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Distinct requirements for activation of NKT and NK cells during viral infection

Aaron J. Tyznik[*], **Shilpi Verma**[†], **Qiao Wang**[†], **Mitchell Kronenberg**[2,3,*], and **Chris A. Benedict**[3,†]

[*]Division of Developmental Immunology, La Jolla Institute for Allergy & Immunology, La Jolla, California 92037, USA.

[†]Division of Immune Regulation, La Jolla Institute for Allergy & Immunology, La Jolla, California 92037, USA.

Abstract

Natural killer (NK) cells are key regulators of innate defense against mouse cytomegalovirus (MCMV). Like NK cells, NKT cells also produce high levels of IFNγ rapidly after MCMV infection. However, whether similar mechanisms govern activation of these two cell-types, and the significance of NKT cells for host resistance, remain unknown. Here we show that although both NKT and NK cells are activated via cytokines, their particular cytokine requirements differ significantly, in vitro and in vivo. IL-12 is required for NKT cell activation in vitro, but is not sufficient, while NK cells have the capacity to be activated more promiscuously in response to individual cytokines from innate cells. In line with these results, GM-CSF-derived DC activated only NK cells upon MCMV infection, consistent with their virtual lack of IL-12 production, while Flt3L-derived DC produced IL-12 and activated both NK and NKT. In vivo, NKT cell activation was abolished in IL-12$^{-/-}$ mice infected with MCMV, while NK cells were still activated. In turn, splenic NK cell activation was more IL-18 dependent. The differential requirements for IL-12 and IL-18 correlated with the levels of cytokine receptor expression by NK and NKT cells. Finally, mice lacking NKT cells showed reduced control of MCMV, and depleting NK cells further enhanced viral replication. Taken together, our results show that NKT and NK cells have differing requirements for cytokine-mediated activation and both can contribute non-redundantly to MCMV defense, revealing that these two innate lymphocyte subsets function together to fine-tune antiviral responses.

INTRODUCTION

Host defense to mouse cytomegalovirus (MCMV, a β-herpesvirus) involves multiple cell types of both the innate and adaptive immune systems (1). Two innate-like lymphocyte populations, natural killer (NK) cells and natural killer T (NKT) cells; are major producers of IFNγ early during MCMV infection (2, 3). NKT cells are a T lymphocyte subset that is characterized by expression of an invariant T cell antigen receptor (TCR) α chain, formed by a Vα14 to Jα18 rearrangement in mice. When paired with several β chains, prominently Vβ8.2, this α chain imparts specificity for glycolipids presented by CD1d, a class I-like antigen-presenting molecule. These cells are commonly referred to as Type I or invariant natural killer T (*i*NKT) cells. The *i*NKT cell TCR is capable of recognizing several types of glycolipid antigens derived from microbial, environmental or endogenous sources (4-7).

[2]Corresponding Authors: Chris A. Benedict, Ph. (858)- 752-6732, Fax. (858)-752-6986, benedict@lji.org Mitchell Kronenberg, Ph. (858)- 752-6540, Fax. (858)-752-6990 mitch@lji.org.
[3]C.A.B. and M.K. share senior authorship.

While NK cells are truly innate lymphocytes, we refer to *i*NKT cells as innate-like cells, because although they are *bona fide* T cells that mature in the thymus, they carry out very rapid effector responses.

In addition to TCR/CD1d-dependent activation of *i*NKT cells, antigen-independent activation of these lymphocytes can also occur, for example in response to viruses or TLR ligands, and this typically results in their exclusive production of IFNγ, while TCR stimulations results in IL-4 and other cytokines as well. This 'indirect' *i*NKT cell activation has been shown to involve dendritic cells (DC), and potentially other antigen presenting cells (APC), which produce cytokines in response TLR-triggering (3, 8-10). To this point, the key cytokines promoting *i*NKT cell activation in these experimental systems have been IL-12, IL-18, and type I interferons (IFN-I), with IL-12 playing a dominant role. This also holds true in the case of MCMV infection, with IL-12$^{-/-}$ mice being severely compromised for *i*NKT activation, while IL-18 or IFN-I signaling-deficient mice show only very modest reductions (3, 10). In these cases, where no foreign lipid antigen is present to contribute to *i*NKT activation, IL-12 may synergize with TCR recognition of self-antigens presented by CD1d (11). However, *i*NKT cells are activated normally in MCMV-infected mice where CD1d expression is lacking or blocked (3, 10), providing evidence for a purely cytokine-driven activation process.

