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Activation of Invariant NKT Cells by Toll-like Receptor 9-Stimulated Dendritic Cells Requires Type I Interferon and Charged Glycosphingolipids

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SUMMARY

Invariant natural killer T (iNKT) cells are a subset of innate lymphocytes that recognize lipid antigens in the context of CD1d and mediate potent immune regulatory functions via the rapid production of interferon-γ (IFN-γ) and interleukin-4 (IL-4). We investigated whether diverse Toll-like receptor (TLR) signals in myeloid dendritic cells (DCs) could differentially stimulate iNKT cells. Together with the lipopolysaccharide-detecting receptor TLR4, activation of the nucleic acid sensors TLR7 and TLR9 in DCs were particularly potent in stimulating iNKT cells to produce IFN-γ, but not IL-4. iNKT cell activation in response to TLR9 stimulation required combined synthesis of type I interferon and de novo production of charged β-linked glycosphingolipid(s) by DCs. In addition, DCs stimulated via TLR9 activated both iNKT cells and NK cells in vivo and protected mice against B16F10-induced melanoma metastases. These data underline the role of TLR9 in iNKT cell activation and might have relevance to infectious diseases and cancer.

INTRODUCTION

Invariant natural killer T (iNKT) cells, which are defined by the canonical Vα14-Jα18 T cell receptor (TCR)-α chain in mice (Vα24-Jα18 in humans), recognize lipid antigens (Ags) bound to the MHC class I-related molecules CD1d expressed by Ag-presenting cells (APCs), including myeloid dendritic cells (herein referred as DCs) (Bendelac et al., 2006; Godfrey and Kronenberg, 2004). Upon primary stimulation, in particular in response to α-galactosylceramide (α-GalCer), a nonmammalian glycosphingolipid (GSL), iNKT cells produce large amounts of interferon (IFN)-γ and interleukin (IL)-4 that leads to downstream activation of DCs, NK cells, B cells, and conventional T cells (Bendelac et al., 2006; Van Kaer and Joyce, 2005). Through this unique property, iNKT cells modulate autoimmune diseases, inflammation, resistance to tumors, and antimicrobial host responses (Bendelac et al., 2006; Godfrey et al., 2004; Van Kaer and Joyce, 2005). During infection, iNKT cells can become activated, although the underlying mechanisms are not fully understood, in particular during nonbacterial infection. In the case of bacteria, the proposed scenario is that microbes activate iNKT cells either by providing CD1d-restricted lipids and/or by producing conserved molecular patterns able to engage innate sensors and to trigger an appropriate maturation program in DCs (Bendelac et al., 2006). Indeed, it has been recently demonstrated that a subset of Gram-negative, lipopolysaccharide (LPS)-negative α-proteobacteria, such as *Sphingomonas*, *Ehrlichia*, *Rickettsia*, and *Borrelia*, express iNKT cell ligands, including α-linked glycuronylceramide (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005) and glycosyldiacylglycerol (Kinjo et al., 2006), able to directly activate iNKT cells. In contrast, for other Gram-negative bacteria (such as *Salmonella*), LPS has been proposed to indirectly activate iNKT cells, via Toll-like receptor (TLR)4 expressed by DCs (Brigl et al., 2003; Mattner et al., 2005). In this process, although bioactive IL-12 might be sufficient to activate autoreactive iNKT cells independently from CD1d-TCR engagement (Nagarajan and Kronenberg, 2007), it might also act in concert with self β-linked GSL(s) to enhance the response (Brigl et al., 2003; Mattner et al., 2005). Among self lipids, the neutral GSL isoglobotrihexosylceramide (iGb3) has been proposed as a potential candidate (Mattner et al., 2005;

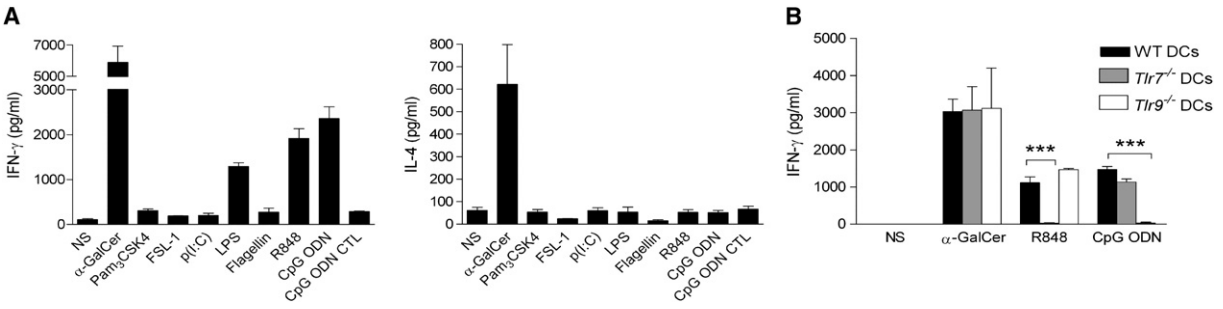

Figure 1. LPS-, R848-, and CpG ODN-Sensitized DCs Activate Liver MNCs to Produce IFN-γ

(A) α-GalCer (10 ng/ml), Pam_3CSK_4 (0.5 μg/ml), FSL-1 (100 pg/ml), p(I:C) (10 μg/ml), LPS (1 μg/ml), flagellin (1 μg/ml), R848 (100 nM), CpG ODN, or non-CpG ODN (2 μg/ml) were incubated with DCs for 16 hr. After extensive washing, DCs were cocultured for 48 hr with liver MNCs, and then cytokine production was quantified by ELISA. Data represent the mean ± SEM of five independent experiments performed in triplicates.

(B) α-GalCer, R848, or CpG ODN were incubated with WT, *Tlr7*$^{-/-}$, or *Tlr9*$^{-/-}$ DCs and then DCs were cocultured with WT liver MNCs. Shown is a representative experiment out of three performed. Results represent the mean of triplicate cultures ± SD. ***$p < 0.001$.

Zhou et al., 2004), although recent data have called this into question (Porubsky et al., 2007; Speak et al., 2007).

Activation of TLRs is the main pathway by which pathogens induce DC maturation and immunostimulatory functions (Takeda and Akira, 2005). The TLR family consists of more than ten members able to distinguish distinct microbial molecular patterns from invading pathogens. TLR2, TLR4, and TLR5, which are predominantly expressed on the cell surface, recognize an array of bacterial components including lipoproteins, peptidoglycans, and lipoteichoic acids (for TLR2 in cooperation with TLR1 or TLR6), LPS (for TLR4), and flagellin (for TLR5). In contrast, TLR3, TLR7 (TLR8 in humans), and TLR9, which are located in endosomal compartments, are committed to the recognition of nucleic acids from intracellular pathogens. Whereas TLR3 recognizes double-stranded RNAs and TLR7 and TLR8 recognize single-stranded RNAs of viral origin, TLR9 senses bacterial and viral DNAs with hypomethylated CpG motifs. Although common intracellular pathways are generated in DCs in responses to TLR agonists, major differences can also occur according to the TLR(s) engaged (Takeda and Akira, 2005). These differences might differentially affect the activation of bystander cells, including iNKT cells. In the present study, with canonical agonists, we aimed to investigate the respective role of each TLR member expressed by mouse DCs on iNKT cell activation. Our data showed that, along with LPS, R848 (TLR7) and CpG oligodeoxynucleotide (ODN) (TLR9) activated DCs to selectively induce IFN-γ (but not IL-4) release by iNKT cells. In response to CpG ODN-treated DCs, the synthesis of both type I IFN (but not IL-12) and charged β-linked GlcCer-based GSL(s) by DCs is essential for this process. In vivo, CpG ODN-treated DCs activated both iNKT cells and NK cells and protected mice against B16F10-induced melanoma metastases. Our finding might help to better understand iNKT cell activation during pathological situations (including infection) and to better exploit the iNKT cell-NK cell axis for the improvement of DC-mediated immunotherapy by means of TLR activation.

