Immunoregulatory Role of CD1d in the Hydrocarbon Oil-Induced Model of Lupus Nephritis


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Immunoregulatory Role of CD1d in the Hydrocarbon Oil-Induced Model of Lupus Nephritis


Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is accompanied by the emergence of autoreactive T cells and a reduction in regulatory T cells. Humans and mice with SLE have reduced numbers of CD1d-restricted NK T cells, suggesting a role for these cells in the regulation of SLE. In this study, we show that CD1d deficiency exacerbates lupus nephritis induced by the hydrocarbon oil pristane. This exacerbation in disease is associated with: 1) reduced TNF-α and IL-4 production by T cells, especially during the disease induction phase; and 2) expansion of marginal zone B cells. Strikingly, inoculation of pristane in wild-type mice resulted in reduced numbers and/or functions of NK T cells and CD1d-expressing dendritic cells. These findings suggest that CD1d may play an immunoregulatory role in the development of lupus in the pristane-induced model. The Journal of Immunology, 2003, 171: 2142–2153.

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5 Abbreviations used in this paper: SLE, systemic lupus erythematosus; α-GaLCer, α-galactosylsphingoceramide; DC, dendritic cell; KBS, kidney biopsy score; GAS, glomerular activity score; TIA, tubulointerstitial activity score; BUN, blood urea nitrogen; CLS, chronic lesions score.

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Oil-Induced Model of Lupus Nephritis

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ystemic lupus erythematosus (SLE, lupus) is a systemic autoimmune disease characterized by the loss of tolerance to a variety of self-Ags. Development of SLE is associated with the emergence of autoreactive Th cells (1–4) accompanied with a reduction in regulatory T cells (5–7). The nature and specificity of regulatory T cells that inhibit autoantibody production and development of lupus remain largely undefined. Some regulatory T cells are γδ T cells or V_{H} peptide-reactive CD8^{+} T cells that inhibit autoantibody production by ablating autoreactive B cells (3, 7). Recent reports show that humans and mice with lupus and related autoimmune diseases have reduced numbers of NKT cells (8–11), which recover with improvement in disease activity in patients with SLE (10). These observations suggest that NKT cells may be part of a regulatory T cell network that inhibits the induction of SLE.
blotting (25) or by PCR using the neo (sense, 5′-CTTGGTGAGGAG GCTATTC-3′; antisense, 5′-AGGGTGAAGGACGGGATGAC-3′) and CD1d primers (sense, 5′-ATAATACCTTCCCGTCGCC-3′; antisense, 5′- CTTGGTAGGTGAGCTTGGT-3′) under the following conditions: 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min for 35 cycles. The N9 CD1d+ BALB/c mice were intercrossed to establish CD1d+ BALB/c mice. The CD1d+ phenotype was further confirmed by flow cytometry of PBLs stained with a conjugated anti-CD1 mAb, 1B1 (BD PharMingen, San Diego, CA). Anti-DNA Ab were measured by ELISA, as previously described (6, 7). Anti-DNA Ab titers are expressed as units per milliliter using a reference-positive standard of pooled serum from MRL-lpr/lpr mice.

**Flow cytometry**

For liver NKT cells, liver was perfused with PBS via the portal vein until opaque and pressed through a 70-μm cell strainer (BD Biosciences, Mountain View, CA). Hepatocytes were pelleted by centrifugation at 30 × g for 3 min. The remaining liver cells in the supernatant were pelleted at 300 × g for 5 min and then resuspended in a 40% isotonic Percoll solution (Amersham Pharmacia Biotech, Piscataway, NJ). This suspension was underlaid with a 60% isotonic Percoll solution. After centrifugation for 20 min at 1500 × g, mononuclear cells were isolated at the 40%/60% interface, and then washed once with RPMI 1640 medium (Life Technologies, Grand Island, NY) with 5% FCS (HyClone Laboratories, Logan, UT). The cells were stained with murine CD1d/α-GalCer tetrarmers that were generated as described elsewhere (18) or with human CD1d/α-GalCer tetrarmers that also recognize murine NKT cells (17). Stained cells were analyzed by flow cytometry. Spleen or thymus cells were incubated with anti-CD16/32 (2.4G2; BD PharMingen) to block FcγRII/III, followed by staining with various conjugated mAbs (all BD PharMingen), as indicated in the figure legends. Stained cells were analyzed using a BD Biosciences FACSCalibur flow cytometer and CellQuest software.

**Activation of NKT and T cells**

For in vitro NKT cell activation, spleen cells (1–2 × 10^6/ml) were incubated with titrated doses of synthetic α-GalCer (KRN7000; Kirin Brewery, Gunma, Japan) (15). For T cell activation, splenocytes (2 × 10^5/well) were stimulated with plate-bound anti-CD3 mAb (1–10 μg/ml) and supernatants were collected after 48 h of culture for the measurement of cytokines.