Despite being distinct cell lineages, NK and *i*NKT cells are similar in some respects, including the shared expression of NK receptors, dependence upon IL-15 signaling, overlapping tissue localization and the ability to rapidly release copious amounts of cytokines, particularly IFNγ, following infection. Consistent with these shared properties, both cell types have been implicated in the immune response to viral, bacterial, and parasitic infections. Further linkage comes from evidence showing that *i*NKT cells promote the secondary activation of NK cells through *i*NKT cell expression of CD40L, which mediates the activation of APC (9, 12). Taken together, these data suggest that *i*NKT and NK cells could play similar, and potentially redundant, roles in innate defense to infection.

The critical role of NK-mediated protection during MCMV infection has been studied extensively (13, 14). In C57BL/6 (B6) mice, recognition of the MCMV m157 protein by NK cells expressing the Ly49H activating receptor results in enhanced control of early replication (15). In turn, the lack of a Ly49H homologue in BALB/c mice results in significantly enhanced levels of early MCMV replication (16). At very early times of MCMV infection (8-12h), a burst of IFN-I synthesis emanating from infected stromal cells promotes the activation of NK cell cytolytic activity (17-19). By ~36h, MCMV has completed its first replication cycle, and subsequently triggers DC populations to produce IL-12 and IL-18, in addition to a second wave of IFN-I (20, 21). While *i*NKT do show markers of activation at 12h (3), it is only at ~36h when a large proportion of NK and *i*NKT produce IFNγ (2, 3, 10). Notably, plasmacytoid DC (pDC) are the primary source of innate cytokines at 36h leading to NK and *i*NKT IFNγ production, through TLR9-dependent recognition of MCMV (3, 22, 23).

In this study, we explored the regulation of NK and *i*NKT cells by APC-produced cytokines, and determined if they have distinct roles in antiviral control. We find that *i*NKT cells and NK cells do have different cytokine-mediated activation requirements, and they contribute to MCMV innate defense in a non-redundant fashion.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology (AP112-MK2-0410 and AP087-CB2-0110). Our institute and animal care committee adheres to NIH OLAW (office of lab animal welfare) and AAALAC guidelines. Our institute's OLAW assurance number is A3779-01 and our AAALAC accreditation number is #000840.

Mice

C57BL/6 (B6), B6.129S1-*Il12b*^{tm1Jm}/J (*IL-12p40*$^{-/-}$), B6.129P2-*Il18*^{tm1Aki}/J (*IL-18*$^{-/-}$) and BALB/c mice were purchased from The Jackson Laboratory. *CD1d1*$^{-/-}$ mice were a kind gift from Dr. L. Van Kaer (Vanderbilt University, Nashville TN). *Tlr9*^{cpg1} mice were a kind gift from Dr. B. Beutler (UT Southwestern, Dallas TX). BALB/c *Jα18*$^{-/-}$ mice were a kind gift from Dr. M. Taniguchi (Riken Research Center for Allergy and Immunology, Yokohoma, Japan), and were maintained as *Jα18*$^{+/-}$ heterozygotes. *Jα18*$^{+/+}$ and *Jα18*$^{-/-}$ littermates were used for MCMV infection experiments. 4get mice on the B6 background were a kind gift from Dr. R. Locksley (University of California San Francisco, San Francisco CA). All mice were housed in specific pathogen-free conditions.

Reagents and Abs

mAbs to the following mouse antigens were purchased from BD Biosciences, as purified or conjugates to FITC, Alexa 488, phycoerythrin (PE), PerCP- cyanin (Cy)5.5, PE-Cy7, allophycocyanin, Becton Dickenson Horizon V450, or V500: TCR-β, CD11b, CD8α, NK1.1, CD11c, CD44, CD25, CD69, CD4, CD212 (IL-12 receptor β 1), IL-4, TNF, DX5, and IFNγ. A mAb to CD218a (IL-18 receptor α) conjugated to Alexa Fluor 647 was purchased from BioLegend. PE-conjugated αGalCer-CD1d tetramers were generated in our laboratory as previously described (9) and used to stain cell suspensions. Recombinant mouse IL-12 and IL-18 were purchased from R&D Systems. IFNβ was purchased from PBL Interferon Source.

