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CD1d-Dependent NKT Cells Play a Protective Role in Acute and Chronic Arthritis Models by Ameliorating Antigen-Specific Th1 Responses

Anna Teige,* Robert Bockermann,*† Maruf Hasan,*† Katarina E. Olofsson,*‡ Yawei Liu,*‡ and Shohreh Issazadeh-Navikas*†

A protective and anti-inflammatory role for CD1d-dependent NKT cells (NKTs) has been reported in experimental and human autoimmune diseases. However, their role in arthritis has been unclear, with conflicting reports of CD1d-dependent NKTs acting both as regulatory and disease-promoting cells in arthritis. These differing modes of action might be due to genetic differences of inbred mice and incomplete backcrossing of gene-modified mice. We therefore put special emphasis on controlling the genetic backgrounds of the mice used. Additionally, we used two different murine arthritis models, Ag-induced arthritis (AIA) and collagen-induced arthritis (CIA), to evaluate acute and chronic arthritis in CD1d knockout mice and mice depleted of NK1.1+ cells. CD1d-deficient mice developed more severe AIA compared with wild-type littermates, with a higher degree of inflammation and proteoglycan depletion. Chronic arthritis in CIA was also worse in the absence of CD1d-dependent NKTs. Elevated levels of Ag-specific IFN-γ production accompanied these findings rather than changes in IL-17α. Depletion of NK1.1+ cells supported these findings in AIA and CIA. This report provides support for CD1d-dependent NKTs being suppressor cells in acute and chronic arthritis, likely via inhibition of arthritogenic Th1 cells. These results make CD1d-dependent NKTs an attractive target for therapeutic intervention. The Journal of Immunology, 2010, 185: 345–356.

The CD1 protein family comprises Ag-presenting molecules found on the cell surface of mainly hematopoietic-derived cells (1). The human genome encodes five CD1 genes (CD1A-E) with four corresponding proteins (CD1a–d) expressed on the cell surface (2) and a fifth (CD1e) expressed as a soluble form in lysosomal compartments (3). Based on shared sequence homology, the CD1 molecules are divided into groups 1 (CD1a, CD1b, CD1c) and 2 (CD1d) (4). The group 1 CD1 molecules are found in humans but not mice, whereas group 2, CD1d, is found in both species (5).

Antigen-Specific Th1 Responses

The type I NKTs are further subdivided into CD4+ and CD4– CD8– double-negative (DN) categories in mice, and in humans a population of CD8+ NKTs has been identified (7–9). These cells have been im-

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autoimmune diseases remains disputed, as several conflicting reports point toward a dichotomous influence of NKTs as being both beneficial or detrimental for the host (40, 41).

During the past several years, studies have been published on the role of CD1d-dependent NKTs in experimental arthritis. Some of them have focused on possible therapeutic effects of treatment with CGalCer, whereas others have worked with different knock-out (ko) approaches to elucidate the intrinsic role of NKTs in arthritis. Puzzlingly enough, the results from these studies have shown discrepancies, with some results pointing toward a suppressive role for NKTs also in experimental arthritis (42–44) and others suggesting that NKTs are drivers of arthritogenic inflammation (45–48).

Due to the discrepant results from various groups, we investigated further the physiological role of endogenous CD1d-dependent NKTs in controlling versus promoting arthritis by using different means to deplete CD1d-dependent NKTs. We investigated their role in acute joint inflammation utilizing Ag-induced arthritis (AIA) (49) and their role in a chronic arthritis model, collagen-induced arthritis (CIA) (50). Both are frequently used models for the human disease rheumatoid arthritis. We show in this study that CD1d-deficient (CD1d ko) mice, lacking CD1d-dependent NKTs, develop more arthritis in both AIA and CIA compared with wild-type (wt) littermates. Hence it was of interest to clarify whether the lack of CD1d-dependent NKTs also affects severity during disease progression.

**Materials and Methods**

**Animals**

Animals were kept and bred at the conventional animal facility of the University of Lund, Lund, Sweden and the University of Copenhagen, Copenhagen, Denmark. All animal experiments were done in accordance with the ethical committees in Malmo¨-Lund, Sweden, and Denmark. CD1d ko mice (13) on a C57BL/6J (B6) background (10-generation backcross) were obtained and further backcrossed to B6 in our animal facility for five generations. These mice were also backcrossed to C57Bl/10-H2b (BQ) for >10 generations. Both heterozygote CD1d+/− and homozygote CD1d−/− littermates were used in the experiments as controls. As no differences were detected between these two groups, they will hereafter be referred to as CD1d wt mice. Animals were sex- and age-matched (14–20 wk) in all experiments.

