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Recent advances in processing and presentation of CD1 bound lipid antigens

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It is well established that different populations of $\alpha\beta$ T lymphocytes can recognize not only peptides in the context of MHC class I and class II molecules, but also foreign and self-lipids in association with CD1 proteins, which share structural similarities with MHC class I molecules. CD1 molecules are comprised of five isoforms, known as group 1 (CD1a, b, c, e) and group 2 (CD1d) CD1, presenting lipid antigens to conventional T lymphocytes or innate-like T cells bearing an invariant T cell receptor (TCR) and known as invariant NKT (iNKT) cells. During the last couple of years, several papers have been published describing important aspects of the mechanisms controlling the processing and presentation of endogenous and exogenous CD1 lipid antigens, which will be the main focus of this review.

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CD1 trafficking and lipid antigen uptake

Lipid antigen uptake by CD1 positive cells

Because lipids are water-insoluble molecules that are either embedded in lipid bilayers or associated with plasma lipoproteins in the extracellular milieu, their bio-physical properties require a number of mechanisms controlling their uptake into cells, extraction from membranes and loading onto CD1 molecules. It has been demonstrated that antigenic lipids presented by CD1b, CD1c and CD1d molecules can be incorporated into VLDL and taken up by LDL receptors via ApoE-mediated recognition [1,2^{••}]. LDL-receptor mediated uptake is very efficient, as demonstrated by the observation that iNKT cell activation in response to intravenously administered iNKT cell agonists was significantly reduced in ApoE-deficient mice [2^{••}]. Furthermore, den-

dritic cells (DC) pulsed with iNKT cell agonists in ApoE depleted human serum had a lower iNKT cell stimulatory capacity [2^{••}]. In addition to LDL receptors, several other cell surface receptors (including DC-SIGN, the mannose-receptor and Langerin, as reviewed by Barral and Brenner [3]) have been shown to bind microbial lipids and deliver them to the endolysosomal compartments for loading onto different CD1 molecules. More recently, it has been shown that B cell receptor mediated uptake of iNKT cell agonists either directly conjugated to B cell antigens [4^{••}] or coupled to particles coated with B cell antigens [5^{••}] can selectively induce maturation and differentiation of antigen-specific B cells. Coupling iNKT cell agonists to particles coated with B cell antigens overcomes LDL-mediated antigen uptake [1], hence abolishing non-specific iNKT cell dependent B cell activation, while jump-starting antigen-specific B cell differentiation and antibody secretion.

After being taken up by different mechanisms, lipid antigens traffic through different intracellular compartments depending on their bio-physical properties. Specifically, lipid antigens with short or unsaturated alkyl chains traffic to the endocytic recycling compartment, while lipids with long saturated tails are delivered to late endocytic compartments, thus intersecting with different CD1 molecules [6]. In addition, certain lipids can be directly loaded onto CD1 molecules at the cell surface, for example the analogue of the iNKT cell agonist α -GalactosylCeramide (α -GalCer) C20:2 on CD1d [7] and mycobacterial glucose monomycolate (GMM) that has short (C₃₂) acyl chains on CD1b [8]. The combination of the short lipid tail and saturation of the acyl chain, which facilitates positioning of the C20:2 in the A' CD1d channel [9], may account for the ability of these two lipids to bind to surface CD1 molecules, without any need for accessory proteins and/or low pH.

CD1 lysosomal trafficking

Similarly to MHC class II molecules, CD1 molecules survey the endocytic pathway for incoming exogenous antigens, which are capable of displacing self-lipids (such as CD1d bound phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine [10[•],11[•]]) acquired in the secretory pathway. While MHC class II molecules may reach the endocytic compartment directly from the trans-Golgi-network (TGN), CD1 molecules first traffic to the cell surface and are subsequently internalized through mechanisms that depend on the characteristics of the different CD1

