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CD1d1-Dependent Control of the Magnitude of an Acute Antiviral Immune Response

Tonya J. Roberts, Yinling Lin, Philip M. Spence, Luc Van Kaer, and Randy R. Brutkiewicz

CD1d1-restricted NK T (NKT) cells rapidly secrete both Th1 and Th2 cytokines upon activation and are therefore thought to play a regulatory role during an immune response. In this study we examined the role of CD1d1 molecules and NKT cells in regulating virus-induced cytokine production. CD1d1-deficient (CD1KO) mice, which lack NKT cells, were infected with lymphocytic choriomeningitis virus, and spontaneous cytokine release from splenocytes was measured. We found that CD1KO mice produce significantly higher amounts of IL-2, IL-4, and IFN-γ compared with wild-type controls postinfection. Depletion studies of individual lymphocyte subpopulations suggested that CD4+ T cells are required; however, isolation of specific lymphocyte populations indicated that CD4+ T cells alone are not sufficient for the increase in cytokine production in CD1KO mice. Splenocytes from lymphocytic choriomeningitis virus-infected CD1KO mice continued to produce enhanced cytokine levels long after viral clearance and cleared viral RNA faster than wild-type mice. There was no difference in the number of splenocytes between uninfected wild-type and CD1KO mice, whereas the latter knockout mice had an increased number of splenocytes after infection. Collectively, these data provide clear evidence that the expression of CD1d1 molecules controls the magnitude of the cell-mediated immune response to an acute viral infection. The Journal of Immunology, 2004, 172: 3454–3461.

N atural killer T (NKT) cells comprise a specialized subset of T lymphocytes that express cell surface markers characteristic of NK cells (reviewed in Refs. 1–3). NKT cells express an invariant TCR α-chain (Vα14α281), which is associated with Vβ-chains of limited diversity. NKT cells recognize glyco- and phospholipids, rather than peptide Ags, presented by the MHC class I-like molecule, CD1d1. Upon activation, by interaction with the appropriate CD1d1/Ag complex or by stimulation with Abs to CD3 or the TCR, NKT cells rapidly secrete IFN-γ and IL-4 (1). These two cytokines are important for the differentiation of Th1 and Th2 subsets, respectively (4, 5). As a significant source of IFN-γ and IL-4, NKT cells are thus believed to be able to modulate the immune response during certain infections or other disease conditions. For example, several studies have implicated a role for NKT cells in the control of many autoimmune diseases (6–10) and in the induction of systemic tolerance (11), whereas other reports suggest that NKT cells play an important role during the immune response to Plasmodium yoelii (12), Plasmodium berghei (13–15), and Toxoplasma gondii (16) by virtue of their production of IFN-γ or IL-12. In contrast, NKT cells have been shown to be dispensable for IgE responses induced by Nippostrongylus brasiliensis (unlabeled, in which case they are suppressive) (17), and although an earlier report suggested that IgG responses to the Plasmodium berghei circumsporozoite GPL-anchored protein was CD1d-dependent (18), Tsuji and colleagues (19) found no role for such a response. Rather, MHC class II-dependent CD4+ T cells appear to be the major players in the circumsporozoite-specific IgG response after immunization. This latter observation was independently confirmed (15). More recently, the Tsuji laboratory (20) showed that a C-glycoside analog of the CD1d-binding and NKT cell-activating α-galactosylceramide (α-GalCer) was more effective than the parental compound at inducing a protective antimalarial response in vivo that was dependent on TH1 cytokines. Other studies have indicated a role for NKT cells in the induction of Th2-mediated responses (21, 22). During bacterial or intracellular parasitic infections, the immune response polarizes to either a Th1 or a Th2 phenotype (4, 5), although work in the malaria system, as recently reviewed by Tsuji and colleagues (23) shows that, at least with this parasite, NKT cells importantly serve as a bridge between the innate and adaptive immune responses through the production of IFN-γ. Unlike many nonviral pathogens, virus infections will generally elicit both Th1 and Th2 cytokines (24, 25), with a major emphasis on Th1 cytokines early during infection. In this study we investigated the potential immunoregulatory role of NKT cells during an acute virus infection. The infection of adult mice with a natural pathogen, the arenavirus lymphocytic choriomeningitis virus (LCMV), has been well characterized and provides an excellent model system for investigating antiviral T cell responses and cytokine production (24, 26–29). In fact, the Biron laboratory (28), by analyzing spontaneous cytokine release after LCMV infection in vivo, has shown that IL-2 is produced early and is followed by IL-4 and IFN-γ production. These findings suggest that both CD4+ and CD8+ T cells are

