Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation

Mariolina Salio*[†], Anneliese O. Speak[‡], Dawn Shepherd*, Paolo Polzella*, Petr A. Illarionov[§], Natacha Veerapen[§], Gurdyal S. Besra[§], Frances M. Platt[‡], and Vincenzo Cerundolo*

*Tumor Immunology Group, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS, United Kingdom; [‡]Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom; and [§]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

Invariant natural killer T (iNKT) cells are a subset of nonconventional T cells recognizing endogenous and/or exogenous glycolipid antigens in the context of CD1d molecules. It remains unclear whether innate stimuli can modify the profile of endogenous lipids recognized by iNKT cells on the surface of antigen-presenting cells (APCs). We report that activation of human APCs by Toll-like receptor ligands (TLR-L) modulates the lipid biosynthetic pathway, resulting in enhanced recognition of CD1d-associated lipids by iNKT cells, as defined by IFN-γ secretion. APC-derived soluble factors further increase CD1d-restricted iNKT cell activation. Finally, using soluble tetrameric iNKT T cell receptors (TCR) as a staining reagent, we demonstrate specific up-regulation of CD1d-bound ligand(s) on TLR-mediated APC maturation. The ability of innate stimuli to modulate the lipid profile of APCs resulting in iNKT cell activation and APC maturation underscores the role of iNKT cells in assisting priming of antigen-specific immune responses.

APC | glycosphingolipids | innate immunity | NKT cells

Natural killer T (NKT) cells are a subset of nonconventional T cells recognizing lipid antigens presented by the nonpolymorphic CD1d molecule (1). The majority of human and murine NKT cells, referred to as invariant NKT cells (iNKT), display a highly restricted TCR repertoire, consisting of a semiinvariant TCR α chain (Vα24Jα18 in humans and Vα14Jα18 in mice) and a more variable β chain (Vβ11 in humans and Vβ2, Vβ7, and Vβ8 in mice). iNKT cells play a role in the control of bacterial, parasitic, viral infections, and natural immunosurveillance (2). A number of synthetic glycolipids have been shown to activate iNKT cells. Although the best-characterized agonist to date is α-galactosylceramide (α-GalCer) (3), it has recently been shown that iNKT cells also recognize bacterial glycosphingolipids (GSLs), glycolipids, and cellular phospholipids (4–9).

CD1d molecules have a restricted pattern of expression limited to APCs, including dendritic cells (DCs), B cells, macrophages, cortical thymocytes, and some epithelial cells (2). Professional APCs, such as DCs, are essential players in eliciting adaptive immune responses. DCs reside in peripheral tissues and can be activated by inflammatory stimuli, direct recognition of microbial components via pattern recognition receptors [such as Toll-like receptors (TLR)] or T cell-derived signals (10). Activation of iNKT cells via exogenous CD1d ligands, such as α-GalCer, induces the maturation of DCs and B cells, resulting in powerful adjuvant activity, leading to enhanced CD4, CD8, and antibody responses to coinjected protein antigens (11, 12). In this setting, iNKT cell mediated recognition of bacterial lipids may serve as a "surrogate pattern recognition receptor" for pathogens lacking TLR-ligands (5–7). For some pathogens, however, recognition of exogenous microbial antigens is not required, and iNKT cells are activated after amplification of weak self-reactivity by IL-12 released from DCs upon TLR signaling (6, 13). A TCR-independent mode of bacterial recognition has also been recently reported (14).

Recognition of endogenous antigens is essential for iNKT cell development and selection by double positive cortical thymocytes (1). The identity of the selecting lipid ligand(s) remains unknown. Evidence from a β-glucosylceramide synthase mutant cell line unable to stimulate Vα14 iNKT cells has suggested that the natural ligand(s) is a GSL (15). More recently, it has been proposed that the natural selecting ligand presented by cortical thymocytes and LPS matured DCs is the neutral GSL isoglobotrihexosylceramide (iGb3) (6, 8). However, there is no biochemical evidence for the presence of iGb3 in mouse or human thymus or DCs (16), and iGb3 synthase deficient mice have normal number and subsets of iNKT cells (17). Further studies are therefore required to identify other lipid species responsible for iNKT thymic selection and/or peripheral expansion.

Because it has been shown that iNKT cells can rapidly license DCs for T cell priming via CD40L signaling (11, 12), it is important to assess whether TLR-dependent DC maturation also results in the up-regulation of natural lipid ligands that can be directly recognized by iNKT cells via CD1d. To address this question, we set up an experimental system to study the relative contribution of soluble factors versus self-lipid antigens in eliciting CD1d-restricted human iNKT cells activation after stimulation of APCs with a panel of TLR ligands.