RESULTS

LPS-, R848-, and CpG ODN-Stimulated DCs Activate Liver Mononuclear Cells to Produce IFN-γ

To investigate whether iNKT cells respond differentially and indirectly to various TLR activators, bone-marrow-derived DCs were exposed to distinct TLR agonists and, after extensive washing, were cocultured with liver mononuclear cells (MNCs), a rich source of iNKT cells. At the doses used, all agonists induced substantial DC maturation, as assessed by the surface upregulation of costimulatory and presenting (including CD1d) molecules and by the synthesis of inflammatory cytokines (Figure S1 in the Supplemental Data available online). Interestingly, DCs stimulated with LPS (TLR4), R848 (TLR7, 8), or CpG ODN (TLR9) promoted IFN-γ secretion by hepatic cells whereas DCs stimulated with Pam_3CSK_4 (TLR2-1), FSL-1 (TLR2-6), polyinosine-polycytidylic acid (poly(I:C)) (TLR3), or flagellin (TLR5) were inactive (Figure 1A). Notably, although α-GalCer-pulsed DCs, used here as a positive control, promoted IL-4 synthesis by liver cells, DCs stimulated with the various TLR agonists failed to do so. The effect of CpG ODN was CpG specific, as shown by the fact that DCs exposed to the GC control ODN did not trigger IFN-γ release by hepatic MNCs (Figure 1A). Moreover, *Tlr7*$^{-/-}$ and *Tlr9*$^{-/-}$ DCs failed to induce IFN-γ production in response to R848 or to CpG ODN, respectively, whereas they maintained their ability to activate hepatic cells in response to α-GalCer (Figure 1B). Thus, among the TLR agonists tested, LPS, R848, and CpG ODN were the most potent inducers of IFN-γ release by liver MNCs. Because TLR7 and TLR9, which belong to the same TLR subfamily, exerted similar effects in this setting, we focused on CpG ODN for the rest of the study.

CpG ODN-Stimulated DCs Directly Activate iNKT Cells

We next investigated whether IFN-γ production by liver MNCs, in response to CpG ODN-stimulated DCs, was dependent on CD1d expression by the latter and was due to

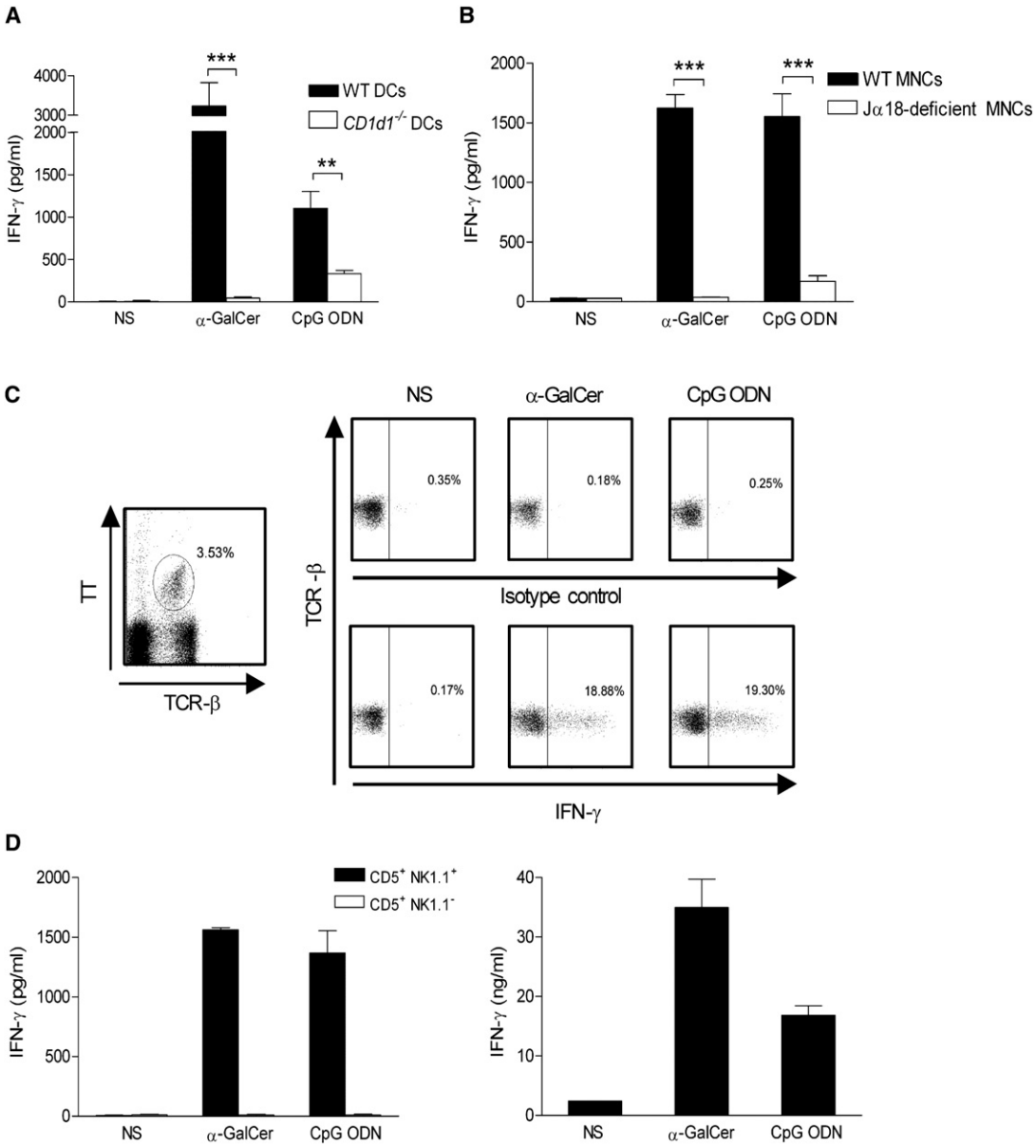

Figure 2. CpG ODN-Stimulated DCs Directly Activate iNKT Cells

(A) α-GalCer or CpG ODN was incubated with WT or *CD1d1*$^{-/-}$ DCs and then cocultured with liver MNCs from WT mice for 2 days.

(B) Stimulated DCs were cocultured with liver MNCs isolated from WT or Jα18-deficient mice.

(C) Intracellular FACS staining of iNKT cells after stimulation with α-GalCer- or CpG ODN-activated DCs. Stimulated DCs were cocultured with liver cells for 12 hr, and afterwards brefeldin A was added for another 4 hr. Cells were labeled with TCRβ mAb and TT, fixed, and permeabilized for intracellular cytokine staining. Cells were analyzed by flow cytometry, and TT$^+$ TCRβ$^+$ cells were gated and screened for intracellular IFN-γ production. Gates were set based on the isotype control. The percentages of TT$^+$ TCRβ$^+$ cells positive for IFN-γ are represented. One representative experiment out of three is shown.

(D) α-GalCer- or CpG ODN-stimulated DCs were cocultured with purified liver-derived CD5$^+$ NK1.1$^+$ or CD5$^+$ NK1.1$^-$ cells (left) or with TT$^+$ TCRβ$^+$ cells (right) for 2 days. Of note, exposure of purified CD5$^+$ NK1.1$^+$ or TT$^+$ TCRβ$^+$ cells with CpG ODN alone did not result in IFN-γ production (not shown).