**Detection of cytokines**

A standard sandwich ELISA was used to measure cytokines (Figs. 3 and 5a, b), as previously described (4). TNF-α levels were measured using the BD PharMingen mouse cytokine cytometric bead array kit (Fig. 4) according to the manufacturer’s instructions. To examine the cellular sources of cytokines in α-GalCer-stimulated cultures (Fig. 5, b–e), a cytokine secretion assay was performed using the MACS cytokine secretion assay kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol with some modifications. Briefly, stimulated or control spleen cells (1 × 10^7) were incubated with titrated doses of synthetic α-GalCer (KRN7000; Kirin Brewery, Gunma, Japan) (15). For T cell activation, splenocytes (2 × 10^5/well) were stimulated with plate-bound anti-CD3 mAb (1–10 μg/ml) and supernatants were collected after 48 h of culture for the measurement of cytokines.

**Statistical analysis**

Levels of Abs and cytokines, lymphocyte percent and numbers, and renal scores were compared using Student’s t or Mann-Whitney U test. Frequencies of Abs, proteinuria, and BUN were compared using the two-sided Fisher’s exact test.

**Results**

CD1d deficiency accelerates pristane-induced lupus nephritis

Inoculation of hydrocarbon oils such as pristane induces lupus-like autoantibody production and mild glomerulonephritis in otherwise normal mouse strains such as BALB/c (Refs. 23 and 24, and see data in CD1d+ mice in Figs. 1 and 2). To determine whether CD1d is involved in the development of pristane-induced lupus, we backcrossed CD1d+ 129/B6 mice onto the BALB/c background for nine generations and inoculated the final CD1d+ and control BALB/c mice with pristane or PBS. All mice were bled before and at 3 and 6 mo postinoculation and monitored for proteinuria.

Proteinuria developed early and was more severe in the pristane-inoculated CD1d+ mice than in the CD1d- littermates (Fig. 1a). At 6 mo postinoculation, 54% of the mice in the CD1d+ group, but none in
the CD1d⁺ group, had ≥100 mg/dl (moderate to severe) proteinuria (p < 0.0001). The frequency of moderate to severe proteinuria was still high in CD1d⁻ mice at 10 mo (74% in CD1d⁻ vs 16% in CD1d⁺; p = 0.005). None of CD1d⁺, but 63% of CD1d⁻, mice developed severe (≥300 mg/dl) proteinuria at 10 mo postinoculation (p = 0.0001).

BUN was also elevated in pristane-inoculated CD1d⁻ mice (Fig. 1b), suggesting an advanced renal disease in these mice. Nine (82%) of 11 CD1d⁻, but 0 of 7 CD1d⁺, mice had elevated (>15 mg/dl) BUN (p = 0.002). Two (18%) of 11 CD1d⁻ mice had a severely elevated BUN (≥30 mg/dl).

Mice were sacrificed at 10 mo of age to harvest kidneys and their renal histology was analyzed (Fig. 1, c and d). Mild and focal mesangio-proliferative glomerulonephritis was found in 60% of pristane-inoculated CD1d⁺ mice (Fig. 1d, middle panels), the remaining 40% of mice had no evidence of nephritis by light microscopy, and none of the CD1d⁻ mice had diffuse proliferative or chronic lesions. CD1d⁻ mice, however, developed diffuse proliferative glomerulonephritis with fibrous crescents, glomerulosclerosis, tubular atrophy, and interstitial fibrosis in 50% of mice (Fig. 1d, right panels); another 25% of pristane-inoculated CD1d⁻ mice had mild to moderate mesangio-proliferative lesions; and the remaining 25% of mice had...
active (GAS) as well as chronic kidney lesions (CLS) in CD1d o mice (Fig. 1b). None of the PBS-injected mice (six CD1d c mice, three CD1d o mice) had proteinuria or renal histological changes (Fig. 1c). Pristane-inoculated BALB/c mice develop autoantibodies to several cellular Ags (23, 24). We detected these Abs with an immunoprecipitation assay using cell extract from an erythroleukemia cell line, K562, as a source of autoantigens. Overall, reactivity to cellular Ags was higher in sera from pristane-inoculated CD1d o mice than in sera from pristane-inoculated CD1d + mice or PBS-injected CD1d + mice (Fig. 2a). Anti-ribosomal P (Fig. 2a) and anti-OJ (isoleucyl tRNA synthetase complex) Abs were significantly increased in the CD1d o mice as compared with wild-type littermates (p < 0.01). Serum IgG anti-dsDNA Ab levels, as measured by ELISA, were also higher in CD1d o mice at 6 mo postinoculation (p < 0.05, Fig. 2c).