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4 Abbreviations used in this paper: NKT, NK T; CD1KO, CD1d1-deficient; EMCV, encephalomyocarditis virus; α-GalCer, α-galactosylceramide; LCMV, lymphocytic choriomeningitis virus; LCMV-gp, LCMV glycoprotein; NP, nucleoprotein; p.i., postinfection; RPA, RNAse protection assay; Treg, regulatory T cells.

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needed for optimal cytokine production. With the reported immunoregulatory role of CD1d1-restricted NKT cells in other model systems (2, 30) and to extend the report by Biron and colleagues (28), we have investigated the role of CD1d1 molecules in the immunoregulatory control of antiviral immune responses to LCMV. Our findings indicate that CD1d1 molecules play a critical role in the control of cytokine production after an acute virus infection.

Materials and Methods

Mice

Male and female C57BL/6 wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CD1d1-deficient (CD1KO) mice (31) were backcrossed 10 times onto a C57BL/6 background and bred in specific pathogen-free facilities at Indiana University School of Medicine. All mice were infected between 6–12 wk of age and were age- and sex-matched. All experiments were performed in accordance with procedures approved by Indiana University School of Medicine animal use and care committee.

Virus and infection of mice

The Armstrong strain of LCMV was provided by Dr. R. Welsh (University of Massachusetts Medical Center, Worcester, MA). Viral stocks were prepared in BHK cells. Mice were infected i.p. with 1–2 × 10^6 PFU of LCMV as previously described (32, 33). On the indicated days postinfection (p.i.), spleens were harvested for analysis of T cell subpopulations and cytokine production as described below.

Splenocyte isolation

Spleens were harvested from uninfected and LCMV-infected mice and processed into single-cell suspensions. Erythrocytes were lysed by hypotonic shock in 0.84% NH_4Cl. The remaining cells were washed twice with IMDM supplemented with 5% FBS (complete medium), then resuspended in the same medium.

Spontaneous cytokine release

To measure virus-induced spontaneous cytokine production, 5 × 10^4 splenocytes were incubated in 200 μl of IMDM containing 5% FBS for 20–24 h in a 96-well, U-bottom plate (Costar, Corning, NY). The supernatants were harvested, and cytokine production was measured by ELISA, as previously described (34).

ELISPOT assay

ELISPOT assays were performed to enumerate IFN-γ-producing cells. Ninety-six-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml purified anti-IFN-γ mAb (BD PharMingen, San Diego, CA) in coating buffer (0.1 M Na_2CO_3 and 0.1 M NaHCO_3, pH 8.5). Plates were incubated with complete medium for 2 h at 37°C to block nonspecific binding. Cell dilutions in 100 μl of complete medium were added in triplicate wells. After incubation at 37°C for 20–24 h, plates were washed with PBS/0.05% Tween 20. Twenty and incubated with 2 μg/ml of a biotinylated anti-mouse IFN-γ mAb (BD PharMingen). A peroxidase-conjugated avidin (Sigma-Aldrich, St. Louis, MO) was then added at a 1:400 dilution in PBS/10% FBS at 100 μl/well and incubated at 37°C for 1.5 h. Plates were washed six times, then an aminoethylcarbazole substrate solution was added. The resulting brown spots were enumerated under low power magnification (×20) using a dissecting microscope.

RNase protection assay (RPA)

Total RNA samples were isolated from the cell populations indicated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). RPA samples were performed according to the manufacturer’s instructions (BD PharMingen). Measurements of cytokine and chemokine transcripts were determined using a RiboQuant Multiprobe Rnase protection system (BD PharMingen) following the instructions provided by the manufacturer. A gel imager (ChemiImager 4000; Alpha Innotech, San Leandro, CA) with molecular software (AlphaEase version 3.3a; Alpha Innotech) was used to compare the levels of mRNA. The values obtained for each level of cytokine mRNA measured were normalized against the combined levels of expression obtained for mRNA from L32 and GAPDH housekeeping genes within the same lane on the RPA gel.