(A, B, D) Cytokine production was measured by ELISA. Shown is a representative experiment of four (A, B) or three (D) performed. Results represent the mean of triplicate cultures ± SD. ***$p < 0.001$; **$p < 0.01$.

iNKT cells. CD1d deficiency in DCs resulted in a complete abrogation of IFN-γ secretion in response to α-GalCer-sensitized DCs. These DCs also showed a ~70% decrease (average of four independent experiments) in the secretion of IFN-γ in response to CpG ODN-treated DCs (Figure 2A). This was confirmed by the neutralizing CD1d antibody (Ab) 1B1, added during the DC-liver MNC coculture (not shown). In addition, IFN-γ release was strongly reduced (by ~80%) when CpG ODN-treated DCs were cocultured with hepatic MNCs isolated from iNKT

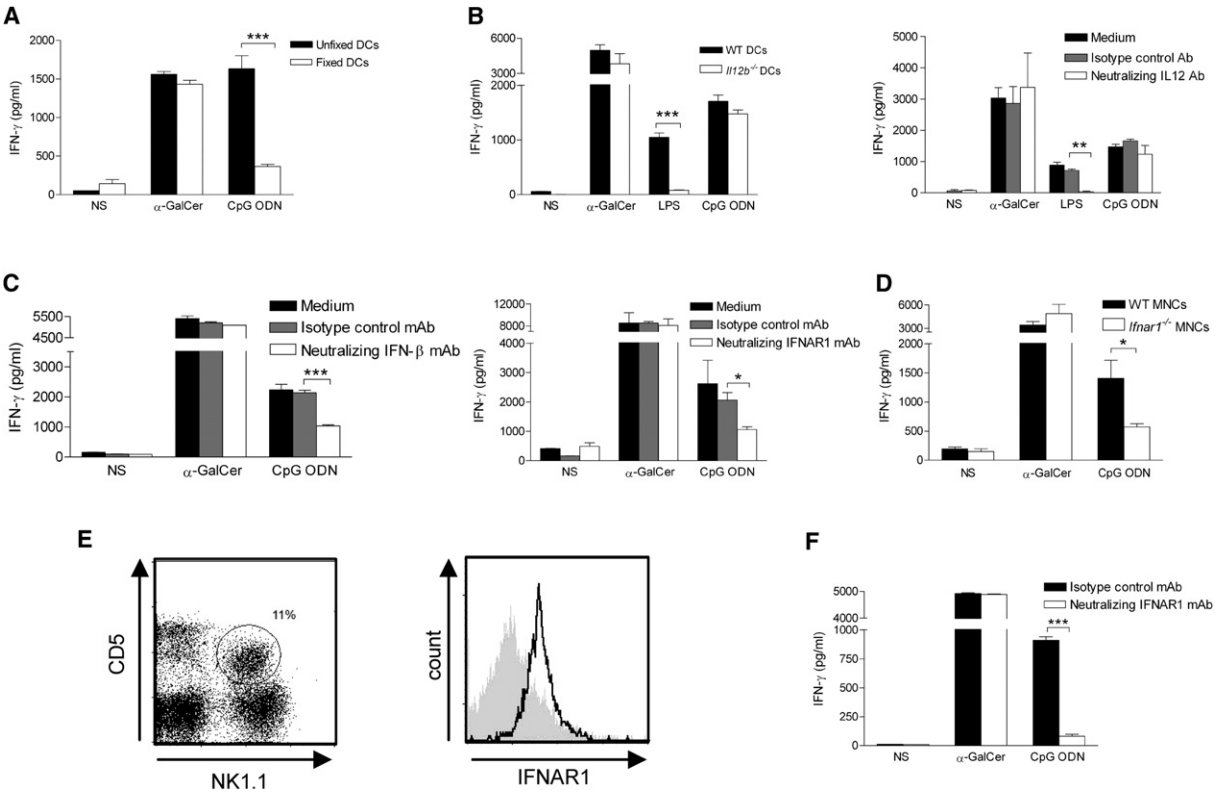

Figure 3. Type I IFN Production by CpG ODN-Stimulated DCs Is Essential in the Activation of iNKT Cells

(A) α-GalCer- or CpG ODN-stimulated DCs were fixed with glutaraldehyde, washed, and cocultured with liver MNCs.

(B) α-GalCer-, LPS-, or CpG ODN-stimulated WT or *Il12b*$^{-/-}$ DCs were cocultured with hepatic MNCs (left). Stimulated WT DCs were cocultured with MNCs in the absence (medium) or the presence of a neutralizing IL-12 Ab or an isotype control Ab (0.1 μg/ml) (right).

(C) α-GalCer- or CpG ODN-stimulated WT were cocultured with MNCs in the absence or the presence of neutralizing IFN-β (left), IFNAR1 (right), or isotype control mAbs (5 μg/ml).

(D) WT DCs were cocultured with hepatic MNCs isolated from WT or *Ifnar1*$^{-/-}$ mice.

(E) IFNAR1 expression by liver iNKT cells as assessed by FACS analysis. Liver cells were gated based on the double expression of CD5 and NK1.1. A representative profile is shown. No labeling was found on cells isolated from *Ifnar1*$^{-/-}$ mice (not shown).

(F) DCs were cocultured with purified hepatic CD5$^+$ NK1.1$^+$ cells in the presence of neutralizing IFNAR1 or isotype control mAbs.

(A–D, F) Shown is a representative experiment of three performed. Results represent the mean of triplicate cultures ± SD. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

cell-deficient mice (Jα18-deficient mice) (Figure 2B). To further confirm that iNKT cells produce IFN-γ in this setting, intracellular staining was performed. After DC-liver MNC culture (16 hr), cells were labeled with the α-GalCer-CD1d tetramer (TT), a probe that exclusively stains iNKT cells, plus a TCRβ mAb. As shown in Figure 2C, TT$^+$ TCRβ$^+$ cells produced IFN-γ intracellularly in response to CpG ODN-stimulated DCs. Similar results were obtained when liver CD5$^+$ NK1.1$^+$ cells (~90% TT$^+$ TCRβ$^+$ pure) were analyzed (not shown). Notably, CD5$^-$ NK1.1$^+$ (NK) cells also produced IFN-γ intracellularly in this setting, but the percentage of NK cells positive for IFN-γ was reduced by ~60% when cells from iNKT cell-deficient mice were examined (Figure S2). Thus, CpG ODN induced in CD1d-expressing DCs a process capable of activating iNKT cells.

To investigate whether TLR9-stimulated DCs directly activate iNKT cells, cells were sorted from liver MNCs based on NK1.1 and CD5 staining. As expected, α-GalCer-treated DCs promoted IFN-γ and IL-4 (not shown) secretion by CD5$^+$ NK1.1$^+$, but not by CD5$^+$ NK1.1$^-$, hepatic cells (Figure 2D, left). In addition, CpG ODN-sensitized DCs induced IFN-γ (but not IL-4) secretion by CD5$^+$ NK1.1$^+$ cells whereas CD5$^+$ NK1.1$^-$ cells failed to do so. Although this procedure partially activated iNKT cells, these results were confirmed by sorting hepatic TT$^+$ TCRβ$^+$ cells (Figure 2D, right). Collectively, these data show that, in response to CpG ODN, DCs directly activate liver iNKT cells to produce IFN-γ in vitro.

iNKT Cell Activation in Response to CpG ODN-Treated DCs Requires Type I IFN but Not IL-12

To study the potential requirement of soluble DC-derived factors in iNKT cell activation, CpG ODN-stimulated DCs were fixed with glutaraldehyde just before DC-MNC contact. α-GalCer-treated fixed DCs maintained their ability to activate liver MNCs to produce IFN-γ whereas CpG ODN-treated fixed DCs had a strongly reduced (~85%) capacity to do it (Figure 3A). Thus, in response to CpG ODN, DC-derived soluble factors are required to fully activate iNKT cells.

IL-12 and type I IFN, two major cytokines produced by DCs after TLR9 triggering (Figure S1), have recently been shown to cooperate with the CD1d-TCR pathway to activate iNKT cells (Brigl et al., 2003; Marschner et al., 2005; Mattner et al., 2005; Montoya et al., 2006). In response to CpG ODN, DCs deficient in IL-12p40 maintained their ability to activate liver cells (Figure 3B, left), and this result was confirmed via a neutralizing IL-12 Ab, added during the DC-MNC coculture (Figure 3B, right). In contrast, in agreement with previous studies (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007), IL-12 was essential in the ability of LPS-stimulated DCs to promote IFN-γ release by liver MNCs. To investigate the contribution of IFN-β, which is released by CpG ODN-stimulated DCs (Figure S1), a neutralizing Ab against it was utilized. Blockade of IFN-β reduced by ~50% the CpG ODN-induced production of IFN-γ by hepatic MNCs (Figure 3C, left). To confirm this finding, a neutralizing Ab directed against the IFNAR1 subunit of the type I IFN receptor or liver MNCs isolated from *Ifnar1*$^{-/-}$ mice were used. Compared to controls, this resulted in a strong reduction of IFN-γ production by liver cells (~60% and 70% reduction, respectively) (Figure 3C, right, and Figure 3D). In contrast, when IFNAR1 was blocked or absent in liver cells, the degree of iNKT cell activation after exposure to α-GalCer-treated DCs (Figures 3C and 3D) or to LPS-stimulated DCs (not shown) was not reduced. FACS analysis showed that liver CD5$^+$ NK1.1$^+$ expressed IFNAR1 (Figure 3E). Interestingly, blockade of IFNAR1 on sorted iNKT cells resulted in a total abrogation of IFN-γ production by these cells (Figure 3F). These data argue that, in response to CpG ODN-stimulated DCs, iNKT cell activation fully requires type I IFN but not IL-12.