**CD1d deficiency enhances pristane-induced autoantibody production**

Pristane-inoculated BALB/c mice develop autoantibodies to several cellular Ags (23, 24). We detected these Abs with an immunoprecipitation assay using cell extract from an erythroleukemia cell line, K562, as a source of autoantigens. Overall, reactivity to cellular Ags was higher in sera from pristane-inoculated CD1d o mice than in sera from pristane-inoculated CD1d + mice or PBS-injected CD1d + mice (Fig. 2a). Anti-ribosomal P (Fig. 2a) and anti-OJ (isoleucyl tRNA synthetase complex) Abs were significantly increased in the CD1d o mice as compared with wild-type littermates (p < 0.01). Serum IgG anti-dsDNA Ab levels, as measured by ELISA, were also higher in CD1d o mice at 6 mo postinoculation (p < 0.05, Fig. 2c).

**T cell cytokine responses in pristane-injected CD1d-deficient BALB/c mice**

Abnormalities in cytokine production contribute to the development of lupus (24). To determine whether exacerbation of lupus in CD1d o animals is related to abnormalities in cytokine production, we measured cytokine responses in the spleens of pristane-inoculated CD1d o and CD1d + BALB/c mice. After 12–24 h, 10–12 days, 6 wk, and 6 mo of pristane inoculation, spleen cells were
CD1d deficiency or pristane injection on T cell production of IFN-γ/H9253 signiﬁcantly decreased, whereas IFN-γ/H9253 production was signiﬁcantly lower in pristane-inoculated CD1d−/H9251 mice as compared with CD1d+/H9251 mice, while there was no effect of pristane inoculation itself on CD1d+/H9251 mice; there was no difference in the levels of these cytokines between PBS vs pristane-inoculated mice at 12 h and 11 days after pristane inoculation. This change in cytokine proﬁle in CD1d−/H9251 mice was not associated with signiﬁcant alterations in various spleen cell populations, including CD4, CD8, DC, B220, macrophages, and neutrophils (Ref. 25 and data not shown). At 6 mo, IL-4 and IL-13 production was lower in pristane-inoculated than in PBS-inoculated animals (Fig. 4). Such selective deﬁciency in T cell production of TNF-α during the initial phases of disease development may contribute to the development and exacerbation of disease in wild-type and CD1d−/H9251 mice, respectively.

NKT cell functions and numbers are reduced in pristane-injected animals

Although the lack of cytokine production upon α-GalCer stimulation was expected in CD1d−/H9251 mice, it was surprising that in wild-type mice α-GalCer-induced TNF-α production was signiﬁcantly lower in pristane-inoculated than in PBS-inoculated animals (Fig. 4). This suggested that pristane inoculation itself alters NKT cell functions. To further evaluate the effect of pristane on NKT cell functions, spleen cells were harvested from BALB/c mice at various time points (12–24 h, 10–12 days, 6 wk, and 6 mo) after pristane or PBS injection and stimulated with α-GalCer for 48 h. Culture supernatants were assayed for various cytokines, which were signiﬁcantly decreased in the pristane group at 10–12 days (p < 0.05 to < 0.0001, n = 3–7 mice/group).
FIGURE 5. α-GalCer-induced cytokine responses are markedly reduced in pristane-inoculated mice. a, Effect of α-GalCer stimulation on cytokine production: 10- to 14-wk-old BALB/c mice were inoculated with pristane or PBS (n = 24/group). Left panels, Ten- to 11-day after inoculation, spleens were harvested and single spleen cells were cultured with α-GalCer for 40–48 h. Culture supernatants were assayed for cytokines (IFN-γ, IL-2, IL-4, IL-5, and IL-10). Values of p < 0.05–0.01 at all α-GalCer concentrations, n = 4–5 mice/group. Cytokine levels in control cultures containing synthetic β-GalCer or vehicle in which galactosylceramides were dissolved were similar to or <2-fold of the baseline value (cells with medium alone). Right panels, Twelve hours, 10 days, or 6 mo after PBS or pristane inoculation, wild-type and CD1d−/− mice were injected i.v. with 4 μg α-GalCer, bled 2 h later, and serum samples were assayed for IFN-γ, IL-2, IL-4, IL-10, and IL-13. *p < 0.05; **p < 0.001, n = 3–5 mice/group, ±SE. In pristane-inoculated CD1d−/− mice at 6 mo postinoculation, the mean IFN-γ, IL-2, IL-4, IL-10, and IL-13 levels were 0.3, 0.1, 0.03, 0.3, and 0.02 ng/ml, respectively (data not shown). The background serum cytokine levels in wild-type mice injected with vehicle alone ranged from undetectable (<0.02) to 0.15 ng/ml. Data are from one of three similar experiments. b–e, Cytokine-secreting cells that are mostly NKT cells in α-GalCer-exposed animals are reduced after pristane injection. PBS- or pristane-inoculated mice were injected i.v. with α-GalCer (b–d). Two hours after injection, mice were sacrificed; their spleen cells were stained for IFN-γ (upper two rows), IL-2, IL-4, IL-10, and IL-13. *p < 0.05; **p < 0.001, n = 3–5 mice/group, ±SE. In pristane-inoculated CD1d−/− mice at 6 mo postinoculation, the mean IFN-γ, IL-2, IL-4, IL-10, and IL-13 levels were 0.3, 0.1, 0.03, 0.3, and 0.02 ng/ml, respectively (data not shown). The background serum cytokine levels in wild-type mice injected with vehicle alone ranged from undetectable (<0.02) to 0.15 ng/ml. Data are from one of three similar experiments. b–e, Cytokine-secreting cells that are mostly NKT cells in α-GalCer-exposed animals are reduced after pristane injection. PBS- or pristane-inoculated mice were injected i.v. with α-GalCer (b–d). Two hours after injection, mice were sacrificed; their spleen cells were stained for IFN-γ (upper two rows), IL-2, IL-4, IL-10, and IL-13. *p < 0.05; **p < 0.001, n = 3–5 mice/group, ±SE. In pristane-inoculated CD1d−/− mice at 6 mo postinoculation, the mean IFN-γ, IL-2, IL-4, IL-10, and IL-13 levels were 0.3, 0.1, 0.03, 0.3, and 0.02 ng/ml, respectively (data not shown). The background serum cytokine levels in wild-type mice injected with vehicle alone ranged from undetectable (<0.02) to 0.15 ng/ml. Data are from one of three similar experiments.

