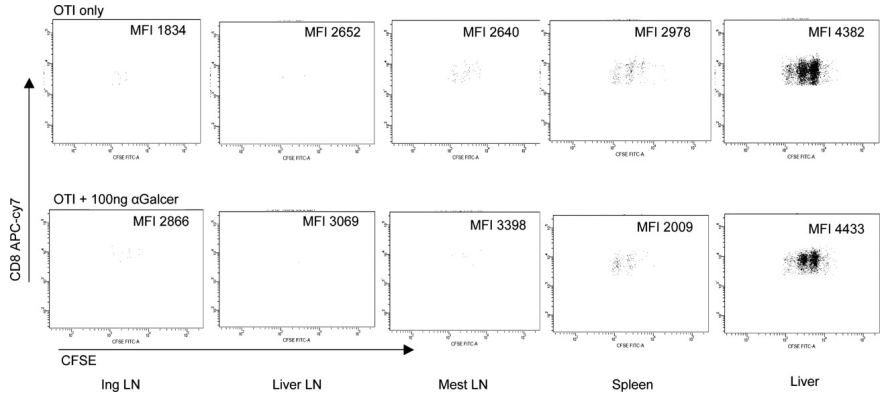

FIGURE 3. OTI T-cell proliferation in Tf-mOVA mice is unaffected by activation of iNKT cells using αGalCer. CFSE-labeled OTI T cells were injected into Tf-mOVA mice in the presence (top panels) or absence (bottom panels) of αGalCer. The number in the top right corner of each dot plot reflects the CFSE mean fluorescence intensity (MFI) of OTI T cells recovered from the organs indicated below each plot at 36 hours after transfer. Data shown are from a representative experiment of 5 experiments with 4 mice each.

The activation of iNKT cells using αGalCer induces leukocyte recruitment to the liver, including NK cells, T cells, and iNKT cells [8,24]. Treatment of Tf-mOVA mice with αGalCer indeed induced a profound increase of intrahepatic leukocytes, and a large fraction consisted of NK cells and iNKT cells (Figure 5A). However, αGalCer treatment did not result in a significant increase in intrahepatic OTI T cells: coinjection of αGalCer changed the median OTI T-cell number in Tf-mOVA livers from 1.8×10^6 to 1.6×10^6 (day 5 post-transfer). From αGalCer-coinjected CD1d$^{-/-}$ Tf-mOVA mice, we retrieved 1.4×10^6 intrahepatic OTI T cells ($P > .05$) (Figure 5B). Does αGalCer treatment induce apoptosis of recruited OTI cells? Only a small fraction of OTI T cells in livers of mice that did not receive αGalCer was undergoing apoptosis, which was only slightly increased after αGalCer treatment. Apoptotic OTI T cells were characterized by Vα2 costaining for active caspase 3 or the probe DEVD-FMK (Figure 5C). Taken together, in Tf-mOVA mice, iNKT-cell activation by αGalCer does not stimulate proliferation or liver-directed migration of antigen-specific CD8 T cells. Instead, iNKT-cell activation facilitates the intrahepatic effector function of OTI T cells.

Activation of iNKT Cells May Promote Hepatitis Induced by OTI T Cells

To determine whether iNKT-cell activation promotes liver damage in Tf-mOVA mice, we analyzed serum levels of ALT at 5 days post-OTI transfer in the presence or absence of αGalCer (Figure 6A). Transfer of OTI T cells induced mild hepatitis (median ALT level, 37.5 IU/mL). Injection of αGalCer alone caused a modest increase of serum ALT levels in Tf-mOVA and WT mice, which is most likely due to nonspecific liver damage after activation of iNKT cells [25]. Coinjection of both factors in Tf-mOVA mice increased the median serum ALT level (57 IU/L; $P > .05$). Furthermore, severe hepatitis (>5× upper limit of normal) only occurred in Tf-mOVA mice that received both OTI and αGalCer. Analysis of liver histology revealed that activation of OTI T cells by transfer into Tf-mOVA mice

induced severe hepatitis by day 5, regardless of αGalCer presence (Figure 6B). Treatment with αGalCer in the absence of OTI T cells caused only mild inflammation.

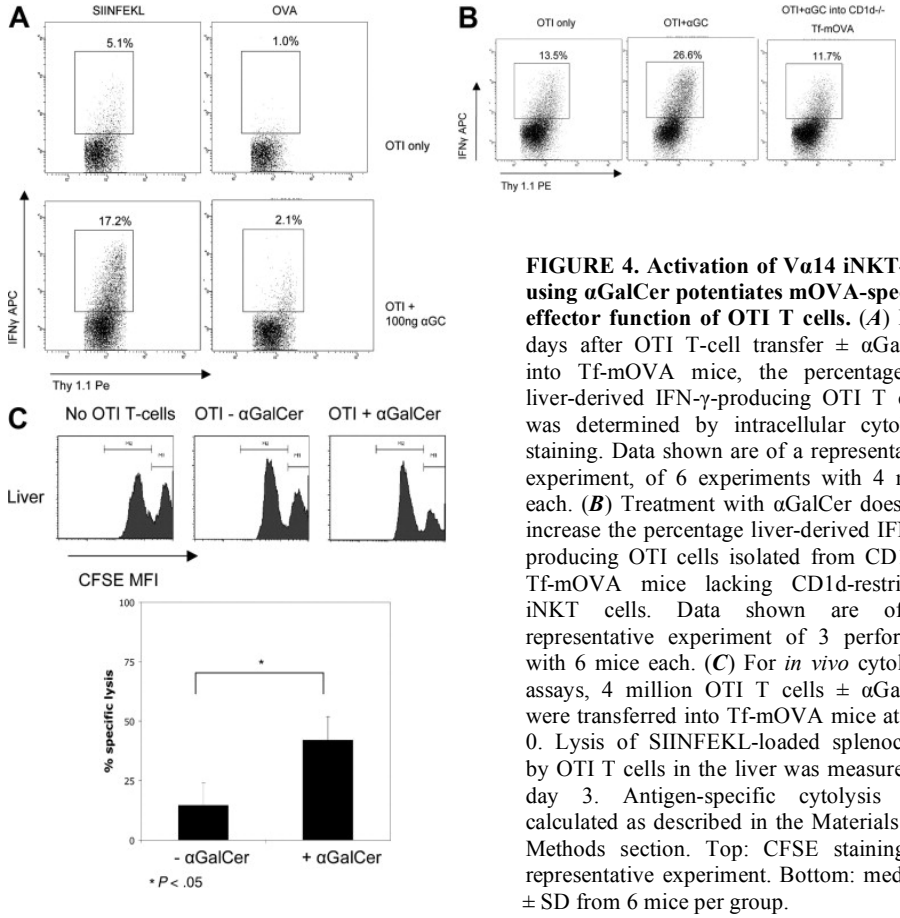

FIGURE 4. Activation of Vα14 iNKT-cell using αGalCer potentiates mOVA-specific effector function of OTI T cells. (***A***) Five days after OTI T-cell transfer ± αGalCer into Tf-mOVA mice, the percentage of liver-derived IFN-γ-producing OTI T cells was determined by intracellular cytokine staining. Data shown are of a representative experiment, of 6 experiments with 4 mice each. (***B***) Treatment with αGalCer does not increase the percentage liver-derived IFN-γ-producing OTI cells isolated from $CD1d^{-/-}$ Tf-mOVA mice lacking CD1d-restricted iNKT cells. Data shown are of 1 representative experiment of 3 performed with 6 mice each. (***C***) For *in vivo* cytolysis assays, 4 million OTI T cells ± αGalCer were transferred into Tf-mOVA mice at day 0. Lysis of SIINFEKL-loaded splenocytes by OTI T cells in the liver was measured at day 3. Antigen-specific cytolysis was calculated as described in the Materials and Methods section. Top: CFSE staining of representative experiment. Bottom: medians ± SD from 6 mice per group.

Neutralization of TNF-α and IFN-γ Function Inhibits αGalCer-Mediated Potentiation of Intrahepatic Antigen-Specific CD8 T Cells

Treatment with αGalCer induces iNKT cells to secrete TNF-α and IFN-γ [26,27] Indeed, shortly after αGalCer injection, intrahepatic iNKT cells produced TNF-α and IFN-γ (Figure 7A). Is potentiation of intrahepatic OTI T-cell function by αGalCer injection mediated by TNF-α and IFN-γ? Antibody-mediated neutralization of both cytokines significantly reduced the frequency of intrahepatic IFN-γ-producing OTI T cells compared with levels observed using isotype control Ab (Figure 7B). Interestingly, only anti-TNF-α Ab and not

anti-IFN-γ Ab pretreatment could prevent liver damage after transfer of OTI T cells + αGalCer as measured by increased serum ALT levels (Figure 7B) and liver damage (Figure 7C).

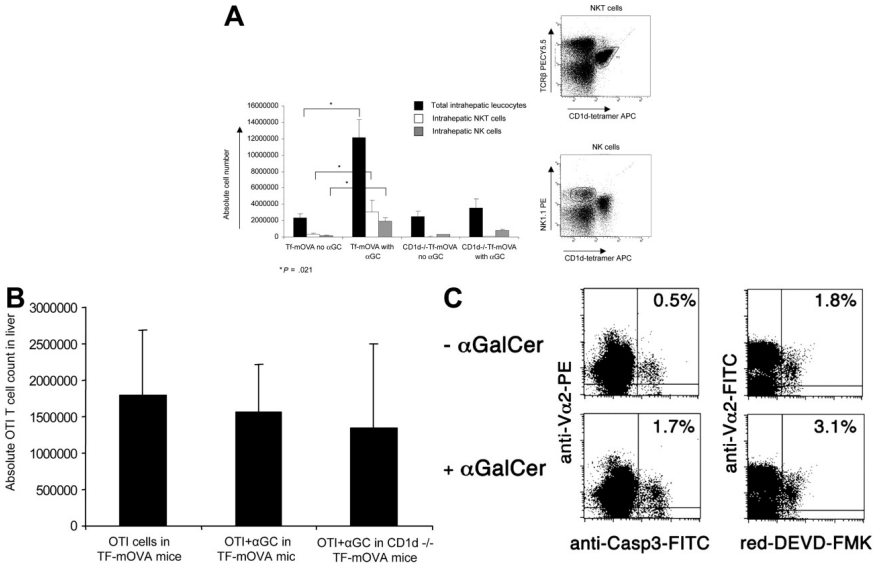

FIGURE 5. iNKT-cell-mediated increase in leukocyte numbers in livers of Tf-mOVA mice after transfer of OTI T cells ± αGalCer. (***A***) α-GalCer was injected in Tf-mOVA and CD1d$^{-/-}$ Tf-mOVA mice, and, 5 days later, the absolute numbers of total lymphocytes, Vα14 iNKT cells (TCRβ+ and CD1d-tetramer+; gated population in top right panel), and NK cells (NK1.1+ and CD1d-tetramer−; gated population in bottom right panel) in the liver were compared with their counterparts in the livers of mice that had not received αGalCer. Data shown are medians ± SD of 4 mice per group. α-GalCer treatment induced a significant influx of Vα14 iNKT cells and NK cells (P = .021), which was absent in CD1d$^{-/-}$ Tf-mOVA mice. (***B***) OTI cells ± αGalCer were transferred into Tf-mOVA or CD1d$^{-/-}$ Tf-mOVA mice, and, 5 days later, their absolute intrahepatic cell counts were determined. Shown are medians ± SD of 6 mice per group. (***C***) OTI cells ± αGalCer were transferred into Tf-mOVA mice, and, at day 3, the frequencies of intrahepatic apoptotic OTI cells were determined by analyzing caspase-3 activity. OTI cells were defined as CD8+ Vα2+ cells, and the dot plots show representative data of 3 experiments, after gating on the CD8+ cell population. At day 5 after transfer of OTI T cells in the presence or absence of αGalCer, caspase-3 activity was minimal and no significant difference was observed (data not shown).

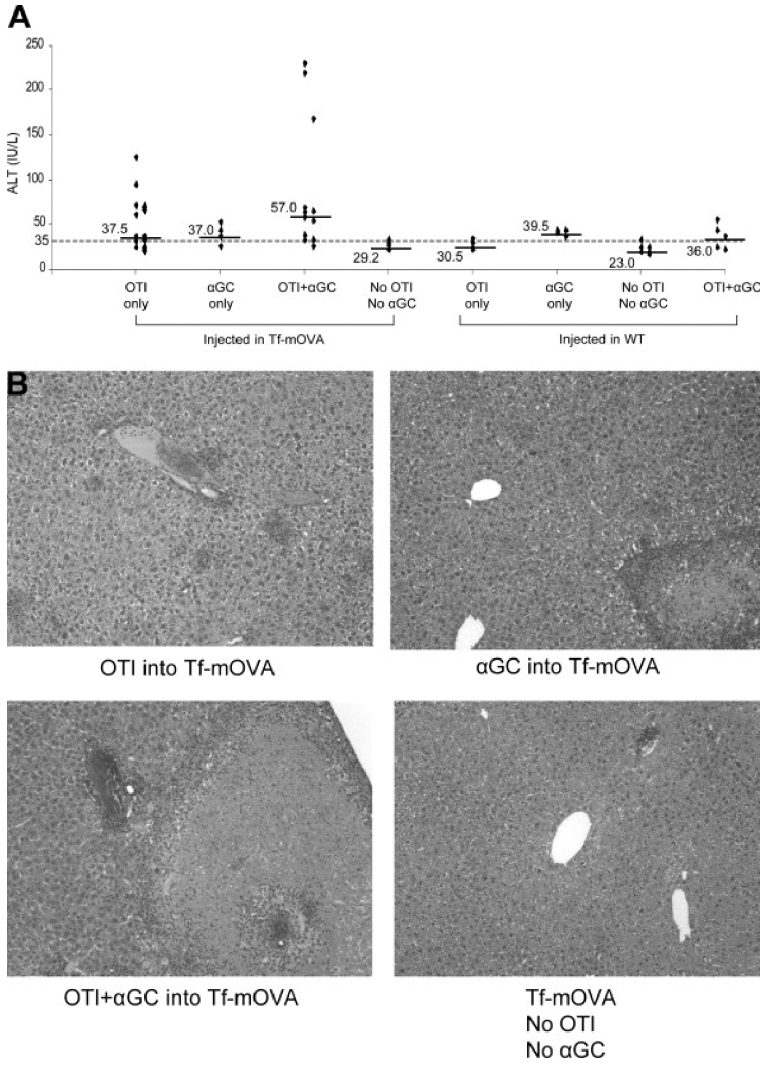

FIGURE 6. Analysis of liver damage induced by transfer of OTI T cells in Tf-mOVA mice. (***A***) Analysis of serum ALT levels 5 days after the transfer of 4×10^6 OTI T cells in the presence or absence of αGalCer in Tf-mOVA and wild-type mice. The dotted line represents the upper limit of normal (35 IU/L). The dots represent ALT levels of individual mice, and the small lines with the corresponding numbers represent the median ALT level of a population. (***B***) Analysis of liver histology by H&E staining at 5 days posttransfer of OTI cells ± αGalCer. The photographs shown are representative images of liver tissue obtained from 3 mice per group. In Tf-mOVA mice that received OTI T cells ± αGalCer, hepatitis was induced that involved mixed inflammatory cell infiltrates especially in the portal areas. αGalCer without OTI cells caused intrahepatic infarction but only mild inflammation. In Tf-mOVA mice that received neither OTI cells nor αGalCer, no hepatitis or infarction was observed.

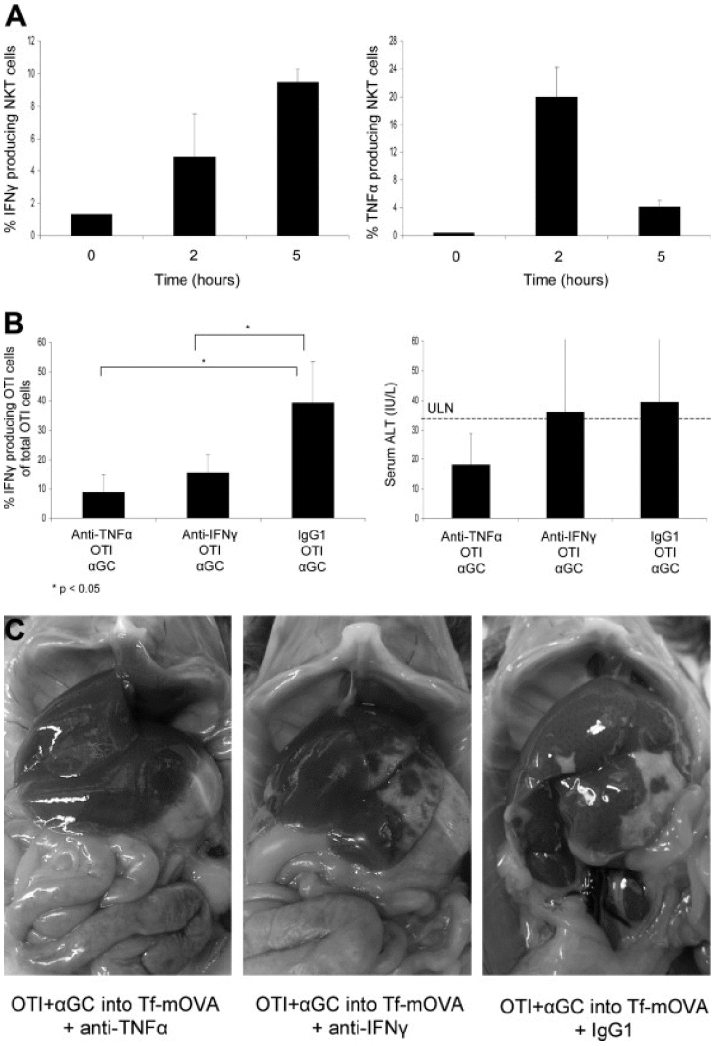

FIGURE 7. Pretreatment with neutralizing antibodies to TNF-α and IFN-γ inhibits αGalCer-mediated potentiation of effector function of liver-resident OTI T cells. (***A***) Wild-type mice were treated with αGalCer, and liver-derived Vα14 iNKT cells were analyzed for production of TNF-α and IFN-γ by intracellular cytokine staining at 2 and 5 hours. Shown are medians ± SD of 10 mice per group. (***B***) Left panel. Tf-mOVA mice were pretreated with anti-TNF-α, anti-IFN-γ, and isotype control Ab prior to transfer of OTI cells + αGalCer, and, 5 days later, the percentage of liver-derived IFN-γ-producing OTI T cells was determined by intracellular cytokine staining. Shown are medians ± SD of 4 mice per group of 1 representative experiment of 2 performed. Right panel. Additionally, in these mice, serum ALT levels were determined. (ULN, upper limit of normal). (***C***) Livers of mice were scored blindly by 2 individuals for the appearance of liver damage, as visible macroscopically. Shown are representative images of mice treated with anti-TNF-α, anti-IFN-γ, and isotype control Ab, as indicated (6 mice for each group).