Cell sorting

Splenocytes were immunomagnetically separated using the MACS system (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer’s protocol. In brief, to block nonspecific Ab binding, splenocytes were incubated with hybridoma supernatant from the 2.4G2 cell line (murine FcRγ-specific) for 10 min at 4°C. To deplete the various leukocyte subpopulations, splenocytes were incubated with PE-conjugated Abs specific for mouse B220, 1A-8, CD4, or CD8 (BD PharMingen) for 15 min at 4°C. The cells were washed, incubated with anti-PE mAb-coated magnetic beads for 15 min at 4°C, washed again, and passed through magnetic columns. The purity of the magnetically sorted populations was determined by FACS. For positive selection, cells were first pretreated with hybridoma supernatant from the 2.4G2 cell line and stained with PE- or FITC-conjugated mAb as indicated. The cells were then electronically sorted using a FACStar Plus (BD Biosciences, Mountain View, CA). The purified subpopulations (CD4+, CD8+, and B220+) were used to measure spontaneous cytokine release. Analysis was performed using ELISA, as described above.

FACS analysis

Splenocytes from uninfected or LCMV-infected mice were pretreated with 2.4G2 supernatant and stained with FITC-conjugated anti-mouse CD25 and PE-labeled anti-mouse CD4, CD8, CD94, or IFN-γ, and allophycocyanin-conjugated anti-mouse CD62L (all purchased from BD PharMingen). The cells were washed and fixed in 1% paraformaldehyde in PBS. The samples were resuspended in HBBS/0.1% BSA containing 0.02% sodium azide and analyzed on a FACSCalibur flow cytometer (BD Biosciences) as previously described (33). For intracellular staining, splenocytes were cultured with 1 μg/ml of the indicated LCMV immunodominant peptides (nucleoprotein-derived, NP_368–404 or gp39–41; Sigma-Aldrich) (35) for 5 h at 37°C in the presence of 10 μg/ml brefeldin A. The cells were then washed, treated with 2.4G2, and stained for CD8 cell surface expression. After fixation, the cells were treated with permeabilization buffer (0.5% saponin in HBSS/BSA) for 10 min at room temperature. The cells were then stained with a PE-conjugated anti-mouse IFN-γ (or isotype control) mAb diluted in permeabilization buffer, washed, and analyzed by FACS.

RT-PCR analysis

Semiquantitative RT-PCR. Splenic RNA was extracted from uninfected and LCMV-infected mice using Tri-Reagent and reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. Amplification of LCMV glycoprotein (LCMV-gp)-specific and β-actin control sequences was performed as previously described (33, 36). PCR products were run on a 1% agarose gel, stained with ethidium bromide, and photographed.

Real-time RT-PCR. Primers and probes for LCMV-gp were designed using Primer Express software (all from PE Applied Biosystems, Foster City, CA). Primers and probe for GAPDH were derived from TaqMan rodent GAPDH control reagents (PE Applied Biosystems). For LCMV-gp, the forward and the reverse primers were 5’TGC CTG ACC AAA TGG ATT-3’ and 5’TGC CTG TGT FCC CAA AAC ACT-3’, respectively, and the TaqMan MGB probe was 6FAM-ATT-3’ and MGBNFQ. RNA was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega). The cDNA was amplified using TaqMan Universal PCR Master Mix (PE Applied Biosystems). The PCR cycle parameters were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. GAPDH PCR reactions were performed in triplicate using different tubes from those used for LCMV-gp. The threshold cycle, representing the fractional cycle at which the amount of the amplified target achieves a fixed threshold, was used in subsequent calculations. The relative difference in the level of LCMV-gp transcripts among samples from uninfected and infected mice was determined using the threshold cycle method as outlined in the PE Applied Biosystems protocol for real-time PCR. The data are presented as the fold change in LCMV-gp transcript.

Statistics

Unpaired Student’s t tests (two-tailed) were performed using GraphPad Prism software (version 3.00 for Windows; GraphPad, San Diego, CA). A value of p < 0.05 was considered significant. The error bars in the bar graphs show the SD.