**AIA: induction and clinical evaluation**

AIA was induced in the first experiment (Fig. 1A) by s.c. injection of 100 μg methylated BSA (mBSA) (Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in 50 μl PBS (Life Technologies, Paisley, U.K.) and emulsified in CFA (2 mg/ml heat-killed Mycobacterium tuberculosis, strain H37RA) (Difco Laboratories, Detroit, MI) at the base of the tail at days −21 and −14. In addition, 500 ng pertussis toxin (Bordetella pertussis; Sigma-Aldrich Chemie) was injected i.p. at days −21 and −14. At day 0, 100 μg mBSA in 25 μl PBS was injected into the left knee joint space. As a control, 25 μl PBS was injected into the right knee. Sizes of the knees were measured daily until day 9 and thereafter three times a week.

As this protocol resulted in severe joint inflammation, we widened the window for the detection of disease increase by applying a milder immunization protocol. Therefore, AIA was further induced using a milder protocol by injecting mBSA in CFA and pertussis toxin (as above) at day −14 only (data in Figs. 1B and 7). At day 0, 50 μg mBSA in 5 μl 0.9% saline solution (saline being less of an irritant than PBS) was injected into the joint space of the left knee. As a control, 5 μl 0.9% saline solution was injected into the right knee. Sizes of the knees were measured daily. Knee joint swelling was measured with an Oditest vernier caliper (H.C. Kro¨ plin, Schluettern, Germany) and was expressed in millimeters as Δ values between the right and left knee.

**CIA: induction and clinical evaluation**

For induction of CIA, mice were immunized s.c. at the base of the tail with 100 μg rat collagen type II (rCII) (Chondrex, Redmond, WA) emulsified 1:1 in CFA (Difco Laboratories). Mice were given booster injection at day 30 with 50 μg rCII emulsified 1:1 in IFA (Difco Laboratories). Clinical scoring was performed as described earlier (51). Briefly, each inflamed toe or knuckle gave one point, whereas an inflamed wrist or ankle counted for five points, resulting in a score of 0–15 (5 toes + 5 knuckles + 1 wrist/ankle) for each paw and 0–60 points for each mouse. The mice were examined one to four times per week for at least 2 mo postimmunization. The following parameters were used in data analysis:

- frequency of diseased animals was calculated as number of diseased animals divided by total number mice in group; mean score was calculated as the sum of all individual scores on one day divided by group size and embedded under the curve (AUC) is the sum of each scoring point of one individual covering the whole experimental period. Clinical scoring in all experiments was performed blindly, often by at least two independent investigators.

**In vivo NK1.1 cell depletion**

To deplete NK1.1+ cells in vivo, mice were injected i.p with 500 μg anti-NK1.1 (PK136, hybridomas originally obtained from Dr. M. Kronenberg, La Jolla Institute for Allergy and Immunology, La Jolla, CA) or isotype-matched control Ab (L243, BD Pharmingen, San Diego, CA) in 50 μl PBS.

**Histology**

Both knee joints were dissected at day 5 of AIA, fixed in 4% formaldehyde for 3 d, and then decalcified in EDTA. The knee joints were then dehydrated and embedded in paraffin, sectioned, and stained with hematoxylin and erythrosine for inflammation evaluation and with Safranin O staining for scoring of proteoglycan depletion. Six slides per group were stained and three areas per slide were scored. Histology scoring was performed using a scale from 0–3; for inflammation, defined as the influx of inflammatory cells, 0 = no inflammation to 3 = severely inflamed joint; for matrix proteoglycan depletion, 0 = no abnormalities to 3 = destained/complete loss of staining. Data analysis was done on the mean scores from three areas scored per slide.

**Proliferation assay using thymidine incorporation**

Single-cell suspensions were prepared from spleens at day 5 of AIA and cultured in DMEM with glutamate-1 (Life Technologies) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 0.5% MEM nonessential amino acid (#11140, Life Technologies), 0.16 μM penicillin (Sigma-Aldrich Chemie), and 0.03 μg/ml streptomycin (Sigma-Aldrich Chemie) in 96 flat-bottom plates (Nunc, Roskilde, Denmark) with concentrations of 1 × 106 cells/ml. The cells were cultured in medium with 25 μg/ml of mBSA (Sigma-Aldrich Chemie) or 10 μg/ml purified protein derivative (PPD; Serum Institute, Copenhagen, Denmark) as positive control or without Ag as negative control. Cells were pulsed with 1 μCi [3H]thymidine per well (Amersham Biosciences, Buckinghamshire, U.K.) after 72 h, harvested after an additional 18-h culture, and counted using a β scintillation counter (Matrix 96 direct β counter, Packard, Meriden, CT) to assay T cell proliferation. Data were expressed as the mean from duplicate wells, and the proliferation ratio was calculated (i.e., β counts from Ag-stimulated cultures divided by background counts from cultures in medium only).
**Sandwich cytokine ELISAs**