Discussion

We here describe the role of Vα14 iNKT cells in stimulating intrahepatic antigen-specific CD8 T-cell responses using a recently established mouse model that allows for study of intrahepatic immune responses *in vivo* [18]. The presence of the antigen on Tf-mOVA hepatocytes led to retention of antigen-specific CD8 T cells in the liver. These intrahepatic CD8 T cells were fully functional because they produced IFN-γ after brief restimulation with SIINFEKL, for induction of hepatitis *in vivo*. Using a different transgenic mouse model in which alloantigen was present within both liver and lymph nodes, it was recently suggested that the site of primary T-cell activation determines their functional faith: CD8 T cells activated in the periphery were able to cause liver injury, whereas intrahepatically activated T cells exhibited defective function [28]. As recently suggested by Derkow et al in their study describing the Tf-mOVA model [18], we here show that, whereas Tf-mOVA antigen is only expressed in hepatocytes [18], DCs in lymphoid organs presented this antigen to OTI T cells (Figure 1A). However, when lymph node homing and exit were blocked, the large majority of transferred CD8 T cells was recovered from livers. Thus, even though the antigen is present in the periphery, in Tf-mOVA mice, OVA-specific CD8 T cells are most likely primed in the liver, and this is in accordance with previous studies [28-30].

Several studies have investigated the role of iNKT cells in intrahepatic immunity. αGalCer-activated iNKT cells can cause extensive liver damage [25] and inhibited tumor metastasis to the liver [8,31], which involved the activation of tumor nonspecific CD8 T cells [8,31]. Very little was known, however, about whether iNKT cells mediate antigen-specific hepatitis whereby antigen-specific conventional T cells instigate liver injury. Here, we demonstrate that activation of CD1d-restricted iNKT cells with αGalCer facilitates the effector function of intrahepatic antigen-specific CD8 T cells. Activation of iNKT cells tripled the frequency of intrahepatic IFN-γ-producing OTI T cells and increased their cytolytic capacity, contributing to liver damage.

αGalCer-mediated iNKT-cell activation did not appear to increase intrahepatic effector function of OTI T cells by promoting their proliferation or influx from the periphery. Neither could we demonstrate a switch in polarization phenotype of OTI T cells as measured by IL-4 production: regardless of αGalCer coinjection, only very few (<1%) intrahepatic OTI T cells produced IL-4 (data not shown). Instead, iNKT-cell activation influences the cytolytic ability and IFN-γ production of intrahepatic mOVA-specific CD8 T cells primed in the liver. Because αGalCer did not promote mOVA-specific IFN-γ production in $CD1d^{-/-}$ mOVA mice, its enhancement of OTI T-cell effector function is CD1d dependent.

Surface expression of CD1d in liver is detected on hepatocytes [32], DCs [32], Kupffer cells [33] and Ito cells [34]. Display by DCs of both CD1d/αGalCer and specific peptide-loaded class I MHC molecules can trigger iNKT cells to enhance T-cell responses to soluble antigen [6]. Kupffer cells and hepatic stellate cells are additional candidate cells that may mediate the CD8 T-cell stimulation we report here because these cells are potent Vα14 iNKT-cell activators [33,34].

We show that αGalCer induces iNKT cells to produce TNF-α and IFN-γ. The production of these cytokines by iNKT cells can be crucial in mediating antimetastatic effects of αGalCer

treatment [26,27,35]. In our study, pretreatment with neutralizing antibodies to either anti-TNF-α or anti-IFN-γ reduced the frequency of IFN-γ-producing OTI T cells in liver to the levels observed in the absence of αGalCer. Therefore, both cytokines appear to be potent effectors of αGalCer-mediated stimulation of intrahepatic CD8 T cells, either directly or indirectly by stimulating surrounding lymphocytes such as NK cells [8,31]. It is yet unclear whether iNKT cell-derived cytokines stimulate intrahepatic CD8 T cells directly or through stimulation of antigen presentation by APCs such as Kupffer cells, Ito cells, and DCs. Importantly, anti-TNF-α treatment, but not anti-IFN-γ treatment, prevented serum ALT elevations and liver damage in all animals. This is in accordance with previous studies that suggest that activated iNKT cells use the production of IFN-γ and TNF-α as different effector tools, whereby especially the latter plays an essential role in the hepatic injury induced by αGalCer [27,36].

The observed stimulatory effect of iNKT-cell activation on the intrahepatic antigen-specific immune response suggests the possibility to use αGalCer in immune-modulatory therapy for human conditions characterized by insufficient Th1 responses in the liver. αGalCer-mediated iNKT-cell activation may stimulate weak virus-specific immune responses generally observed in patients with chronic HBV and HCV infection (reviewed in Bertoletti and Ferrari, 37). However, as shown by others previously [8,24,25] and also shown here, αGalCer-mediated iNKT-cell activation can cause liver injury. Moreover, use of especially repeated dosages of αGalCer somehow causes redirection of the immune response toward T helper cell 2 rather than T helper cell 1 reactivity (reviewed in Van Kaer, 38). Therefore, several obstacles need to be overcome before αGalCer can be used as treatment to stimulate inadequate T helper cell 1 immune responses in human liver.

In conclusion, functional antigen-specific CD8 T cells can be activated in the liver. Stimulation of iNKT cells with 1 single low dose of αGalCer facilitates the intrahepatic effector function of these antigen-specific T cells. Activation of iNKT cells may stimulate effector function of antigen-specific CD8 T cells that are primed in the liver, leading to hepatitis and subsequent liver damage. Thus, cross talk between CD1d-restricted Vα14 iNKT cells and APCs in the liver may be critically involved in controlling intrahepatic MHC-restricted T-cell responses.

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Notes

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Supplementary data

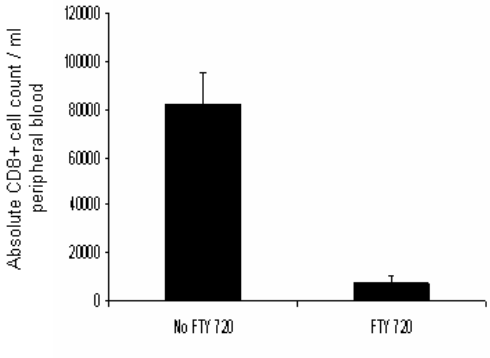

FIGURE S1. FTY720 blocks T-cell exit from lymph nodes. To block lymph node exit of T cells, FTY720 was injected into mice. To make sure FTY720 treatment blocks the number of circulating CD8+ T cells, in every experiment using FTY720-treated and nontreated mice, the absolute number of total CD8+ cells/mL peripheral blood was determined (bar graph).

Chapter 6

Summarizing discussion

Role of CD1d in the immune system

The major part of the adaptive immune system is based on the presentation of exogenously acquired peptide- antigen (Ag) by Class II major histocompatibility (MHC) molecules [1] and on Class I MHC molecules by cross-presentation [2,3]. This Class I or II mediated peptide-Ag presentation activates naïve and effector T cells, thereby inducing an immune response, which is essential for host defense against invading pathogens and tumor cells. However, some pathogens and tumor cells have evolved evasive mechanisms to escape from peptide-specific T- and B-cell immune response [4-10] and **Chapter 2** [11]. To overcome this type of immune evasion the immune system has evolved an independent Ag-presentation system organized around the presentation of lipid Ags by CD1 molecules to CD1-restricted Natural Killer T (NKT) cells [12,13]. Activated CD1-restricted NKT cells amplify the innate response [14] and trigger cells from the adaptive immune system, thereby functioning as a bridge between the innate and adaptive immune responses via the rapid release of cytokines [15,16]. There are five different isotypes of CD1 categorized in group 1 (CD1a, CD1b, CD1c) group 2 (CD1d) and group 3 (CD1E) [17-19]. Mice function as a model to study group 2 CD1d molecules and CD1d-restricted NKT cells. The mouse studies in this thesis focused on clarifying the role of intracellular transport (of CD1d and pathogen or Ag) on CD1d-restricted NKT cell development and activation during autoimmune hepatitis and *Mycobacterium tuberculosis* (*Mtb*) infection.

CD1d is constitutively expressed by many cells and in particular antigen presenting cells (APCs) [20-24]. CD1d molecules are assembled in the endoplasmic reticulum (ER) [25-27]. With the assistance of several adaptor and chaperone molecules CD1d molecules are then loaded with lipid Ags [19,28-34] and guided through secretory and endosomal pathways [35-42], eventually to be presented on the plasma membrane for the activation of NKT cells. The current status of research on endosomal processing of lipid-Ags for CD1d-mediated presentation is summarized in **Chapter 2** [11]. It is also reviewed how integrated signals from inflammatory cytokine receptors and pattern recognition receptors (PRR) instigate the rearrangement of the antigen processing and presentation machinery in endosomes for induced cell-surface display of antigenic lipid-CD1d complexes. In addition, it is covered how certain pathogens such as *Mtb* can evade the CD1d-lipid immune recognition through inhibition of lipid-Ag presentation of phagosomal origin. Finally, it is addressed how deviations in endosomal lipid antigen processing and presentation affect the immune system in patients suffering from lysosomal glycosphingolipid storage diseases. These events emphasize the need to understand the endosomal processes that underlie the display of Ag-loaded CD1d complexes and the activation of CD1d-restricted NKT cells for the development of therapeutic strategies against autoimmune and pathogenic diseases.

Trafficking of CD1d and invariant NKT cell development

Vα14 iNKT cells are key effector cells activated via the presentation of both exogenous and endogenous lysosomal lipids on CD1d displayed on the surface of cells including professional APCs [43-47]. In mice, these T cells are called after their mostly invariant T-cell receptor, in which Vα14 is coupled with Vβ8.2, Vβ7 or Vβ2. The positive selection of iNKT cells in mice requires CD1d-mediated Ag presentation by $CD4^+CD8^+$ double positive (DP) thymocytes [48-53]. Maturation of newly selected iNKT cells continues in the periphery and depends on CD1d expression [51,54,55]. CD1d molecules traffic through endosomal compartments where lipid-Ag sampling occurs [47,56-58] via their intrinsic

CD1d-encoded tyrosine motif and by association with the Class II MHC chaperone, invariant chain (Ii) [40,41]. Previously designed mouse models [40,47] were so far insufficient to clarify the roles that the CD1d-intrinsic sorting motif plays in CD1d-mediated positive selection, terminal maturation and stimulation of matured iNKT cells.

Using CD1d-EYFP/EYFP mice, a new mouse model in which all CD1d is replaced by CD1d-enhanced yellow fluorescent fusion protein (EYFP), we confirmed that endosomal sorting mediated by the CD1d-intrinsic tyrosine motif is critical for thymic selection of Vα14 iNKT cells ([59] and **Chapter 3**). CD1d-EYFP molecules were stable, presented lipid Ags, and had near normal subcellular distribution. Yet, the internalization rate of CD1d-EYFP molecules from the cell surface to endosomes was specifically reduced in T cells. Consequently, CD1d-EYFP molecules mediated positive selection of iNKT cell precursors at decreased efficiency, with an overrepresentation of $V\beta7^+$ iNKT cells. The display of endogenous ligands by CD1d shapes the selection of iNKT cells through the use of different T cell receptor (TCR) Vβ domains [60]. Vβ7 confers higher avidity binding, and is preferentially selected in situations where endogenous ligand concentrations are suboptimal [60], as appears the case in the CD1d-EYFP/EYFP mice. The suboptimal endogenous antigen presentation during thymic positive selection of iNKT cells also caused a delay in their thymic maturation, judged by their expression of CD44, NK1.1 and CD69. Maturation and activation of iNKT cells in the periphery requires the presence of CD1d [54]. We thus studied the effects of altered intracellular trafficking of CD1d on peripheral maturation and activation of iNKT cells. On the one hand, although CD1d-EYFP molecules supported normal peripheral maturation of iNKT cells, their Th2 (IL-4), but not their Th1 (IFN-γ) effector function was inhibited. On the other hand, CD1d-EYFP molecules on professional APCs (dendritic cells) from the periphery could not activate wild-type iNKT cells as wild-type CD1d could.

Thymocytes do not express Ii, and CD1d therefore strictly relies on its own CD1d-encoded tyrosine-based sorting motif for localization in lysosomes. In professional APCs, however, endosomal sorting of CD1d could be mediated by the CD1d-encoded tyrosine-based sorting motif, or via association of CD1d with Ii [40-42]. In **Chapter 3** we studied whether in professional APCs, the CD1d-encoded tyrosine-based sorting motif and Ii-encoded sorting motif could supplement for each other in mediating lysosomal localization, Ag acquisition and presentation by CD1d. To this end we compared the Ag presentation capacities of dendritic cells expressing CD1d-EYP, lacking Ii or harboring both mutations ($Ii^{-/-}$CD1d-EYFP/EYFP). The results demonstrated that both CD1d-encoded and Ii-encoded endosomal sorting motifs are necessary to induce the activation of iNKT cells by professional APCs in the periphery.

In **Chapter 4** we continued our study of endosomal sorting requirements for CD1d-mediated Ag presentation and resultant iNKT cell development in greater detail, by use of Ii-deficient and Ii-processing enzyme cathepsin S (CatS)-deficient mice. We found that iNKT cells in $Ii^{-/-}$ mice are delayed in maturation. Invariant NKT cells in both $Ii^{-/-}$ and $Cats^{-/-}$ mice have a decreased usage of Vβ7 TCR in the thymus but not in the periphery, which is known to occur upon overexpression of CD1d and/or endogenous lipid-Ag [61]. In addition, peripheral iNKT cells from Ii-deficient mice were unable to suppress *Mtb* growth in infected macrophages. Since DP thymocytes do not express Ii, our data confirm previous suggestions that thymic selection [61-63] and consequently iNKT cell function involves the presentation of lipid/CD1d complexes by Ii and CatS-expressing professional APCs.

All together, using these different mouse models, we clarified that the intrinsic CD1d-encoded sorting motif mediates thymic selection, while for peripheral terminal differentiation the intrinsic CD1d sorting motif is dispensable. The endosomal sorting motifs of both CD1d and Ii are necessary for activation of iNKT cells by professional APCs. In addition, we demonstrate that thymic selection of iNKT cells not only occurs on DP thymocytes but also involves CD1d-Ag presentation by Ii and CatS-expressing professional APCs.

CD1d-restricted NKT cell activation during mycobacteria infections

Invariant NKT cells play a role in the early immune response against *Mycobacterium tuberculosis* (*Mtb*) by suppressing intracellular bacterial growth when activated by antigenic CD1d/lipid complexes on *Mtb*-infected cells [64-66]. *Mtb* causes chronic tuberculosis in at least nine million people and more than 1,7 million deaths each year [67-69]. The Bacille Calmette–Guérin (BCG) vaccine against tuberculosis affords insufficient protection against *Mtb* [summarized in [68,70,71]]. It is therefore necessary to understand the mechanisms that are critical in the protective immunity to *Mtb* in order to develop better prophylactic strategies against *Mtb*. *Mtb* is an intracellular pathogen that survives in phagosomes of myeloid cells [72] by preventing fusion of phagosomes with lysosomes, thereby avoiding acidic pH and exposure to lysosomal hydrolases and proteases [72-79].