Results

CD1d1-deficient mice produce higher levels of IL-2, IL-4, and IFN-γ than wild-type mice in response to LCMV infection

We have previously reported that following an acute infection with LCMV, there is a rapid and selective decrease of ~60–80% in

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spleenic NKT cells (33). These data suggest a role for CD1d1-restricted NKT cells early in infection. To examine the role of CD1d1 molecules after LCMV infection, splenocytes from wild-type and CD1KO mice (31, 37, 38) were harvested at different time points after infection. To assess the immune response to the virus, spleenic leukocytes were cultured in medium without any additional stimuli. Supernatants were harvested 20–24 h later, and spontaneous cytokine production was measured by ELISA. In agreement with data reported by Biron and colleagues (28), we found that spontaneous production of IL-2, IFN-γ, and IL-4 could be detected from splenocyte cultures after LCMV infection (Fig. 1). In addition, we found that there was a significantly higher level of each of these cytokines produced by splenocytes from CD1KO mice. Early after an LCMV infection (day 6), IL-2 production was 7- to 10-fold higher in CD1KO mice (Fig. 1A). Later, at 10 days p.i., IFN-γ production was decreasing in wild-type mice, whereas secretion of this cytokine remained at high levels in CD1KO mice. IL-4 was expressed at low to undetectable levels in wild-type mice. In striking contrast, in LCMV-infected CD1KO mice, IL-4 levels were substantially higher. At earlier times p.i. (e.g., day 3), there was no detectable spontaneous cytokine release in splenocyte cultures from either wild-type or CD1KO mice (data not shown). Overall, these data suggest that CD1d1 molecules may influence LCMV-induced spontaneous cytokine production.

We next investigated whether LCMV-infected CD1KO mice express alterations in the levels of other cytokines using an RNAse protection assay. As was observed in the splenocyte cultures, the expression levels of mRNA for IL-6, IL-10, IL-15, and IFN-γ were higher in CD1KO than in wild-type mice. Remarkably, in each case the cytokine mRNA was significantly higher at later time points p.i., particularly on day 10 p.i., with p values ranging from 0.003 (IFN-γ) to 0.04 (IL-6). These data demonstrate that the levels of several cytokines after an acute LCMV infection are (at least in part) regulated by the expression of CD1d1 molecules.

**Analysis of the splenic lymphocyte subpopulations after LCMV infection**

An LCMV infection is cleared within 8–10 days, and this is mostly due to the CTL population (39). The effector phase of the host immune response to LCMV declines sharply ~20–30 days p.i., but a stable population of memory cells is maintained (27, 40, 41). In a previous study analyzing the LCMV-specific CTL response in wild-type and CD1KO mice, we reported that the cytolytic activities of CD8+ T cells from these two groups of mice were comparable (32). However, in the current study we observed a striking difference in spontaneous cytokine production between wild-type and CD1KO mice (Figs. 1 and 2). Thus, these observations prompted us to examine splenic lymphocyte populations at later time points after an LCMV infection. In uninfected mice, there was no difference in the number of total splenocytes between wild-type and CD1KO mice (Fig. 3A). Upon infection, however, the number of splenocytes was slightly (but consistently) higher in CD1KO mice. This included total T cells (Fig. 3B). To determine whether the increased number of splenic T cells in infected CD1KO mice resulted in an elevation in the number of those cells spontaneously producing cytokines, ELISPOT analyses were performed. On day 10 p.i., the number of IFN-γ-producing cells was dramatically higher in CD1KO mice (Fig. 3C); also, the spots were qualitatively larger in diameter in LCMV-infected CD1KO mice than in wild-type mice (data not shown). Collectively, these data indicate that not only was the actual number of IFN-γ-producing splenocytes higher in CD1KO mice, but these cells appeared to produce more IFN-γ on a per cell basis.