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molecules' cytoplasmic tails [3]. CD1a molecules, which lack a tyrosine-based internalization motif, are internalized to the early endosomes through a clathrin/dynamin-independent mechanism and recycle back to the cell surface through a mechanism that is dependent on the small GTPases Rab22 and ARF-6 [12]. Conversely, CD1b, CD1c and CD1d have a tyrosine-based motif in their cytoplasmic tail and are internalized through clathrin-coated pits via the adaptor protein AP-2 and further directed along the endolysosomal compartments depending on their ability to bind AP-3 [3]. The ability of CD1d molecules to traffic from the cell surface to the lysosomal compartment is important for the acquisition of self-antigens and subsequent activation of murine, but not human, self-reactive iNKT cells [3,13[•],14]. However, this conclusion is based on a limited number of human iNKT cell clones [13[•],14], and detailed analysis of the antigenicity of lipids selectively eluted from lysosomal human CD1d molecules, using protocols described by Yuan *et al.* [11[•]], may reveal a higher degree of complexity.

CD1d molecules can also gain access to the endocytic compartment directly from the TGN by association with MHC class II-invariant chain complexes, which may regulate human CD1d-mediated presentation of extracellular antigens, but not autoreactivity of iNKT cell clones [14].

It has been shown that genetic defects leading to lysosomal accumulation of lipids (such as glycosphingolipid storage disorders and Niemann-Pick C1 deficiency) affect the optimal presentation of both exogenous and endogenous iNKT cell ligands, resulting in lower frequencies of iNKT cells in mice [15,16^{••}]. These results are consistent with the possibility that abnormal accumulation of lipids in lysosomes prevents optimal loading of iNKT cell agonists onto CD1d molecules trafficking through lysosomes. It is of interest, however, that patients with a defective α -Galactosidase A (i.e. Fabry's patients) were shown to have normal number of iNKT cells in peripheral blood lymphocytes [17], a result which is consistent with the differences described above between the trafficking of human and mouse CD1d molecules [3,13[•],14].

CD1 surface distribution and stability

CD1 molecules have been shown to be associated with plasma membrane detergent-resistant membrane microdomains (hereafter referred to as rafts), which are domains enriched in cholesterol and lipids, influencing the localisation and hence the function of antigen presenting molecules and the assembly of the immunological synapse. CD1a molecules reside in rafts, where they can associate with invariant chain thereby enhancing the efficiency of presentation of CD1a-sulfatide complexes to lipid-specific T cells [12,18].

Murine CD1d molecules are also associated with lipid rafts, and this association is important for efficient iNKT cell activation, particularly at low antigen concentrations [19,20^{••}]. Several investigators have reported that analogues of α -GalCer — differing either in the polar head or in the length and saturation of the sphingosine and acyl chains — elicit biologically different responses, possibly because of their presentation by different subsets of antigen presenting cells and/or different stability of the CD1d-lipid complexes as reviewed by Cerundolo *et al.* [21]. Recently, the ability of the CD1d-lipid complexes to reside within detergent-resistant membrane microdomains has been shown to correlate with the biological outcome of CD1d-lipid/iNKT cell recognition [20^{••}]. Ligands inducing a strong Th1-cytokine response, such as α -GalCer, preferentially load onto CD1d molecules in the endolysosomal compartment and are raft associated. Conversely, Th2-type cytokine biasing ligands, such as C20:2, are loaded rapidly and directly at the cell surface onto a pool of CD1d molecules that is non-raft associated [20^{••}]. Similar kinetics of loading have been observed for human CD1d molecules, although association with lipid rafts has not been investigated [22]. In addition, the stability of human CD1d-lipid complexes is controlled by the length of the sphingosine chain occupying the CD1d F' channel [23[•]], while filling of the CD1d A' channel by self-lipids can compensate for shorter acyl chains and stabilize the CD1d-lipid complex [24].