0.05–< 0.01; Fig. 5a, left panel), 6 wk, and 6 mo postinoculation (data not shown); TNF-α (Fig. 4) and IL-2 levels (data not shown) decreased as early as 12–24 h postinoculation in the pristane group as compared with the PBS group.

We then assessed the effect of pristane on in vivo NKT cell functions. Mice were injected with pristane or PBS and 12–24 h, 10–11 days, or 6 mo later injected i.v. with 4 μg α-GalCer and bled 2 h later for detection of cytokines in serum samples (Fig. 5a,
observed in CD1do mice injected with levels of the expression of activation markers were 88% were NKT cells (TCR
H9252
were cultured with spleens were harvested 2 h later, and the spleen cells
H11001
ther, PBS- or pristane-inoculated mice were injected i.v. with 4 µg α-GalCer. Two hours later, mice were sacrificed and their spleen cells were enumerated for cytokine-secreting CD1α-GalCer tetramer⁺ cells using a cytokine secretion assay. Less than 2% of live B220⁻ lymphocytes in α-GalCer-primed (Fig. 5b) and few cells in unprimed (Fig. 5e) animals were positive for IFN-γ, IL-2, or IL-4. Most cytokine⁺ cells were tetramer positive (Fig. 5b). To further confirm this, spleen cells (1 x 10⁶) were enriched for cytokine⁺ cells (Fig. 5c), which was highly efficient (>98.5%) for all three cytokines tested. Among all IFN-γ⁺ cells in PBS-injected mice, 88% were NKT cells (TCRβ⁺ tetramer⁺) and ~6% each were conventional T cells (TCRβ⁻ tetramer⁻) or NK cells (TCRβ⁺DX⁵⁺) (Fig. 5d). Only a few cytokine-secreting tetramer⁺ cells expressed DX5 (Fig. 5d). Intriguingly, IL-4-secreting cells expressed more α-GalCer-induced responses shown in Fig. 5a may reflect its direct effect on NKT cells as well as the secondary effects of NKT cell activation on other immune cells (27). To investigate this further, PBS- or pristane-inoculated mice were injected i.v. with 4 µg α-GalCer. Two hours later, mice were sacrificed and their spleen cells were 157 vs 202 for IL-4 and 220 vs 194 for IFN-γ in pristane- vs PBS-injected mice, respectively.

α-GalCer stimulation induces the expression of activation markers on spleen cells (27). To assess whether this function of NKT cells is also compromised in pristane-induced lupus, α-GalCer-stimulated spleen cells from PBS- or pristane-inoculated wild-type and CD1d⁻ mice were analyzed for activation and memory markers, CD25, CD44, CD69, CD80, and CD86, by flow cytometry (Fig. 6 and data not shown). Significant decreases in the induction of CD25 and CD69 on B and T cells were observed at 6 mo postinoculation (p = 0.03–0.0005; Fig. 6), but not significantly at earlier time points, i.e., 12 h and 10–12 days postinoculation (data not shown). On CD1dα-GalCer tetramer⁺ cells, however, decreases in CD25 and CD69 expression were observed as

right panel). All cytokines tested were markedly reduced at 6 mo postinoculation (p = 0.002–0.00003, pristane vs PBS-injected mice). Serum IL-2 and IL-13 levels decreased as early as 12–24 h after pristane inoculation, while serum levels of other cytokines were variable at 12 h and 10 days postinoculation. Thus, NKT cell cytokine responses, as assessed by brief in vivo or in vitro exposure to α-GalCer, markedly decline after pristane inoculation.