In **Chapter 4** we investigated the possible requirement for *M. tuberculosis* to traffic from phagosomes to lysosomes and for endosomal multivesicular morphology, to support pathogen replication and induce CD1d-mediated immune defense. For this purpose we made use of mice deficient in Ii and CatS. Ii regulates endosome fusion and architecture, and is required for endosomal transport [80-83]. Cells lacking CatS exhibit enlarged endosomal compartments without multivesicular structures [80,84]. *Mtb* replication and CD1d Ag loading and presentation does not require multivesicular structures within endosomal compartments, since infected CatS-deficient macrophages supported normal *Mtb* growth and could fully activate CD1d-restricted iNKT cells. Despite the finding that *Mtb* have normal ability to infect Ii-deficient macrophages, they exhibit a significant defect in pathogen replication. Infected Ii-deficient macrophages selectively retained *Mtb* in mature $EEA1^+$ phagosomes, did not upregulate CD1d upon infection and did not elicit CD1d-mediated iNKT cell activation. These findings imply that while obstruction of endosomal transport prevents *Mtb* immune-recognition, *Mtb* replication does require phagosomal fusion to lysosomes. *Mtb* growth benefits from endosomal trafficking, which possibly involves transport of *Mtb* growth factors (i.e., nutrients), localization of *Mtb* to compartments harboring co-factors or an membrane architecture that facilitate *Mtb* replication. Lysosomes but not phagosomes possess endosomal architecture conducive for *Mtb* replication. Lysosomal presence of *Mtb* is, however, equally pivotal for induction of CD1d-dependent iNKT cell responses in the host. We thus highlight the lysosome as a potential therapeutic target, due its importance in both *Mtb* growth and for CD1-restricted iNKT cell response against *Mtb*.

Invariant NKT cells and autoimmune hepatitis

Invariant NKT cells also regulate tolerance [85,86] and prevent autoimmunity and inflammation [87-91]. Yet, sometimes iNKT cells rather exacerbate autoimmunity [92-95],

as we found in **Chapter 5** in a model of autoimmune hepatitis. We describe a role for iNKT cells in facilitating 'conventional' $CD8^+$ T cell responses to liver-expressed Ag, using transferrin-ovalbumin (Tf-mOVA) mice [96]. The presence of Ag on Tf-mOVA hepatocytes leads to the retention of Ag-specific $CD8^+$ T cells in the liver, which induce hepatitis upon restimulation. Naïve mOVA-specific Class I MHC-restricted T cells (OTI) were adoptively transferred into Tf-mOVA mice in the presence or absence of iNKT-cell agonist α-galactosylceramide (αGalCer). Transfer of OTI cells resulted in robust intrahepatic, Ag-specific proliferation of T cells. OTI T cells were activated in the liver, and their Ag-specific effector function was stimulated by coactivation of iNKT cells using αGalCer. The activation of iNKT cells increased IFN-γ production of intrahepatic OTI T cells and increased their cytolytic capacity, contributing to liver damage. These effects were absent in $CD1d^{-/-}$ Tf-mOVA mice, which lack iNKT cells indicating that the enhancement of OTI T cell effector function is CD1d dependent. Blocking interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) produced by iNKT cells, demonstrated that both cytokines are potent effectors of αGalCer-mediated stimulation of intrahepatic $CD8^+$ T cells. During this process TNF-α specifically plays an essential role in the hepatic injury. We thus demonstrate how rapid cytokine production by activated CD1d-restricted iNKT cells stimulates intrahepatic $CD8^+$ T cell effector responses to liver -expressed Ag. Our findings highlight the rapid cytokine production by iNKT cells as an intervention point for targeted immunotherapy to autoimmune and possibly infectious liver diseases.

Clinical implications and future perspectives

Since iNKT cell numbers vary widely between individuals and reduced iNKT cell numbers or complete absence of iNKT cells are associated with many different disease states in mice and humans, it is crucial to understand iNKT cell development, homeostasis and function [97]. In addition, CD1d-restrictive iNKT cells represent highly potent immunoregulatory cells with conserved specificity, which makes them attractive targets for immunotherapy. However, before these cells can be safely and effectively manipulated in the clinic, it is crucial to understand the mechanisms behind their development and dichotomous behavior. Our results contribute to the understanding of the role of intracellular transport on CD1d-restricted NKT cell development and activation during autoimmune hepatitis and *Mycobacterium tuberculosis* (*Mtb*) infection.

Clinical implications for altered CD1d trafficking

The data in **Chapter 3** and partly **Chapter 4** demonstrate that the tyrosine-based sorting motif of CD1d and Ii are differentially important for the development and function of iNKT cells. This might have implications for patients with mutations in the CD1-encoded sorting motifs or deficiencies in Ii expression and other accessory molecules that interact with CD1. Human CD1b displays similar trafficking pathways and protein interactions as mouse CD1d [98]. It is known that patients with Hermansky-Pudlak syndrome type 2 (HSP-2) are deficient for adaptor complex-3 (AP-3), which causes defects in CD1b trafficking, antigen presentation and NKT cell selection, accounting for recurrent bacterial infections [38]. Invariant NKT cells are selected on CD1d molecules of which tyrosine-based motif in humans does not bind to AP-3 for lysosomal localization, but does bind to AP-2 for internalization from the plasma membrane [99]. Similar to the effect of AP-3 deficiency on CD1b-restricted immune responses it is possible that mutations in AP-2 or the CD1d-

tyrosine-based motif itself lead to iNKT cell-deficiencies and susceptibility to pathogen infections in humans.

Class II MHC deficiency, in the most extreme case resulting in bare lymphocyte syndrome (BLS) is a severe combined immunodeficiency caused by defects in MHC-specific transcription factors [100,101]. BLS patients with mutations in the RFX5 gene not only lack expression of specific HLA genes, but also lack Ii expression [102]. It is known that BLS patients are deficient in Class II MHC Ag-presentation [102]. However, since we now show that Ii is also important for the selection and function of iNKT cells ([59] and **Chapter 4**), it is most likely that these patients also exhibit iNKT cell deficiencies and therefore may be more susceptible to specific pathogen infections.

NKT cells and lysosomes as therapeutic targets against Mycobacterial infections

In **Chapter 4** we demonstrate that the lysosome is a crucial compartment for pathogen replication and the loading and presentation of lipid antigens on CD1d during *Mtb* infection. These lipid antigens activate CD1d-restricted iNKT cells, which can suppress intracellular pathogen growth and contribute to host defense against *Mtb* infection [64-66]. There are studies indicating the potential therapeutic use of activating iNKT cells with αGalCer for enhanced protection against *Mtb* infections [103-105]. This αGalCer-mediated activation of iNKT cells could be enhanced by addition of lenalidomide (LEN) or other thalidomide (Thal) analogues, which have already proved beneficial in myeloma patients [106]. Engagement of $P2X_7$ receptors with nicotinamide adenine dinucleotide (NAD) also enhances cytokine production by activated iNKT cells, which proves destructive during autoimmune hepatitis [107], but might be beneficial for the treatment of *Mtb* infections. Further studies are needed to define the balance between utility and side effects of αGalCer and other ligands for adjuvant therapy to the current *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccination, for αGalCer-modified BCG [105] or chemotherapy for tuberculosis in humans.

With the emergence of multidrug resistant (MDR)-*Mtb*, it is imperative to develop new intervention strategies. Our data highlights the lysosome as an important therapeutic target during *Mtb* infections. Promoting phagolysosomal fusion and optimizing Ag processing and loading conditions in the lysosomes are therefore important therapeutic strategies against *Mtb* infection. One such target could be the immunity-related GTPase (IRG) Irgm1 and its human ortholog IRGM via class I phosphatidylinositol-3-OH kinases PI(3)Ks. Stimulation of Irgm1/IRGM and class I PI(3)Ks by IFNγ treatment, or engagement of Toll-like receptor 4 (e.g. by lipopolysaccharide, LPS) or cell starvation, results in the assembly of a signaling platform that can orchestrate early phagosome events including autophagy and thereby facilitate the delivery of bacteria to lysosomes [108-111]. Autophagy, through its capacity to sequester and degrade unwanted contents and organelles, can also be induced pharmacologically with rapamycin [108]. Activating autophagy with the drug rapamycin could therefore potentially facilitate the eradication of *Mtb*-containing phagosomes by inducing autolysosome formation.

Mycobacteria activate certain host protein kinases to control actin dynamics and prevent phagolysosomal fusion for intracellular survival [112]. Targeting these protein kinases with inhibitors (e.g. AKT inhibitor compound H89) could counteract this bacterial inhibition of

phagolysosomal fusion and thus prevent intracellular growth of various bacteria including MDR-*Mtb* [112,113].

Cyclic AMP could also be a therapeutic target to influence phagolysosomal fusion and control of *Mtb* infection. Phagosomal protein kinase A (PKA), which regulates phagosome actin and maturation in macrophages, is cAMP-dependent [114]. Inhibition of adenyl cyclase, the enzyme that synthesizes cAMP with for example compound SQ22539, causes reduced levels of cAMP in phagosomes, which leads to phagolysosomal fusion and acidification in cells and consequently inhibition of *Mtb* intracellular growth [113]. A new treatment for *Mtb* infection could therefore also be based on cAMP inhibition, using compounds such as SQ22539, through its ability to block *Mtb* localization to lysosomes.

Phagolysosomal fusion to control *Mtb* infection could also be promoted by targeting the host phosphoinositide 3-kinase signaling pathway. For example, by stimulating this pathway with vitamin D3, phagosome maturation and phagolysosomal fusion is rescued during *Mtb* infection [115]. Supplementing anti-mycobacterial treatments with vitamin D3 might thus facilitate lysosomal localization and consequently eradication of *Mtb*.

However, because these host factors often play a role in other life-essential mechanisms it is crucial to target these therapeutic drugs specifically to mycobacteria-containing phagosomes. Alternative strategies promote phagolysosomal fusion by targeting *Mtb*-encoded proteins. For example, AX20017 a tetrahydrobenzothiophene inhibits mycobacterial Ser/Thr protein kinase G (PknG), which intervenes with host cell signaling pathways and blocks phagolysosomal fusion [116].

New therapeutic strategies to promote phagolysosomal fusion could also be combined with controlling phagosome/lysosome acidification. This would stimulate the activity of numerous host defenses such as reactive oxygen, nitrogen intermediates and lysosomal hydrolases (proteases, DNases, lipases etc.), the latter that degrade *Mtb* and self-lipids and thus provide antigens for the presentation on CD1 molecules. For example, addition of specific lipids (arachidonic acid, ceramide, sphingosine, sphingomyelin or phosphatidylinositol-4,5-phosphate [PTdIns(4,5)P2]) to *Mtb* infected macrophages stimulates phagosome actin assembly and phagosome fusion with late endocytic organelles [117]. This also leads to a significant increase in the fraction of acidified phagosomes and consequently inhibits intracellular pathogen growth [117]. Another approach could be activating the NF-κB pathway, which increases the synthesis and membrane trafficking of lysosomal enzymes to phagosomes resulting in pathogen killing [118]. Other supplementary drugs could be targeted directly at the activation of lipid processing enzymes such as α-galactosidase A [56] or lipid-transfer-proteins (LTPs), such as saposins or GM2 –activator protein (GM2A) to promote the enzymatic degradation of glycolipids by hydrolases and presentation on CD1 molecules [28,31]. Again, here lies the challenge to target these drugs directly to the phagolysosome to avoid unintended side activities.

NKT cells as potential therapeutic targets against autoimmune hepatitis

In **Chapter 5** we demonstrated a critical role for CD1d-restricted iNKT cells in $CD8^+$ T cell-mediated autoimmune hepatitis [96]. By blocking IFN-γ and TNF-α production by iNKT cells with monoclonal antibodies we could inhibit their negative influence on autoimmune hepatitis and thus elucidated a previously unknown intervention point for

targeted immunotherapy to autoimmune and possibly infectious liver diseases. Since the CD1d-dependent antigen-recognition pathway is highly conserved from mice to humans, findings in experimental models can be relatively easily translated to the clinical setting. Indeed, a recent report demonstrates the successful treatment of difficult-to-treat autoimmune hepatitis by TNF-α blockade in a human patient [119]. The biologic antagonists of TNF-α, infliximab, etanercept and adalimumab are approved for use in treating selected autoimmune diseases. Care should be taken while considering an intensive immunosuppressive treatment in patients with autoimmune hepatitis and infectious implications as occurred in the patient with difficult-to-treat autoimmune hepatitis, should be anticipated [119]. Indeed to date, reactivation of latent tuberculosis (TB) infection is a major complication of TNF-α antagonists. Screening and treatment of latent and active TB infection is therefore recommended before immunosuppressive treatment is initiated [120].

Our data demonstrates that activation of iNKT cells by a single low-dose of αGalCer exacerbates $CD8^+$ T cell-mediated autoimmune hepatitis. It has been observed that a single high-dose and repeated stimulation with αGalCer is followed by long-term anergy in iNKT cells, which become unable to proliferate and produce IFN-γ, but which retain the ability to secrete IL-4 [121]. One therapeutic strategy to ameliorate autoimmune hepatitis could therefore be to make iNKT cells anergic by repeated αGalCer injections, thereby making them non-responsive cells. However, it should be carefully studied whether the initial αGalCer injections do not cause irreversible liver damage by activating iNKT cells before they become anergic. In addition, observations suggest that although IL-4 is considered a prototypic TH2 (i.e. anti-inflammatory) cytokine, in the setting of certain liver diseases IL-4 has pro-inflammatory properties [122-124]. Thus rendering iNKT cells TH1-anergic through repeated αGalCer stimulations, might not have always have the desired effect. Moreover, since stimulation of iNKT cells results in either exacerbation or protection depending on the type of disease, care should be taken when using αGalCer as immunotherapy in patients with multiple disease syndromes.

Other therapeutic strategies could be focused on the regulation of (type I) iNKT cells by (type II) diverse NKT cells. In another murine autoimmune hepatitis model based on Concanavalin A (ConA), type I invariant NKT cell contribute to pathogenesis, whereas activation of sulfatide-reactive type II diverse NKT cells protects from disease [125]. Sulfatide-mediated activation of type II NKT cells resulted in IL-12 secretion and MIP-2 upregulation by plasmacytoid dendritic cells and consequent recruitment and induction of anergy in type I iNKT cells in the liver [125]. In turn, these anergic iNKT cells prevented ConA-induced hepatitis by specifically blocking effector pathways, including the cytokine burst and neutrophil recruitment that follow ConA injection [125]. Thus the administration of IL-12, sulfatide (a self-glycolipid) or the adoptive transfer of sulfatide-activated type II NKT cells could alter the destructive influence of type I iNKT cells in autoimmune hepatitis and possibly avert infectious liver diseases.

Skewing the cytokine profile of iNKT cells towards a, for autoimmune hepatitis, protective response is another therapeutic strategy. For example, administration of IL-15 protects against ConA-induced liver injury via an iNKT cell-dependent mechanism by reducing their production of IL-4, IL-5, TNF-α and infiltration of eosinophils [126].

Blocking NKT cell stimulatory receptors such as NK1.1, NKG2D, Ly49D and Ly49H, may also inhibit NKT cell function during autoimmune hepatitis. This could be achieved with chemical inhibitors, blocking antibodies against the stimulatory receptors [127], and potentially in the future with RNAi-mediated silencing [128]. Of course, indirect effects of these approaches exist and have to be considered when designing therapeutic protocols with genetically engineered effector cells or monoclonal blocking antibodies.

Alternatively, iNKT cell function during autoimmune hepatitis could be suppressed by an altered diet. Soy, which consumption increases globally, is a major source of isoflavonoids and sphingolipids and plays a complicated role in various aspects of the immune system. This sometimes occurs with positive effects on inflammatory disorders, but often with detrimental effects, as in the case of autoimmune hepatitis [129]. The administration of β-glycolipids could overcome these negative effects. Both a soy-free diet or the administration of β-glycolipids, even during a soy-based sphingolipid-rich diet, decreased intrahepatic iNKT cells and alleviated ConA-induced hepatitis by inhibiting IL-10 secretion and increasing IL-12 serum levels in mice [129]. In addition, administration of β-glycolipids prevented the ConA-induced trapping of intrahepatic CD8 T cells, which cause liver injury in autoimmune hepatitis [129]. Thus, alteration of the glycolipid background of the host, by administration of specific β-glycolipids, or by a soy-free diet, may serve as a novel therapeutic tool in autoimmune hepatitis and other disorders.

A more radical approach to ameliorate autoimmune hepatitis would be to completely eliminate iNKT cells. Absence of iNKT cells in CD1d$^{-/-}$ mice [96] or depletion of iNKT cells with monoclonal antibodies [126] abolished liver injury in autoimmune hepatitis models. However, since an iNKT cell exclusive marker has not yet been identified, most antibody-dependent depletion methods would also delete other cell types that might be necessary for immune response against other diseases.

Concluding remarks

In this thesis we demonstrated that intracellular trafficking of CD1d mediated by its intrinsic sorting motif, or by the invariant chain affects iNKT cell development and function, with implications for patients with mutations or deficiencies of these molecules. In addition, we highlight the lysosome as a crucial organelle for *Mycobacterium tuberculosis* (*Mtb*) replication and CD1d-antigen loading and presentation to iNKT cells. This emphasizes the need to continue research towards iNKT cell-based therapies and strategies focused on increasing phagolysosomal fusion during *MTb* infection. Finally, we demonstrated the destructive role of CD1d-restricted iNKT cells during autoimmune hepatitis. Our results emphasize the usefulness of research focused on blocking, altering or eliminating these iNKT cells to alleviate liver injury. CD1d/iNKT cell-mediated immune responses have lately received an increasing amount of attention in the immunological field because of their crucial influence on many disease outcomes. With the studies in this thesis we have been able to contribute to understanding the role of intracellular transport on CD1d-restricted NKT cell development and activation, thereby highlighting therapeutic targets against autoimmune hepatitis and *Mtb* infection.