Results

DC Activation with TLR Ligands Enhances NKT Cell Basal Autoreactivity. Immature human DCs were incubated with a panel of TLR ligands at different concentrations, and DC activation was studied in the presence or absence of iNKT cells. Stimulation through TLR-2 (Pam3Cys), TLR-3 [polyinosinic:polycytidylic acid (poly I:C)], TLR-4 (LPS), TLR-5 (flagellin), and TLR-8 (ssRNA-40 and R-848) led to different degrees of DC activation, as evidenced by secretion of IL-12 (Fig. 1A). In most cases, the presence of iNKT cells resulted in enhanced DC activation. In addition, coculture of TLR-L matured DCs and iNKT cells resulted in iNKT cell activation, as shown by IFN-γ secretion (Fig. 1B) and up-regulation of activation markers (data not shown). Subsequent experiments were performed with LPS and R-848, prototype ligands of a cell surface (TLR-4) and an endosomal (TLR-8) receptor and of two different signaling pathways. Both TLR-4 and TLR-8 signal through the adaptor molecule MyD88, whereas TLR-4 also utilizes the TRIF adaptor molecule (18). Of note, R-848 also signals through the endosomal receptor TLR-7, however, human monocyte-derived DCs only express TLR-8 (19).
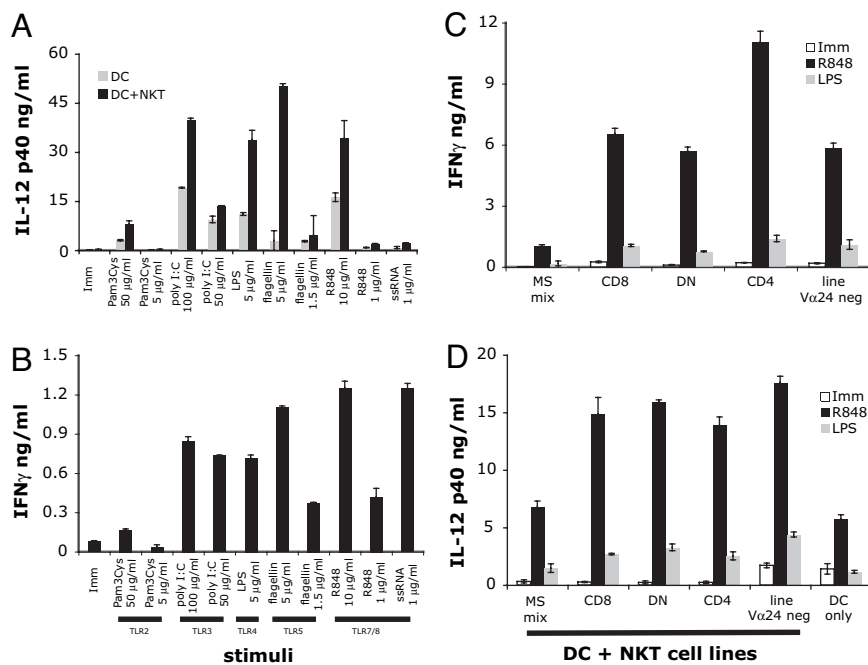
Fig. 1. DC maturation by TLR-L enhances *i*NKT cells basal recognition. (*A* and *B*) Human DCs were left untreated (Imm) or matured with a panel of TLR-L at the indicated concentrations in the presence or absence of the *i*NKT line MS mix. The means of triplicate values and standard deviation of IL-12 p40 (*A*) and IFN-γ (*B*) of one ELISA experiment representative of three are shown. IL-12 p70 was detected only in samples stimulated with R-848 (data not shown). Soluble factors, like type I IFN, may influence the overall secretion of IL-12 by DC, hence the differences between different TLR-L. (*C* and *D*) Activation of different subsets of NKT cells by TLR-L-matured DC. Four *i*NKT cell lines and a noninvariant Vα24-negative NKT cell line, were incubated with DCs and R-848 or LPS. The means of triplicate values and standard deviation of IFN-γ (*C*) and IL-12 p40 (*D*) of one ELISA experiment representative of three are shown. DCs from different donors were used in *A* and *B* and *C* and *D*, hence the variability in the absolute amounts of IFN-γ released by *i*NKT cells.

Recognition of R-848, and to a certain extent of LPS-matured DCs, was not restricted to any *i*NKT cell subset but could be observed with a panel of different *i*NKT lines and clones (CD4+, CD8+, and DN), including a Vα24-negative Vβ11+ NKT line (Fig. 1 *C* and *D*). In all experiments, *i*NKT cell activation by TLR-L stimulated DCs resulted in IFN-γ but not IL-4 secretion (data not shown).