Charged GSL(s) Derived from CpG ODN-Stimulated DCs Act(s) in Concert with Type I IFN to Activate iNKT Cells

We next addressed the possibility that, after TLR9 triggering in DCs, generation of β-GlcCer-derived GSLs, which represent potential self ligands for iNKT cells (Stanic et al., 2003), may be of importance in iNKT cell activation, in concert with type I IFN. DCs were pretreated with *N*-butyl-deoxygalactonojirimycin (*N*B-DGJ), an inhibitor of GlcCer synthase (the first committed step in GSL biosynthesis [Platt et al., 1994]). Preincubation of DCs with *N*B-DGJ before α-GalCer stimulation had no effect on IFN-γ release by liver MNCs (Figure 4A), a process that does not require de novo synthesis of GSLs by DCs. In marked contrast, pretreatment of DCs with *N*B-DGJ before CpG ODN stimulation reduced by ~50% their ability to promote IFN-γ release by liver MNCs. Of note, at the dose tested, *N*B-DGJ was not toxic, did not modulate the CpG ODN-induced maturation process of DCs (including CD1d expression), and did not affect the DC-mediated activation of conventional peptide-specific T cells (not shown).

We next determined whether lipids isolated from CpG ODN-stimulated DCs could contain iNKT cell ligand(s). For this purpose, total lipids extracted from CpG ODN-stimulated DCs were incubated with DCs prior to coculture with liver MNCs, performed in the presence or absence of type I IFN (IFN-β). Total lipid fractions or IFN-β alone failed to induce IFN-γ release by liver MNCs (Figure 4B, left). In the presence of IFN-β, DCs incubated with total lipids extracted from CpG ODN-stimulated, but not from nonstimulated, DCs promoted IFN-γ release by liver cells, an effect that was dose dependent (Figure 4B, right). Of note, the lipid fraction prepared from *N*B-DGJ-treated, CpG ODN-stimulated DCs had a reduced ability to activate hepatic cells. Finally, the activating effect of the lipid fraction required CD1d expression by DCs and was dependent on iNKT cells (Figure 4C). To further examine the nature of the activating molecule(s), fractions containing either neutral or charged lipids were separated from the total lipid fraction (Figure S3). Interestingly, DCs incubated with the charged fraction (CpG ODN-stimulated DCs) induced IFN-γ release by liver MNCs, whereas the neutral fraction was nonactivating (Figure 4D). To confirm the involvement of GSLs in iNKT cell activation, the charged fraction was base treated, a process known to eliminate all lipids (such as phospholipids) except those based on β-GlcCer and β-GalCer (Figure S3; Butters et al., 1981). This procedure did not reduce IFN-γ production by liver cells (Figure 4E). In contrast, digestion of the charged fraction with ceramide glycanase, an enzyme that releases oligosaccharides from the GlcCer-based GSLs (Figure S3), fully abrogated liver MNC activation (Figure 4E). Taken together, these data strongly suggest that charged β-GlcCer-derived GSL(s) is (are) synthesized in DCs upon CpG ODN stimulation and that, in concert with type I IFN, it (they) activate(s) iNKT cells to release IFN-γ.

TLR9 Activation in DCs Enhances the Expression of mRNAs for Several Glycosyltransferases Involved in the Synthesis of GSLs

Because CpG ODN generates new or increasing amounts of charged GSLs in DCs able to activate iNKT cells, we investigated the possibility that it could modulate the expression of genes involved in the GSL biosynthetic pathway, by quantitative real-time PCR (Figure 5). Compared to unstimulated cells, CpG ODN moderately upregulated the mRNA expression of GlcCer synthase (*Ugcg*) (~2.1-fold increase at both 4 and 18 hr) but not lactosylceramide (LacCer) synthase (*B4galt6*) (Figure 5A). The enzymes utilizing LacCer as a substrate are involved in the genesis of the ganglio, (iso)globo, and (neo)lacto series of GSLs. Increased amounts of transcripts for GM3 synthase (*St3gal5*), iGb3 synthase (*A3galt2*), Gb3 synthase (*A4galt*), and lactotriaosylceramide (Lc3) synthase (*B3gnt5*) were observed (~2.2- to 5-fold), whereas that for asialo-GM2 (GA2) synthase (*B4galnt1*) was unchanged (Figure 5A). We next focused on enzymes involved in the synthesis of gangliosides, which represent a major source of charged GSLs. In addition to GM3 synthase (*St3gal5*) (a-series), mRNA levels for GD3 synthase (*St8sia1*) (b-series) and GT3 synthase (*St8sia5*) (c-series) were dramatically increased in response to CpG ODN (~8.5-fold at

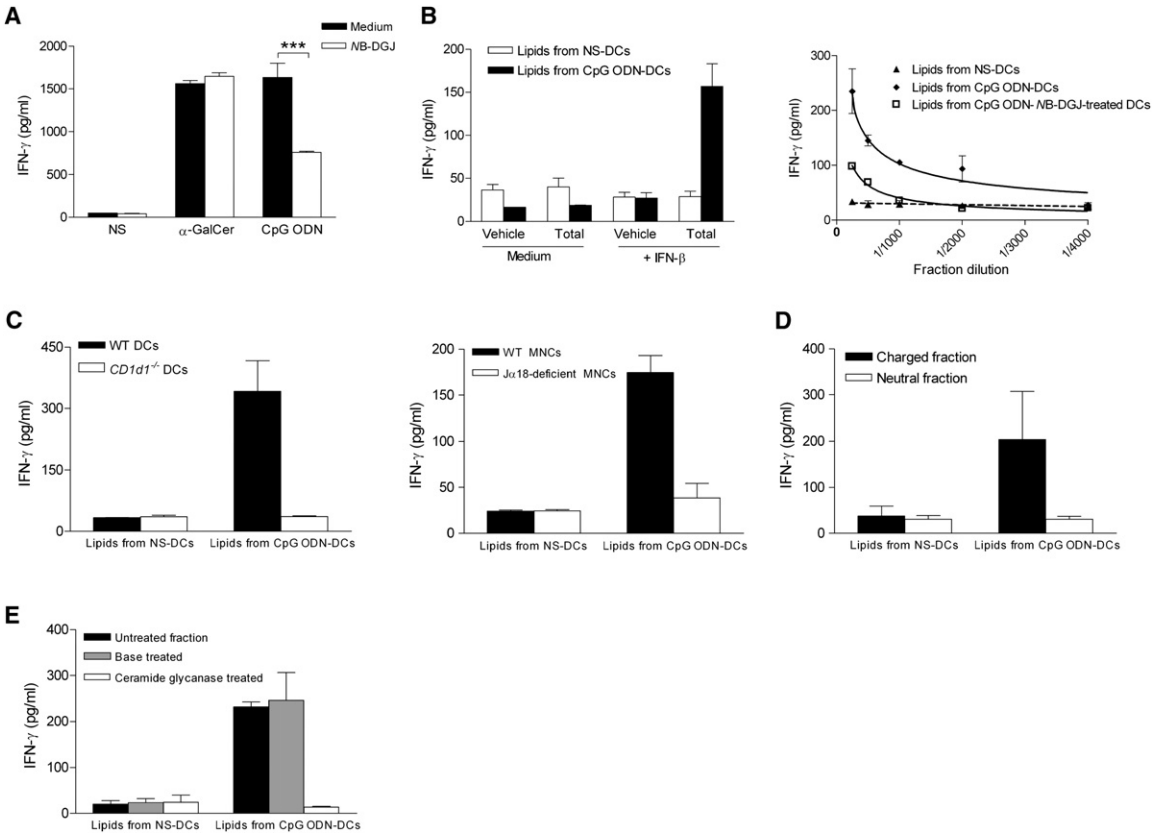

Figure 4. Charged GSLs Extracted from CpG ODN-Stimulated DCs Activate iNKT Cells to Produce IFN-γ, via CD1d-Competent DCs

(A) DCs were treated for 24 hr with *N*B-DGJ (50 μM) and then stimulated with α-GalCer or CpG ODN. After washing, DCs were cocultured with liver MNCs for 48 hr. ***$p < 0.001$.