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Fenna

Curriculum vitae

Fenna C.M. Sillé was born March 18th, 1980, in Willemstad, Curaçao, Netherlands Antilles. In 1998 she completed high school (VWO) at the Peter Stuyvesant College in Curaçao. Subsequently, she studied Biology at the University of Groningen in the Netherlands. Dr. W. Helfrich supervised her during her Master's education. She first rotated in the Department of Medical Microbiology and Molecular Virology at the University of Groningen under the supervision of Dr. J.M. Smit and Prof. Dr. J.C. Wilschut. In the final year of the Master's program she rotated in the laboratory of Prof. Dr. D. Kershenobich under the direct supervision of Dr. S. Sixtos at the Instituto Nacional de Ciencias Medicas y Nutrición Salvador Zubirán, Mexico City, Mexico. Prof. Dr. C.H. Gips at the International school of Hepatology and Tropical Medicine (GISH-T), University of Groningen, supervised the exchange from the Netherlands. This exchange was partly funded by international fellowship awards from the Marco Polo fund and the Groninger University Fund. In April 2004 she received her Master of Science degree with specialty in Molecular Virology and Immunology. After graduating from the University of Groningen she started as a Ph.D. student in the laboratory of Dr. M.L. Boes in the Department of Dermatology at the Brigham and Women's Hospital/ Harvard institute of Medicine in Boston, USA. In 2005 she received a pre-doctoral fellowship from the Boehringer Ingelheim Foundation. This thesis booklet is the end result of her Ph.D. research.

List of publications

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Abbreviations

Ag	antigen
αGalCer	α-galactosylceramide
ALT	alanine aminotransferase
AP	adaptor protein complex
APC	antigen presenting cell
BCG	Bacille Calmette-Guérin
BLS	bare lymphocyte syndrome
β2m	β2-microglobulin
cAMP	cyclic adenosine monophosphate
CatS	cathepsin S
CD	cluster of differentiation
CD1d-EYFP	CD1d linked with enhanced yellow fluorescent fusion protein
CFSE	carboxyfluorescein diacetate succinimidyl ester
CFU	colony-forming units
ConA	concanavalin A
DC	dendritic cell
DDM	didehydroxymycobactin
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
EAE	experimental autoimmune encephalomyelitis
EEA1	early endosomal autoantigen 1
ER	endoplasmic reticulum
EYFP	enhanced yellow fluorescent fusion protein
FCS	fetal calf serum
GD3	disialoganlioside
GM1	monosialoganglioside
GMM	glucomonomycolate
GPI	glycosylphosphatidylinositol
GTPase	guanosine-5'-triphosphatase
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSP-2	Hermansky-Pudlak syndrome type 2
IFN	interferon
Ig	immunoglobulin
iGb3	Isoglobotrihexosylceramide
Ii	invariant chain
IL	interleukin
Ing LN	inguinal lymph node
iNKT cell	invariant Natural Killer T cell
IP	intraperitoneal
IRG	immunity-related GTPase
IV	intravenous
LAM	lipoarabinomannan
LAMP-1	lysosomal-associated membrane protein 1
LDL-R	low-density lipoprotein receptors
LEN	lenalidomide
LN	lymph node

LPS	lipopolysaccharide
LTP	lipid transfer protein
mAb	monoclonal antibody
MDR	multidrug resistant
Mest LN	mesenteric lymph node
Mϕ	macrophage
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MOI	multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MTP	microsomal triglyceride transfer protein
Mya	million years ago
Neo	neomycin
NAD	nicotinamide adenine dinucleotide
NF-κB	necrosis factor-κB
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
NOD	non-obese diabetic
OTI	mouse OVA-specific class I MHC-restricted T cells
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI(3)K	phosphatidylinositol-3-OH kinases
PIM	phosphatidylinositol mannoside
PG	phosphatidylglycerol
PKA	protein kinase A
PRR	pattern recognition receptor
PTdIns(4,5)P2	phosphatidylinositol-4,5-phosphate
PVDF	polyvinylidene difluoride
SD	standard deviation
SEM	standard error of the mean
TB	tuberculosis
TCR	T cell receptor
Tf	transferrin
Tf-mOVA	mice with ovalbumin expressed under the transferrin receptor
Tfr	transferrin receptor
Th1/Th2	type-1/type-2 T helper cell
Thal	thalidomide
Tk	thymidine kinase
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF related-apoptosis-inducing ligand
Treg	regulatory T cell
ULN	upper limit of normal
WT	wild-type

Appendices

Appendix 1

T cell priming by tissue-derived dendritic cells: New insights from recent murine studies.

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Abstract

Dendritic cells (DCs) act as sentinels in peripheral tissues, continuously scavenging for antigens in their immediate surroundings. Their involvement in T cell responses is generally thought to consist of a linear progression of events, starting with capture of antigen in peripheral tissues such as the skin followed by migration to draining lymphoid organs and MHC-restricted presentation of antigen-derived peptide to induce T cell priming. The role of tissue-derived DCs in the direct priming of immune responses has lately been challenged. It now appears that, at least in some instances, a non-migratory subtype of DCs in the secondary lymphoid tissue presents tissue-derived antigen to T cells. Here, we review recent developments in research on DC function in the priming of immune responses.

Keywords: DC migration; DC subsets; T cell priming

Introduction

Dendritic cells (DCs) are a motile population of cells that are efficient at antigen capture (1) and that migrate out of the peripheral tissues towards lymphoid tissues upon activation by a variety of stimuli (2), (3) and (4). The degradation of internalized antigen by DCs triggers the segregation of pathogen-derived protein and lipid moieties in endosomal compartments, followed by the possible acquisition by products of the major histocompatibility complex (MHC) locus and CD1, respectively (5). Upon arrival in peripheral lymph nodes, antigen-experienced DCs present processed antigen in the form of peptide/MHC or lipid/CD1 complexes to CD4 T cells.

During T cell development, T cells are taught to recognize antigen presented by MHC or CD1 complexes. Accordingly, adaptive immune responses can only be initiated upon the triggering of the T cell antigen-specific receptor (TCR) when pathogen-derived fragments are displayed as complexes with MHC Class I or Class II, or CD1 (6) and (7). A further restriction to prevent inappropriate T cell activation (which would pose a risk for the development of autoimmune disease) is that only a few selected cell types called professional antigen-presenting cells (APCs) are capable of naïve T cell activation: B cells, macrophages, and DCs. DCs in particular are specialized in priming naïve T cells by virtue of their exquisite ability to generate peptide/MHC complexes (through their attenuated proteolysis as compared with that of macrophages (8) and (9)), high expression of adhesion and co-stimulatory molecules, their preferred location at sites of pathogen entry, and migratory capacities.

The skin is the body's largest exposed interface with the environment and has evolved as a barrier to shield external agents, including pathogens, from entering the body. When pathogens do succeed in breaching the skin, potent phagocytic cells, most notably DCs, are strategically present near barrier surfaces to initiate immune defense strategies. Well-known examples are Langerhans cells (LCs) in the epidermis and dermal DCs (dDCs) in the dermis. LCs and dDCs are thought to play important roles in priming immune responses initiated in the skin (10), (11) and (12). LCs were the first DC subset to be described, by Paul Langerhans in 1868, but were considered DCs only in 1985 (13). After that, LCs became the preferred model for studying DC biology in vivo.

Antigen uptake

Encounter of pathogens by epidermal LCs is optimized by their scattered presence that forms an extensive network. Antigen uptake is stimulated by the expression of endocytic receptors such as DEC205, langerin, and other C-type lectins, which recognize conserved carbohydrate structures on pathogens (14). Another mechanism by which epidermal LCs now appear to stimulate antigen uptake is by extension and retraction of their dendrites, a behavior called dSEARCH, which is enhanced upon receiving maturation stimuli (Nishibu et al., submitted manuscript, personal communication MB).

The migration of DCs that carry antigen to the secondary lymphoid tissues where most T lymphocytes reside is important for increasing the chances of cognate interaction between DCs and T cells. Equally important, prior to the migration of DCs out of their residential tissue, antigen needs to be internalized from the inflamed tissue. The formation of an ATP gradient may transiently inhibit the migration of local DCs, thus prolonging the time of exposure to antigen and inflammatory mediators (15). Expression of the adhesion molecule JAM-A also inhibits DC migration, as there is increased DC migration from skin to lymph nodes in the absence of JAM-A (16), perhaps to optimize antigen uptake efficiency.

Migration

DCs can be triggered to migrate out of their native location by a variety of stimuli, which may be mechanical in nature (i.e., burns or other wounds (17)), or chemical (skin-permeable toxins such as poison ivy (18)), or represent the encounter with pathogen-derived material (i.e., lipopolysaccharide (LPS), by insect bite or infected wound). DC migration can be accomplished via receptors to conserved pathogen-associated structures (such as Toll-like receptors (TLRs)) or by endogenous signals released during tissue injury and necrosis (19) and (20). These stimuli may induce rapid activation of the transcription factor NFκB and the upregulation of chemokine-receptor expression to assist migration to lymphoid tissues (19), (20), (21) and (22). DC migration can also be stimulated directly by chemokines, signaling lipids, cytokines, and other molecules without inducing overt DC maturation, perhaps resulting in more tolerogenic T cell responses (19), (21), (23), (24), (25) and (26). The differentiation steps that distinguish mature from immature DCs remain poorly defined (27). DCs that appear mature, based on surface levels of MHC complexes and co-stimulatory molecules, may not always have reached full functional maturity (28) or been ‘licensed’ to activate T cells (29). For example, LCs that reach the lymph node upon inflammatory signaling display a mature phenotype expressing high levels of MHC Class II complexes, CD80, and CD86 but maintain the capacity to capture and process antigen during their subsequent presence in the lymphoid tissue (28).

LC precursors are bone marrow-derived cells that migrate to the epidermis around the time of birth (30). Cognate interaction of antigen-laden LCs (and DCs in general) and rare T cell clones is facilitated by the migration of DCs to secondary lymphoid tissues (1). Many factors can influence migration whether DCs are mature or immature and in steady-state or inflammatory conditions. The most well-known factors are chemokines (31) and (32) which regulate DC migration by virtue of their expression of chemokine receptors (CCR) (21) and (22). In particular, CCR7 on DCs and the ligation of its ligands CCL19 and

CCL21 in the lymph node paracortex (33) and (34) are important in the migration of DCs under both steady-state and inflammatory conditions (21), (22), (35), (36), (37), (38) and (39). CCL21 is also expressed by afferent lymphatic endothelium (33). Expression of CCR7 is induced in LCs as they leave the epidermis (40) and is required for lymph node-directed trafficking of LCs, although CCR7 appears to be dispensable for the mobilization of migration of LCs from the epidermis to the dermis (36). Under steady-state conditions, LCs are maintained locally, but inflammatory changes in the skin result in their replacement by blood-borne LC progenitors in a CCR2-dependent manner (41). CCL1 (also known as I-309 and a ligand for CCR8) acts synergistically with the homeostatic chemokine CXCL12 (SDF-1α) for recruitment of activated T cells and $CCR8^+$ LCs to the skin (42). DC migration is often dependent on the maturation-induced responsiveness to distinct sets of chemokines (i.e., by ligation-induced downregulation of the chemokine receptor as was demonstrated for LCs (31), (43) and (44)). The migratory capacity of DCs is also variable among different subtypes: plasmacytoid DCs and myeloid DCs differ in their migratory properties and in their capacity to recruit different cell types at inflammation sites (19).

Besides chemokines, the presence of inflammatory cytokines in the epidermis (i.e., tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-18) stimulates migration of LCs from the epidermis and their accumulation in draining lymph nodes following skin sensitization (3), (45), (46), (47) and (48). TNFα accomplishes this by the downregulation of E-cadherin on epidermal LCs, which results in the loosening of cells from keratinocytes (49). TGFβ1 is essential to LC migration, as shown by the deficiency of epidermal LCs in TGFβ1 knock-out mice (50). Moreover, antigens in the epidermis and dermis at steady-state are trafficked into regional lymph nodes in a TGFβ1-dependent manner, possibly by DCs (51). Prostaglandins (52) and leukotriene C_4 (LTC_4) (20) are mediators of inflammation that also influence LC migration. The transporter MRP1 is involved in the migration of LCs from epidermis by promoting chemotaxis to CCL19, probably by stimulating MRP1-mediated release of LTC_4. Indeed, MRP1 and the related multidrug resistance protein 1 (MDR1, p-glycoprotein, ABCB1) may control the intracellular and extracellular accumulation of key signaling lipids that regulate DC migration (20), (24) and (26).

Inherent DC factors important for DC migration are adhesion molecules such as E-cadherin (53) and (54), ICAM-1 (53), (55), (56) and (57), α6 integrins (58), and CD44 (59). Cytoskeletal rearrangements in LCs are critical to their ability to dislodge and transmigrate. For example, epidermal LCs deficient in the Wiskott–Aldrich syndrome protein (WASp) show impaired migration to lymph nodes following contact sensitization (60). Finally, matrix metalloproteinase-9 (MMP-9), capable of degrading type IV collagen in the basal membrane, is involved in LC migration across the basement membrane and through connective tissue (61) and (62) to allow access to afferent lymph vessels, from which they travel to draining lymph nodes.

The classical paradigm

The classical model describes a linear route between antigen uptake in the periphery and T cell priming in the lymph node. DCs that take up antigen in the periphery were considered the same entity as DCs involved with transport of the antigen to lymph nodes and eventually the priming of T cells. This model was based primarily on observations of LCs

from contact hypersensitivity (CHS) models. These CHS models involve skin-sensitizing agents such as fluorescein isothiocyanate (FITC), dinitrofluorobenzene (DNFB) (63) and (64), and trinitrochlorobenzene (TNCB) (65) or carcinogens such as dimethylbenzanthracene (DMBA) (65). CHS studies in mice made LC-deficient by ultraviolet light treatment suggested that LCs are important for effective hypersensitivity responses (63). Upon CHS, skin-derived LCs appeared to pick up (haptenated and sometimes fluorescent) antigen in the skin, migrate into lymph nodes, and initiate an immune response (28), (64), (66) and (67). Models of *Leishmania major* infection provided further evidence for the classical paradigm. LCs were shown to take up *L. major* upon subcutaneous or intradermal infection with *L. major* and to subsequently migrate to lymph nodes, resulting in T cell priming (68).

The migration of DCs from the skin to the draining lymph nodes was also studied by gene-gun-based experiments (69). LCs were tracked using gene-gun-mediated transfection of cre recombinase into mouse skin. Mice that were transfected expressed a loxP-flanked neomycin cassette between the promoter and lacZ gene. Therefore, expression of lacZ was accomplished only after cre recombination (69). This study showed that, transfected LCs migrated to the draining lymph nodes and resided there for approximately two weeks (69). Other elegant studies involved transgenic mice that express a keratin 14 promoter-driven mini-gene encoding an immunodominant MHC class I epitope of ovalbumin. These mice developed a lethal autoimmune disease after transfer of antigen-specific CD8 T cells, which were activated spontaneously (70). This implied that LCs have a cross-priming potential when loaded exogenously with preprocessed antigenic peptides. LCs purified from normal human skin appeared to be efficient in presenting not only peptide antigens (71) but also lipid antigens (72), resulting in MHC Class II- and CD1a-restricted T cell priming, respectively (71) and (72). Together, these studies suggested a major role for LCs in antigen uptake and transport to the lymph node and perhaps T cell priming. Extending the classical paradigm, CD8α-negative epidermal LCs were found to upregulate CD8α upon arrival in the draining lymph nodes (66). Accordingly, LCs may be among the CD8α-expressing cells localized in the T cell areas of the lymph nodes (66).

Although the classical paradigm is widely accepted, solid proof that LCs are indeed in general responsible for both antigen uptake and presentation in the lymph nodes is still scarce. Most of the previously mentioned studies focused on individual aspects of LC behavior and only provided indirect evidence for the direct stimulation of T cells by LCs. The approaches used so far for tracking DCs in the epidermis (i.e., by FITC painting) have been expedient and informative, but such marking of DCs has the caveat of being transient because of dilution of fluorochrome and the possibility of FITC transfer to other DCs. Furthermore, the application of irritants may trigger mechanisms of LC migration different from those associated with the normal processes of antigen presentation. Other studies used subcutaneous immunization to study antigen uptake and transport to lymph nodes. This method likely bypasses the epidermis-resident LCs. While the gene-gun study provides convincing evidence that LCs were transfected and migrated to the draining lymph nodes (69), it is possible that β-galactosidase was transferred from LCs to other DCs in the lymph node. Moreover, this study provided only an indirect and coincidental link between antigen expression and presentation to T cells.

It has become clear that DCs are not a homogeneous cell lineage. There are different DCs subtypes, which can be distinguished based on surface markers (73), (74), (75), (76), (77) and (78). LCs in lymph nodes are generally recognized through a combination of their high expression of MHC Class II, low expression of CD8α, intermediate expression of E-cadherin, and high expression of DEC205. Discriminating LCs from dermis-derived DCs in skin-draining lymph nodes has been particularly difficult because the levels at which they express relevant cell surface molecules differ only slightly. The only LC-specific marker identified to date is the C-type lectin langerin (79), (80) and (81). Until recently, however, the unavailability of a commercial antibody for langerin hampered research on the role of LCs in priming immune responses. Moreover, with the identification of a number of different subpopulations of DCs comes the realization that distinct DC subsets may not perform every function attributed to them by in vitro studies. Recent research, which has looked more profoundly into T cell priming by migrating cells, has suggested that LCs may not be the APCs that induce most T cell priming to skin-derived antigens.