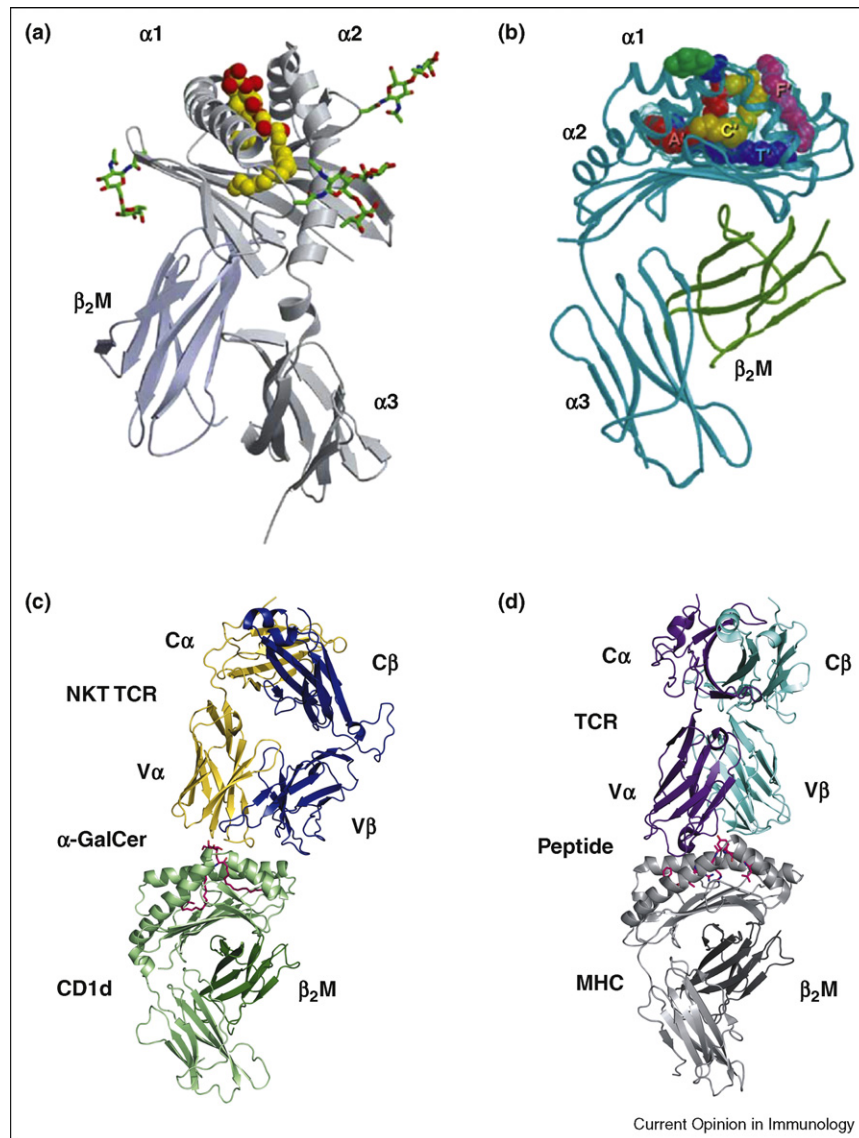
Finally, although the trafficking of CD1 and MHC class II molecules is similar, their cellular distribution is regulated differently during DC maturation [3]. MHC class II molecules are rapidly translocated to the cell surface during DC maturation, because of the downmodulation of the ubiquitin ligase March1 [25]. Although this has not been observed for CD1 molecules, the possibility remains that a member of the March family of ubiquitin ligases might also modulate CD1 levels of expression during DC maturation. Consistent with this possibility are the findings that: firstly, the ubiquitin ligase MIR proteins of the Kaposi sarcoma herpesvirus have been shown to down-regulate CD1d cell surface expression [26] and secondly, ubiquitination also regulates CD1e trafficking from the TGN to the endolysosome [27] (Box 1).

Lipid loading onto CD1 molecules

Lipid transfer molecules

The crystal structures of several CD1 molecules have revealed a common binding mode of lipids, whereby the hydrophobic lipid chains are buried within the CD1 groove and the polar moieties are exposed for TCR recognition [9,15,24] (Figure 1 and Box 2). Because of the deep and narrow nature of the CD1 groove, an important question remains as to how lipids are efficiently loaded onto CD1 molecules; recent studies have highlighted the role of several lipid transfer proteins in this process.