(B) Total lipids extracted from nonstimulated (NS) or from CpG ODN-stimulated DCs were exposed to DCs (1/500 in DMSO) for 16 hr, and afterwards washed DCs were cocultured with liver MNCs for 48 hr, in the presence or absence of IFN-β (1000 U/ml). Data represent the mean ± SEM of five independent experiments (five different lipid preparations). A dose-dependent response is shown in the right panel (one representative experiment). In this case, DCs were treated or not with *N*B-DGJ. Of note, excepted CD1d, which was upregulated in response to IFN-β, the lipid fractions used did not induce DC maturation, even in the presence of IFN-β (not shown).

(C) WT or *Cd1d1*$^{-/-}$ DCs sensitized with the lipid fractions were cocultured with liver MNCs isolated from WT or Jα18-deficient mice.

(D) The neutral or charged fractions obtained from total lipids isolated from nonstimulated or CpG ODN-stimulated DCs were added to DCs.

(E) The charged fractions were treated, or not, with sodium hydroxide (base treatment) or with ceramide glycanase and were then incubated with DCs.

(C–E) IFN-β was added during the coculture. One representative experiment out of four is shown. Results represent the mean of triplicate cultures ± SD.

18 hr), whereas the other enzymes were not modulated. Thus, CpG ODN enhanced the synthesis of transcripts encoding for glycosyltransferases of the GSL biosynthetic pathway, in particular for sialyltransferases involved in the first step of the a, b, and c series of gangliosides. Although measurement of transcript expression does not necessarily predict the intracellular GSL content, these data confirmed that CpG ODN impacts the expression of genes that encode the enzymes responsible for the biosynthesis of charged GSLs in DCs.

CpG ODN-Sensitized DCs Activate iNKT Cells In Vivo

We then determined whether CpG ODN-stimulated DCs could activate iNKT cells in vivo. For this, DCs were injected i.v., and the activation state of liver iNKT cells was determined by intracellular FACS staining. Both α-GalCer-sensitized and CpG ODN-treated DCs induced an apparent decreased frequency of iNKT cells (Figure 6), an effect likely to be due to TCR downmodulation (Wilson et al., 2003). Despite this, and compared to the control (nonstimulated DCs), α-GalCer-sensitized DCs and CpG ODN-stimulated DCs promoted an increased frequency of iNKT cells positive for IFN-γ. Of importance, after injection of CpG ODN-sensitized DCs, $CD5^{-}$ $NK1.1^{+}$ (NK cells) also labeled positively for IFN-γ and this partially depended on iNKT cells (~65% reduction of positive cells in iNKT cell-deficient mice) (Figure S4).

CpG ODN-Stimulated DCs Prevent B16F10 Metastasis, in part via iNKT Cells

It is known that, when stimulated in vivo with α-GalCer or with α-GalCer-pulsed DCs, iNKT cells exert potent

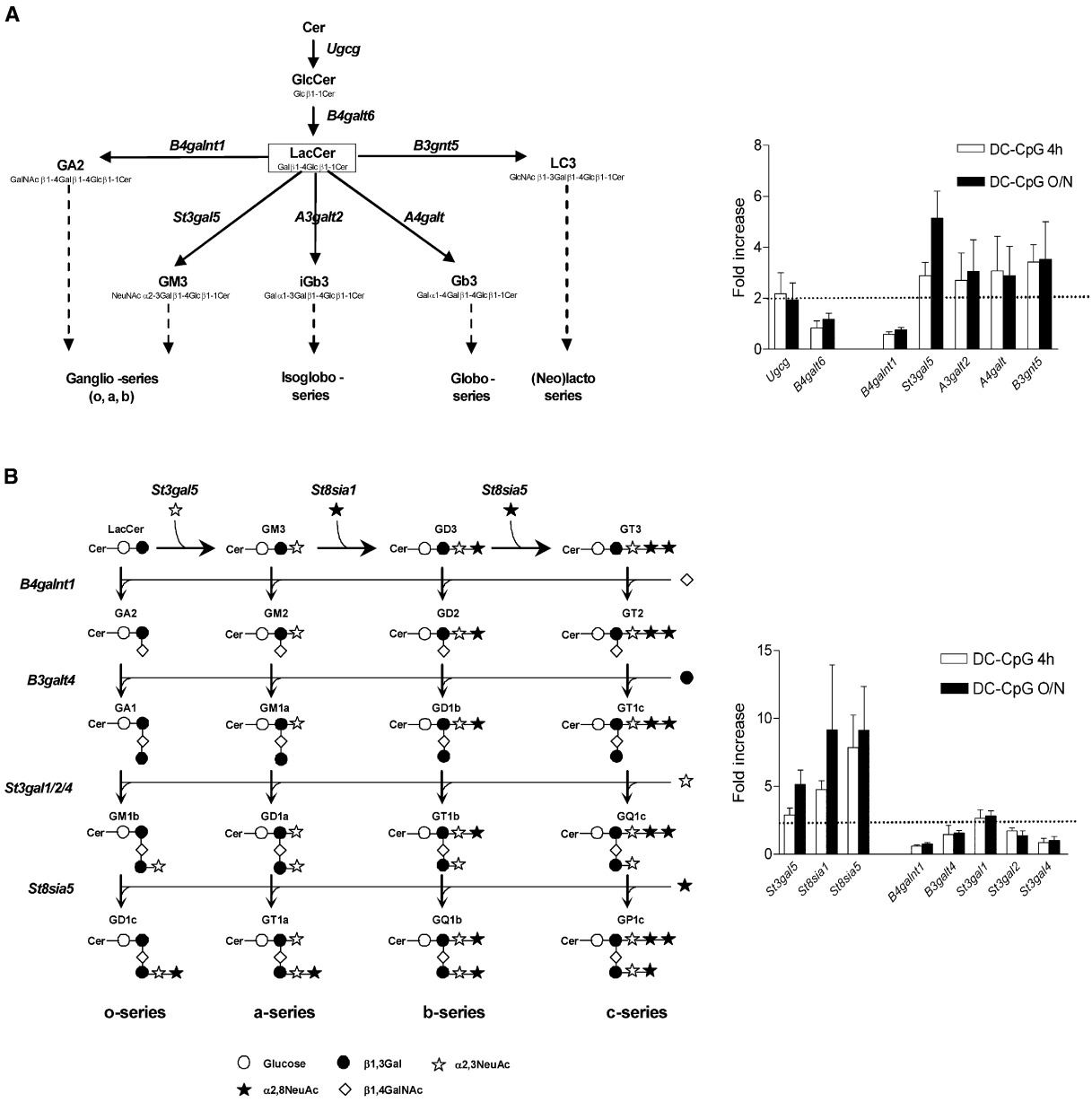

Figure 5. CpG ODN Increases mRNA Transcripts for Some Sialyltransferases in DCs

(A) Synthetic pathway of GSLs from ceramide with the full structures of the ganglio, (iso)globo, and (neo)lacto series of GSLs (left). RNAs from unstimulated or CpG ODN-stimulated DCs were harvested after 4 or 18 hr stimulation, and GlcCer synthase (*Ugcg*), LacCer synthase (*B4galt6*), asialo-GM2 (GA2) synthase (*B4galnt1*), GM3 synthase (*St3gal5*), iGb3 synthase (*A3galt2*), Gb3 synthase (*A4galt*), and lactotriaosylceramide (Lc3) synthase (*B3gnt5*) mRNA copy numbers were measured by quantitative real-time PCR (right).

(B) Synthetic pathway of the ganglio o, a, b, and c series GSLs (left). After stimulation, GM3 synthase (*St3gal5*), GD3 synthase (*St8sia1*), GT3 synthase (*St8sia5*), *B4galnt1*, *B3galt4*, *St3gal1*, *St3gal2*, *St3gal4* mRNA copy numbers were measured by quantitative real-time PCR (right).