FIGURE 6. α-GalCer-induced expression of activation markers on spleen cells is reduced in pristane-inoculated mice. Six months after pristane inoculation, mice were injected with 4 µg of α-GalCer i.v., their spleens were harvested 2 h later, and the spleen cells were cultured with α-GalCer for 40 h. Expression of activation markers, CD25, CD44, CD69, CD86, and CD62 ligand, on B and/or T cells is shown. The (mean ± SE) percentage of cells expressing these markers is shown in the table, *, p < 0.05 and **, p < 0.01 compared with PBS-injected wild-type mice (WT/PBS/α-GC group); n = 3–5 mice/group. Similar levels of the expression of activation markers were observed in CD1d⁻ mice injected with α-GalCer and wild-type mice injected and cultured with vehicle alone (WT/PBS/Vehicle group). Results are from one representative of three independent experiments.

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*Not shown in the FACS plot.
early as 10 days postinoculation. For example, the percent CD69+ tetramer+ cells (6.6 ± 0.6 vs 12 ± 1% of gated TCRβ+ cells, p < 0.05) as well as the mean fluorescent intensity of CD69 on tetramer+ cells (51 ± 2.3 vs 63 ± 5.7, p < 0.05) were significantly reduced in pristane-inoculated as compared with PBS-injected mice. Pristane inoculation also affected the induction of other activation and memory markers. For example, the induction of CD44 and CD86 expression was significantly decreased on TCRβ+ and B220+ cells, respectively (p < 0.01–0.001; Fig. 6). As expected, induction of all activation markers was markedly lower in CD1d+ mice (Fig. 6).

Results shown in Figs. 4–6 describe the effect of pristane on α-GalCer-induced NKT cell responses. To examine whether NKT cell numbers decline spontaneously (i.e., without any α-GalCer priming) in pristane-injected mice, we enumerated CD1d/α-GalCer tetramer+ TCRβ+ cells in the thymus, spleen, and liver of BALB/c mice at 12–24 h, 10–12 days, 6–8 wk, or 12 mo after pristane injection. We found that the most severe deficiency in NKT cells found was in thymus 6–8 wk after (Fig. 7a), but not at earlier time points (12–24 h and 10–12 days) after pristane injection (data not shown). The percent and total thymic tetramer+ cells were significantly reduced at 6–8 wk in pristane-inoculated mice as compared with PBS-injected control animals (p < 0.05–0.01); the percent and total splenic tetramer+ cells, however, were not significantly different between the two groups (p = 0.06–0.08; Fig. 7a). In the liver, results on tetramer+ cells at 12 mo postinoculation were difficult to interpret (data not shown) because of pristane-induced changes in the liver architecture and cellularity.

NKT cells comprise diverse phenotypes of T cells (19); some subsets that may contribute to immune regulation and protection against autoimmunity might be particularly deficient in lupus mice. To examine such a possibility, we analyzed CD4 and CD8 expression on tetramer+ cells at 6–8 wk after pristane injection (Fig. 7b). In the thymus, the reduction in percent and total tetramer+ cells in pristane-injected animals was seen in all three subsets, CD4+, CD4−CD8+, and CD4+CD8− double-positive cells. In the spleen, the percent, but not total, CD4+ and CD4−CD8+ tetramer+ cells were reduced in pristane-inoculated mice (p < 0.05). Thus, all subsets of invariant NKT cells decline in pristane-injected animals. Thus, primary NKT cell functions as well as secondary effects of NKT cells on other immune cells are markedly compromised in pristane-inoculated BALB/c mice. Although a significant decrease in NKT cell numbers and most NKT cell functions are detectable 6 wk after pristane inoculation, some impairment of NKT cell functions, such as TNF-α and IL-2 production upon α-GalCer stimulation, begins as early as 12–24 h after pristane exposure.