Figure 1



Crystal structures of the CD1 family of antigen presenting molecules. **(a)** The crystal structure of CD1a was determined to 2.15 Å resolution in the presence of the self-antigen sulfatide [48]. Shown is a front view of CD1a with light grey ribbons indicating CD1a $\alpha 1$ – $\alpha 3$ domains and $\beta_2 M$. In green are three N-linked sugar moieties while yellow spheres are ligand (sulfatide) carbon atoms. **(b)** CD1b was crystallized in complex with both phosphatidylinositol (PI) and a ganglioside GM2 at 2.3 and 2.8 Å, respectively [66]. Shown is the CD1b structure ($\alpha 1$ – $\alpha 3$ domains in aqua and $\beta_2 M$ in green) with bound PI (alkyl chains in yellow and red, phosphate in lilac, inositol in green) and detergent molecules (pink and violet). Ligands within the binding channels are shown as A' (red), C' (yellow), F' (pink) and T' (violet). **(c)** The first co-crystal of a CD1 family member with a TCR was generated for human CD1d– α -GalCer in complex with the iNKT TCR to 3.2 Å, revealing a novel binding mode that had not previously been seen with classical MHC class I-restricted and II-restricted TCR [62**]. As a comparison, in panel **(d)** is shown the HLA-B8–peptide–TCR (LC13) complex originally described by [67]. Human CD1d is shown in green with α -GalCer in magenta. The iNKT TCR α -chain and β -chains are shown in yellow and blue, respectively. The TCR–pMHC-I complex is shown as MHC in grey, peptide in magenta and TCR α -chain and β -chain, purple and cyan, respectively. A higher resolution (2.5 Å) structure of the iNKT TCR/CD1d complex has recently been described [63*]. Permission to reproduce the image was obtained from Elsevier (licence n 2303031406995); Nature (2303030760948); Nature Immunology (2302031149701 and 2302021035727).

The endoplasmic reticulum (ER) resident chaperone microsomal triglyceride transfer protein (MTP) has been shown to regulate the loading of self-lipids onto CD1 molecules in the ER [28*]. MTP, which is able to transfer lipids between vesicles, associates with CD1d in the ER

and directly transfers lipids (such as phosphatidylethanolamine) to CD1d [28*,29]. Conditional deletion of MTP reduces surface CD1d expression and abrogates presentation of α -GalCer to iNKT cells in hepatocytes, epithelial cells and DC [28*,29]. A splice variant of murine

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MTP also regulates development of iNKT cells, as shown in fetal thymic organ cultures [30]. Unexpectedly, it has been shown that despite its ER localization, MTP also regulates presentation of lipids requiring lysosomal processing and CD1d trafficking between the lysosome and the plasma membrane [31]. MTP also regulates endogenous and exogenous presentation of lipids by CD1a, b and c, which requires lysosomal trafficking [32]. However, it remains to be determined how this ER-lipid transfer protein can affect CD1 antigen presentation in the lysosomal compartment.

Once delivered to late endocytic and lysosomal compartments, some complex lipids require processing by resident lipases and glycosidases, such as α -Galactosidase A, mannosidase and hexosaminidase [33–35]. Lipid processing occurs in intraluminal vesicles and is assisted by sphingolipid activator proteins (such as saposins, the GM2-activator protein and the Niemann-Pick C2 protein (NPC2)), which have been shown to solubilize membrane-embedded polar lipids and/or present them to lysosomal enzymes [36].

NPC2. NPC2 is a small, soluble lysosomal protein important for cholesterol and sphingolipid transport in the lysosomes [36]. It has been shown that NPC2 dimers can also load gangliosides, such as GM3 and iGb3 to CD1d molecules [37].