Data are normalized to expression of *Gapdh* and are expressed as n-fold increase in mRNA level compared to unstimulated DCs. Data represent the mean ± SEM of six independent experiments performed in triplicates. Genes varying at least 2-fold were considered as markedly modulated.

antitumor properties against B16F10 melanoma, a phenomenon that depends on IFN-γ production (Fujii et al., 2002, 2006; Smyth et al., 2005). In agreement with previous reports (Fujii et al., 2002, 2006), the number of lung metastases was dramatically decreased in mice injected with α-GalCer-sensitized DCs (~85% reduction), and this protective effect was fully dependent on iNKT cells (no reduction of lung metastases in iNKT cell-deficient mice) (Figure 7). Interestingly, CpG ODN-treated DCs also dramatically reduced the number of B16F10 colonies in WT mice (~85% reduction, compared to nonstimulated DCs). Although CpG ODN-treated DCs still lowered the number of metastases in iNKT cell-deficient mice, the effect was not significant. Thus, CpG ODN-treated DCs

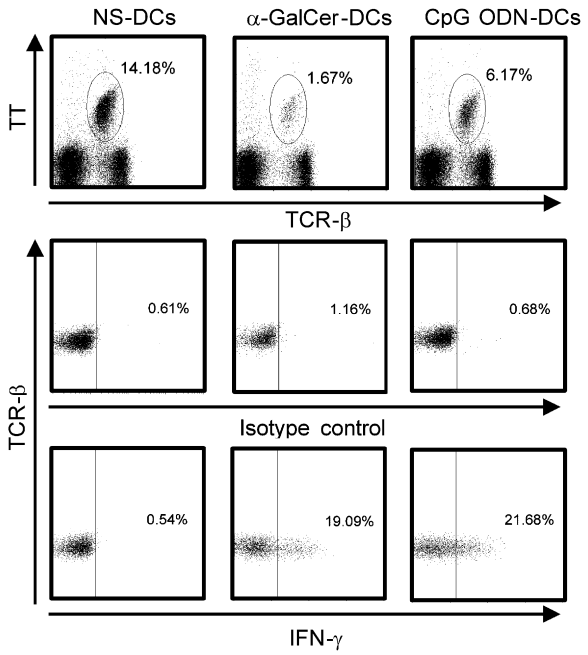

Figure 6. iNKT Cells Produce IFN-γ after In Vivo Stimulation with CpG ODN-Treated DCs

WT mice were i.v. injected with α-GalCer- or CpG ODN-stimulated DCs and were sacrified 2 hr or 16 hr later, respectively. Liver MNCs were treated with brefeldin A for another 2 hr and were labeled with TCR-β mAb and TT, fixed, and permeabilized for intracellular cytokine staining. Cells were analyzed by flow cytometry, and gated TT^+ $TCR\text{-}\beta^+$ cells were screened for intracellular IFN-γ production. Gates were set based on the isotype control. The percentages of TT^+ $TCR\text{-}\beta^+$ cells positive for IFN-γ are represented. One representative experiment out of three is shown.

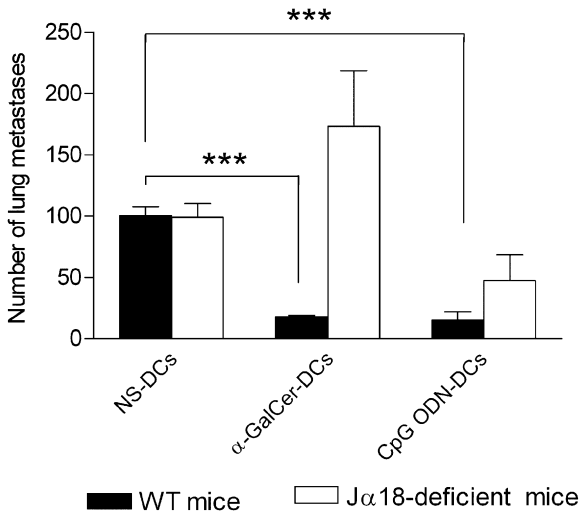

Figure 7. CpG ODN-Treated DCs Protect against Lung Metastases of B16 Melanoma

WT or Jα18-deficient mice were inoculated i.v. with 5×10^5 B16F10 cells at day 0. At days 0, 4, and 8, mice were injected i.v. with 10^6 DCs previously treated or not with α-GalCer or CpG ODN. 14 days after tumor inoculation, lungs were harvested and B16F10 colonies were counted and recorded as the mean number ± SEM. Data are pooled from three independent experiments (n = 9). ***$p < 0.001$.

protect mice against B16F10 lung metastases, in part through iNKT cell activation.

DISCUSSION

Because TLRs relay maturation signals to DCs and play a role in Ag processing and presentation (Blander and Medzhitov, 2006; Takeda and Akira, 2005), we herein compared several major known TLR ligands for their capacity to trigger DC-mediated activation of iNKT cells. We first show that, among the panel of TLR agonists tested, only LPS, R848, and CpG ODN were potent inducers of IFN-γ (but not IL-4) release by hepatic MNCs. Furthermore, we show that TLR9- (and TLR4- and TLR7-, not shown) stimulated DCs directly activate purified iNKT cells to produce IFN-γ. Thus, as described for the LPS-detecting receptor TLR4 (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007), activation of the nucleic acid-recognizing receptors TLR7 and TLR9 in DCs biased iNKT cells toward Th1 type-producing cells. The reason why TLR2- and TLR5-treated DCs were not active might be explained by the lack of type I IFN and bioactive IL-12 synthesis after stimulation, the latter cytokine being known to drive autoreactive iNKT cells to produce IFN-γ (Takeda et al., 1996). In agreement with our previous observation (Mallevaey et al., 2006), TLR3 stimulation in DCs had no effect on iNKT cell activation, although its engagement induced type I IFN release (but not bioactive IL-12) by DCs. It is possible that the lack of TLR3 activity on iNKT cells is due to its inability to induce appropriate signals required for the de novo synthesis of lipid(s) and/or for its (their) loading onto CD1d. Our data confirm those from other reports stating that TLR4 activation in DCs results in iNKT cell activation (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007). It is particularly interesting to note that in this setting, IL-12 was required, but not type I IFN. This suggests that according to the TLR engaged (e.g., TLR4, which responds to extracellular pathogens versus TLR7 and TLR9, which detect intracellular pathogens), alternative mechanisms of iNKT cell activation can occur. Whether DCs stimulated through TLR4 or through TLR7,9 activate the same subpopulation(s) of iNKT cells is still unknown and worthy of future study. Although our data reveal a clear preference for TLR7 and TLR9 (and TLR4) in iNKT cell activation, we however do not fully eliminate the possibility that other TLRs expressed by DCs could be involved in iNKT cell activation during pathological situations. For instance, the TLR agonists used in our study may not accurately reflect the activity of TLR ligands found in vivo, in particular during infection. Moreover, it is known that pathogens can be sensed by several TLRs expressed by DCs and that their combined activation influences both the magnitude and functional polarization of bystander immune cells (Napolitani et al., 2005). In this context, non-TLR7,9 TLR members might cooperate with each other or with TLR7,9 (or TLR4) to influence iNKT cell functions in vivo.