To explore the mechanisms of decline in NKT cell functions and numbers after pristane inoculation, we examined the effect of pristane inoculation on CD1d-expressing APC (Fig. 8). Overall, the expression of CD1d was decreased in spleen cells of pristane-inoculated mice as compared with untreated control animals (mean fluorescence intensity, 79 ± 0.8 vs 98 ± 3.6, p < 0.05). Specifically, the number of CD1d-expressing CD11c+ DCs was significantly decreased in pristane-injected mice as compared with control animals (6.7 ± 0.2 vs 9.4 ± 0.3, p < 0.01). The CD1d+CD11c+ cells could be subdivided into three subsets, namely, CD1dCD11c+CD11b+, CD1dCD11c+CD11b−, and CD1dCD11c− cells. Significant differences between the pristane-inoculated and control mice were seen in the numbers of CD1d+CD11c+CD11b+ (1.7 ± 0.2 vs 25 ± 0.3, p < 0.05) and CD1d+CD11c+CD11b− subsets (3.3 ± 0.3 vs 5.2 ± 0.1, p = 0.02) of CD11c+ cells. The CD1d-expressing B220+ cells were also slightly decreased in pristane-inoculated mice (p = 0.04–0.06). There was no significant effect of pristane injection on the numbers of CD1d-expressing macrophages (CD11b−) and T cells. Thus, pristane inoculation results in decreased numbers of CD1d-expressing DCs and B

FIGURE 7. Decreased NKT cell numbers in pristane-inoculated mice. Eight weeks after inoculation with pristane or PBS, 16-wk-old BALB/c mice were sacrificed and their thymus and spleen cells were analyzed for CD1d/α-GalCer tetramer+ cells. a, TCRβ+ tetramer+ cells are expressed as the mean ± SE (percent) of gated lymphocytes or as the absolute numbers ×104; b, NKT cell subsets (CD4+ tetramer+, CD4−CD8+ tetramer+, and CD4−CD8− tetramer− cells) are expressed as the mean percentage of gated lymphocytes. Note that TCRβ+ tetramer+ thymocytes (a) and both CD4+ and CD4− subsets of tetramer+ thymocytes or splenocytes (b) were significantly lower in pristane-injected than in control animals. A minor population of double-positive (CD4+CD8+) tetramer− thymocytes was also decreased in pristane-injected mice as compared with control mice (b). *, p < 0.05; **, p < 0.01, n = 3–6 mice/group, ±SE. Results are representative of three independent experiments.

FIGURE 8. Reduced numbers of CD1d-expressing DCs in pristane-inoculated mice. Twelve days after inoculating with pristane or PBS, BALB/c mice were injected i.v. with 4 μg of α-GalCer and sacrificed 2 h later. Total spleen cell numbers were similar between the two groups of animals. CD1d+CD11c+, CD1d−B220+, CD1d−CD11b+, and CD1d−TCRβ+ cells are expressed as the mean percentage of spleen cells. *, p < 0.01, n = 5 mice/group. Results are representative of three independent experiments.
cells, which, in turn, may be responsible for the decreased NKT cell functions in pristane-induced lupus.

**Effect of CD1d deficiency on marginal zone B cells in pristane-inoculated BALB/c mice**

Results in Fig. 8 show that, while overall CD1d expression was decreased on B cells, the CD1d<sup>hig</sup>B220<sup>+</sup> cells that correspond to marginal zone B cells (29) were increased in pristane-injected mice in two separate experiments (total number of cells (×10<sup>6</sup>) were 3.7 ± 0.7 and 8.1 ± 0.9 in PBS- vs pristane-injected mice, p < 0.05). To further examine the effect of pristane inoculation and CD1d deficiency on marginal zone B cells, we enumerated CD21<sup>hig</sup>CD23<sup>low</sup> B cells in the spleens of CD1d<sup>−/−</sup> and CD1d<sup>+/+</sup> mice on day 11 or 30 (Fig. 9, a and b) or at 6 or 12 mo (data not shown) after pristane or PBS injection. We found that marginal zone B cells were increased by ~1.5- to 2-fold in the pristane group at 1, 6, and 12 mo after injection, regardless of the CD1d status of mice. Interestingly, this expansion of marginal zone B cells was restored to normal in pristane-injected BALB/c mice treated with a CD1d ligand, α-GalCer (A. K. Stanic, J.-Q. Yang, L. V. Kaer, and R. R. Singh, manuscript in preparation). Additionally, although serum IgG1 and IgG2a levels were similar between the two groups of animals (data not shown), serum levels of IgG3 isotype that is known to be preferentially secreted by marginal zone B cells (29) were significantly higher in pristane-injected CD1d<sup>−</sup> mice than in pristane-injected CD1d<sup>+/+</sup> mice (Fig. 9c). Previous studies have reported reduced CD23 expression on B cells in lupus-prone NZB mice (30). We found that the mean fluorescent intensity of CD23 expression on B cells was also reduced in pristane-injected mice as compared with PBS-injected mice, regardless of the CD1d status of mice, although the differences were not statistically significant (data not shown).