 Supernatants from in vitro stimulation of T cells (as described above) were collected after 72 h and assayed for IFN-γ. IFN-γ ELISA was performed using 5 μg/ml capturing Ab R46-A2 and 0.5 μg/ml biotinylated detection Ab, Anti-β2M (BD Biosciences, San Jose, CA). Ninety-six well plates (FluorolNunc Maxisorp, Nunc) were coated with capturing Ab in coating buffer (pH 9) at 4°C overnight. Plates were blocked with 1% BSA (Sigma-Aldrich Chemie) in PBS (Life Technologies). Samples and recombinant cytokines were added in duplicate and incubated at room temperature before detection Ab was added. Plates were thereafter incubated with europium-avidin (Wallac Oy, Turku, Finland) and washed, and enhancement buffer (Wallac Oy) was added and fluorescence intensity measured as relative luminescence units (RLUs) with a Victor/Wallac (Wallac Oy, Turku, Finland). For washing between incubations, PBS-based ELISA buffer with 0.1% Tween (Sigma-Aldrich Chemie) was used. Data were expressed as the mean from duplicate wells, and the cytokine release ratio was calculated (i.e., RLU from Ag-stimulated cultures divided by background RLU from cultures in medium only).

 Cytokine ELISAs for the CIA experiment were performed using sandwich ELISA kits following the manufacturer’s instruction manuals: IFN-γ (#551866, BD OptEIA, BD Biosciences); IL-17α (#14-7175-68, eBioscience, San Diego, CA); IL-13 (Duoset #DY413, R&D Systems, Minneapolis, MN), and TNF-α (Ready-SET-Go! mouse TNF-α, #88-7324, eBioscience). As substrate TMB ONE (#4380L, Kem-En-Tec Diagnostics, Taastrup, Denmark) was used, the reactions were stopped with 0.2 M sulfuric acid and plates were read at 450 nm in a photometer (BioTek Instruments, Winooski, VT). KJ-junior software was used, and the concentration was calculated based on the provided recombinant cytokine standards.

**FACS staining and sorting of PK136-depleted splenocytes**

B6 wt and B6 CD1d ko male mice were injected with 500 μg functional grade PK136 (#16-5941-85, eBioscience); control B6 wt mice and B6 CD1d ko mice received i.p. injections with carrier solution. Spleens were collected 2 d later for FACS analysis. Single-cell suspensions were made, and erythrocytes were lysed with RBC-lysis buffer (#00-4333-57, eBioscience), washed in PBS, and resuspended in PBS-based ELISA buffer (2% BSA, 0.01% sodium azide in PBS); cells were incubated with anti-FeR Ab (2.4G2) for 20 min on ice. Thereafter, splenocytes were incubated with biotinylated, FITC- or PE-labeled Abs. For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences). All Abs were purchased from BD Pharamingen and were used at 1–5 μg/ml unless stated otherwise. Abs were allowed to bind for 20 min on ice. Abs were PE-Cy5–anti-CD4 (OKT15), FITC–anti-CD3 (145-2C11), APC–anti-CD8a (53-67), PE–anti-IFN-γ (XMGI.1, eBioscience), PE–anti–IL-17a (TCL1-18H10), PE–anti-CD49b (DX5), FITC, and PE–anti-NK1.1 (PK136). Dead cells were discriminated in all stainings using the LIVE/DEAD Fixable Dead Cell Stain Kit for 405 nm excitation (L34955, Invitrogen, Carlsbad, CA). Cells were acquired with an FACSARia (BD Biosciences) using the FACSDiva software for acquisition after exclusion of duplets, and FlowJo 8.8.6 (Tree Star, Ashland, OR) was used for further analysis. The experiment was repeated twice with similar results, and hence, the shown data are the summary of these two experiments.

**Statistical analysis**

All statistical evaluations were done using nonparametric Mann-Whitney U tests or Student unpaired t tests with StarView software and Prism 4 for Macintosh (GraphPad San Diego, CA). FACS data were analyzed using ANOVA with Newman-Keuls multiple comparison test. Values of \( p < 0.05 \) were considered significant. Data are shown with respective mean ± SEM.