Refining the classical paradigm

Recent papers have resulted in a shift in the paradigm concerning the dynamics and function of LCs in vivo. Contrary to the commonly accepted notion, several studies in the past few years have indicated that LCs may not play a leading role in activating T cell-mediated defenses against viral infection of the skin and mucosa (82) and (83). During murine herpes simplex virus 2 (HSV2) infection via the vaginal epithelia, LCs did not perform antigen-presenting functions. Instead, $CD8\alpha^{high}$ $CD45RA^{-}$ $CD11b^{+}$ submucosal DCs, which are thought to be the functional equivalent of dDCs, were responsible for the induction of protective $CD4^{+}$ Th1 responses to HSV2 (82) and (84). However, LCs may be inhibited from performing antigen-presenting functions in this situation as a result of the destruction of the epithelial layer by HSV2.

Further evidence against the priming of naïve T cells by LCs came from a study of cutaneous HSV infection in mice (83). Exploiting chimeric mice that contained HSV presentation-competent LCs but HSV presentation-incompetent $CD8\alpha^{+}$ DCs, it was shown that LCs were incapable of priming HSV-specific $CD8^{+}$ T cells in vivo (83). Taken together, these studies showed that LCs do not prime virus-specific $CD4^{+}$ or $CD8^{+}$ T cells during certain epidermal infections (82), (83) and (84). The research was taken a step further by the demonstration that lymph node-resident $CD8\alpha^{+}$ DCs are the principal DC subset that initiates CTL immunity to subcutaneous and intravenous infection by three different viruses: influenza, herpes simplex, and vaccinia (85). A role for LCs in immunity against these viral skin infections was excluded and it was suggested that non-LC $CD8\alpha^{+}$ DCs may represent the major DC subset responsible for priming CTL immunity to these types of viral infection.

Several groups have also discarded a role for LCs in initiating immunity upon subcutaneous injection with *L. major* (86), (87) and (88). Studies using a mouse model in which MHC Class II expression is restricted to $CD11b^{+}$ and $CD8\alpha^{+}$ DCs showed that control of cutaneous *L. major* infection is dependent on these non-LC DCs (86). An interesting consideration is that *Leishmania* lipophosphoglycan alters the expression of certain adhesion molecules (53), hence impairing LC migration out of the skin. If *L. major* inhibits LC migration and thereby a potentially LC-mediated immune response, other DC

subsets may take over and present *L. major* antigens to T cells in the lymph nodes. The lack of LC involvement may thus be particular to this type of infection. Furthermore, most of the aforementioned studies used subcutaneous injection of the parasite, thereby possibly not addressing a role for epidermis-resident DCs in antigen presentation. The exact nature of the DC subset(s) that perform most T cell priming in the *L. major* models remains to be shown.

A recent study exploited a novel EGFP-tagged diphtheria toxin (DT) receptor (DTR)/DT-based system, in which LCs could be inducibly removed without affecting the skin microenvironment (89). In LC-depleted mice, CHS responses were significantly decreased, although some ear swelling still occurred, indicating that dDCs alone are able to mediate CHS (89). Others independently used the same ablation technique, showing that most EGFP-labeled cells were sessile under steady-state conditions, whereas skin inflammation induced LC motility and emigration to lymph nodes (90). After epicutaneous application of TRITC, dDCs ($EGFP^-$) arrived in lymph nodes first (after 24 h) and colonized areas within the paracortical T cell-rich zone distinct from slower migrating LCs ($EGFP^+$ LCs peaking after four days). TRITC-laden dDCs migrated into the outer paracortex, just beneath the B cell follicles, whereas TRITC-laden LCs migrated into the inner paracortex. Moreover, LCs appeared to be dispensable for induction of T cell priming in draining lymph nodes as well as for activation of specific effector T cells in skin immunization in this study (90).

Antigen uptake and T cell priming seem to be separated events not only in LCs but also in other DC types. To further complicate matters, multiple DC subtypes can present antigen at the same time. During lung infection with influenza virus or HSV1, both an airway-derived $CD8\alpha^-$ $CD11b^-$ DC subset and a distinct $CD8\alpha^+$ lymph node-resident DC subset can present MHC Class I-restricted antigens in the lung-draining mediastinal lymph node (85). Some DC subsets, such as TNF/iNOS-producing (Tip)-DCs (91) or plasmacytoid DCs (92), might not even be involved in antigen uptake and T cell priming but instead be important for cytokine production and the orchestration and mediation of innate immune defenses (91).

Transfer of antigen and T cell priming

After DCs have internalized antigen and migrated to the lymph nodes, the antigen needs to be presented as peptide/MHC or lipid/CD1 complexes to appropriately restricted T lymphocytes. It now appears that T cell priming in lymph nodes is already initiated within 24 h of epicutaneous or subcutaneous application of antigen (93), (94), (95) and (96), whereas LCs enter the lymph node no earlier than on day 3 under these conditions (90), (94) and (97). Naïve T cell priming likely represents a complex interplay among distinct DC subsets involved in the process of antigen transport and presentation, making the classical paradigm, mentioned earlier, less attractive. In the past few years, several studies have discarded a role for LCs in the conventional priming of naive T cells (82), (83), (84), (86), (87), (88), (89), (90) and (98). Instead, dDCs were described to be most important for presentation of skin-derived antigen in lymph nodes (89) and (90).

Currently, it is unclear what contribution LCs make to skin immunity, although several potential roles have been proposed. Perhaps, LC migration or antigen uptake is inhibited (as a pathogen-induced immune evasion strategy, as in *L. major* infections (53)) in specific

infections and other DC subsets may take over in such cases. Other plausible models for DC migration and T cell priming are that either different DC subsets sequentially guide the progression of naïve T cells toward fully differentiated effector cells or that each DC subset participates uniquely in the priming of a particular T cell population (91). The T cell area of lymph nodes, the paracortex, is occupied by three subsets of DCs that enter lymph nodes from the blood and three that enter via the lymph from tissue (78), (95), (99) and (100). CD4 T cells do not need sustained interaction with one DC to be fully primed. Instead, the sum of a lower threshold level of activation signals (possibly acquired from different antigen-presenting DCs) allows for acquisition of effector function (101). Thus, priming of T cells in vivo may well require the interplay of a T cell with more than one DC.

Lately, several lines of evidence have emerged for antigen transfer between DCs under both steady-state conditions and during immune activation. The occurrence of antigen transfer is evident from studies showing the ability of APCs to acquire antigen by phagocytosis and then present this antigen by means of MHC Class I molecules, a process called 'cross-presentation' (102), (103) and (104). Mice rendered transgenic for the poliovirus receptor sustain productive infection with poliovirus, a pathogen normally incapable of infecting mice. When these animals were lethally irradiated and reconstituted with bone marrow from donors that did not express the poliovirus receptor, a robust response involving $CD8^+$ T cells was nonetheless observed. This required the presence of a functional TAP (transporter associated with antigen presentation) complex in the donor bone marrow used for reconstitution. It provides strong evidence that bone marrow-derived APCs can indeed access the MHC Class I pathway of antigen presentation, even when not infected with the virus themselves (105). Furthermore, it was demonstrated that keratinocyte-expressed ovalbumin is captured and presented on MHC Class I molecules of bone marrow-derived cells (106).

Other evidence that tissue-associated "self-antigens" can be cross-presented in the draining lymph nodes includes the finding that ovalbumin expressed on β-cells in the pancreas could be presented by bone marrow-derived APC to OT-I cells (107). Likewise, the $CD8^+$ subset of DCs is reported to be responsible for cross-presentation in the spleen (108). In the gastric lymph node, interstitial DCs were suggested to transfer antigen, derived from gastric mucosa, to $CD8^+$ DCs residing in the lymph node, so that both subsets could present antigen to T helper cells (109). Another form of antigen transfer was shown in tolerance studies in which induction of tolerance to parenchymal self-antigens requires antigen transfer to a bone marrow-derived APC that presents it to T cells in a tolerogenic fashion (110). Antigen transfer also occurs in Peyer's patches. Peyer's patch DCs can process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice and activate primed T cells (111). While transfer of antigen fragments (complexed with heat-shock proteins or as apoptotic cell parts) or peptide/MHC complexes appears most likely, the transfer of unbound cytosolic peptides may occur as well. In fact, a sophisticated mechanism for cross-presentation that was recently described involves cell-to-cell transfer of peptides across gap junctions (112).

DC subsets

It has been attempted to couple T cell priming to specific DC subsets. For example, several studies suggest that CD8α-positive DCs are uniquely capable of priming $CD8^+$ T cells (83) and (108), whereas CD8α-negative DCs are better at priming $CD4^+$ T cells (82), (113), (114), (115) and (116). Distinct DC subsets may selectively prime distinct T cell subsets with the corollary that certain DCs leave other T cell populations unaffected (91), but it is also possible that full differentiation of T cells requires sequential interactions with DC subsets (94). General statements are difficult to make however, due to the high degree of variability in model systems and the different criteria used to distinguish the various DC populations.

One way to categorize lymph node-resident DCs is by their activation stage. Both immature and mature DCs exist in skin-draining lymph nodes (27) and (75). This distinction might be important, as mature DCs are thought to induce immunity, whereas the persistence of an immature phenotype could lead to a state of tolerance, possibly caused by a lack of co-stimulatory molecules (23), (117), (118) and (119). However, it has also been suggested that tolerance is induced by DCs that are mature but quiescent, whereas immunity is induced by DCs that are fully activated (78) and (120). Direct DC activation via Toll-like receptor (TLR) signaling results in DCs that prime an effective T helper response. Indirect activation by inflammatory mediators, such as IFNγ, alone generates mature DCs that support $CD4^+$ T cell clonal expansion but lack the ability to produce IL-12 and thus fail to support full T helper cell differentiation (29). Immature and mature DCs may therefore be distinguished on the basis of the classical phenotypic and functional features (using surface markers, morphology, endocytic capacity, and antigen presentation) but only 'licensed' DCs can induce immunity (121). These discrepancies have made it difficult to appoint T cell priming to any specific DC subset. Further studies are needed to improve our understanding of the various DC subsets and their function during each maturational stage. It would be wise to extend these findings with in vivo imaging studies. Recently, it has become possible to visualize the migration of endogenous DCs within lymph nodes in live mice through the use of two-photon microscopy (122) and (123). So far, however, the few studies that have specifically looked at single-cell dynamics of T cells by DCs in lymph nodes (122), (123) and (124) did not specify the DC subset.

Concluding remarks

The fortuitous observation that DCs extracted from immunized mice contain internalized antigen does not necessarily imply their direct involvement in mediating T cell priming in vivo. Instead, T cell priming may occur via several not mutually exclusive pathways, the details of which are only beginning to be elucidated. It now appears that different DC subsets may guide the progression of naïve T cells towards fully differentiated effector cells in a sequential manner, each DC type providing essential information such as peptide/MHC, inflammatory cytokine or tissue-derived TLR ligands. At the same time, selective DC subsets may participate in the priming of particular T cells. In those instances, selectivity for antigen uptake is conveyed by the expression of endocytic pathogen-recognition molecules (i.e., C-type lectins such as langerin, DEC205 and DC-SIGN). Not all DCs may be equipped equally well at proteolysis of pathogen-derived antigens. Macrophages differ from DCs in their ability to process antigen (8) and (9), which may

prove to be the case for DC subtypes as well. Therefore, not all DC types may be able to generate peptide/MHC complexes from endocytosed antigen with the same efficiency, which may introduce variegation in the peptide/MHC complexes produced and their ability to activate rare antigen-specific T cells. Lastly, internalized, tissue-derived antigen may be carried into draining lymph nodes by way of tissue-derived DCs, where antigens are transferred to a second, non-migratory type of DC that may perform most of T cell priming (77).

Most of what is now known about the migratory pathways of DCs in regard to T cell priming ability was learned from skin studies. LCs only arrive in the lymph nodes three days after epicutaneous dye application, while dDCs prime T cells after 24 h (90), (94) and (97) (as illustrated in Fig. 1). Lymph node-resident DCs may acquire soluble antigen directly from the lymph within hours of subcutaneous injection (94) and (125) or antigen transferred from dDCs after a day, but may not be fully licensed (29). Such DCs may therefore not have the full capacity to mediate the differentiation of naïve CD4 T cells into true T helper cells able to support class switching of B cells.

What role may there be for LCs that arrive after initial T cell activation has occurred by dDCs or lymph node-resident DCs? The importance of LCs may lie in mediating the full differentiation of primed T cells that are undergoing clonal expansion. There is some data to support such a mechanism. Recently primed T cells only start to express intracellular IFNγ (a measure of their acquisition of effector function) after several cell divisions, which coincides with the arrival of LCs, three days after antigen exposure (29), (126) and (127). The significance of LCs in mounting full-fledged immune responses was shown in contact hypersensitivity studies (89). Inflammatory cytokines are important to support T cell priming, and IFNγ can be produced by DCs themselves (128). The presence of IFNγ receptors in the immunological synapse appears to direct the earliest stages of T helper commitment, perhaps to concentrate a IFNγ at a critical location (129). Thus, perhaps tissue-derived DCs are critical for providing additional signals not restricted to antigen (but perhaps entailing cytokines) necessary for the differentiation of T cells into fully potent effector T cells. The prevalence and importance of such processes will need to be determined by future research. The combined use of intravital imaging and new mouse models in which specific DC subtypes are conditionally eliminated or fluorescently labeled will be valuable tools in this respect.

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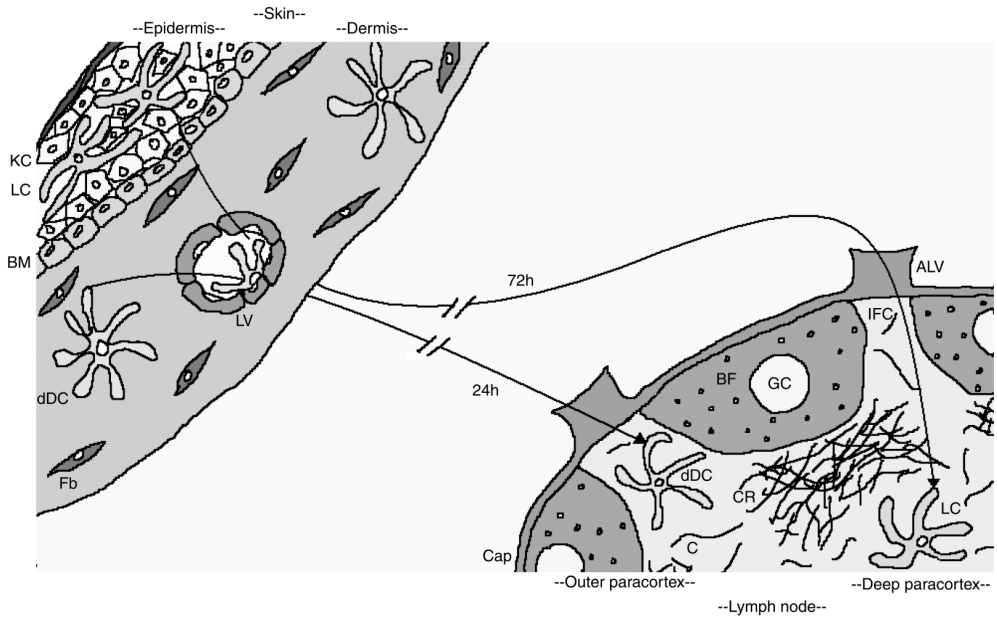

FIGURE 1. Complementation of DC subtype function to optimize T cell priming. We hypothesize that DC subtypes may each have specialized roles, to join forces for elicitation of fully effective T cell responses. In this specific example, the migration of murine skin-derived DCs to a skin-draining lymph node is projected. dDCs migrate out of the dermis and into the outer paracortex of the draining lymph nodes within 24 h. There they prime T cells by direct antigen presentation or indirectly, via antigen transfer to lymph node-resident, non-migratory DCs. LCs complement this dDC-initiated T cell priming by bringing in tissue-derived signals after three days, when they arrive from the epidermis into the deep paracortex. These signals are necessary for activated T cells to become full effector T cells capable of producing effector cytokines and support class switching of B cells. LCs may also prime distinct T cell subsets specific to the same pathogen, by providing additional peptide/MHC complexes of similar or distinct composition to those generated by dDCs and non-migratory DCs. Proteolysis capabilities in the various DC subtypes has not been investigated in full at this time. Finally, LCs may support T cell priming in a manner that does not directly involve antigen presentation, for example, by cytokine release to activate other cell types, such as NK cells and NKT cells, to release inflammatory cytokines. (ALV, afferent lymphatic vessel; BF, B cell follicle; BM, basal membrane; C, collagen; Cap, capsule; CR, cortical ridge or superficial T cell area; dDC, dermal dendritic cell; Fb, fibroblast; GC, germinal center; IFC, interfollicular channel; KC, keratinocyte; LC, langerhans cell; LV, lymphatic vessel).

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Appendix 2

Protein kinase C delta stimulates antigen presentation by Class II MHC in murine dendritic cells.