Next, the CD4+ and CD8+ T cell subpopulations were examined. Classically, the CD4:CD8 ratio is ~2:1 in splenocytes from uninfected wild-type mice, and this reverses to 1:2 (or greater) after LCMV infection (29, 40, 42, 43). The data from the ELISPOT assays suggested that there might be a difference in the number and/or phenotype of the T cell subpopulations. Between wild-type and CD1KO mice, the relative CD4:CD8 ratios on day 10 were comparable (Fig. 4A). In other experiments, individual CD4 and CD8 populations at earlier time points (days 3 and 6) were analyzed. With regard to CD4+ T cells, there were no differences between wild-type and CD1KO mice at any time point examined (data not shown). Analysis of the CD8+ T cell population showed elevated levels of CD8+ T cells in CD1KO mice compared with wild-type mice on day 3 (10.0 vs 5.6%, respectively) and day 6 (19.2 vs 15.9%, respectively), but not on day 0. In this particular experiment, day 10 differences between CD1KO and wild-type mice were present as well (33.9 vs 25.3%, respectively). It has been reported that an up-regulation in the CD94/H11011 population occurs after LCMV infection (44). We found that acute LCMV infection induces the expression of CD94 similarly in wild-type and CD1KO mice (Fig. 4A). In additional experiments, as expected, the increase in CD94+ T cells p.i. was detectable as early as day 6 p.i.; on this day, ~50% of the CD8+ were CD94+ (data not shown). Also, we were able to detect some differences in the CD4+CD8+ double-positive population between wild-type and CD1KO mice on day 6 (9.4% for wild-type vs 12.1% for CD1KO), which were occasionally greater on day 10 as well. However, as in the wild-type animals, the majority of CD8+ T cells in CD1KO mice were CD94+ during late stages of an acute infection. Another cell surface marker, CD62L, has been reported to be down-regulated in effector cells after a virus (i.e., LCMV) infection (43). When the cell surface expression of CD62L in CD8 and CD4
subpopulations was examined, it was found that expression decreased similarly in wild-type and CD1KO mice (Fig. 4B).

To determine whether there was a difference in the LCMV-specific CTL population between wild-type and CD1KO mice, splenocytes from both groups of mice were harvested on day 10 p.i. and stimulated with the LCMV-immunodominant peptides, gp33 and NP396 (35), and intracellular IFN-γ was analyzed by FACS. These experiments showed a slight increase in the CD8+ IFN-γ+ population from CD1KO compared with wild-type mice (Fig. 4C).

To further analyze the lymphocyte subpopulations, CD4+, CD8+, and B220+ populations were purified by electronic cell sorting. Compared with the unsorted, LCMV-infected population, the purified subpopulations produced lower amounts of IFN-γ (Fig. 5A). These data suggest that more than one lymphocyte subpopulation contributes to the enhanced cytokine production in CD1KO mice and/or that cell-to-cell cross-talk is required for optimal cytokine responses. Next, we evaluated whether the CD8+ population is important for cytokine production by comparing spontaneous cytokine release from LCMV-infected, wild-type, CD1KO, and TAP1KO mice. In agreement with data published using LCMV-infected C57BL/6-β2-microglobulin KO mice (28), we found almost undetectable levels of IFN-γ produced by infected TAP1KO mice compared with infected wild-type or CD1KO mice (data not shown). Although not important for the control of LCMV infection (45), IL-12 has been shown to be important for CD1d1-restricted, NKT cell-mediated activities, such as inhibition of tumor metastases (46–49). We found that spontaneous IL-2 and IL-4 production from LCMV-infected IL-12KO splenocytes was comparable to that in infected wild-type mice (data not shown), although, as expected, the level of LCMV-induced spontaneous IFN-γ was barely detectable in IL-12-deficient mice (45) (data not shown). Taken together, these data suggest that along with CD8+ T cells, other subpopulations are required for cytokine production.