**Results**

**B6 CD1d ko mice develop more severe acute AIA with higher levels of Ag-specific IFN-γ**

To investigate the inflammatory response in the absence of CD1d-dependent NKTs, AIA was induced in B6 CD1d ko mice and their heterozygote and homozygote littermates. Swelling of knee joints was measured, and \( \Delta \) values of left and right knees were compared between the groups of mice. No difference was detected between heterozygote and homozygote wt littermates, and data from the groups were hence collated and will be referred to as the wt group.

As seen in Fig. 1A, both groups developed knee joint inflammation with an acute phase lasting for \( \sim 1 \) wk, after which the swelling receded in both groups. B6 CD1d ko mice developed significantly more severe acute inflammation compared with their wt littermates (\( p < 0.001, \) days 1–7), with almost twice as large Ag-specific swelling of the knee joints as the wt mice. After the
acute phase, the extent of joint inflammation subsided and became comparably low in both groups. The experiment was repeated with similar results (Fig. 1B). Histopathological analysis of the knee joints on day 5 supported the clinical observations and revealed a significantly higher degree of inflammation (Fig. 1C, 1D) based on scoring numbers of infiltrating cells (CD1d ko, 2.67 ± 0.4; wt, 1.28 ± 0.5; p < 0.001) and proteoglycan depletion score (CD1d ko, 2.03 ± 0.5; wt, 1.31 ± 0.5; p < 0.05) in affected joints from B6 CD1d ko mice compared with B6 wt. Evident bone destruction was not observed in this milder form of the AIA model at this time point.

Ag-specific T cell responses were investigated using in vitro splenocyte cultures dissected on day 5 post AIA. Data presented in Fig. 2A show that Ag-specific T cells from B6 CD1d ko mice released much higher levels of IFN-γ in response to mBSA compared with T cells from B6 wt mice (Ag-specific IFN-γ release, negative control ratio: CD1d ko, 28 ± 10; wt, 7 ± 2; p < 0.01). However, they did not significantly differ in their proliferation (Fig. 2B), indicating that individual mBSA-specific T cells from B6 CD1d ko mice had a higher capacity to release IFN-γ when re-encountering Ag. Interestingly enough, no difference was observed between B6 CD1d ko and wt mice in regards to T cell response toward PPD (Fig. 2) or Con A (data not shown), suggesting that the elevated T cell IFN-γ release in mice lacking CD1d-dependent NKTs was not due to a general higher activation status of T cells in these mice. Even though IFN-γ has recently been implicated as a regulatory cytokine in arthritis (52), others have shown that IFN-γ worsens CIA and is important for cartilage destruction during immune complex-mediated arthritis (53, 54).

Nevertheless, arthritis is commonly believed to be a Th1-driven disease, and hence, IFN-γ is an appropriate marker to investigate T cell effector function in models of arthritis. The significant increases in joint inflammation, measured by joint swelling and histopathological scoring, and increased Ag-specific effector T cell functions indicate an elevated cellular immune response in B6 CD1d ko mice. This is in agreement with the conclusion that CD1d-dependent regulation is active in the acute inflammation in AIA.

**BQ CD1d ko mice have a higher incidence and severity of CIA**

To investigate the role of CD1d-dependent NKTs in a chronic arthritis model, we immunized BQ CD1d ko and wt mice with rCII to induce CIA. The B6 and BQ mouse strains differ mainly in the MHC haplotypes, with H-2^q strains having been shown to be more susceptible to CIA and hence are more widely used in this chronic model (55). No differences in numbers or phenotype of CD1d-dependent NKTs have been reported for the strains, and our preliminary unpublished data do not support any such differences.

As shown in Fig. 3 and Table I, BQ CD1d ko mice developed arthritis more frequently than wt mice (p < 0.05 over experimental period) and also had significantly higher severity scores (AUC over experimental period: CD1d ko, 54.5 ± 21.5; wt, 3.4 ± 1.4; p = 0.01). Single-cell preparations of splenocytes revealed that the BQ CD1d ko mice had significantly higher numbers of splenocytes (Fig. 3C). The higher disease outcome in the BQ CD1d ko mice was also reflected by the significantly higher Ag-specific IFN-γ production upon in vitro restimulation, shown in Fig. 4. For other measured cytokines (IL-17α, IL-13, and TNF-α), no significant differences in the Ag-specific responses could be detected between the BQ CD1d ko and wt groups.

The observed increased arthritis was hence accompanied by an increased level of Ag-specific IFN-γ production, which supports the conclusion that CD1d-dependent NKTs are not only regulators in the acute joint inflammation in AIA, but also operate in the chronic arthritis model, CIA, by dampening the arthritogenic Th1 response, but without a major influence on Ag-specific Th2 or Th17 responses.