Requirements for *i*NKT Cell Activation by TLR-L Matured DCs. To investigate the relative contribution of DC metabolic processes and soluble factors released during DC maturation, we compared *i*NKT cell recognition of live versus fixed DCs (Fig. 2*A*). DC fixation, before exposure to either LPS or R-848, prevented *i*NKT cell activation, demonstrating the requirement for an active APC metabolic process. These data also rule out a direct effect of the two TLR ligands on *i*NKT cells. By contrast, when DCs were fixed after overnight maturation with the TLR-8 ligand R-848, we observed a 30–50% reduction in their *i*NKT cell stimulatory capacity compared with live DCs.

To establish whether the *i*NKT cell stimulatory effect on TLR-L activation was mediated by CD1d-restricted antigen presentation, we demonstrated that, in the presence of anti-CD1d blocking antibodies, *i*NKT cells recognition of R-848 matured DCs was reduced by 50% (Fig. 2*B*). The reduction of *i*NKT cell stimulation after fixation of R-848 matured DCs (Fig. 2*A*) also suggested a contribution of DC-derived soluble factors. Indeed, we confirmed the role of IL-12 in amplifying *i*NKT cell autoreactivity (13) by demonstrating that anti-IL-12 antibodies resulted in 50% reduction of DC recognition. The combination of anti-CD1d and anti-IL-12 antibodies abrogated DC recognition (Fig. 2*B*).

Soluble Factors Amplify *i*NKT Cell Basal Autoreactivity. To further investigate the role of soluble factors in mediating *i*NKT cell

activation, we incubated *i*NKT cells with supernatants from immature or matured DCs, in the presence of suboptimal amounts of plate bound anti-CD3 antibodies, to mimic weak TCR stimulation. Anti-CD3 stimulation alone resulted in minimal *i*NKT cell activation, which was not enhanced by supernatants from immature or LPS-matured DCs, most likely because of a lack of bioactive IL-12 [supporting information (SI) Fig. 6*A* and data not shown]. Conversely, supernatants from R-848 matured DCs markedly enhanced *i*NKT cell activation in the presence of anti-CD3 stimulation (8-fold) and induced minimal activation, even in the absence of TCR triggering.

We excluded direct stimulation of *i*NKT cells via TLR-7/8 or TLR-4, because neither R-848 nor LPS were capable of enhancing anti-CD3 stimulation (SI Fig. 6*B*). IL-12 p70 alone or in combination with IL-15 and IL-18 potently enhanced anti-CD3 stimulation (7- and 30-fold, respectively). The combination of IL-12 p70, IL-15, and IL-18 induced IFN-γ secretion (but not IL-4) by *i*NKT cells even in the absence of suboptimal TCR stimulation (SI Fig. 6*B* and data not shown). Conversely, IFNα-2a and IFNβ did not enhance anti-CD3 stimulation and had no effect in the absence of TCR triggering.

***i*NKT Cell Autoreactivity Is Associated with Increased GSL Biosynthesis.** To investigate the possibility that *de novo* biosynthesis of GSLs is important for *i*NKT cell activation, we treated human DCs for 4 days with the specific inhibitor of GSL biosynthesis, *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) (20). *N*B-DGJ treatment reduced the stimulation by R-848 and LPS matured DCs by 50% and 33%, respectively (Fig. 3*A*). The antigen-presenting capacity of the same DCs was unaffected when the exogenous antigen C20:2 [analogue of α-GalCer (21)] was used to stimulate *i*NKT cells and when DCs were used to stimulate alloreactive T cells (SI Fig. 7), ruling out a nonspecific effect of *N*B-DGJ. Consistent with TLR-L-dependent up-regulation of GSL anti-
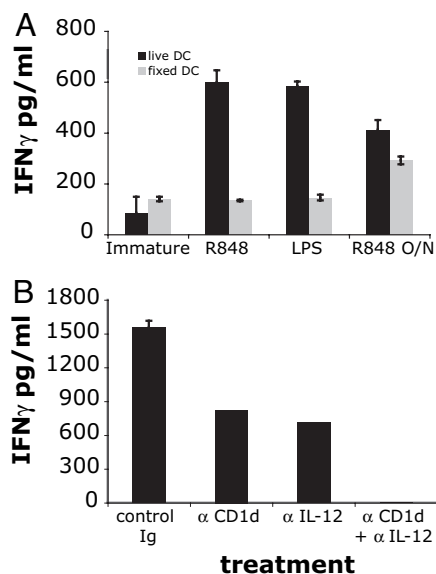
Fig. 2. The role of soluble factors and CD1d in TLR-dependent *i*NKT cell activation. (*A*) Fixation of DCs reduces *i*NKT cell activation. Live human DCs (black bars) were either matured with R-848 or LPS in the presence of the *i*NKT cell line MS mix or matured overnight with R-848, then washed and incubated with *i*NKT cells (R848 O/N). As a control, DCs were fixed with glutaraldehyde (gray bars) and then incubated with *i*NKT cells in the presence or absence of the indicated TLR-L. Only live cells secreted IL-12 p40 (SI Fig. 6*A*). (*B*) *i*NKT cells' recognition of R-848-matured DCs is CD1d- and IL-12-dependent. Human DCs were matured with R-848 in the presence of the *i*NKT cell line MS mix and each of the indicated blocking antibody. The means of triplicate values and standard deviation of IFN-γ of one ELISA experiment representative of three are shown.