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Abstract

Maturation of dendritic cells (DCs) regulates protein sorting in endosomal compartments to promote the surface expression of molecules involved in T cell activation. MHC Class II complexes are mobilized to the surface via intracellular effector molecules that remain largely unknown. We here show that protein kinase C (PKC) stimulates Class II antigen surface expression, using knock-in mice that express a Class II–green fluorescent protein fusion protein as a read out. Selective inhibition of PKC counteracts the ability of DCs to stimulate Class II MHC-restricted antigen-specific T cells. Activation of PKCδ does not affect antigen uptake, peptide loading and surface display of Class I MHC and transferrin receptor in DCs. We show that activation-induced Class II MHC surface expression is dependent on activation of PKCδ and conclude that this event is pivotal for optimal CD4 T cell activation.

Keywords: antigen presentation/processing, antigens/peptides/epitopes, dendritic cells, rodent

Introduction

The initiation of a productive adaptive immune response requires the surface display of peptides bound to products of the MHC locus on antigen-presenting cells (APCs). Activation of naive T cells is accomplished most effectively when dendritic cells (DCs) display sufficient peptide–MHC complexes to T cells in the simultaneous presence of accessory molecules (1–3). Class II MHC molecules are expressed by professional APCs, in contrast to Class I MHC complexes which are more widely distributed (4). Class II MHC complexes acquire antigenic cargo for presentation to CD4 T cells by intersection of the endocytic and Class II biosynthetic pathways (5). The biosynthesis of Class II MHC and their mode of peptide acquisition are well established (6–9), while the mechanisms underlying the later stages of peptide–Class II trafficking are still poorly defined. Efficient display of peptide–Class II MHC complexes involves the proteolysis of both internalized protein antigen and Class II-associated invariant chain (Ii). During biosynthesis of Class II α–ß complexes, Ii associates with Class II dimers to achieve proper folding, occupying the peptide-binding cleft (10, 11). Recognition of targeting motifs in the Ii cytoplasmic domain guides trafficking of Ii–Class II MHC complexes from the endoplasmic reticulum via the trans-Golgi network to late endosomal antigen-processing compartments for binding of antigen-derived peptides (12). Peptide-loaded Class II MHC complexes can then be transported to the cell surface for inspection by appropriately restricted CD4 T cells.

DCs are highly effective at stimulating naive T cells in comparison to other APCs such as macrophages and B cells. Activation of DCs can lead them to undergo a process called maturation, for example when microbial products trigger surface receptors to pathogen-associated molecular patterns [i.e. Toll-like receptors (TLRs)] (13). During DC activation, endosomal sorting and trafficking of endosomal compartments are adjusted to favor peptide loading, as well as surface display of loaded peptide–Class II MHC complexes (14, 15). Other activation-mediated changes include down-regulation of endocytosis, up-regulation of chemokine receptors and induced formation of membrane extensions thought to optimize T cell activation (16). Cytoskeletal rearrangements, trafficking of membrane proteins and cell adhesion can all be regulated by members of the protein kinase C (PKC)

family of serine–threonine protein kinases (17, 18). What are the mechanisms that mediate activation-induced surface display of Class II MHC complexes in DCs?

Members of the PKC family of serine–threonine kinases are activated when cells that express TLR4 are exposed to its ligand LPS (19, 20). We hypothesized that the activity of PKC family kinases is involved in up-regulating Class II MHC during DC activation. The observation that TLR4-mediated LPS signaling stimulates the transcription of IL-12 subunits p35 and p40 is in agreement with this notion (21, 22). Conversely, PKC activation is inhibited in macrophages derived from mice that carry a mutation in the TLR4 gene and that are therefore hyporesponsive to LPS (23). Endosomal trafficking of proteins can also be regulated by PKC activity, presumably by phosphorylation of serine–threonine residues near endosomal targeting motifs of the substrate (24–26). However, whether the activation-induced surface display of Class II MHC in DCs involves PKC activity has not been studied. We here describe the role of PKC activation in Class II antigen presentation by DCs, using bone marrow-derived DCs from Class II–green fluorescent protein (GFP) knock-in mice. We define PKCδ as the isoform responsible for stimulating Class II MHC plasma membrane expression.

Methods

Mice

Class II–GFP knock-in mice have been described (14, 27, 28). Class $II^{-/-}$ mice were obtained from Jackson Laboratories (29). All mice were backcrossed to C57Bl/6 for at least 10 generations. Mice were housed in a barrier facility and studies were performed according to the institutional guidelines for animal use and care.

DC culture and treatment

Bone marrow was extracted from the bone marrow cavity of murine femurs. The bone marrow was re-suspended until a single-cell suspension was generated. Cells were plated at 2.5 x 105 cells per well in 200 μl of DMEM/10% FCS/2 mM glutamine/200 U ml–1 penicillin/200 μg ml–1 streptomycin without phenol red and in the presence of 10 ng ml–1 granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA) and 1 ng ml–1 IL-4 (Roche Molecular Biochemicals, Somerville, NJ, USA). Bone marrow precursors were cultured for 4 days at 37°C in plastic dishes or cover slip dishes (Lab-Tek, to allow for live-cell confocal imaging) in a 5% CO2 and 95% air incubator. Inclusion of GM-CSF and IL-4 induces the selective outgrowth of CD11c+ Class II MHC+ DCs (14, 30, 31). The presence of elaborate endosomal compartments expressing large amounts of Class II–GFP was used as an additional DC marker. Where indicated, DCs at day 4 of culture were stimulated with 100 ng ml–1 LPS, 25 ng ml–1 phorbol 12-myristate 13-acetate (PMA) (Sigma–Aldrich), 500 ng ml–1 ionomycin (Sigma–Aldrich) or rottlerin (2 μM in 3-h experiments or 10 μM when analyzed after 18 h, Calbiochem). After culture at 37°C, DCs were harvested by vigorous re-suspension and transferred to 1.2 ml FACS tubes (USA Scientific) for staining for flow cytometry.

Flow cytometry

Harvested DCs were incubated 5 min on ice with Fc block 1:200 (BD Biosciences) in FACS buffer: PBS (GIBCO BRL) supplemented with 5% FCS. Immunostaining was performed for 15 min on ice with fluorophore-conjugated antibodies against CD11c, I-Ab, CD86, H2-Kb, CD4, CD69 and Vß5. All antibodies for flow cytometry were directly fluorophore-conjugated and were obtained from BD Biosciences. After immunostaining, the cells were pelleted by centrifugation (1200 x g, 5 min, 4°C) and washed three times with cold FACS buffer. FACS analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

Antigen endocytosis assay

DCs at day 4 of culture were treated with LPS, PMA and ionomycin or left untreated for 3 h at 37°C. Half of the DCs were then moved to 4°C and half the DCs were kept on 37°C, and FITC-conjugated ovalbumin (OVA) was supplemented for a further 30-min incubation (5 µg ml–1, Molecular Probes). After the 60 min of incubation at 4 or 37°C, DCs were washed and stained with anti-CD11c–APC antibody and immediately analyzed by flow cytometry as described above.

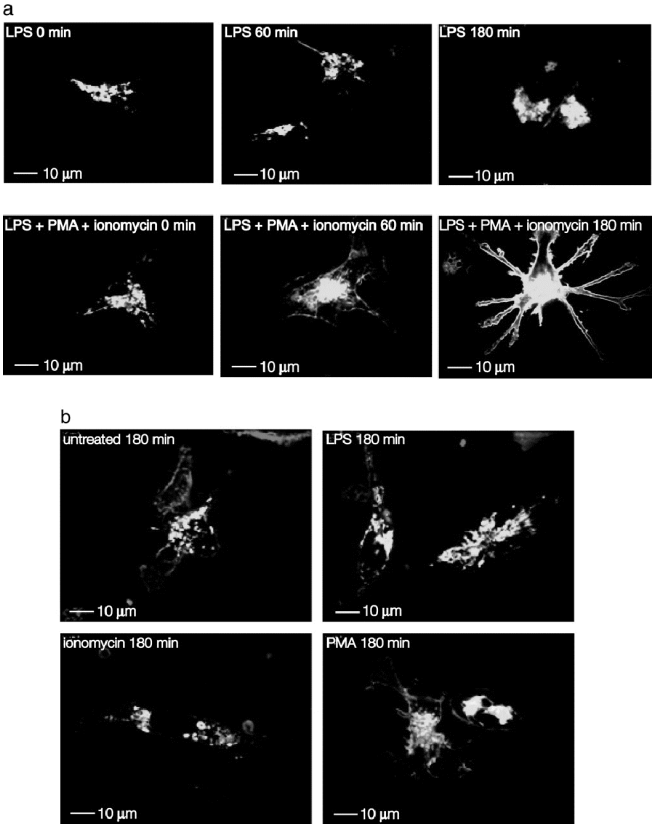


FIGURE 1. PKC agonists rapidly induce Class II MHC surface display and significant changes in DC morphology. (***a***) Bone marrow-derived DCs (day 4 of culture in the presence of GM-CSF and IL-4) were cultured in cover slip dishes and analyzed by confocal microscopy after 0, 1 and 3 h of treatment with LPS alone (top panels) or LPS, PMA and ionomycin (bottom panels). (***b***) Increased surface display of Class II MHC upon treatment with PMA. Bone marrow-derived DCs (day 4 of culture) were cultured for 3 h in the presence of LPS, ionomycin and PMA or left untreated. Treatment with PMA alone stimulates both Class II surface display and acquisition of mature DC morphology. Images are representative of three independent experiments.

T cell activation assay

DCs at day 4 of culture were cultured in round-bottom 96-well plates in the presence of 0–40 μM OVA (Sigma) for 3 h in conjunction with various combinations of LPS, PMA and ionomycin (at the concentrations described above), and then washed three times with DC culture medium. In assays where rottlerin pre-treatment was done, DCs were pulsed with 4 μM OVA antigen O/N at day 3 of culture. At day 4, DCs were washed three times, rottlerin was added (10 μM) for 30 min and then LPS or PMA were added for 3 h. DCs were washed three times and OTII T cells (5 x 105) were added to the stimulated DC cultures for 4 or 18 h in normal media. At the end of the culture, the T cells were harvested and transferred to 1.2 ml FACS tubes on ice for antibody staining and flow cytometry analysis.

Pulse–chase analysis and immunoprecipitations

Pulse–chase experiments and immunoprecipitations were performed as described (32). Briefly, DCs were starved in cysteine/methionine-free media for 45 min and labeled for 45 min with 0.1 mCi ml–1 (35S) cysteine/methionine (PerkinElmer) in the presence of the indicated drug treatments. Cells were chased for the indicated times in complete DC media in the presence of drug treatments. Cells were lysed in NP-40 lysis buffer: 1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.5 mM Tris, pH8.0 and complete protease inhibitor cocktail (Roche), pH 7.6. Lysates were pre-cleared with normal mouse serum together with Staph A. Class II MHC complexes were immunoprecipitated using an antibody to Class II molecules, N22 (33), and recovered with Staph A. After washing, the immunoprecipitates were split in half, re-suspended in sample buffer and incubated at room temperature, or boiled for 5 min. Samples were then analyzed by SDS–PAGE.

Reverse transcription–PCR

DCs were grown from mouse bone marrow as described above. Mouse cerebrum served as a control tissue in which most PKC isoforms are expressed (34). Cells were treated with DNAse to dissipate contaminating DNA. Template mRNA was extracted using an RNA extraction kit according to the manufacturer's recommendations (Ambion). The concentration of RNA was determined by densitometry by UV spectrofotometer (260 nm, Beckman). The amount of mRNA template used was 100 ng (cerebrum) or 250 ng (DCs). PKC primer sequences and annealing temperatures are described (34). Reverse transcription (RT)–PCR was done as recommended by the manufacturer (Stratagene). PCRs were allowed to proceed for 45 cycles (cerebrum) or 30 cycles (DCs).

Cell lysis and western blotting

Bone marrow-derived DCs (day 4 of culture) were re-suspended in cold lysis buffer: 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4 and complete protease inhibitor cocktail (Roche, 30 min, 4°C). Protein concentrations were normalized using the BCA Protein Assay Kit (Pierce) and were run on 4–20% SDS–PAGE gradient midigels (Bio-Rad). After transfer to polyvinylidene difluoride (PVDF) membrane (Bio-Rad), proteins were blocked for 1 h at 25°C with 5% milk block in Tris-buffered saline/0.1% Tween 20 (TBS-T) (Sigma). Membranes were blotted with mAbs specific to PKC isoforms α, ß, δ and λ (BD Biosciences, 1:500) or with phospho-PKCδ/θ (Ser643/676) antibody (Cell Signaling Technology, Inc., 1:1000). After three washes with TBS-T (10

min each), membranes were incubated for 30 min with anti-mouse (for PKCα, ß, δ and λ blots) or anti-rabbit (for anti-phospho PKCδ/θ) HRP-conjugated secondary antibodies (Jackson ImmunoResearch) (1:10 000 in 2% milk:TBS-T). Blots were developed with a chemiluminescent substrate (Bio-Rad) and x-ray film (Amersham Biosciences).

Immunofluorescence staining of mouse PKCδ in DCs

DCs were generated from bone marrow in cover slip bottom dishes (Lab-Tek II, Nalge Nunc) for 4 days. DCs were fixed using 4% PFA by adding an equal volume of PFA (8%, 37°C, 10 min) to cultured cells. DCs were permeabilized using 0.1% Triton-X in PBS (5 min, RT) and washed three times using PBS. Cells were then blocked using 10% BSA:PBS (1 h, RT) and stained 2 h using the anti-mouse PKCδ mAb (BD Biosciences, PKC Sampler Kit, 1:100 in 10% BSA:PBS) and developed using 1:400 secondary Alexa 568-conjugated goat anti-mouse antibody (Molecular Probes). Imaging was performed by spinning disc confocal microscopy at the microscopy center of the Department of Pathology, Harvard Medical School.

Statistical analyses

An unpaired two-tailed t-test was used for statistical analyses.

P-values below 0.05 are considered statistically significant and are indicated in the figure legends as *$P < 0.05$ or $P < 0.01$ where indicated and **$P < 0.005$.

Results

PKC stimulation induces surface display of Class II MHC in mouse DCs
To visualize whether activation of PKC is involved in the activation-induced surface display of Class II MHC complexes, we made use of bone marrow-derived DCs from Class II–GFP knock-in mice (14, 35, 36). Untreated, resting DCs were compared with DCs treated with PMA/ionomycin, a commonly used activator of conventional and novel forms of PKC. As a positive control for activation, we treated DCs from Class II–GFP mice with LPS (100 ng ml–1), in the presence or absence of PMA/ionomycin, and analyzed them by confocal microscopy (Fig. 1a). Untreated cells exhibit an immature phenotype, as characterized by the presence of the majority of Class II MHC in endosomal compartments (Fig. 1a). After 3 h of LPS treatment, surface display of Class II MHC is seen (Fig. 1a, top panels) (37, 38). However, combined treatment of LPS/PMA/ionomycin shows rather different kinetics of activation. Robust surface display of Class II MHC can be visualized already after 1 h of treatment, further enhanced after a 3 h of treatment (Fig. 1a, bottom panels). This rapid activation is also evident by acquisition of a mature morphological phenotype of the DCs (39, 40).

Conventional PKC isoforms (α, ßI, ßII and γ) require both diacyl glycerol (DAG) (or phorbol ester) and a rise in cytosolic calcium ions (Ca2+) for their activation, while the novel PKC isoforms δ, ε, τ and η are DAG dependent but Ca2+ independent (41–44). Atypical PKC isoforms ζ and λ appear regulated independently of DAG and Ca2+ fluxes (42). To investigate whether a conventional or novel PKC isoform is involved in stimulating the translocation of Class II MHC complexes to the plasma membrane, we

treated DCs with PMA or ionomycin separately or with LPS as control. During the last 5 min of culture, Alexa 594-conjugated cholera toxin B subunit was added to stain ganglioside (GM1)-containing lipid domains (45) and so facilitate the visualization of Class II MHC expression at the cell surface. Untreated DCs have low levels of Class II MHC complexes at the cell surface and an immature morphology (Fig. 1b). After 3 h of stimulation, LPS- or ionomycin-treated DCs generally express modest surface levels of Class II MHC. PMA treatment induces surface display of Class II MHC, as visualized by co-localization of Class II MHC (GFP, green) with the GM1-containing lipid domains in the plasma membrane (Alexa 594, red) (yellow color, Fig. 1b). Since PMA treatment in the absence of ionomycin induces Class II MHC surface display, we conclude that activation of novel PKC isoforms induces surface display of Class II MHC in mouse DCs.