No touch iNKT cell isolation

Spleens from 4*get* mice were isolated at 8-10 weeks of age and dissociated into single cell suspensions and RBC were lysed using red cell lysing buffer (Sigma-Aldrich), washed, filtered and counted. Single cell preparations were stained with a custom cocktail of biotin-labeled Abs containing anti-CD8α, CD11b, CD19, CD24, CD62L, B220, F4/80, Gr-1, and Ter119. Labeled cells were loaded onto a RoboSep cell separation system using a custom enrichment reagent kit and protocol according to the manufacturer's instructions (Stemcell Technologies, Vancouver BC). *i*NKT cells were enriched up to 20% and eGFP bright cells were then sorted by FACSAria II (BD Biosciences). Sorted cells were 99% GFP^{+} with the purity of the cells collected being typically >91% *i*NKT cells, as assessed using CD1d tetramers loaded with αGalCer **(**Supplementary Fig. 1**)**.

Cell preparation and cell culture

All cell preparations were conducted using complete IMDM (Invitrogen Life Technologies) supplemented with 10% FBS, penicillin-streptomycin-glutamine, and 2-ME. DCs were prepared using 2 systems. For GM-CSF derived DCs, bone marrow was harvested and cultured in 10 cm bacterial dishes in complete IMDM supplemented with 20 ng/ml recombinant mouse GM-CSF (provided by Kyowa Hakko Kirin). On day 4, half of the

media was replaced with complete IMDM containing GM-CSF (20 ng/ml). GM-CSF derived BMDCs were harvested on Day 7. For Flt3L derived DCs, bone marrow was harvested and cultured with 100 ng/ml recombinant human Flt3L (Amgen) for 8 days in T75 flasks at a concentration of 45×10^6 cells/25 ml. Special attention was paid not to disturb the cultures during the entire incubation period. DCs were treated with 10 μg/ml type B CpG ODN 1826, control ODN 1982 (Alexis Biochemicals), infected with MCMV Smith strain (ATCC VR-1399) at a multiplicity of infection of 3, or mock infected, for 2 h. A total of 2.5×10^5 BMDCs were cultured with or without 5×10^4 sorted *i*NKT cells or NK cells for 48 h in a volume of 200ul in a round bottom 96 well plate at 37 ° C and 5% CO2 before cytokine detection as described (24). Cell-cell contact assays were performed by culturing 5×10^4 sorted *i*NKT cells or NK cells with 200ul of conditioned BM DCs culture supernatant. MCMV stocks were prepared from NIH-3T3 fibroblasts. NK Cells were purified from splenocytes using NK1.1 PE labeled mAb followed by positive selection on RoboSep cell separation system with a PE Selection kit (Stemcell Technologies) and then sorted for $NK1.1^+$ $TCR\beta^-$ cells on a FACSAria II. NK cells were typically >98% pure.

ELISA

A standard sandwich ELISA was performed to measure mouse IFNγ, IL-12p70, (R&D Systems), IL-18 (MBL International Corporation) and Type I IFNs (PBL Interferon Source) following the manufacturer's instructions.

Viral infections

Salivary gland stocks of MCMV Smith strain were prepared essentially as described (25). BALB/c mice were infected with 1×10^4 PFU of virus in 500 μl of PBS via i.p. injection. 4 days post-infection, mice were killed by CO_2 asphyxiation and organs were harvested and immediately snap frozen in liquid nitrogen. To determine viral titers, organs were weighed, homogenized and serial dilutions of homogenates were added to monolayers of NIH 3T3 fibroblasts plated in 24 well plates. Plates were spun at 524x *g* for 10 min prior to incubation at 37°, which increased the sensitivity of the plaque assays ~6-10 fold. Cells were fixed with formalin and plaques were visualized with 0.1% crystal violet and quantified.

Cell depletion

$J\alpha18^{+/+}$ BALB/c and $J\alpha18^{-/-}$ BALB/c mice were depleted of NK cells by injecting 50 μl of anti-asialo-GM1 rabbit polyclonal antibody (Wako) on day -1 of MCMV infection. NK cell depletion was verified in individual mice by flow cytometry after bleeding and just prior to infection, using a DX5-specific mAb, and non-specific cellular depletion was not observed. Additionally, control mice were antibody depleted of $CD8^+$ T cells (clone 2.43). Depletion was verified by flow cytometric staining with anti-CD8 clone 53-6.7 after bleeding and just prior to infection.