gen(s), by quantitative PCR (Q-PCR) we observed that R-848 matured DCs increase the expression of genes encoding enzymes responsible for the synthesis of GlcCer and LacCer (Fig. 3 *B* and *C*). There was a slight reduction in the gene encoding for the enzyme leading to GSLs of the lacto/neolactoseries and a marked increase in genes encoding for enzymes synthesizing GSLs of the globo and ganglio series. We did not detect any amplification of iGb3 synthase, consistent with reports of it being a pseudogene in humans (data not shown and ref. 22). Similar results were obtained by using LPS matured DCs (data not shown).

The increased *i*NKT cells stimulatory capacity of R-848-treated DCs could also be due to increased cell surface expression of CD1d and co-stimulatory molecules. The results of our experiments indicated that CD1d up-regulation does not play a major role. Using three different antibodies in flow cytometry assays, CD1d expression was undetectable on the surface of immature and R-848-matured human DCs (data not shown). In addition, Q-PCR analysis of CD1d mRNA confirmed equal levels of expression of CD1d transcripts between immature and mature DCs (Fig. 3*C*).

Myelomonocytic Cell Line THP-1 Elicits *i*NKT Cell Activation. To further investigate the contribution of CD1d and GSLs to *i*NKT cell activation, we extended our investigations to the myelomonocytic cell line THP-1. Unlike monocyte-derived DCs, THP-1 cells have low but detectable levels of CD1d expression (data not shown and SI Fig. 8*A*). Preliminary experiments revealed that THP-1 cells responded only to TLR-4 and TLR-8 ligands, but not to other TLR-L, including the TLR-7 ligand R-837, which was used as negative control. THP-1 cells stimulated with R-848, and two other TLR-8 ligands, CL097 and CL075 (23), induced efficient *i*NKT cell activation (Fig. 4*A* and
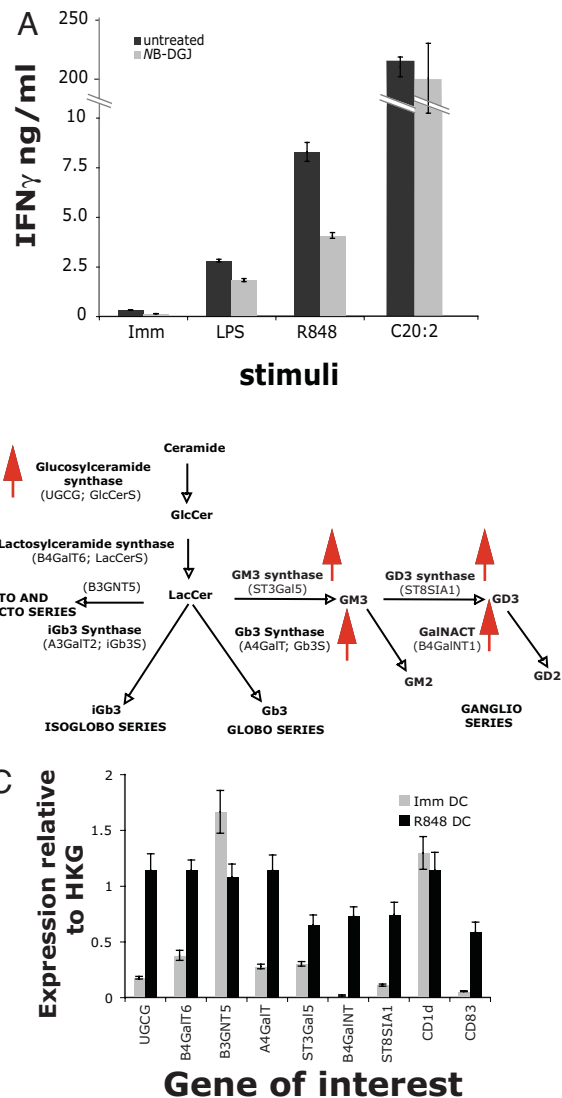
Fig. 3. Increased GSL biosynthesis in TLR-L-matured DCs contributes to *i*NKT cell activation. (*A*) Inhibition of GSL biosynthesis reduces *i*NKT cell activation by TLR-L-matured DCs. Monocytes were differentiated to DCs in the presence of *N*B-DGJ. Untreated and *N*B-DGJ-treated DCs were incubated with the *i*NKT line MS mix in the presence or absence of LPS or R-848. As control, untreated and *N*B-DGJ-treated DCs were pulsed with C20:2. The means of triplicate values and standard deviation of IFN-γ of one ELISA experiment representative of five are shown. (*B* and *C*) Q-PCR analysis of enzymes involved in the GSL biosynthetic pathway. (*B*) Diagram of the GSL biosynthetic pathway. Red arrows indicate genes that have been up-regulated. (*C*) The expression of the genes involved in the GSL biosynthetic pathway and of CD1d and CD83 is shown in immature and R-848-matured DCs, relative to two housekeeping genes (HKG), β-actin, and ubiquitin C. Data represent mean values of triplicates and standard deviation of one experiment representative of three.