The use of genetically deficient mouse cells and neutralizing Abs indicated that type I IFN (probably IFN-β) is essential in iNKT cell activation induced by CpG ODN- (as well as R848-, not shown) stimulated DCs. This finding is in line with a recent study reporting that IFN-α, produced by CpG ODN-stimulated plasmacytoid DCs, potentiates α-GalCer-induced IFN-γ production by iNKT cells, at least in the human system (Marschner et al., 2005). Furthermore, a recent study showed that IFN-β induces a higher expression of CD1d on DCs and that, through this mechanism, it could contribute to activate iNKT cells during infection (Raghuraman et al., 2006). In contrast, it was reported that exogenous IFN-β decreased the release of IFN-γ by mouse iNKT cells cocultured with α-GalCer-pulsed DCs (Trobonjaca et al., 2002). In this phenomenon, it appeared that IFN-β targeted DCs to reduce IL-12-dependent responses of iNKT cells. Thus, to our best knowledge, our report is the first that shows that type I IFN, a key cytokine implicated in antiviral defense and tumor immunity (Dunn et al., 2006; Stetson and Medzhitov, 2006), cooperates with self Ag(s) to promote iNKT cell cytokine release. Notably, IFN-β alone did not trigger IFN-γ production by iNKT cells cocultured with nonstimulated DCs, indicating that the inherent autoreactivity of iNKT cells toward self Ag is not amplified by this factor. We hypothesized that, along with type I IFN, CpG ODN could induce the generation of new or increasing amounts of self lipids in DCs. Indeed, stress-inducible lipid Ags (including sulfatide and GM1 ganglioside) are increasingly generated in human APCs, in response to some bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) or to LPS, to activate CD1(a, b)-restricted cells (De Libero et al., 2005). Two approaches were employed to investigate this hypothesis in our system. First, pretreatment of DCs with *N*B-DGJ, an inhibitor of GlcCer synthase, profoundly affected the capacity of CpG ODN-stimulated DCs (or lipids extracted from these cells) to activate iNKT cells. Second, DCs incubated with total lipids extracted from CpG ODN- (as well as from R848-, not shown) exposed DCs exerted a stimulatory, CD1d-dependent, effect on iNKT cells, whereas lipids extracted from nonstimulated cells failed to do so. As discussed previously, iNKT cell activation required the presence of type I IFN in this setting. Interestingly, charged, but not neutral, lipids activated iNKT cells via DCs. Moreover, although treatment of the lipids with sodium hydroxide (to eliminate all lipids except β-linked GSLs) had no impact on iNKT cell activation, digestion with ceramide glycanase (cleaves the glycan from ceramide) abrogated it. These data strongly suggest that charged β-GlcCer-derived GSL(s) is (are) generated in DCs in response to CpG ODN to stimulate iNKT cells, in concert with type I IFN. Although unlikely, we cannot fully rule out the possibility that the active charged GSL(s), together with IFN-β, might have an effect on CD1d trafficking and/or loading of a different self Ag, perhaps by stimulating lipid exchange. Of interest, we also found that the charged fraction activated, via DCs, the iNKT cell hybridoma 2C12 to produce IL-2 (not shown). Our results rule out iGb3 as a potential candidate because this GSL is not charged and also because we did not detect its presence in stimulated DCs (not shown). Attempts are now underway to characterize the stimulatory compound(s), the search of which represents an intense area of investigation at the moment. Among potential candidates is GD3, a charged (melanoma-derived) GSL able to activate a subset of iNKT cells (Wu et al., 2003). The consequences of TLR activation on the synthesis of enzymes involved in the genesis of GSLs are presently unknown. We show that, in response to CpG ODN, some transcripts encoding enzymes responsible for GSL synthesis were enhanced in DCs, albeit at a moderate amount. Interestingly, the most marked increase in transcript expression corresponded to three GSL-specific sialyltransferases involved in the production of a, b, and c series of gangliosides, namely *St3gal5*, *St8sia1*, and *St8sia5*. Together, these results confirmed that CpG ODN modulates GSL biosynthesis in DCs.

Suzuki and coworkers recently reported that CpG ODN encapsulated in cationic liposomes activates splenic $CD3^+$ $NK1.1^+$ cells to produce IFN-γ in vivo (Suzuki et al., 2004). Although IFN-γ production by iNKT cells was not firmly shown, and despite the fact that CpG ODN could also act via other cells than myeloid DCs (for instance plasmacytoid DCs), our data are in accordance with and extend those from this report. Indeed, administration of ex vivo CpG ODN-stimulated DCs to naive mice activated liver iNKT cells to produce IFN-γ. As observed in vitro, CpG ODN-stimulated DCs also activated NK cells to produce IFN-γ intracellularly, an effect that partially depends on iNKT cells. It is likely that the residual NK cell activation in iNKT cell-deficient mice is due, at least in part, to direct action of CpG ODN-stimulated DCs on NK cells (Zanoni et al., 2005) (data not shown). It was described that the iNKT cell-NK cell pathway can promote potent cell-mediated antitumor responses (e.g., rejection of B16F10 melanoma) when stimulated with α-GalCer (Smyth et al., 2005) or α-GalCer-sensitized DCs (Fujii et al., 2002, 2006), a phenomenon that depends on IFN-γ. In addition, it has been widely reported that CpG ODN or CpG ODN-stimulated DCs can exert highly effective antitumor immune responses in vivo (Hafner et al., 2001; Suzuki et al., 2004). However, although suspected, the role of iNKT cells in this setting has not yet been formally demonstrated. Here, we show that DCs stimulated through TLR9 can protect against B16F10 melanoma and that it depends, at least in part, on iNKT cells. The lack of total iNKT cell dependency might be explained by the (in)direct effects of CpG ODN-stimulated DCs on NK cells (Zanoni et al., 2005) as well as on other effector cells.

The finding that engagement of TLR7 or TLR9 in DCs activates iNKT cells might be relevant in other pathological situations, such as infection. In the mouse system, these receptors are not only expressed in myeloid DCs but also in plasmacytoid DCs, a cell population participating in innate responses to several different types of viruses, via type I IFN production. Thus, TLR7 and TLR9 might serve a special function in iNKT cell activation during

infection, in particular for subgroups of intracellular pathogens. Indeed, early after mouse cytomegalovirus infection, splenic NK1.1$^+$ TCRβ$^+$ (NKT) cells as well as NK1.1$^+$ TCRβ$^-$ (NK) cells produce IFN-γ intracellularly and that it is largely dependent on TLR9 (Tabeta et al., 2004). Whether myeloid DCs and/or plasmacytoid DCs participate in this process is still unknown and deserves further investigation. Moreover, although it is known that infected DCs can directly activate NK cells to produce IFN-γ and/or to promote their cytotoxic functions (Degli-Esposti and Smyth, 2005), it would be interesting to assess the contribution of TLR-based DC-iNKT cell crosstalk on NK cell functions during infection. In certain circumstances, TLR7 and TLR9 can also be activated by self components (small ribonucleoproteins and auto-DNAs, respectively) to promote autoimmune diseases (Deane and Bolland, 2006). Here, too, whether TLR-stimulated DCs contribute to the pathology by impacting iNKT cell functions is unknown and worthy of further investigation. Along with its therapeutic potential to improve resistance to cancer and infection, CpG ODN (as well as TLR7/8 ligands) can also protect against some inflammatory diseases, including asthma (Klinman, 2004; Krieg, 2006). It is possible that the mechanisms by which activators of TLR7 or TLR9 provide therapeutic benefit in these pathological situations is dependent on the iNKT cell-NK cell axis.

In conclusion, we have described a mechanism of iNKT cell activation and provided evidence that TLR9 (as well as TLR7) triggering in myeloid DCs is important in the primary activation of iNKT cells. We also report that type I IFN can drive iNKT cells to produce IFN-γ in concert with TLR-inducible lipid(s) expressed by DCs. Our results may lead to the identification of new CD1d-restricted Ag(s) involved in the peripheral activation of iNKT cells.

EXPERIMENTAL PROCEDURES

Reagents and Abs

Pam_3CSK_4 and FSL-1 were purchased from EMC Microcollections (Tuebingen, Germany) and p(I:C), ultrapure LPS (from *Escherichia coli* serotype 0111:B4), resiquimod (R848), and type B CpG ODN (ODN 1826) were from Cayla (Toulouse, France). Non-CpG ODN (Cayla), in which the CG sequence was changed to GC, was used as a control. Flagellin was kindly provided by J.C. Sirard (Institut Pasteur de Lille). α-GalCer was purchased from Axxora Life Sciences (Coger S.A., Paris, France), and *N*B-DGJ was from Calbiochem (San Diego, CA). Monoclonal Abs against mouse CD5 (APC-conjugated), NK1.1 (PE- or PerCP-conjugated), IFN-γ (PE-conjugated), isotype controls, and PE-conjugated streptavidin were purchased from BD PharMingen (Le Pont de Claix, France). APC-conjugated TT was from ProImmune (Oxford, UK). The neutralizing goat IgGs directed against mouse IFNAR1 and IL-12 was from R&D Systems (Abingdon, UK), and rat monoclonal anti-mouse IFN-β Ab was from PBL Biomedical Laboratories (Piscataway, NJ). Isotype control Abs were from Sigma (Lyon, France). The biotin-conjugated IFNAR1 mAb (MAR1-5A3) and the isotype control mAb (GIR-208) used for FACS staining have been described (Sheehan et al., 2006). Recombinant IFN-β was from PBL Biomedical Laboratories (Piscataway, NJ).