**No changes in IL-17α and IFN-γ+ T cell or NKT subsets in B6 CD1d ko versus PK136 depleted mice**

The observations of clinical and immune response differences could be due either to an altered immune homeostasis from the ontogenetic absence of CD1d-dependent NKTs or to immune deviation of the immune response mounted in the presence or absence of CD1d-dependent NKTs. To investigate this further, CD1d-dependent NKTs need to be selectively depleted at the time of immunization for arthritis. As there is currently no superior method available to selectively deplete CD1d-dependent NKTs, we chose to investigate their role in arthritis models using the PK136 Ab (anti-NK1.1 IgG) to deplete NK1.1^+ cells. In the B6 mouse, the NK1.1 marker is believed to be expressed by the majority of mature CD1d-dependent NKTs in the periphery (41, 56), and the majority of T cells expressing NK1.1 are CD1d reactive (40). However, classical NK cells also express NK1.1 and have been reported to control the early-onset phase of CIA (57), which could influence the results in such an experiment. Nevertheless, using NK1.1 depletion could further elucidate and verify the findings from the CD1d ko mice. However, as mentioned above, the peripheral immune cell homeostasis could be different between genetically CD1d-deficient mice and NK1.1-depleted mice. Therefore, an FACS analysis of splenocytes was conducted to compare B6 wt male mice and B6 CD1d ko, both injected i.p. with the NK1.1-depleting Ab PK136 (500 μg/mouse), to B6 wt

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** T cells from B6 CD1d ko mice release elevated levels of IFN-γ in response to mBSA in AIA. Ag-specific T cells in spleen cell cultures from B6 CD1d ko mice released significantly higher amounts of IFN-γ in response to mBSA, but not to PPD, compared with wt mice (p < 0.01) (A) but did not proliferate significantly more vigorously in response to Ag (p = 0.13) (B). Bars represent mean cytokine release ratio (IFN-γ levels in culture with Ag divided by IFN-γ levels in culture with medium only) ± SEM and mean stimulation ratio (proliferation in culture with Ag divided by proliferation in culture with medium only) ± SEM; n = 12 per group. **p < 0.01
mice and B6 CD1d ko mice receiving only carrier solution (n = 3–6/group). Two days postinjection, single-cell suspensions were made from spleens and stained for FACS analysis. As shown in Fig. 5, the analysis revealed that the percentages of DN (gated on CD3+CD42CD82) T cells, single-positive CD4 and CD8 T cells, and the CD4/CD8 ratio were similar among the groups studied. As expected, the main differences among wt and CD1d ko groups were found to be in the NKT population (gated on CD3+NK1.1+), which was also reduced as a result of PK136 treatment. Classical NK cells (gated on DX5+NK1.1+) were, as anticipated, also significantly reduced as a result of NK1.1 depletion in both wt and CD1d ko mice and B6 CD1d ko mice receiving only carrier solution (n = 3–6/group). Two days postinjection, single-cell suspensions were made from spleens and stained for FACS analysis. As shown in

Table I. **BQ CD1d ko mice show a higher severity and incidence of CIA**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Arthritis Incidence (%)</th>
<th>Maximum Score [mean (SD)]</th>
<th>Day of Onset (Affected Mice Only) [mean (SD)]</th>
<th>Accumulative Score (Until Day 50) [mean (SD)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BQ-CD1d2/2</td>
<td>20</td>
<td>70</td>
<td>9.8 (15.2)</td>
<td>35.5 (6.4)</td>
</tr>
<tr>
<td>BQ-CD1+/+</td>
<td>20</td>
<td>35</td>
<td>0.85 (1.6)</td>
<td>36.5 (7.3)</td>
</tr>
<tr>
<td>Statistics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p = 0.02a</td>
<td>p = 0.008b</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Arthritis incidence indicates if the mouse was disease affected during the time course of the experiment. Maximum score indicates the highest scoring value of a mouse during the disease course. Day of onset applies to mice that got disease only (healthy mice are excluded). Accumulative score until day 50 (AUC day 50) is the sum of all scores during the experimental period.

aχ2 test.
bMann-Whitney U test.
The percentage of NK cells gated on CD3^−NK1.1^+ showed similar results: mean ± SD of wt, 7.5 ± 1.4; wt PK136-treated, 0.18 ± 0.05; CD1d ko, 5.1 ± 0.94; and CD1d ko PK136-treated, 0.24 ± 0.41; PK136 treatment in both groups reduces NK cells significantly \( (p < 0.001) \).