Saposins. Saposins are a family of lipid transfer proteins involved in glycosphingolipid metabolism [36]. They are ubiquitously expressed and synthesized as the precursor protein prosaposin, which is targeted to the lysosome for cleavage into four small proteins (A–D) by any of the three following receptors, mannose-6-phosphate receptors, low-density lipoprotein receptor related-protein (LPR) or mannose-receptors [36]. Saposins share lipid-

Box 1 Evasion of CD1 antigen recognition

Lipid-specific T cells contribute to innate and adaptive immune responses to pathogens [54]. Pathogens have therefore developed evasion mechanisms from CD1 recognition, in a manner similar to that observed for class I presentation, as reviewed by Van Kaer [55]. A number of viruses have adopted different mechanisms to evade CD1 presentation by reducing expression of surface CD1: Herpes simplex virus-1, Human cytomegalovirus [56], Human immunodeficiency virus-1, Vaccinia and Kaposi sarcoma-associated herpes virus. Blockade of endosomal acidification and prevention of phagolysosome fusion are invasion mechanisms used by some microbes, allowing for escape from both CD1b and MHC class II antigen recognition simultaneously. *Mycobacterium tuberculosis* (MTB) exploits the p38 pathway to interfere with CD1 expression in differentiating DC [57], although on mature DC MTB interferes with peptide but not lipid antigen presentation [58]. However, cytokine and microbial signals may also upregulate CD1 expression [59] and modulate biosynthesis of potentially antigenic endogenous lipids [51,52]. Therefore the final effect of any pathogen on lipid antigen presentation will depend on the fine balance between positive and negative signals.

Box 2 Recognition of ligands by the semi-invariant TCR

Crystal structures of iNKT TCR/CD1d-ligand complexes together with mutagenesis studies have provided significant information about the molecular mechanisms controlling recognition of glycolipid ligands by human and murine iNKT cells [60,61*,62**]. Ligand recognition by iNKT cells is dramatically different from any previously characterized $\alpha\beta$ TCR/MHC-peptide complexes in terms of both the orientation of the TCR on the complex, and of the mode of ligand recognition. The recent high-resolution (2.5 Å) crystal structure of the human V α 24 iNKT TCR/ α -GalCer-CD1d complex confirmed that recognition of the sugar head group is mediated predominantly by the invariant TCR α -chain loops while the limited repertoire of β -chains selected interacts mainly with the exposed surface of CD1d [62**,63*]. The crystal structure of the murine iNKT TCR containing the V β 8.2 chain in complex with α -GalCer-mCD1d revealed that, as in humans, the murine V β 8.2 chain interacted mainly with the α 1 helix of mCD1d via the CDR2 β loop [63*]. The galactose head group of α -GalCer underwent a relatively small shift on binding the TCR, indicating a rigid interaction between the iNKT TCR/ α -GalCer-CD1d complex. The interaction of murine CD1d with the V β 7-containing iNKT TCR was similar [63*], although the different β -chain modified the position of the invariant α -chain and consequently influenced its interaction with the ligand head group.

Two recent studies have demonstrated that the iNKT TCR β -chain plays a greater role in ligand recognition than was previously appreciated. Recognition of different iNKT cell ligands including α -GalCer, α -Galactosyldiacylglycerol (α -GalDAG, from *Sphingomonas*), isoglobotrihexosylceramide (iGb3) and a number of derivatives of these ligands was critically influenced by β -chain usage, correlating with the affinity of the iNKT TCR for CD1d-lipid complexes [64*,65*]. Particular residues in the CDR2 β loops were shown to be important for the recognition of α -GalCer-CD1d, while residues in other TCR loops in both α -chains and β -chains had more of an effect on recognition of more complex ligands such as iGb3. CDR2 β residues were also found to be important for the positive selection of iNKT cells *in vivo*, demonstrating that the sequence of the CDR2 β is important for determining whether the TCR is functional.