data not shown). Overexpression of CD1d by lentiviral transduction (SI Fig. 8*A*) enhanced the basal *i*NKT cell autoreactivity and led to increased response to R-848 and LPS but not to R-837 (Fig. 4*A*). In all experiments, *i*NKT cell activation by TLR-L stimulated THP-1 cells resulted in IFN-γ but not IL-4 secretion (data not shown). The *i*NKT cell autoreactivity to untreated THP-1 CD1d cells was completely blocked by anti-CD1d antibodies (data not shown). Reactivity to R-848-treated THP-1 cells was blocked by a combination of anti-CD1d and anti-IL-12 antibodies, consistent with release of IL-12 upon maturation (data not shown). These results confirmed our previous findings with DCs.
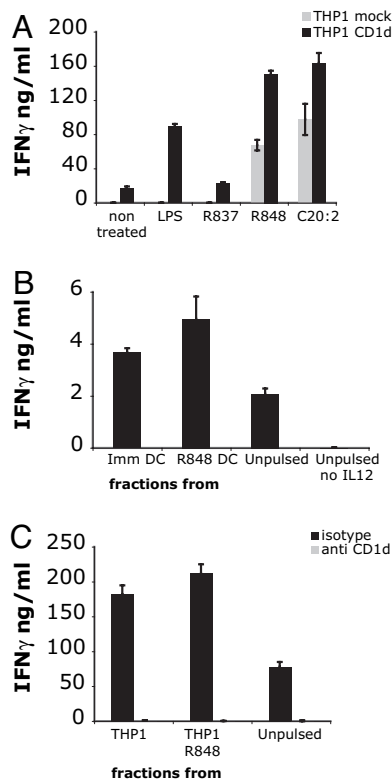
Fig. 4. THP-1 cells and DCs express ligands recognized by *i*NKT cells. (*A*) THP-1 cells transduced with control (mock, gray bars) or with human CD1d lentivirus (black bars) were stimulated with LPS, R-848, or R-837 in the presence of the *i*NKT line MS mix. (*B*) Total lipid fractions extracted from immature and R-848 matured DCs were pulsed on immature DCs. Cells were incubated with the *i*NKT line MS mix in the presence of IL-12 p70. Controls are unpulsed DCs in the presence or absence of IL-12 p70. (*C*) Total lipid fractions extracted from immature and R-848 matured THP-1 cells were pulsed on immature THP-1 cells. Activation of the *i*NKT line MS mix was assessed in the presence of IL-12 p70 and isotype control antibody or blocking anti-CD1d. The means of triplicate values and standard deviation of IFN-γ of one ELISA experiment representative of five are shown.

Total APC-Derived Lipid Fractions Sensitize CD1d-Positive Targets. To further investigate the contribution of endogenous lipids to *i*NKT cell activation, we isolated the total lipid fraction from immature and R-848-treated DCs and THP-1 cells. DCs and THP-1 cells pulsed with a total lipid extract were capable of activating both *i*NKT cells and the Vα24-negative line in the presence, but not in the absence, of soluble recombinant bioactive IL-12 (Fig. 4 *B* and *C* and data not shown). As a specificity control, *i*NKT cell activation was abolished in the presence of blocking anti-CD1d antibodies (Fig. 4*C*). Consistent with the observed autoreactivity against immature DCs and THP-1 cells, lipid fractions isolated from immature DCs and untreated THP-1 cells were only marginally less potent that those isolated from R-848-treated cells at inducing *i*NKT cell activation. These results also underscore the important role of soluble factors released during maturation in amplifying *i*NKT cell autoreactivity.