**Effect of α-GalCer-activated NKT cells on autoantibody production in vitro**

Our findings suggest that the development of lupus-like disease in pristane-inoculated wild-type mice and its exacerbation in CD1d<sup>−</sup> animals is related, at least in part, to the induced or genetic deficiency of NKT cells, respectively. To provide further support for this possibility, we tested whether NKT cell activation can suppress autoantibody production by B cells from pristane-injected mice. For this purpose, B cells isolated from the spleens of pristane-inoculated BALB/c mice were cocultured with T cells isolated from the spleens of PBS-injected control animals, with or without α-GalCer. Supernatants collected on day 6 were tested for IgG anti-DNA Ab and rheumatoid factor. IgG anti-DNA Ab, and rheumatoid factor levels in these cultures were generally low and were not affected by the addition of α-GalCer (data not shown). However, in LPS-stimulated cultures, which had high levels of rheumatoid factor, addition of α-GalCer significantly decreased rheumatoid factor levels (Fig. 10).

**Discussion**

In this article, we report that lupus nephritis, which is generally mild in pristane-inoculated BALB/cJ mice, is quite severe and advanced in CD1d<sup>−</sup> mice. Anti-ribosomal P and anti-OJ Abs that are not normally induced in pristane-inoculated BALB/cJ mice (26) are detected in CD1d<sup>−</sup> mice. This exacerbation of disease activity is associated with: 1) reduced TNF-α and IL-4 production by T cells, especially during the disease induction phase; and 2) expansion of marginal zone B cells. Strikingly, pristane inoculation by itself results in reduced NKT cell numbers and functions and decreased numbers of CD1d-expressing DCs and B cells.

Our observations suggest a regulatory role of CD1d-restricted events and that depletion of NKT cells or reduction in their functions may participate in the development of lupus. Indeed, patients with lupus and other systemic autoimmune diseases have reduced numbers of NKT cells (9–11). The CD161, a marker of NK/NKT cells, is the most significantly decreased lineage marker in the

![FIGURE 9](http://www.jimmunol.org/)  
*Effect of pristane inoculation and CD1d deficiency on marginal zone B cells. CD1d<sup>−</sup> or CD1d<sup>+/+</sup> mice were injected with PBS or pristane and sacrificed on day 11 or 30. Their spleen cells were stained with CD21, CD23, and B220 and analyzed by flow cytometry. Marginal zone B cells (CD21<sup>hig</sup>CD23<sup>low</sup>) are shown as the mean ± SE (percent) of B220<sup>+</sup> cells on day 30 (a) or on days 11 and 30 (b) after injection with PBS or pristane. Note 1.5- to 2-fold expansion of marginal zone B cells on day 30, but not on day 11, in pristane-injected mice. Results represent three independent experiments (n = 2–4 mice/group). c, Serum total IgG3 levels (mean ± SE, µg/ml) in CD1d<sup>−</sup> and CD1d<sup>+/+</sup> BALB/c mice 9–12 mo after pristane injection (n = 13–16 mice/group, p < 0.02).*

![FIGURE 10](http://www.jimmunol.org/)  
*α-GalCer-stimulated T cells can decrease autoantibody production by LPS-activated B cells from pristane-inoculated mice in vitro. Splenic B cells from BALB/c mice 6 mo after pristane inoculation were cocultured with splenic T cells from PBS-injected control animals, with or without α-GalCer. Supernatants collected on day 6 were tested for rheumatoid factor, which are expressed as the mean ± SD triplicate OD.*
Peripheral blood cells of patients with SLE, as revealed in a recent gene expression study (31). Importantly, lupus disease activity appears to inversely correlate with numbers of circulating NKT cells in patients with SLE (10). Lupus-prone NZB/NZW F1 mice, when rendered deficient in CD1d, also experience an exacerbation of lupus nephritis (J.-Q. Yang, L. V. Kaer, and R. R. Singh, manuscript in preparation), albeit less profound than in the pristane model. Old (>1.5 years) CD1d<sup>−/−</sup> BALB/c mice also have increased serum anti-DNA Ab levels as compared with age-matched controls (S. Porcelli, personal communication). A more direct evidence of NKT cell involvement in regulation of lupus-like autoimmunity comes from aging B6 Jax18 knockout mice which have elevated anti-DNA and anti-cardiolipin Abs and renal IgG and complement deposition as compared with age-matched B6 controls (S. Porcelli, F. Dieli, and G. Sireci, personal communication). In MRL-<i>lpr/lpr</i> mice, however, CD1d deficiency does not worsen kidney disease or anti-DNA Ab production (32). The regulatory effect of CD1d-restricted events on nephritis may require intact Fas signaling that is absent in MRL-<i>lpr/lpr</i> mice or the antiapoptotic effects of mutant Fas ligand are able to bypass the role of CD1d-reactive T cells. Furthermore, mechanisms of tissue damage appear to differ between different manifestations of lupus in the same or in different animal models of lupus (22, 32), which probably represent different subsets of this heterogeneous disease in humans. For example, mouse strains that develop anti-DNA Abs and kidney disease represent ~50% of patients with lupus nephritis, whereas NZB/NZW F1 and MRL-<i>lpr/lpr</i> mice, which develop diffuse proliferative nephritis (7, 22), may represent ~30% patients with lupus nephritis (reviewed in Ref. 33). It is, therefore, not surprising that different mechanisms may operate in the development of lupus in various animal models. It is also possible that many functionally distinct subsets of CD1d-reactive T cells may exist; while some may promote autoimmune responses (34), others inhibit autoimmune disease (34, 35). For example, implantation of transgenic T cells that express the TCR-<i>α</i> and -<i>β</i> chain genes from a T cell clone, which is CD1d specific but does not express the invariant V<sub>α</sub>14 NKT TCR, induces a lupus-like disease in irradiated BALB/c nude recipients, whereas another subset of CD1d-reactive T cells prevents the development of lupus in the same model (34).