These three papers suggest that despite the semi-invariant nature of the recognition unit, antigen recognition by iNKT cells is mediated by the limited repertoire of β -chains with different CDR loops. Both directly, and indirectly through interaction with the invariant α -chain, the iNKT TCR β -chains may allow recognition of low affinity agonists. Despite the usage of a germ-line encoded invariant α -chain there is a degree of inherent flexibility, allowing iNKT cells to recognize a relatively broad spectrum of different ligands with different affinities bound to the non-polymorphic CD1d molecules.

binding properties and membrane-perturbing activity, although the individual saposins display substrate specificity. By disrupting membrane integrity in a pH-dependent manner, saposins render glycolipids more accessible to hydrolases. The crystal structure of saposin B revealed a shell-like homodimer, capable of binding lipids in an internal hydrophobic cavity [38]. The importance of saposins in glycosphingolipid catabolism is underscored by the severe, life-threatening lipid storage disorders resulting from saposin deficiency [36].

Recently, the involvement of saposins in CD1 antigen presentation has been demonstrated. Saposin B is important for loading lipids onto CD1d molecules, and is

particularly efficient at acidic pH [39,40[•],41[•]]. Prosaposin deficient mice are unable to positively select iNKT cells, as a consequence of altered loading of CD1d molecules with selecting endogenous lipids [40[•]]. However, auto-reactivity of a limited number of human iNKT cell clones towards prosaposin deficient fibroblasts is preserved [39,41[•]], indicating different requirements for loading self-antigens on human and murine CD1d molecules. Saposin C was shown to be important in loading mycobacterial antigens to CD1b molecules [42]. Although the molecular mechanisms by which saposins facilitate loading of lipids on CD1 molecules are still to be defined, it was shown that they form complexes with murine CD1d [40[•]] and CD1b molecules [42].

GM2-activator protein. GM2-activator protein (AP) is a small lysosomal lipid transfer protein essential for the hydrolysis of the ganglioside GM2 to GM3 by hexosaminidase A. GM2-AP was shown to transfer α -GalCer and iGb3 to murine CD1d [40[•]] and — unlike saposin B — it was also shown to be capable of unloading lipids from CD1d molecules [40[•]].

CD1e. Unlike other lipid transfer proteins, expression of CD1e molecules is restricted to immune cells, consistent with their specific role in lipid antigen presentation. CD1e molecules never reach the cell surface, as they accumulate in the TGN of immature DC and move to the late endosomes following DC maturation [43]. Ubiquitination of the cytoplasmic portion of CD1e controls exits from the Golgi and transport to the lysosomes [27]. In the lysosome, CD1e molecules are cleaved to a soluble form that facilitates processing of complex glycolipid antigens [43]. For example, it has been shown that CD1e facilitates the generation of the antigenic PIM2 from mycobacterial PIM6 by the lysosomal enzyme α -mannosidase [33]. Interestingly, CD1e is the most polymorphic of the CD1 genes and different CD1e alleles vary in their ability to assist lipid antigen presentation by CD1b molecules [44]. Like group 1 CD1 molecules (CD1a, b and c), CD1e is not expressed in mice.

The role of pH in modulating lipid loading onto CD1 molecules

Antigen loading onto CD1 molecules is also enhanced by low pH (4.5–5.5) of the late endosomes or lysosomes, and this is observed particularly for lipids with long alkyl chains binding to CD1b, such as C₈₀ GMM, which cannot be loaded at the cell surface of antigen presenting cells [8]. The acidic pH promotes lipid binding to CD1b molecules by relaxing the conformation of CD1b– β 2m complexes. In an elegant set of experiments, Moody and co-workers demonstrated that mutagenesis of critical residues near the CD1b portal (which is the likely site of antigen entry into the groove) disrupts inter-domain tethers and improves presentation of C₈₀ GMM to antigen-specific T cells [45^{••}]. Similar results were observed

following protonation of these residues as the pH of the lysosomes decreased. Disruption of the ionic interactions and inter-domain tethers presumably increased the flexibility or the volume of the A' pocket of CD1b, facilitating entry, exit and folding of the lipid around the groove [45^{••}]. A similar mechanism may control lipid loading onto CD1d molecules, as the crystal structure of 'empty' human CD1d molecules revealed a more open conformation of the binding groove as compared with α -GalCer-loaded CD1d molecules [9]. In addition, lysosomal pH may differentially affect the stability of CD1d–lipid complexes depending on the biochemical properties of the binding lipids: it has recently been shown that α -GalCer analogues with short and polyunsaturated chains are rapidly dissociated and actively displaced upon recycling to the lysosomes [46].