Interestingly, as shown in Fig. 6, CD4^+NKTs (CD3^+CD4^+ NK1.1^+), CD8^+NKTs (CD3^−CD8^+NK1.1^+), and DN NKTs (CD3^−CD4^−CD8^−NK1.1^+) were similarly affected by genetic knockdown of CD1d and depletion using PK136, all resulting in significantly lower populations compared with the untreated wt mice.

IL-17 production has been reported to be associated with iNKTs (58–60). However, Fig. 7A shows that the percentages of IL-17^+ cells were not affected by either NK1.1 depletion or CD1d deficiency in any of the cell populations analyzed. This is because no differences were observed among the groups in IL-17^+CD4^+ or IL-17^+CD8^− T cells or in any subset of NKTs. Furthermore, there was no evidence that IL-17–producing NK cells were affected by CD1d deficiency or by NK1.1 depletion.

The percentages of IFN-γ^+ cells showed a similar pattern as IL-17^+. No differences in IFN-γ^+ cells were observed in the T cell and NKT subsets or NK cells as a result of genetic modification or NK1.1 depletion (Fig. 7B).

Collectively, the FACS analysis confirms that the major differences among B6 wt, B6 CD1d ko mice, and mice actively depleted of NK1.1 cells are related to the CD1d-dependent NKT subpopulations, including CD4^+NKTs, CD8^+NKTs, and DN NKTs. Although no major effect on NK cells could be seen as a result of genetic knockdown of the CD1d molecule, NK1.1 depletion significantly reduced but did not diminish NK cells in both B6 wt and B6 CD1d ko mice. No significant differences were detected among the groups in the percentages of IL-17^− and IFN-γ^− CD1d-dependent NKTs, other T cell populations, or NK cells.

In summary, it is noteworthy to highlight that the data show that the main differences found among B6 wt and B6 CD1d ko mice in subpopulations of splenocytes can be attributed to the intrinsic defect in generation of CD1d-dependent NKTs. Depletion of NK1.1^+ cells from wt mice using PK136 as compared with CD1d ko mice did, however, result in loss of DN NKTs and NK cells in addition to diminishment of all other NKT subpopulations.

No major differences were found in other cell populations when comparing CD1d-deficient and NK1.1-depleted mice, and depletion of NK1.1^+ cells in CD1d ko mice showed results comparable to wt mice treated with PK136.

Depletion of NK1.1^+ cells prior to AIA induction leads to severe disease and higher Ag-specific inflammatory T cell response

To investigate the effect of depleting NK1.1-expressing cells at the time of AIA induction, B6 wt mice were injected i.p. with depleting Ab 1 d preimmunization and 1 d before knee injection and followed for Ag-specific joint inflammation during the acute phase of the disease. Mice were sacrificed for histopathological evaluation of knee joints and in vitro T cell activation assays.

In concordance with previous observations from B6 CD1d ko mice, B6 wt mice treated with anti-NK1.1 also developed significantly more severe Ag-specific joint swelling when compared with mice receiving control Ab (Fig. 8A). In addition, the acute joint inflammation was associated with significantly higher Ag-specific T cell proliferation and IFN-γ release in NK1.1-depleted mice when splenocytes were restimulated in vitro with mBSA (Fig. 8B, 8C), signifying an elevated inflammatory cellular immune response in NK1.1-depleted mice.

The results indicate that suppression mechanisms operating in AIA in B6 wt mice are absent in the B6 CD1d-deficient mice and that this suppression is mediated by NK1.1^+ cells. However, as there were no differences between B6 CD1d ko and B6 wt NK1.1-depleted mice in regard to AIA development, we believe that this can be attributed to the suppressive function of CD1d-dependent NKTs and not other NKT subsets or NK cells. Our results also indicate that the disease-suppressive effects of NKT cells are not due to an intrinsic imprinting during development of the immune system, but that their regulatory functions operate during the priming of a specific immune response and subsequent inflammation.

Depletion of NK1.1^+ cells upon arthritis induction leads to more frequent and severe CIA