Real-Time PCR Analysis

Total RNA was isolated from sorted splenic *i*NKT cells and NK cells using an RNeasy Plus kit (QIAGEN). After DNase I treatment (Ambion), RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using SYBR Green (Bio-Rad) on a LightCycler 480 Real-Time PCR System with PCR primers pairs for *il-12rb1*, *il-12rb2*, *il-18r1*, *il-18rab*, *ifnar1*, and *ifnar2* from SABiosciences RT^2 qPCR Primer Assays (QIAGEN). Target gene expression was assessed by comparative cycling threshold analysis with L32 mRNA expression as a control. Data are presented in arbitrary units.

Flow cytometry and intracellular cytokine staining

Lymphocytes were stained with αGalCer/CD1d tetramers labeled with streptavidinallophycocyanin, anti-NK1.1-PerCp PE-cyanin (PECy)5, anti-CD8-PECy7, anti-CD11b-PECy7, anti-TCRβ-allophycocyanin-AF750, and CD25-FITC. All Abs and isotype controls, except anti-TCRβ-allophycocyanin-AF750 (eBioscience), were purchased from BD Biosciences. Cells were fixed and permeabilized using Cytofix/Cytoperm buffer and stained for intracellular IFNγ with PE-labeled clone XMG1.2. The data were collected on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Statistics

Differences between groups were evaluated using appropriate statistical tests as recommended by the La Jolla Institute bioinformatics core facility. Results are expressed as mean ± SD, except where differently indicated. p-values of <0.05 were considered significant.

RESULTS

Infected DC subtypes differ in their activation of *i* NKT cells

We have previously demonstrated in co-cultures that MCMV infection of Flt3L-expanded bone marrow DC (Flt3L BMDC) promoted purified *i*NKT cells to secrete IFNγ, but not other cytokines, such as the IL-4 these cells secrete when activated with glycolipid antigens. This did not occur when DCs from IL-12 p40 deficient mice were infected. Together with other studies indicating that IL-12 participates in the activation of *i*NKT cells (9, 24, 26, 27), these data strongly implicated the action of IL-12 rather than IL-23 in promoting *i*NKT cell effector function.

To test whether other DC types can activate *i*NKT cells upon MCMV infection, DC derived from GM-CSF treated bone marrow cultures were also analyzed. GM-CSF or Flt3L BMDCs were infected with MCMV, treated with CpG oligodeoxynucleotides (ODN), because the activation of IL-12 secretion was shown to be TLR9 dependent, or mock treated prior to the addition of purified *i*NKT cells or NK cells, and IFNγ production in the culture supernatants was measured ~48h later. To assess whether GM-CSF BMDCs activated *i*NKT cells in a CD1d-dependent manner, *Cd1d1*$^{-/-}$ BMDC were also tested. In addition, GM-CSF and Flt3L BMDCs generated from *Tlr9*$^{cpg/cpg}$ mice were included as negative controls. For Flt3L BMDCs, MCMV infection and CpG ODN treatment resulted in the production of IFNγ by both *i*NKT cells and NK cells (**Fig. 1a, c**). This activation by MCMV and CpG ODN was independent of CD1d expression, but required TLR9 (Supplementary Fig. 2), as expected based on our previous observations (3). CpG ODN treatment of GM-CSF BMDCs also resulted in TLR9-dependent IFNγ production by *i*NKT and NK cells, and again was independent of CD1d (**Fig. 1b** and Supplementary Fig. 2). However, when GM-CSF BMDCs were infected with MCMV, only NK cells were induced to secrete IFNγ, while *i*NKT cells were unresponsive (**Fig. 1d**).