Conversely, CD1a molecules are loaded within the neutral compartment of early endosomes, without help from known lipid transfer proteins and can be loaded or stabilized even at the cell surface, binding serum glycosphingolipids and phospholipids [47]. This property might be influenced by the structural characteristics of CD1a molecules, which present a relatively large and open F' channel [48].

Protein antigen degradation in endosomal compartments is highly regulated in DC, which maintain a near neutral phagosomal pH as opposed to macrophages, and this has been shown to favor peptide retention and cross-presentation [49]. In addition, DC maturation induces enhanced lysosomal acidification and antigen proteolysis, facilitating efficient formation of peptide–MHC class II complexes [49]. Whether similar mechanisms modulate the generation of CD1–lipid complexes as well is not clear, although presentation of lipid antigens is generally unaffected by DC maturation [3].

Novel insights into the role of iNKT cells during inflammation

Recognition of a limited range of self-lipid(s) by iNKT cells results in iNKT cell activation and CD40 ligand (CD40L)-dependent DC and B cell maturation [21]. These results support a model in which an important function of iNKT cells is to jump-start the expansion of antigen-specific immune responses during inflammatory conditions by providing a rapidly available source of CD40L help. Consistent with this hypothesis is the notion that iNKT cells, unlike conventional $\alpha\beta$ T cells, have a frequency which is orders of magnitude higher than naive MHC class I-restricted and class II-restricted T cells [50], and can therefore be rapidly mobilized during infections and cancer. Recent results have extended this concept by demonstrating that iNKT cells can also assist the expansion of antigen-specific immune responses by abolishing the suppressive activity of myeloid derived suppressor cells (MDSC) expanded during viral infections [16^{••}].

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The notion that iNKT cells play a role during inflammatory conditions by facilitating antigen-specific immune responses is underscored by recent findings linking inflammation with iNKT cell activation. In addition to the enhanced secretion of co-stimulatory cytokines (such as IL-12 and IL-18) [3], TLR-dependent signalling events in DC modulate enzymes involved in the lipid biosynthetic pathway, resulting in iNKT cell activation and enhanced recognition of matured DC [51,52]. These results support the hypothesis that TLR-dependent modulation of the lipid repertoire presented by CD1d molecules upregulates a panel of self-lipids, leading to iNKT cell activation. The observation that inhibitors of the glycosphingolipid (GSL) synthetic pathway can reduce, but not abolish, iNKT cell activation [51,52], is compatible with the possibility that lipids other than GSLs also contribute to the pool of self-lipids capable of activating iNKT cells. Consistent with this possibility, recent results have demonstrated that lysophosphatidylcholine (LPC), generated during an inflammatory response, can stimulate iNKT cells and non-invariant CD1d restricted NKT cells, providing possible mechanistic insights into the activation of iNKT cells during inflammatory processes [13*,53].

As the identity of novel lipids recognized by lipid-specific cells during inflammation is being revealed by state-of-the-art mass-spectrometry analysis [10*,11*] and increasing numbers of CD1 binding lipids are identified from pathogenic and non-pathogenic microbes [54], further studies will be required to understand the molecular requirements for loading and processing of these new antigens.

While this manuscript was under review, two recent papers have further investigated the molecular mechanisms of lipid antigen recognition by iNKT cells. Wang *et al.* [68] show that the number and position of the cis-unsaturation in the fatty acids of the diacylglycerol backbone of *Borrelia burgdorferi* antigens influences the glycolipid orientation in the CD1d binding groove, and hence its antigenicity. In addition, they identify a single amino acid residue in CD1d that is partly responsible for the different responses of human and mouse iNKT cells to the same glycolipid antigen.

Sullivan *et al.* [69] provide evidence that the pharmacokinetic properties of glycolipids, in addition to binding to CD1d and affinity for the iNKT TCR, are important determinants of the ability of glycolipids to elicit Th1 or Th2 responses *in vivo*.

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