To confirm these findings in a chronic arthritis model, BQ wt mice were depleted of NK1.1^+ cells at the time of immunization (day 0).
FIGURE 6. FACS analysis of NKT cell populations after in vivo depletion of NK1.1-positive cells in B6 wt and B6 CD1d ko mice. Percentage of CD4+ NKT cells and CD8+ NKT cells in CD3 gated splenocytes and DN-NKT (CD3+CD4+CD8- NK1.1+) cells for all groups of mice (B6 wt and B6 CD1d ko control-treated mice, B6 wt PK136-treated, and B6 CD1d ko PK136-treated), with representative FACS plots. Graphs show individual values and the mean. One-way ANOVA test with Newman-Keuls multiple comparisons correction was used to evaluate significant differences among the groups; n = 3–6 mice/group.
for CIA and compared with control-treated animals. Both groups developed arthritis (Fig. 9, Table II) with a significantly higher frequency in the NK1.1-depleted group ($p < 0.05$ over experimental period). Also, diseased animals in the NK1.1-depleted group developed a more severe CIA (AUC over experimental period: NK1.1-depleted, 213 ± 70; control, 87 ± 36; $p < 0.05$). The experiment was repeated with similar results (data not shown). These results indicate that NK1.1+ cells are not only operative in the acute inflammatory AIA model, but also during the disease progression in a chronic inflammatory CIA model. Again, as similar results were observed when investigating BQ CD1d ko and B6 wt NK1.1-depleted mice in regard to CIA development, this suggests that the suppression is likely operating through CD1d-dependent NKTs.

**Discussion**

Although CD1d-dependent NKTs have been proven to be a small subset of regulatory lymphocytes, it is not clear whether their influence on the immune system always leads to beneficial suppression in autoimmune diseases, and it seems their function can be two-headed, giving both intensification and moderation of the immune response in different reported model systems (40, 41, 61, 62).

Their function in arthritis models has also been debated, as several contradictory reports have been published (42–48, 63, 64), indicating both protective and disease-promoting roles of CD1d-dependent NKTs in arthritis.

CD1d-dependent NKTs have been implicated as effector cells in Ab-induced arthritis, as serum transfers to induce arthritis in both Jα281−/− mice and CD1d−/− mice resulted in less ankle swelling compared with wt mice (45–47). Pure Ab-induced arthritis models probably do not include adoptive immune responses, but mainly immune complex-induced inflammation (65), and hence, the suppressive role of CD1d-dependent NKTs described in other models with active adoptive immune responses would not be detected in the Ab-induced arthritis model. In contrast, activation of CD1d-dependent invariant NKTs using α-GalCer or its analogs α-C–GalCer and OCH have been reported to protect mice from CIA (42–44), indicating a suppressive role for these cells during the arthritis process involving activation of cellular immune responses. However, also utilizing the CIA model, Jα281−/− mice showed decreased clinical arthritis score compared with wt mice (47, 48). The same has been reported by one of the groups when looking at CIA in CD1d−/− mice (48), whereas the other group has reported similar findings only in Ab-induced arthritis in CD1d−/−.
mice (47). In contrast to the studies activating CD1d-dependent iNKTs with αGalCer or its analogs, these reports would support the notion that NKTs could function as effector cells in driving arthritic inflammation. However, as it has been shown that the TCR-α locus contains several loci modifying arthritis (66), one needs to take into consideration the effect on the linked genetic regions from the ko construct of the Jα281−/− mice when interpreting these results and whether littermates have been used as control animals in these reports.

Taken together, the above-mentioned studies could indicate that CD1d-dependent NKTs have dual functions in arthritis: during the induction phase working as regulators/suppressors of pathogenic T cells and in the effector phase, in which Ab-mediated destruction is prominent, functioning as disease promoters. In our study, by using both chronic and acute arthritis models (CIA and AIA, respectively), we addressed whether CD1d-dependent NKTs could play different roles during distinct phases of the disease. In addition, we aimed to investigate the impact of the congenital lack of CD1d-dependent NKTs compared with their depletion at the induction phase of arthritis.

In the current study, we report that when utilizing CD1d ko mice, which lack thymic-educated CD1d-dependent NKTs, arthritis was significantly more severe throughout the acute phase of AIA. This was seen as augmented swelling of the joint accompanied with histopathological changes such as elevated inflammation and proteoglycan depletion. Prominent Ag-specific IFN-γ production in CD1d ko mice, a predictive disease-promoting factor, was also elevated, implicating CD1d-dependent NKTs as playing an active role in the suppression of inflammatory processes. This is in close agreement with one of our earlier reports in which CD1d ko mice developed more severe experimental autoimmune encephalomyelitis with higher Ag-specific cytokine production (32). Our findings from the acute arthritis model, AIA, were supported when investigating the role of CD1d-dependent NKTs in chronic CIA in CD1d ko mice. These mice developed significantly more frequent and severe arthritis, indicating that CD1d-dependent NKTs are...
functioning by suppression of the inflammatory responses during the disease course. It remains to be clarified if this suppression is direct via cell–cell interaction through surface receptor–ligand interaction with arthritogenic T cells, or via modification of APCs, and/or via modification of tissue-specific cells and hence inflammation. Nevertheless, such a suppressive function of CD1d-dependent NKTs could have implications for the prevention of relapses with further joint destruction during chronic relapsing disease courses. This could hold therapeutic potential in arthritis.