Direct Detection of *i*NKT Cell Ligand(s) at the Surface of CD1d-Positive APCs. To confirm the role of CD1d-lipid complexes in eliciting *i*NKT cell activation, irrespective of any soluble factor released during APCs stimulation with TLR ligands, we probed CD1d-natural ligand complexes at the surface, using tetrameric soluble *i*NKT T cell receptors (TCR) as staining reagents (24). Soluble *i*NKT TCRs stained specifically THP-1 CD1d cells but not THP-1 mock transduced cells (Fig. 5*A*), confirming the ELISA
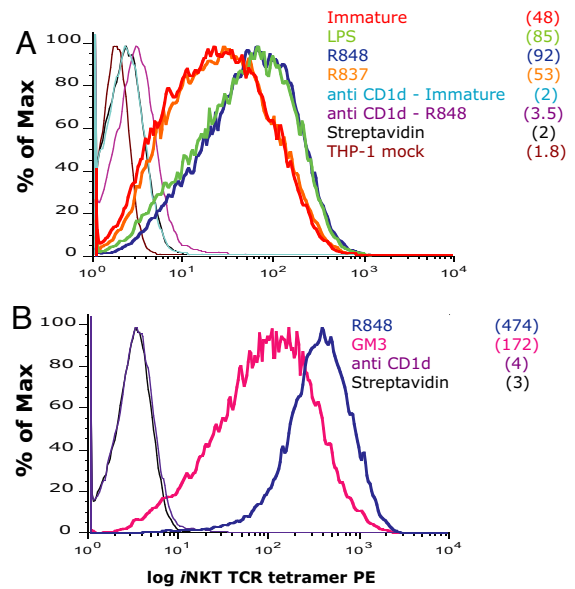

Fig. 5. Direct detection of *i*NKT cell ligand(s) by soluble *i*NKT TCR staining. (*A*) THP-1 CD1d cells were activated for 36 h with LPS, R-848, or R-837. The presence of *i*NKT cell ligands on the surface of THP-1 CD1d cells was revealed by staining with the soluble *i*NKT TCR in the presence of isotype control or blocking anti-CD1d antibodies. (*B*) THP-1 CD1d cells were activated for 24 h with R-848 and incubated with an excess of the ganglioside GM3 overnight or blocking anti-CD1d antibodies before staining with the soluble *i*NKT TCR. Streptavidin staining is included as negative control. THP-1 mock transduced cells and DCs did not show any detectable staining (data not shown). Numbers in brackets represent the mean fluorescence intensity (MFI) values of the *i*NKT TCR staining. CD1d MFI remained unchanged with maturation (data not shown). One experiment representative of six is shown.

results showing an increased *i*NKT cell stimulatory capacity of THP-1 CD1d cells. We did not detect staining of cells expressing low levels of surface CD1d (THP-1 and DCs), consistent with the low-affinity interaction between *i*NKT TCR and CD1d molecules (24, 25). However, activation of THP-1-CD1d cells with R-848 and with LPS, but not with R-837, led to increased staining with the soluble *i*NKT TCR (Fig. 5*A*). The specificity of the staining with the soluble TCR was demonstrated by blocking experiments with anti-CD1d antibody, which reduced the tetramer staining to near baseline levels (Fig. 5*A*). In addition, competition of the natural ligand(s) with excess GM3 ganglioside reduced the intensity of the staining with the soluble *i*NKT TCR, both on immature and R-848-treated THP-1 cells (Fig. 5*B* and SI Fig. 8*B*). No staining of immature or mature THP-1 CD1d cells was observed with an irrelevant soluble TCR (SI Fig. 8*C*).

Discussion

We set up an experimental system to dissect the contribution of APC-derived soluble factors and CD1d/lipid antigens in activating human *i*NKT cells in response to TLR-L engagement of APCs. We demonstrated that DC maturation by TLR-L induces an active metabolic process, which leads to enhanced recognition by *i*NKT cells of CD1d-associated GSLs, as shown by experiments using the specific inhibitor of GSL biosynthesis, *N*B-DGJ (20). Activation of *i*NKT cells by both DCs and the myelo-monocytic cell line THP-1 resulted in secretion of IFN-γ, but not IL-4, as observed with LPS-matured DCs (13).