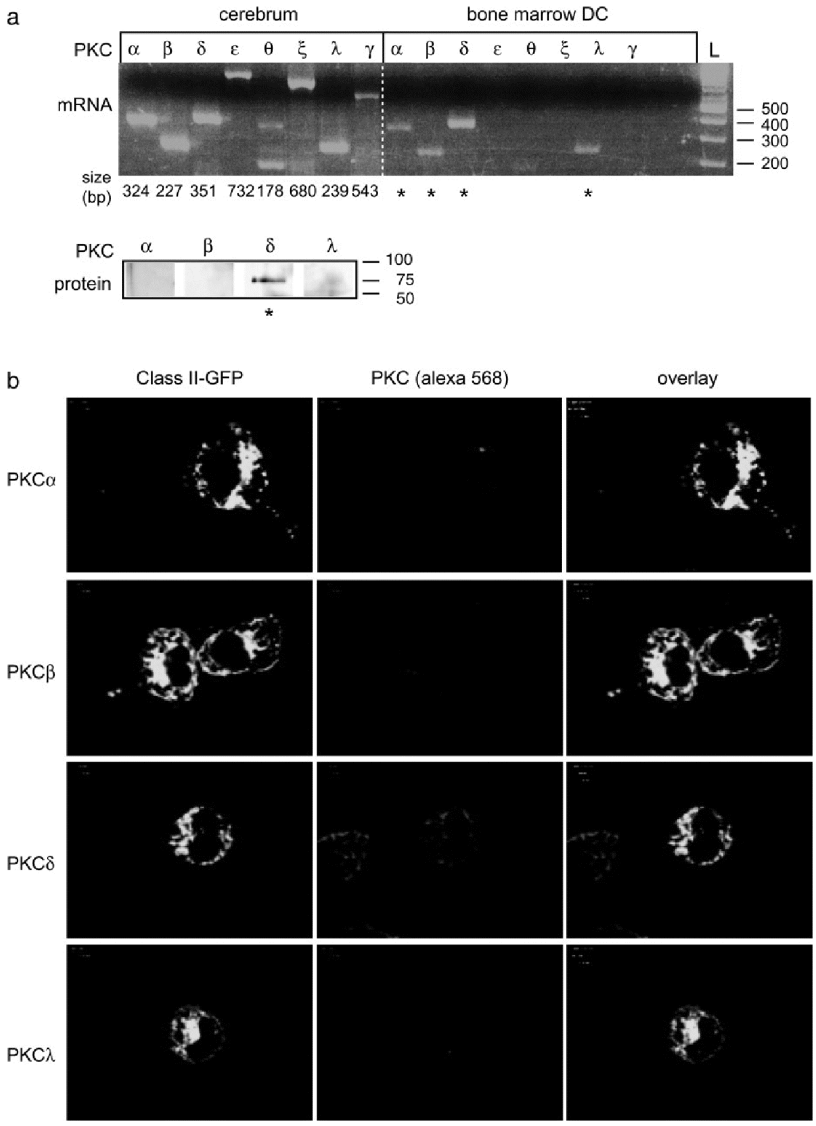
PKCδ is expressed in mouse DCs

Most cells express multiple PKC isoforms and therefore attribution of a specific function to a specific isoform is not straight forward (46). To determine which PKC isoforms are expressed in mouse bone marrow-derived DCs, we extracted mRNA from DCs and from mouse cerebrum as a control. RT–PCR was performed using primer sequences (34) for individual PKC isoforms. In mouse cerebrum, many PKC isoforms are expressed (34): we detected transcripts of PKCα, ß, δ, ε, θ, ζ, λ and γ (Fig. 2a, top, left). Using mRNA from DCs as template, we detected transcripts of PKCα, ß, δ and λ (Fig. 2a, top, right; asterisks indicate the presence of PKC expression), but not γ, ε, θ and ζ. PKCα and ß are conventional isoforms and can thus be excluded as candidates responsible for a PMA-induced process (34, 41). PKCλ is an atypical PKC isoform, which is not stimulated by PMA treatment either (42). As a novel isoform, which can be activated by PMA treatment alone (42), PKCδ must therefore be responsible for the observed rapid Class II MHC surface display.

Acquisition of PKCδ biological activity upon phorbol ester treatment

To establish whether PKCδ is activated upon phorbol ester treatment, we performed western blot and immunofluorescence microscopy studies. The enzymatic activity and biological function of PKCδ is controlled by autophosphorylation of serine 643 of PKCδ (30), which can be measured by western blot. DCs were treated with PMA for the indicated time points and were lysed. Lysates were run on SDS–PAGE and transferred to PVDF for blotting with an antibody specific to phospho-PKCδ/θ (serine 643/676). This antibody detects endogenous levels of PKCδ only when phosphorylated at serine 643 (and PKCθ only when phosphorylated at serine 676). Already at time point zero, at which time PMA was added, some PKCδ was phosphorylated. The level of phosphorylated PKCδ increased over time after PMA treatment, and reached an optimum at 30 min. Antibody reactivity to Glyceraldehyde 3-phosphate Dehydrogenase was used as a loading control, unaffected by PMA treatment (Fig. 2c). We next visualized the intracellular localization of PKCδ in maturing DCs, confocal microscopy was performed on Class II–GFP DCs (green) stained with antibody to PKCδ (red) (Fig. 2d). DCs were left untreated or cultured with LPS, PMA and ionomycin for 3 h, and then fixed in 4% PFA and stained with anti-PKCδ mAb (BD Biosciences). Untreated DCs showed a predominantly round or elongated morphology with most Class II MHC in endosomes and PKCδ in the cytosol. DCs treated with LPS or ionomycin exhibited a predominantly elongated morphology, with a modest surface level of Class II MHC and cytosolic localization of PKCδ. In contrast, PMA-treated DCs

displayed a mature phenotype, with dendritic extensions and increased surface levels of Class II MHC. Moreover, PMA treatment induced the transfer of PKCδ to the plasma membrane and nuclear envelope in DCs (Fig. 2d). Our results show that PMA treatment induces also the membrane translocation of PKCδ, indicative for acquiring its biological function.



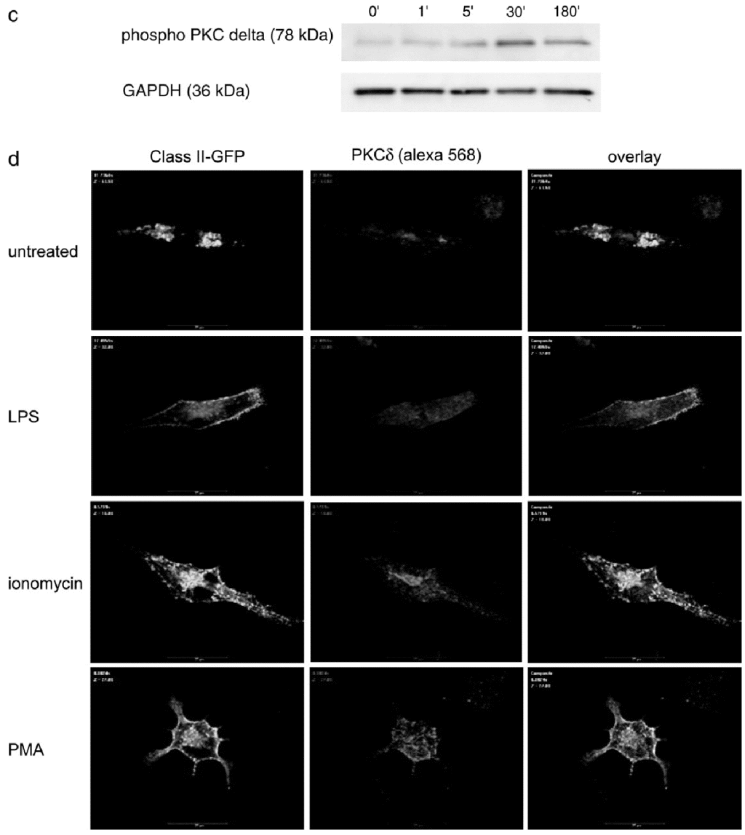


FIGURE 2. PKCδ is expressed in mouse bone marrow-derived DCs and is activated upon PMA stimulation. (***a***) DCs (day 4 of culture in the presence of GM-CSF and IL-4) were purified using CD11c-based MACS purification (Miltenyi). RT–PCR analysis of lysates from mouse cerebrum (left) and purified mouse bone marrow DCs (right). Asterisks indicate the lanes where an RT–PCR product was seen (PKCα, ß, δ and λ). Experiment is representative of four experiments with similar outcome. DC lysates were also analyzed by western blot, after SDS–PAGE and protein transfer to PVDF membrane, using mAbs to PKCα, ß, δ and λ. Only PKCδ was detected, as indicated by an asterisk. (***b***) DCs were grown in glass cover slip dishes, fixed using 4% PFA, permeabilized and stained using unconjugated antibodies against PKC isoforms PKCα, PKCß, PKCδ and PKCλ, followed by Alexa 568-conjugated secondary antibody. Day 4 Class II–GFP-positive DCs as well as Class II–GFP-negative bone marrow cells appear to express PKCδ only, and not the three other PKC isoforms analyzed. (***c***) DCs were stimulated with PMA for 0, 1, 5, 30 and 180 min and were lysed. lysates were run on SDS–PAGE and blotted to PVDF membrane, and were blotted using phospho-PKCδ/θ (ser643/676) antibody. Activation-induced phospho-PKCδ protein was detected most prominently at 30 min after PMA treatment. PKCθ is not detected in mouse DCs. Experiment shown is representative of three independent experiments. (***d***) DCs were grown in cover slip dishes, treated with LPS, ionomycin and PMA or left untreated (3 h, 37°C), and then fixed, permeabilized and stained using PKCδ antibody followed by Alexa 568-conjugated secondary antibody. DCs were analyzed by confocal microscopy (x100 objective, Z-stack analysis, 1 μm step size). Experiment shown is representative of four independent experiments.

To determine at the protein level whether PKCα, ß, δ or λ are expressed in mouse DCs, we performed western blot analysis using antibodies specific to these four PKC isotypes. Whereas PKCδ was readily observed, PKCα, ß and λ were not detected by western blot (Fig. 2a, bottom). To confirm the western blot data, immunofluorescence stainings for PKCα, ß, δ and λ were performed on fixed and permeabilized DCs. Secondary Alexa 568-conjugated antibody was used for visualization of the PKC isoforms (Fig. 2b). Also using immunofluorescence microscopy, only the PKCδ isoform was detected. Taken together, only PKCδ protein is prevalent in mouse DCs.

PKC stimulation of DCs induces increased surface levels of I-Ab, but not of CD86, H2-Kb or transferrin receptor

PKCδ activation induces surface-directed trafficking of Class II MHC and of itself. To study the specificity of this event, we treated DCs with LPS, PMA or ionomycin for 3 h and measured the surface display of Class II MHC (I-Ab), CD86, Class I MHC (H2-Kb) and transferrin receptor (TfR) by flow cytometry (Fig. 3). Changes in surface display of I-Ab, CD86, Kb or TfR after treatment with LPS, PMA or ionomycin are shown as a percentage change to untreated DCs. Class II MHC surface expression was significantly induced by LPS, PMA or ionomycin (40–50% increase compared with untreated DCs, $P < 0.005$, Fig. 3), while confocal imaging had indicated that PMA treatment (but not LPS or ionomycin) induced the most dramatic Class II MHC surface display associated with cytoskeletal rearrangements (Fig. 1b). PMA or ionomycin were unable to induce surface expression of CD86, while LPS treatment did induce significant CD86 up-regulation, as expected (47, 48). Class I MHC was not induced by 3-h stimulation with LPS, PMA or ionomycin, and TfR surface expression was unaffected by LPS or ionomycin treatment. We consistently found that PMA treatment induced significant internalization of TfR, possibly reflecting the internalization of TfR into recycling endosomes (49, 50). Taken together, our data suggest a specific role for PKCδ activation in stimulating the surface display of Class II MHC as opposed to Class I MHC or CD86 trafficking.

PKC stimulation of DCs increases Class II antigen presentation to antigen-specific T cells

Is PKCδ involved in enhancing Class II MHC antigen presentation to antigen-specific T cells under conditions of limited antigen delivery? DCs were treated with 0, 4 and 40 μM OVA for 3 h, in the presence of LPS, PMA and/or ionomycin. DCs were then washed three times prior to addition of OVA-specific naive T cells from transgenic mice that express an OVA-specific I-Ab-restricted TCR (OTII). After 4 and 18 h of co-culture of T cells with DCs, T cells were analyzed for their activation status by flow cytometry (CD4–peridinin chlorophyl protein, (PerCP). Vß5–PE and CD69–FITC, Fig. 4). In the absence of OVA, background levels of T cell activation were seen (except when LPS, PMA and ionomycin were all present, we have not investigated this further). DCs treated for 4 h with 4 μM OVA alone induced activation of 3% of OTII T cells, which increased to 50% when DCs were treated in addition with phorbol ester (Student's t-test, $P < 0.005$ comparing PMA-treated with untreated sample). Comparable data were obtained when DCs were treated with a higher concentration of OVA (40 μM) or when T cell activation was allowed to continue for 18 h instead of 4 h (Fig. 4). Treatment with 40 μM resulted in full activation of most T cells by 18 h. Taken together, we conclude that PKC stimulation enhances the

ability of DCs to stimulate Class II MHC-restricted T cells under conditions of limited antigen availability.

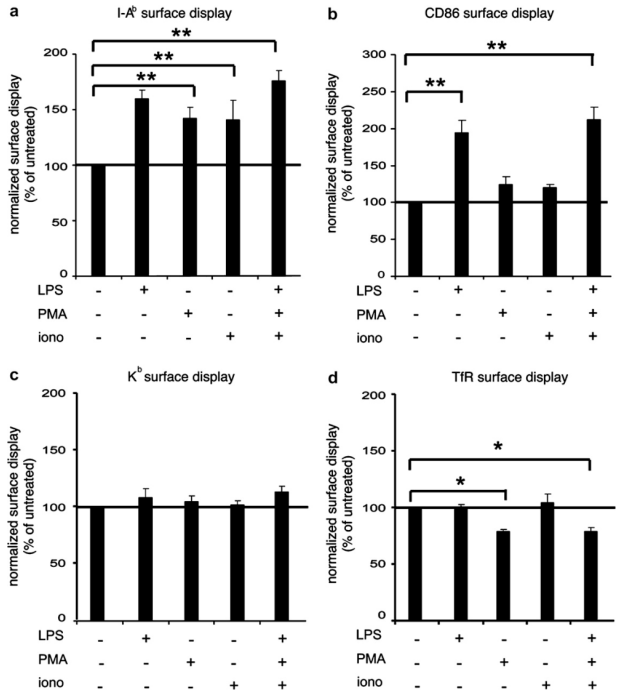


FIGURE 3. PKC activation stimulates surface display of Class II MHC, but not of Class I MHC, CD86 or transferrin receptor. Bone marrow-derived DCs were gated based on high expression of Class II–GFP. DCs were analyzed by flow cytometry analysis of surface display of (***a***) Class II MHC (I-Ab), (***b***) CD86, (***c***) Class I MHC (H2-Kb) and (***d***) transferrin receptor (TfR). Surface expression of H2-Kb, CD86 and TfR are not affected by treatment with PKC agonists, suggesting that LPS and PMA selectively stimulate a surface-directed endosomal pathway, rather than generalized vesicle exocytosis. Values are mean ± SE of at least six experiments (*P < 0.05; **P < 0.005).

Antigen uptake in DCs is independent of PKC activity

Kinases of the PKC family play an important regulatory role in actin reorganization in many cell types, and actin polymerization is involved in the early phases of phagocytosis (51, 52). We therefore investigated whether PKCδ is involved in antigen presentation through mediating antigen uptake. Wild-type DCs were treated with LPS, PMA or ionomycin for 3 h at 37°C (or at 4°C as control), and fluorescein-conjugated OVA was added for one additional hour of culture at 37°C (or at 4°C as control). Uptake was determined by flow cytometry using a marker for DCs (CD11c–APC) and measuring OVA–FITC fluorescence within DCs (Fig. 5). Ionomycin treatment stimulated the uptake of OVA, whereas LPS pre-treatment significantly decreased internalization of OVA by DCs, consistent with induction of TLR-induced DC maturation (53). No significant difference was found in uptake of OVA by 3-h pre-treatment with PMA. When OVA–FITC was added simultaneously with LPS, PMA or ionomycin for 3 h, no difference was seen either (data not shown). We conclude that PKCδ does not promote Class II antigen presentation by stimulating antigen uptake by DCs.

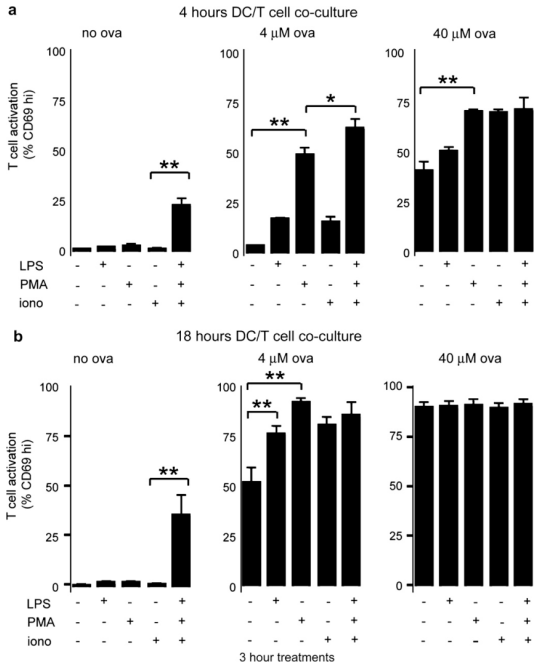


FIGURE 4. PKC activation potentiates antigen-specific T cell activation by antigen-laden DCs. (***a***) Wild-type DCs were treated for 4 h with 4 μM OVA alone or in combination with LPS, PMA or ionomycin, and then washed three times and co-cultured for 4 h with naive OTII T lymphocytes. Activation of OTII T cells was determined by measuring CD69 up-regulation by flow cytometry. (***b***) As in Fig. 5(***a***), DCs from wild-type mice were treated with 40 μM OVA alone, or in conjunction with LPS, PMA, ionomycin or combinations thereof. DCs were then washed three times and co-cultured for 4 h with naive OTII T lymphocytes. Activation of OTII T cells was determined by flow cytometry by analysis of up-regulation of the early activation marker CD69. Significant differences between the mean values of T cell activation by LPS-, iono- and PMA-treated DCs are indicated by asterisks (*P < 0.05; **P < 0.005).