To further delineate which splenic lymphocyte population(s) is necessary for cytokine production, we conducted depletion studies during the peak of the CTL response (i.e., day 8). The removal of B220+, MHC class II+, or CD8+ splenocytes had no effect on spontaneous IFN-γ production (Fig. 5B). As expected, the level of IFN-γ produced by CD1KO was higher than that in wild-type mice. In contrast, removal of the CD4+ T cell population reduced IFN-γ levels to just barely above the limit of detection. We also measured IL-2 production in these supernatants. Notably, IL-2 could be measured in supernatants from splenocyte cultures from LCMV-infected CD1KO (but not wild-type) mice. As observed with IFN-γ, depletion of the CD4+ population completely abrogated IL-2 production (Fig. 5C). Similar results were obtained when CD4+ populations were removed from mice on day 6 p.i., during the peak of IL-2 production (Fig. 1). Interestingly, removal of the CD8+ population resulted in a significant enhancement of the level of IL-2 in CD1KO mice (p = 0.0094) compared with undepleted splenocytes; this was probably due to an increase in the CD4+ population on a per splenocyte basis. These data suggest that early during an LCMV infection, CD4+ T cells are required to initiate cytokine production by CD8+ T cells, indicating a role for CD1d1 molecules in the regulation of virus-induced cytokine production.

As shown above, our data demonstrate a role for the CD4+ population in the enhanced cytokine production in CD1KO mice following infection. In addition to NKT cells, regulatory T cells (Treg), which are defined as CD4+CD25+ (50), have been suggested to play an important role in maintaining immune homeostasis (51). When the CD4+CD25+ T cell subpopulation was examined in wild-type and CD1KO mice after CMV infection, there was an ~50% reduction (0.8–0.4% in this experiment, comparable or slightly less in other experiments) in the Treg population from CD1KO mice on day 10 (Fig. 6). In wild-type mice, there was a modest decrease in this population (1.0–0.8%). However,
the population was comparable in wild-type and CD1KO mice at the time points examined. Notably, when we examined later time points (30 days p.i.), it was found that spontaneous IFN-γ production was comparable in wild-type and CD1KO mice (Fig. 7), suggesting that although changes in the Treg population on day 10 in CD1KO mice cannot be ruled out as contributing to the increase in spontaneous cytokine production observed, it is unlikely that these cells play a role at late stages after infection.

Kinetics of LCMV clearance in wild-type and CD1KO mice

We have previously found that LCMV (as measured by a classical virus plaque assay) is cleared by day 6 p.i. in both wild-type and CD1KO mice (32). However, we did not analyze the kinetics of viral clearance in that study. For the current study we used other methods to more precisely determine whether there are any differences in viral clearance between wild-type and CD1KO mice at the molecular level at various times p.i. RT-PCR is a much more sensitive method of detection for the measurement of mRNA in cells and can be used for the measurement of LCMV RNA (33, 36). Using RT-PCR and supporting our previous study (32), we found that on days 10 and 14 p.i., there was no apparent difference between wild-type and CD1KO in the level of splenic LCMV-gp RNA (Fig. 8A). Later, on day 30 p.i., however, there appeared to be a decrease in the level of LCMV-gp RNA present in CD1KO splenocytes compared with that in wild-type mice. The quantitative real-time RT-PCR approach confirmed this observation (Fig. 8B). Specifically, on day 10 p.i., the fold increase in LCMV-gp RNA in wild-type mice was 4240 ± 0.4 compared with a 1978 ± 0.6-fold increase in CD1KO mice (p < 0.02). On day 30, a difference in the LCMV-gp RNA fold increase was also found between wild-type (739 ± 0.6) and CD1KO mice (256 ± 0.9; p = 0.03). Detectable virus was also lower in CD1KO mice infected with the clone 13 variant of LCMV, which results in a more persistent infection (data not shown), demonstrating that this enhanced clearance effect is not specific for LCMV-Armstrong. Therefore, these data suggest that although both wild-type and CD1KO mice have similar levels of viral RNA that can be detected for the first 2 wk p.i., the increase in number and function of CD1KO splenocytes relative to those from wild-type mice after infection ultimately results in a more rapid clearance of viral RNA in CD1KO mice.
Discussion