Recognition of TLR-L matured DCs was not restricted to specific *i*NKT cell subsets and was also observed with a Vα24-negative Vβ11[+] NKT line. These results are consistent with the similar crystal structure and binding affinities for CD1d-α-GalCer monomers of Vα24 positive and negative NKT TCRs

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(25). In addition, clones derived from both lines display nearly identical CDR3-α sequences, which have been recently shown to directly interact with CD1d α1-, α2- helices, and α-GalCer (26).

Activation of *i*NKT cells by TLR-L matured DCs depended on both the up-regulation of endogenous ligands and on soluble factors, consistent with previous studies (13). Indeed, we observed that *i*NKT cell recognition of self-lipid antigens was abolished by a combination of anti-CD1d and anti-IL-12 antibodies. Moreover, there was a synergistic effect of IL-12, IL-15, and IL-18 in enhancing *i*NKT cell responses to suboptimal TCR-mediated stimulation. In addition, these cytokines were capable of activating *i*NKT cells in the absence of TCR stimulation, suggesting an important role for inducing *i*NKT homeostatic proliferation as reported for murine *i*NKT cells *in vivo* (14, 27).

DC maturation results in increased expression of adhesion and costimulatory molecules, which could also contribute to enhanced *i*NKT cell activation. Although we and others have observed increased CD1d expression in murine DCs and macrophages, after activation by bacteria, cytokines, and TLR-L (data not shown and refs. 28 and 29), we have not been able to detect increased CD1d expression after activation of monocyte-derived DCs by either FACS or Q-PCR. Our results are in agreement with previous reports demonstrating no change in CD1d expression in human DCs matured by TNF-α or CD40L (30) and a predominant effect of MTB and TLR-2 stimulation on group 1 CD1 molecules (31, 32).

To further confirm the role of GSLs in eliciting *i*NKT cell activation, we demonstrated that total lipids from APCs were able to elicit *i*NKT cell activation in the presence of recombinant IL-12, suggesting a low-affinity interaction. We were unable to further characterize the class of lipids responsible for direct activation of *i*NKT cells by either base treatment (to eliminate all lipids except those based on β-GlcCer and β-GalCer) or ceramide glycanase treatment (to release oligosaccharides from the GlcCer-based GSLs) (33), because of reduced viability of DCs pulsed with base-treated fractions and maturation of DCs pulsed with ceramide glycanase-treated factions (data not shown). Likewise, pulsing of DCs with total lipids separated into neutral and charged fractions resulted in DC maturation (data not shown). However, we detected by Q-PCR an up-regulation of transcripts of genes involved in GSL biosynthesis. Although there is no substitute for biochemically measuring GSLs in tissues and cell lines, these results suggest changes in GSL biosynthesis in agreement with previous reports on the effect of LPS on lipid metabolism and presentation of self-antigens in association with CD1a and CD1b molecules (34, 35). In addition, these results do not exclude that other classes of lipids can be *i*NKT-activating ligands, because reduction of up to 80% of the cellular GSL content after *N*B-DGJ treatment does not abrogate *i*NKT cell recognition.

Over-expression of CD1d molecules on THP-1 cells provided the opportunity to assess whether a soluble tetrameric *i*NKT TCR could be used to directly bind to CD1d molecules loaded with natural ligands. The results of these experiments unequivocally demonstrated that THP-1 cells stimulated with both LPS and R-848 could be specifically stained by the soluble *i*NKT TCR at higher levels than unstimulated THP-1 cells. The ability to specifically stain CD1d molecules loaded with natural ligands is of great importance because it validates the *i*NKT cell-based results and provides a tool to biochemically characterize the identity of natural ligand(s) capable of stimulating/selecting *i*NKT cells.

Recent studies have reported that TLR signaling coordinates pathogen recognition with antigen uptake and efficient activation of class II-restricted T cell responses (36, 37). We have now shown that TLR-mediated activation of APCs alters lipid metabolism, resulting in *i*NKT cell activation and enhancing DC maturation. Our data complement results on the effect of TLR agonists on the synthesis of group 1 CD1 molecules and some of their lipid ligands (31, 34). Importantly, these results also suggest that, in addition to directly or indirectly sensing bacteria, *i*NKT cells can be activated during viral infections, despite the absence of unique glycolipid antigens within viruses. Viral recognition by human *i*NKT cells can be accomplished upon triggering of the TLR-8 pathway in myeloid cells in addition to the reported cross-talk between myeloid and plasmacytoid dendritic cells upon TLR-9 engagement (38, 39). Because *i*NKT-dependent DC maturation is an important step in linking innate and adaptive immune responses, understanding the molecular mechanisms and the ligands capable of fine-tuning these interactions is of great importance for harnessing these cells for clinical use in treating and studying infections, autoimmune diseases, and cancer.