Mice

6- to 8-week-old female wild-type C57BL/6 mice were purchased from Janvier (Le Genest-St-Isle, France). The generation of *Cd1d1*$^{-/-}$ and iNKT cell (Jα18)-deficient C57BL/6 mice has been already described (Kawano et al., 1997; Mendiratta et al., 1997). The generation of *Tlr7*$^{-/-}$, *Tlr9*$^{-/-}$, and *Ifnar1*$^{-/-}$ mice C57BL/6 mice has been described earlier (Hemmi et al., 2000, 2002; Muller et al., 1994). *Il12b*$^{-/-}$ mice were from Jackson Laboratories (Bar Harbor, ME). Mice were bred in our own facility in pathogen-free conditions. All animal work conformed to the Pasteur Institute animal care and use committee guidelines.

Preparation of Liver MNCs

Perfused livers from naive mice were harvested and homogenized with a 90 μm pore filter. After extensive washes, liver homogenates were resuspended in a 33% Percoll gradient, and after centrifugation, the cells in the pellet were recovered. Red blood cells were removed by lysis in 155 mM NH_4Cl (pH 7.4) containing 10 mM $NaHCO_3$ and 0.1 mM EDTA.

Generation of DCs and Coculture

In brief, BM-derived cells were cultured in IMDM medium supplemented with 10% FCS and 1% of supernatant from a granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing cell line (J558-GM-CSF) (Mallevaey et al., 2006) (20–30 ng GM-CSF/ml). DCs were used on day 14 of culture. DCs (1×10^6 cells/ml) were stimulated with α-GalCer (10 ng/ml) or with TLR agonists for 16 hr, extensively washed with PBS, and cultured for 48 hr with liver MNCs (10^5 DCs + 5×10^5 MNCs/well) or sorted iNKT cells (4×10^4 DCs + 4×10^4 iNKTs/well) in round bottom 96-well plates in RPMI supplemented with 5% FCS. In some cases, neutralizing or control Abs were added during the coculture. To fix DCs, CpG ODN-stimulated cells were exposed to glutaraldehyde (0.05% in PBS) for 3 min, to 0.1 M lysine, and then extensively washed. To investigate the activity of lipids extracted from TLR-stimulated DCs, cells (10^5 cells/well, 96-well plate) were exposed to lipid fractions or vehicle alone for 16 hr, washed, and then cocultured with liver MNCs (5×10^5) in the presence or absence of recombinant IFN-β (1000 U/ml). Coculture supernatants were collected, and IL-4 and IFN-γ concentrations were measured by ELISA (R&D Systems). Cytokines present in DC supernatants were quantified by ELISAs with commercial kits distributed by R&D Systems and PBL Laboratories (IFN-αA and IFN-β).

Preparation of Sorted iNKT Cells

For sorting of iNKT cells, liver MNCs were labeled with APC-conjugated anti-CD5 and PE-conjugated anti-NK1.1 Abs. After cell-surface labeling, cells were sorted with a FACSVantage (BD PharMingen). Sorted CD5$^+$ NK1.1$^+$ populations were 90% (TT$^+$ TCRβ$^+$) pure. In some case, iNKT cells were sorted from livers with APC-conjugated TT and FITC-conjugated anti-TCRβ Ab (~97% pure).

GSL Extraction and Treatment

Nonstimulated or CpG ODN-stimulated DCs (5×10^7 DCs) were extensively washed, resuspended in 1 ml water (18.2 MΩ), and subjected to three freeze-thaw cycles to lyse the cells. Chloroform: methanol (2:1, v/v) (4 ml) was added to the cell mixture and left to mix gently overnight at 4°C. Lipids were purified over C18 columns (Waters, Elstree, Herts, UK) as previously described (Neville et al., 2004). These purified lipids represent the total fraction. Neutral and charged lipids were then separated with DEAE-sephadex A25 (Sigma Aldrich, Poole, Dorset, UK) column chromatography. DEAE-sephadex in the acetate form was placed in a disposable column (1 ml bed volume) and washed with chloroform:methanol:water (30:60:8, v/v/v). The total fraction was dried under nitrogen and resuspended in chloroform:methanol:water (30:60:8, v/v/v) and applied to the column. The neutral lipid fraction was obtained after several passages of chloroform:methanol:water (30:60:8, v/v/v), and charged lipids were eluted from the column with chloroform:methanol:1 M sodium acetate (30:60:8, v/v/v). To eliminate all lipids except β-linked GSLs, lipid extracts were dried under nitrogen and resuspended in 500 μl chloroform:methanol (1:1, v/v), and 83 μl of 0.35 M sodium hydroxide in methanol was added for 16 hr at room temperature. A phase split was achieved by the addition of 83 μl

water:methanol (9:1, v/v), 166.5 μl water, and 416 μl chloroform. The upper phase was removed and the lower phase was dried under nitrogen. The lower phase was resuspended in 5 μl chloroform:methanol (1:3, v/v) prior to recombining with the upper phase to be desalted over C18 columns as above. To remove the oligosaccharide from β-GlcCer-based GSLs, ceramide glycanase from *Macrobella decora* (EC 3.2.1.123) was used as recommended (Merck Biosciences, Nottingham, UK). All lipid fractions were desalted over C18 columns, dried under nitrogen, and resuspended in DMSO (the equivalent of 1×10^6 DCs/μl).

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total RNA from resting or CpG ODN-treated DCs were isolated with the Trizol reagent (Life Technologies, Grand Island, NY), and cDNA were synthesized from 1 μg of total RNA with random hexamer primers and Superscript reverse transcriptase (Invitrogen, Cergy Pontoise, France) according to standard procedures. cDNAs were used as templates for PCR amplification with the SYBR Green PCR Master Mix (Molecular Probes, Leiden, The Netherlands) and the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers, which are listed in Table S1, were designed by the Primer Express Program (Applied Biosystems) and used for amplification in triplicate assays. PCR amplification of *Gapdh* was performed to control for sample loading and to allow normalization between samples. ΔCt values were obtained by deducting the raw cycle threshold (Ct values) obtained for *Gapdh* mRNA, the internal standard, from the Ct values obtained for investigated genes. For graphical representation, data are expressed as n-fold increase in mRNA level compared to the expression level in nonstimulated cells.

Analysis of iNKT Cell Activation In Vivo

Mice were injected i.v. with nonstimulated, α-GalCer-sensitized, or CpG ODN-stimulated DCs (10^6 DCs/mouse) in 200 μl PBS. Saline-perfused livers were harvested 16 hr later (2 hr for α-GalCer-treated DCs), and liver MNCs were prepared as described above. Cell suspensions were incubated with appropriate dilutions of APC-conjugated TT and FITC-labeled anti-TCRβ for 30 min in PBS containing 2% FCS and 0.01% NaN_3. Cells were then fixed in PBS 1% paraformaldehyde for 10 min, resuspended in PBS plus 2% FCS and 0.1% saponin (permeabilization buffer), and incubated with PE-conjugated mAb against IFN-γ or control IgG mAb in permeabilization buffer. Cells were acquired and analyzed on a FACSCalibur (Becton Dickinson, Rungis, France) cytometer by the CellQuest software.

B16F10 Lung Metastasis Model

B16F10 melanoma cells were maintained as described previously (Smyth et al., 2005). WT or Jα18-deficient mice received 5×10^5 B16F10 cells by way of i.v. injection. 3 hr later, mice were injected i.v with immature DCs, α-GalCer-sensitized DCs, or CpG ODN-stimulated DCs (10^6 DCs/mouse) in 200 μl PBS. On days 4 and 8, mice were reinjected with DCs, having been treated equally than the first day. Mice were killed on day 14, and surface lung metastases were counted with the aid of a microscope.

Statistical Analysis

Results are expressed as the mean ± SD or ± SEM. The statistical significance of differences between experimental groups was calculated by an ANOVA1 with a Bonferroni post test or an unpaired Student's t test (GraphPad Prism 4 Software, San Diego, CA). Results with a p value of less than 0.05 were considered significant. The percentages of inhibition are given as averages of at least three independent experiments and are indicated in the text.

Supplemental Data

Four figures are available at http://www.immunity.com/cgi/content/full/27/4/597/DC1/.

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