CD1d1-restricted NKT cells are a unique subpopulation of T cells that promptly produce both Th1 and Th2 cytokines after activation (1–3). These cells can regulate the differentiation of Th1 or Th2 subsets during the host’s immune response to several infections. Given the wide range of cytokines secreted by activated CD1d1-restricted NKT cells and the immunoregulatory role they play, we examined whether CD1d1 molecules are involved in the regulation of virus-induced cytokine production, using CD1KO mice. The data presented in the current report demonstrate that CD1d1 molecules can regulate cytokine production after an acute LCMV infection. The increase in cytokine production is evident at the level of mRNA, and mice that lack CD1d1 molecules not only produce higher levels of cytokines, they have a larger TCR population that produces more cytokines on a per cell basis. Our data suggest that in wild-type mice, CD1d1-restricted T cells can play an immunoregulatory role on the CD4+ population by controlling cytokine production. However, in the absence of CD1d1 molecules (CD1KO mice), this regulation does not occur, and the CD4+ population produces high levels of IL-2, which, in turn, leads to enhanced proliferation of the CD4+ and CD8+ T cell subsets. This increased T cell population produces high level of cytokines and is therefore able to clear the virus more rapidly. Thus, CD1d1 molecules can influence both arms of the Th1/Th2 paradigm by regulating the CD4+ population.

Consistent with data reported by Su et al. (28), we found that low levels of IL-4 are spontaneously produced after LCMV infection of wild-type mice; however, in CD1KO mice, IL-4 production was substantially higher. Additionally, it was reported that cytokines are produced in MHC class II KO mice, which lack most CD4+ T cells, suggesting that the CD4+ population is not necessary for cytokine production (28). However, in our depletion studies we found that the absence of the CD4+ population reduced IFN-γ production below our level of detection, by either ELISA or ELISPOT (data not shown). It has been previously shown that the majority of CD4+ T cells in MHC class II KO mice are NKT cells (52). Thus, NKT cells and/or other CD1d1-specific cell populations may be able to compensate for the absence of conventional CD4+ T cells in MHC class II KO mice.

It has been reported that CTL have the ability to produce their own IL-2 in the absence of CD4+ cells (53, 54). Further, Varga and Welsh (29, 55) have identified a large population of virus-specific, IL-2-producing, CD4+ T cells during an acute LCMV infection. We have extended these findings by showing that the CD4+ population is important for IL-2 production early in infection and that CD1d1-restricted T cells can negatively regulate this response. In another study, Varga and Welsh (43) examined the CD4+ population during the memory response and found that the number of CD4+ T cells remain elevated throughout long term immunity. When we examined highly purified subpopulations, we found that neither the CD8+, CD4+, nor B220+ subpopulation can produce high levels of cytokine alone. These data suggest that cell-to-cell cross-talk is important for optimal cytokine production.

In CD1KO mice, the increase in the TCR+ population after infection, concomitant with the elevated levels of cytokine production compared with those in wild-type mice, strongly suggest that CD1d1 molecules control the size and function of the TCR+ population.

FIGURE 6. The Treg population in wild-type and CD1KO mice during acute LCMV infection. Wild-type and CD1KO mice were either uninfected or infected with LCMV for the indicated number of days. Splenocytes were harvested and stained for CD4 and CD25 expression. The numbers shown in the upper right quadrant indicate the percentage of the Treg (CD4+CD25+) population in total splenocytes. This experiment was performed twice.

FIGURE 7. Long term analysis of spontaneous splenic IFN-γ production from wild-type and CD1KO mice. Wild-type and CD1KO mice were either uninfected or infected with LCMV for the indicated number of days. Splenocytes from uninfected or LCMV-infected wild-type and CD1KO C57BL/6 mice were cultured for 20–24 h. The error bars correspond to the SD. The supernatants were then harvested, and IFN-γ production was determined by ELISA. This experiment is representative of two independent experiments.

FIGURE 8. Comparative viral RNA clearance in wild-type and CD1KO mice by RT-PCR. A, Splenocytes from uninfected and LCMV-infected wild-type and CD1KO mice were harvested at the indicated time points (in days) p.i. LCMV-gp expression was detected by semiquantitative RT-PCR using specific primer pairs. Actin was used as a control. B, Splenocytes from uninfected and LCMV-infected (days 10 and 30) wild-type and CD1KO mice were used for LCMV-gp detection by quantitative real-time RT-PCR, with GAPDH as the control. The data show the fold difference in the LCMV-gp transcript. This experiment is representative of two performed.
population after an acute infection with LCMV. Another group recently published a study using a different model system (respiratory syncytial virus), and their findings suggest an opposite role for CD1d molecules in NK cells and NKT cells. Therefore, the specific role of CD1d in NK cells and NKT cells remains to be determined.

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