Materials and Methods

Medium and Reagents. The complete medium (CM) used throughout was RPMI medium 1640 (Gibco) supplemented with 10% FCS (Sigma) or 5% human AB serum as described in ref. 40. The following TLR-L were used: poly I:C and LPS from *Salmonella abortus equi* (5 μg/ml) (Sigma); R-848 (10 μg/ml) (PharmaTech); R-837, ssRNA-40, CL097, CL075, and flagellin (InvivoGen); Pam3Cys4K (EMC Microcollections).

Anti-human CD3 and recombinant human IL-12 were purchased from PharMingen, and recombinant human IL-15 and IL-18 were from Peprotech. IFNα-2a (Roferon-A) was purchased from Roche, and IFNβ-1a (Avonex) was purchased from Biogen.

GM3 was purchased from Matreya LLC (Pleasant Gap, PA). α-GalCer and C20:2 were synthesized as described in ref. 41.

APCs and NKT Cells. DCs and NKT cells were generated from healthy blood donors as described in refs. 24 and 40. THP-1 cells [American Type Culture Collection (ATCC)] were transduced with a lentiviral vector encoding for human CD1d (M.S., unpublished data).

Stimulation Assays. APCs were plated at 50,000 cells per well in 96-well plates in CM and incubated with *i*NKT cells (2–3 × 10⁴ per well, in triplicate) in the presence or absence of different concentrations of TLR-L. The TLR-L were maintained in the cocultures for the duration of the assay. In some experiments, DCs were matured overnight with 5 μg/ml R848, washed and used either live or after fixation. DC fixation was performed and controlled as described in ref. 34. For blocking experiments, APCs were preincubated for 2 h with TLR-L and subsequently for 2 h with 25 μg/ml anti human-CD1d [CD1d42.1, a kind gift from S. Porcelli (Albert Einstein College of Medicine, Bronx, NY)], anti-human IL-12 (PharMingen), or anti TNP-isotype control (ATCC), before the addition of *i*NKT cells.

APCs and *i*NKT cell activation was assessed by ELISA (IFN-γ, IL-12 p40, and IL-12 p70, all from PharMingen) on supernatants harvested after 36 h.

Inhibition of GSL Synthesis. *N*B-DGJ (Calbiochem) was added at a final concentration of 50 μM during DC differentiation and maintained at this concentration during the *i*NKT cell stimulation assay. To rule out toxicity of the inhibitors, graded numbers of treated and untreated DCs were irradiated (1,500 rad) and cultured with allogeneic peripheral blood mononuclear cells (250,000 per well). The T cell proliferative response was measured on day 5 by [³H]thymidine (Amersham) incorporation (0.037 MBq per well). The extent of GSL inhibition (≈50%, data not shown) was determined by normal phase HPLC analysis of the GSL content as described in ref. 16.

RNA Extraction and Q-PCR. DCs were untreated or stimulated for 36 h with 1 μg/ml LPS or 5 μg/ml R-848. RNA was extracted with RNAeasy (Qiagen). cDNA was synthesized by using the High-Capacity cDNA Archive kit (Applied Biosystems). Real-time quantitative PCR was performed in triplicate, using the Corbett Research Rotor Gene RG-3000. Conditions for the PCR, primers, and probes are summarized in SI Table 1. Relative quantitation of gene expression was carried out according to the method described by Pfaffl (42).

GSL Extraction and Treatment. Total lipid fractions, base treatment, and ceramide glycanase treatment were performed as described in ref. 33. The total fraction was dried under nitrogen, resuspended in FCS at 5 × 10⁵ cell equivalent per microliter, and extensively sonicated. DCs or THP-1 cells were pulsed with 1 μl of each fraction in a final volume of 200 μl, in the presence

or absence of recombinant IL-12 p70 (0.05 ng/ml), and used to activate the NKT cells.

iNKT TCR Tetramer Staining. The generation of soluble TCR heterodimers has been described (24). Immature and TLR-L matured THP-1 and THP-1 CD1d cells were incubated with 0.5–1 μg of *i*NKT-tetramer or equivalent amounts of streptavidin on ice for 1 h. The irrelevant 1G4-NY-ESO-1 specific soluble TCR (43) was used as negative control at the same concentration. Cells were washed, and samples were analyzed on a Cyan flow cytometer (Dako). Data were processed by using Flowjo software (Treestar). To block the TCR staining, cells were incubated for 30 min on ice with 30 μg/ml of anti-CD1d antibody

(CD1d42.1) or isotype control, washed, and stained with the tetramer. To compete the TCR staining, cells were treated with the TLR-L for 24 h, then the ganglioside GM3 was added to the same wells at 2 μg/ml overnight before staining.

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