Comparable level of peptide loading onto Class II MHC molecules

To determine whether PKC stimulates the rate of intracellular Class II MHC transport and peptide acquisition, we performed pulse–chase analysis and examined the SDS resistance of Class II MHC molecules as a measure of peptide loading. DCs were metabolically labeled with 35S-cysteine/methionine for 45 min and chased for 0, 1, 2, 3 and 6 h. Drug treatments were included during the pulse and chase. After the indicated chase points, cell lysates were prepared and correctly folded αß–peptide complexes were immunoprecipitated using a conformation-specific anti-Class II antibody, N22. Immunoprecipitates were resolved by 12.5% SDS–PAGE (Fig. 6). Immediately after pulse labeling, stable peptide–Class II dimers were not yet formed in either of the DC samples. At the chase time points analyzed, regardless of drug treatment, peptide-loaded stable Class II dimers were detected at 1 h and were most prominent at 3 and 6 h. Regardless of the presence of PMA, ionomycin or LPS, at 6 h of chase, full-length Ii was no longer immunoprecipitated, indicating that all labeled N22-immunoprecipiated Class II MHC had acquired peptide. We conclude that PKCδ does not affect Class II biosynthesis and peptide acquisition, as assessed by this method.

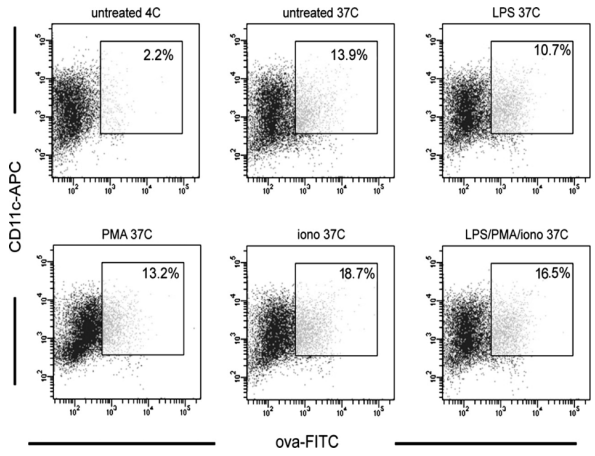


FIGURE 5. PKC activation does not stimulate antigen uptake by DCs. Wild-type DCs were incubated with LPS, PMA and ionomycin for 3 h, and then FITC-conjugated OVA (5 µg ml–1) was added for 1 h of uptake at 4 or 37°C. Background level of FITC–OVA in CD11c+ cells at 4°C was below 5% for all samples. FITC fluorescence was plotted against the DC marker CD11c, showing the percentage of CD11c-positive cells that endocytosed FITC–OVA. A representative experiment is shown out of a total of five independent experiments, each performed in triplicate.

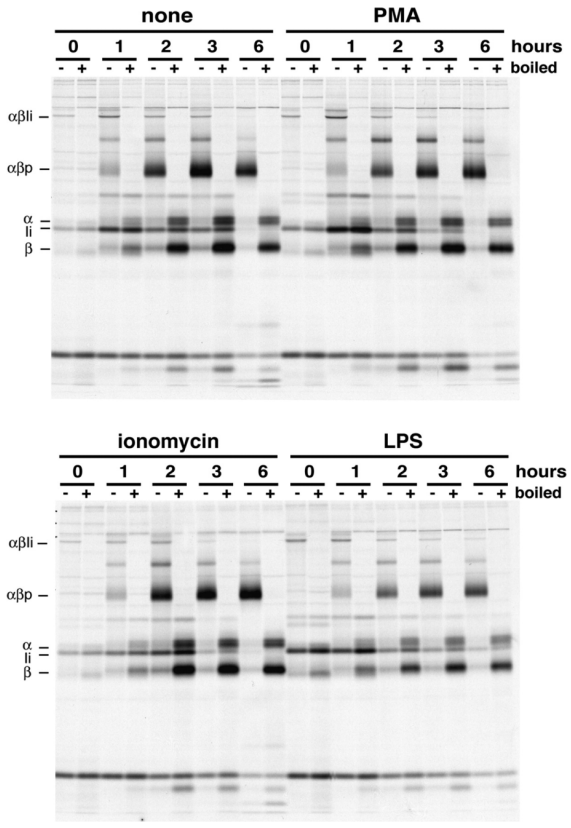


FIGURE 6. PKC activation does not stimulate the formation of peptide–Class II MHC complexes in DCs. Wild-type DCs were pulsed with 35S-cysteine/methionine for 45 min and chased for 0, 1, 2, 3 and 6 h in the presence of PMA, ionomycin or LPS. Class II MHC molecules were immunoprecipitated using conformation-specific antibody, N22, incubated for 5 min at room temperature or boiled and separated on SDS–PAGE. αßIi: Ii-loaded Class II; αßp: stable Class II dimers of αß–peptide; α: alpha chain of Class II; Ii: full-length Ii and ß: beta chain of Class I MHC. A representative experiment of two is shown.

Treatment with rottlerin reduces surface display of Class II MHC

If activation of PKCδ is indeed involved in activation-induced Class II surface display in DCs, conversely, an inhibitor of PKCδ, rottlerin, should inhibit expression of Class II MHC at the cell surface (54, 55). When used in high concentrations (20 μM), rottlerin can inhibit other protein kinases beside PKCδ (56). We therefore used rottlerin only in short-term treatments and at concentrations of 2–5 μM. DCs were pre-treated with rottlerin (2 μM, 30 min, 37°C) after which LPS or PMA was added or cells were left untreated for 3 h of culture. DCs were then stained on ice using antibodies to CD11c (–APC) and I-Ab (–PE) and analyzed by flow cytometry (Fig. 7a). Treatment with rottlerin resulted in a significant decrease in Class II surface display (decrease of 20%, $P < 0.01$ comparing rottlerin-treated with untreated DCs). DCs cultured in the presence of PMA showed an increase in Class II surface display that was inhibited by pre-treatment with rottlerin ($P < 0.01$, comparing PMA treatment with rottlerin–PMA treatment). Three hours of LPS treatment induced only a modest increase in Class II MHC, and rottlerin-mediated inhibition of LPS-dependent Class II up-regulation was not observed (Fig. 7a). This set of experiments differentiates between a LPS-dependent pathway and a PKC-mediated pathway of Class II surface export.

Treatment with rottlerin counteracts T cell activation by DCs

We next investigated the consequences of inhibition of PKCδ in functional antigen presentation assays. DCs were pulsed O/N with 4 μM OVA, washed and then pre-treated with rottlerin (5 μM, 30 min, 37°C) after which LPS or PMA was added for 3 h. DCs were washed and naive OTII T cells were added for 18 h of co-culture in the absence of stimuli or inhibitors (Fig. 7b). T cell activation was measured by up-regulation of CD25 (and CD69, data not shown), by flow cytometry. DCs from Class $II^{-/-}$ mice (29) were included in this assay as controls: OVA-treated Class $II^{-/-}$ DCs induced activation in <4% of OTII T cells, thus excluding the possibility that T cells are stimulated by carry over of PMA.

OVA-loaded wild-type DCs induced T cell activation in 38% of OTII T cells, either when DCs were left untreated or when LPS was included (Fig. 7b). In this experimental setup, the known stimulatory ability of LPS in DC function was not evident. Treatment of OVA-laden DCs with rottlerin (3.5 h, 37°C) resulted in a decrease in T cell activation to ~8% of T cells (Fig. 7b). Three-hour PMA treatment of OVA-loaded DCs resulted in activation of 90% of T cells, which was decreased to 20% by 30-min pre-treatment with rottlerin. Comparable data were obtained for CD69 up-regulation (data not shown). Taken together, these experiments confirm a stimulatory role for PKCδ in Class II MHC-mediated T cell activation.

Discussion

Bone marrow-derived DCs that express fusion proteins of Class II and GFP are now commonly used to study intracellular trafficking of peptide–Class II complexes (14, 15, 35, 36, 57). We here show that PKCδ is pivotal in surface-directed transport of peptide–Class II MHC to the cell surface. Stimulation of PKCδ promotes the surface expression of Class II MHC on the cell surface and Class II-mediated T cell activation. Conversely, inhibition of PKCδ counteracts the ability of DCs to activate naive Class II-restricted T lymphocytes.

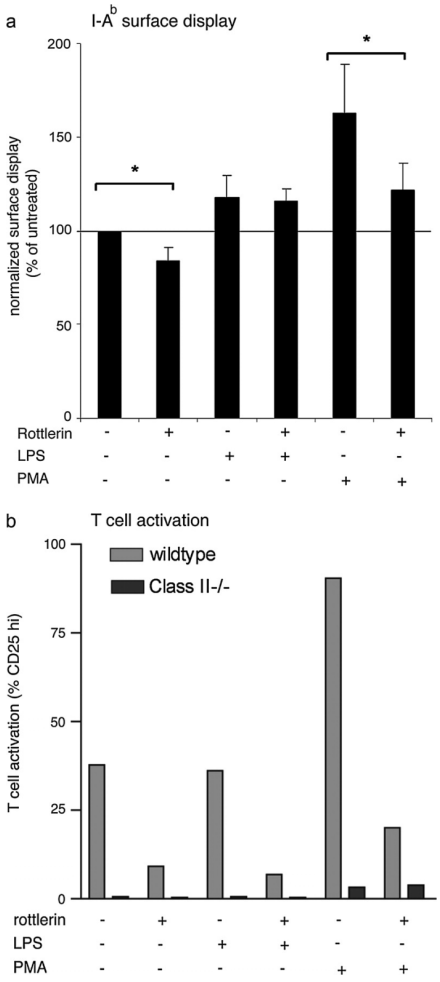


FIGURE 7. Selective inhibition of PKCδ counteracts surface display of Class II MHC in DCs and antigen-specific CD4 T cell activation. (***a***) DCs were pre-treated with rottlerin or left untreated, followed by LPS or PMA treatment for 3 h. Surface display of Class II MHC was measured by flow cytometry using PE-conjugated antibody to I-Ab. Rottlerin pre-treatment counteracts PMA-induced surface display of Class II MHC (*P < 0.01). Experiment shown is representative of four independent experiments. (***b***) DCs from wild-type and MHC Class II$^{-/-}$ mice were cultured in the presence of OVA O/N. DCs were washed twice, pre-treated with rottlerin or left untreated for 30 min, and then LPS or PMA was added where indicated (3 h, 37°C). DCs were washed three times and OTII T cells were added (18 h, 37°C). The effect of rottlerin, LPS and PMA on T cell activation was assayed by flow cytometry measuring up-regulation of CD25–APC on OTII T cells, gated on CD4–PE–Cy7 and Vß5–PE). Class II$^{-/-}$ DCs do not stimulate T cell activation under any of the conditions. Experiment shown is representative of three experiments that were performed.

A role for PKC in Class II MHC-mediated antigen presentation in mouse DCs had not been studied. Different PKC isoforms are involved in exocytosis and endosomal trafficking, as was demonstrated in various other cell types. In CTL, PMA/ionomycin treatment triggers the exocytosis of cytolytic granules (58, 59). Specifically, microtubule-organizing center polarization toward the CTL–target cell interphase was suggested to require PKC phosphorylation of microtubule-associated proteins or of kinesin or dynamin motors (59). Where Class II MHC antigen presentation is concerned, the role of PKC family kinases was investigated earlier in B cell tumor lines. Ligation of the BCR induces a signal transduction cascade via the Igα and Igß co-receptors, and results in a cascade of protein tyrosine kinase activation and in the production of second messengers such as IP3 and DAG (60). In B cells, protein tyrosine kinase pathways and DAG-induced PKC activation converge at the level of the mitogen-activated protein kinase pathway, leading to gene transcription of genes involved in B cell activation (61, 62). PKC activation by PMA treatment of B lymphoma cells delayed the degradation of Class II-associated Ii and

resulted in accumulation of Ii intermediates, equivalent to that seen in leupeptin-treated cells (63). Consequently, PMA treatment impaired the formation of SDS-stable dimers and peptide/Class II presentation in mouse B cells for some T cell epitopes. Thus, PKC activation through PMA treatment reduces peptide/Class II presentation in mouse B cells (63). Our study in mouse DCs now shows that in DCs, PKC activation supports Class II presentation by stimulating Class II MHC surface display rather than inhibiting peptide–Class II dimer formation as seen in mouse B cells. In human B cell lines, PKC directly phosphorylates the Class II-associated Ii, which enhanced the kinetics of Ii degradation and Class II presentation (64). Since Ii is not phosphorylated in murine APCs (63) as it is in humans (64), little effect of PKC activity is expected on Class II biosynthesis and peptide loading in mouse DCs. Our results (Fig. 6) are in line with this conclusion, as confirmed by the demonstration of unchanged kinetics of the formation of SDS-stable dimers.

From studies in multiple cell types it was deduced that the different PKC isoforms are not functionally redundant but mediate discrete biological effects (42, 65). It is therefore important to demonstrate a role for a particular PKC isoform in mediating a specific cell biological process. PKC family members are involved in DC lineage commitment from human hematopoietic progenitor cells or monocytes (66, 67). These differentiation processes require the presence of Ca2+ and DAG and are mediated conventional PKC family members PKCα and PKCß (68–70). In DCs, Class II MHC-mediated antigen presentation was strongly enhanced by phorbol ester treatment alone, which suggested the involvement of a novel rather than conventional PKC isoform. Mouse DCs only express one novel PKC isoform, PKCδ. To directly show that PKCδ is involved in antigen presentation via Class II MHC molecules, we initially considered experiments involving retrovirus-mediated RNAi knockdown of PKCδ in primary bone marrow-derived DCs, but this method proved problematic for technical reasons. We refrained from using PKCδ knock-out mice (71) as PKC isoforms are involved in monocyte or DC development (70, 72, 73). Instead, we made use of rottlerin, a selective inhibitor to PKCδ, which we administered to wild-type end-differentiated DCs. Pre-treatment with rottlerin followed by LPS or PMA stimulation strongly counteracted the ability of DCs to activate naive T cells. PKCδ activity is thus a possible point of interference for MHC Class II-dependent antigen presentation.

Previous studies have addressed a role for PKCδ in DC biology. Apoptosis of mature human DCs as mediated by ligation of Class II MHC is regulated by activation of PKCδ (74). The same laboratory showed that DC maturation induced the recruitment of PKCδ to Class II MHC-positive lipid microdomains in the plasma membrane (75). Culture of human and murine DCs in the presence of bryostatin-1, an anti-neoplastic agent, with calcium ionophore induces DC maturation (76). Bryostatin or PMA/calcium ionophore induced DC-mediated proliferation of allogenic T cells and antigen-specific T cells. Moreover, bryostatin-stimulated T cell activation by DCs was strongly inhibited by pre-incubation with the pan-PKC inhibitor bisindolylmaleimide I or with the PKCδ-selective inhibitor rottlerin (76). Thus, this study already suggested that PKCδ activation under some circumstances stimulate Class II MHC-mediated T cell activation, a mechanism that we investigated here in further detail.

Microbe-derived signals are potent adjuvants that stimulate antigen presentation by DCs (77). LPS can do so via several mechanisms. LPS treatment transiently enhances antigen uptake (78), following which endocytosis is down-regulated (53, 79). LPS treatment

stimulates the degradation of endocytosed antigen (80) and regulates endosomal sorting with the outcome that Class II MHC complexes relocate from internal vesicular membranes of multivesicular bodies to the H2-DM-containing delimiting membranes of Class II-positive endosomal compartments (37). The formation of tubular endosomes for surface-directed trafficking of peptide–Class II complexes is stimulated by the presence of LPS as well (15, 36, 81). Our data indicate that PKCδ-mediated stimulation of Class II antigen presentation is neither mediated by increased endocytosis nor by peptide loading. Others had shown that PKC activation in smooth muscle cells and neuronal growth cones can induce an outgrowth of microtubules from the perinuclear region into the cytoplasm, suggested to be involved in redistribution of vesicular trafficking along microtubules (82, 83). The trafficking of Class II MHC complexes from endosomal structures to the cell surface is also based on microtubule-dependent mechanisms (84, 85). Our findings that phorbol ester treatment induces robust Class II surface display and the appearance of dendritic extensions suggest that PKCδ activity stimulates Class II-mediated antigen presentation via induced surface-directed microtubule-based trafficking of Class II MHC complexes. In conclusion, activation of PKCδ potentiates DC function, by stimulating the display of peptide–Class II complexes at the DC plasma membrane.

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Abbreviations

APC, antigen-presenting cell
Ca2+, calcium ion
DAG, diacyl glycerol
DC, dendritic cell
GFP, green fluorescent protein
GM1, ganglioside M1
GM-CSF, granulocyte macrophage colony-stimulating factor
Ii, invariant chain
OVA, ovalbumin
PKC, protein kinase C
PMA, phorbol 12-myristate 13-acetate
PVDF, polyvinylidene difluoride
RT, reverse transcription
TBS, Tris-buffered saline
TfR, transferrin receptor
TLR, Toll-like receptor
TBS-T, Tris-buffered saline Tween 20

Notes

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