There are multiple mechanisms that may explain why impairment or absence of CD1d-regulated events in pristane-injected wild-type and CD1d<sup>−/−</sup> mice, respectively, would render them more susceptible to lupus (Fig. 11). First, abnormalities in cytokine production may contribute to the induction of autoimmunity in pristane-inoculated mice and its exacerbation in CD1d<sup>−/−</sup> animals. We found that invariant NKT cells in pristane-inoculated mice express lesser amounts of IL-4 and higher amounts of IFN-γ (Fig. 5f). In patients with another autoimmune disease, multiple sclerosis, remission from disease is associated with a Th2 bias of CD4<sup>+</sup> NKT cells (35), supporting our interpretation that lack of IL-4-secreting NKT cells may contribute to the exacerbation of autoimmune disease. Since activation of NKT cells by CD1d and α-GalCer may direct conventional T cells to the acquisition of a Th2 phenotype (27), decrease or absence of NKT cells may result in a Th1 bias of conventional T cells. Indeed, anti-CD3 or Con A-stimulated T cells from pristane-injected CD1d-deficient mice exhibit decreased IL-4 production along with stable IFN-γ production (Fig. 3). Such a cytokine milieu may be detrimental in pristane-inoculated mice, since IL-4 deficiency is known to accelerate autoantibody production in pristane-induced lupus (24). Furthermore, T cells from pristane-inoculated wild-type and CD1d<sup>−/−</sup> mice produce smaller amounts of TNF-α during the early phase of disease development (Fig. 4, middle panel). This finding is important since low levels of TNF-α are believed to trigger an initial step toward the development of renal disease in lupus-prone mice (36, 37).

<ref id="fn1">J.-Q. Yang, V. Saxena, H. Xu, L. Van Kaer, C.-R. Wang, and R. R. Singh. Repeated α-galactosylceramide administration results in expansion of NKT cells and alleviates inflammatory dermatitis in MRL-<i>lpr/lpr</i> mice. Submitted for publication.</ref>
When rendered deficient in TNF-α, NZB mice, which generally develop only a very mild autoimmune disease, exhibit a more severe form of nephritis (38). In our experiments, TNF-α levels increase at 6 mo after pristane injection, i.e., the time when these animals begin to develop kidney disease (Fig. 4, right panel). These findings are consistent with previous reports showing that treatment with TNF-α initiated at a young age improves lupus nephritis (36), whereas administration of TNF-α at a later age accelerates nephritis (39). Thus, reduced TNF-α and IL-4 production by T cells during the early phase of disease development may contribute to the induction of lupus in wild-type mice and to exacerbation of disease in CD1d−/− mice.

Second, NKT cells appear to play a critical role in the development of immune tolerance (40), which is promoted by the expression of CD1d on APC (41). Thus, a decrease in CD1d-expressing DCs in pristane-injected wild-type mice (Fig. 8) and a lack of these cells in CD1d−/− mice might make these mice more susceptible to the loss of immune tolerance and induction of pathologic autoimmunity. In fact, our preliminary results suggest that CD8α−CD11b+CD11c+ cells that correspond to CD1d++CD11c+ cells (42) are reduced in the bone marrow of pristane-injected CD1d−/− mice (data not shown).

Finally, CD1dhigh B cells that correspond to marginal zone (CD21+CD23−) B cells are increased in pristane-injected mice, and IgG3 isotype, which is generally produced by marginal zone B cells (29), is significantly increased in CD1d−/− mice compared with wild-type littermates (Fig. 9). These observations lead us to speculate that NKT cells may regulate the expansion and activation of marginal zone B cells in CD1d, which is highly expressed on T cells during the early phase of disease development may contribute to the induction of lupus in wild-type mice and to exacerbation of disease in CD1d−/− mice.

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References