Looking from a possible treatment perspective, the role for CD1d-dependent NKTs as suppressors of arthritis would be most relevant after disease onset and establishment. This has been addressed in the past with rather promising results in which αGalCer activation of CD1d-dependent invariant NKTs was reported to decrease arthritis, even though it was shown that the protocol for administration was crucial for the outcome (44). Although αGalCer is a very strong activator, it is not a naturally occurring ligand for CD1d-dependent NKTs, and hence results reported in previous studies using such treatment do not address the physiological role of these cells. In addition, it has been suggested that serial exposure to αGalCer could render CD1d-dependent NKTs anergic (67). We instead wanted to investigate if CD1d-dependent NKTs were actors during the course of arthritis development without artificial activation. Moreover, it was of interest to clarify whether our observed CD1d-dependent regulation of arthritis in CD1d ko mice was due to an influence of CD1d-dependent NKTs on the inherited immune homeostasis or rather operated by controlling the priming of the adaptive immune response during disease induction. Hence, we aimed to deplete CD1d-dependent NKTs from adult animals at the time of arthritis induction. According to current knowledge, there is no method available to selectively deplete CD1d-dependent NKTs with stringency. However, it is known that most CD1d-restricted mature NKTs in the periphery express the NK1.1 surface marker (68) and can be depleted by injecting an mAb, PK136. Results from our NK1.1 depletion experiments indicated that the possible suppressive role of CD1d-dependent NKTs in arthritis models was by shaping the adaptive immune responses at onset and thereby controlling the arthritis development. Our observations also suggest that the lack of this subpopulation prevents the control of the disease progression, resulting in stronger disease severity in chronic CIA. However, as the IFN-γ and IL-17α cytokine pattern of the remaining NKT cells before and after PK136 depletion and in CD1d ko mice was unchanged, this suggests that the CD1d-dependent NKTs use other effector mechanisms to change the Ag-specific inflammatory environment, possibly by influencing APCs. The Ag-specific arthritogenic Th1 response was clearly ameliorated by CD1d-dependent NKTs, but without a major influence on Ag-specific Th2 or Th17 responses. Because our current report does not support a role for CD1d-dependent NKTs in suppression of Ag-specific Th17 cells, their function could differ from iNKTs recently reported to prevent development of the Th17 lineage (60).

Classical NK cells also express the NK1.1 marker and have recently been implicated in the regulation of CIA (57). In addition, other NK1.1-expressing cells such as CD1-independent DN NKTs are depleted as a result of PK136 treatment of wt mice, which could influence the results from these experiments. Our FACS results confirm a significant reduction of NK cells in the depletion experiments. In addition, the number of NK cells between CD1d ko mice and wt mice showed no major difference, even though the mice have different disease outcomes. This is suggesting that the arthritis findings reported in this paper are not explained by function of NK cells. In contrast, the CD1d ko experiments and the NK1.1 depletion experiments in wt mice show consistent results regarding arthritis development and Ag-specific IFN-γ production, strengthening the notion that CD1d-dependent NKTs function as suppressors during the development of arthritis. The role of CD1-independent NK1.1-expressing cells in arthritis remains to be clarified, as well as a putative shared alternative function of NK1.1 signaling between CD1-dependent and CD1-independent cells expressing this receptor.

One also needs to take into consideration difficulties in classifying and identifying CD1d-dependent NKTs when interpreting data (e.g., Jα281 ko mice do not lack the same NKT cell population as CD1d ko mice or wt mice depleted with anti-NK1.1 Ab) (69, 70). Jα281 ko mice only lack the invariant type I NKTs, whereas in CD1d ko mice, all CD1-dependent NKTs are absent. When using the NK1.1-specific depleting Ab, all CD1-dependent and CD1-independent NKTs expressing the NK1.1 marker would be depleted in addition to other NK1.1-expressing cells.

Moreover, it is noteworthy to mention that we have in this study used different models of arthritis, used mice with slightly different genetic backgrounds, investigated congenital lack of CD1d-dependent NKTs versus depletion in adult animals, and addressed CD1d-dependent NKT function during an acute joint inflammation and in a later chronic phase—all with consistent results. Our collective data point toward CD1d-dependent NKTs being suppressive cells in arthritis models rather than drivers of inflammation. Our current report is also in line with the findings that CD1d Ag presentation and NKT cell functionality are defective in patients with rheumatoid arthritis (71–73), and the findings point toward CD1d-dependent NKTs as potential therapeutic targets for treatment of chronic arthritis.

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References