Cell contact is not required for *i* NKT cell activation by MCMV infected DC

MCMV has developed numerous mechanisms to avoid detection by the immune system (1). We have previously observed that MCMV-infected GM-CSF BMDCs potently inhibit T cell proliferation through viral modulation of cosignaling pathways (28), raising the possibility that *i*NKT cell activation may be similarly suppressed by MCMV. To test this, or any other mechanism dependent on cell contact, supernatants from MCMV infected Flt3L or GM-CSF derived BMDCs were evaluated for their ability to activate *i*NKT cells. Supernatants from Flt3L BMDCs activated both NK cells and *i*NKT cells, indicating that cell contact was not

required for the induction of IFNγ synthesis by these innate lymphocytes. By contrast, the supernatant from GM-CSF BMDCs only activated NK cells but not *i*NKT cells (**Fig. 2a**). To assess if a soluble factor released from MCMV-infected GM-CSF BMDCs was inhibiting *i*NKT cell activation, Flt3L and GM-CSF BMDC-derived supernatants were mixed prior to *i*NKT cell addition. Even at a 10:1 (GM-CSF:Flt3L) ratio of BMDC supernatants, *i*NKT cells were still activated, demonstrating the absence of a potent inhibitor, and therefore strongly suggesting an activating factor was absent in the supernatants derived from MCMV-infected GM-CSF BMDCs (**Fig. 2b**).

Infected DC subtypes produce different cytokines

Our results indicated that a secreted factor(s) required for activation of *i*NKT cells was absent in supernatants from GM-CSF BMDCs infected with MCMV. Our previous work, and that of others, has indicated that IL-12, IL-18 and IFN-I can all contribute to the activation of *i*NKT cells (3, 9, 10), suggesting that the absence of one or more of these factors could be responsible. To address this, Flt3L and GM-CSF BMDCs were treated with CpG ODN and/or infected with MCMV prior to isolation of supernatants for analysis of IL-12p70, IL-18 and IFNα by ELISA. Following stimulation with CpG ODN, both Flt3L and GM-CSF DCs produced approximately similar amounts of these cytokines (**Fig. 3a**). However, when GM-CSF derived BMDCs were infected with MCMV, virtually no IL-12 was produced. By contrast, IFNα production was similar for the two infected DC types, and the amount of IL-18 was significantly higher from infected GM-CSF DCs compared to Flt3L DC (**Fig. 3b**). Importantly, use of an MCMV-GFP virus indicated that the infection efficiency of these two BMDC cultures was similar (data not shown), a fact also reflected by the high level production of IL-18 and IFNα by both BMDC types. In summary, although GM-CSF DCs have the capacity to produce IL-12 upon TLR9 activation with CpG ODN, they produced virtually none when exposed to MCMV, despite producing both IFN-I and IL-18 at high levels in response to viral infection.

*i*NKT cells and NK cells differ in their sensitivity to cytokines

The data obtained from MCMV infection of the two DC subtypes suggested that *i*NKT cells are more dependent on IL-12 for their activation, while for NK cells, IL-12 may not be essential if other cytokines from innate immune cells are present. To test this, IFNγ production by purified NK and *i*NKT cell populations was measured after treatment with varying amounts of recombinant cytokines in the absence of DC. When the concentrations of IL-12 or IL-18 were limiting (<10 pg/ml), these two cytokines functioned synergistically to activate both *i*NKT cells and NK cells to produce IFNγ. For example, when *i*NKT cells and NK cells were cultured with 10 pg/ml of IL-12, both cell types produced large, and roughly equivalent amounts of IFNγ across a wide range of IL-18 concentrations (**Fig 4a**). These data suggest that this dose of IL-12, with IL-18 at or below the detection limit of the ELISA assay, induced a maximal response of both *i*NKT and NK cells. In contrast, fixing the IL-18 at 10 pg/ml and titrating in very limiting amounts of IL-12 (~0.1-10 pg/ml), revealed that *i*NKT cells were even more sensitive than NK cells to synergistic activation by combinations of these two cytokines (**Fig 4d**).

When a dose titration of IFNβ was performed in the presence of 10 pg/ml IL-12, similar to what was done for IL-18, an increase in IFNγ production by both NK cells and *i*NKT cells was again observed. However, in these circumstances, NK cells were more sensitive to the synergistic effects of IFNβ and IL-12 than *i*NKT cells (**Fig. 4b**). IFNβ alone could not induce IFNγ secretion from *i*NKT cells, regardless of the dose used, while it could promote NK cells to secrete IFNγ when added at higher levels (**Fig. 4e, right panel**). Surprisingly, a similar observation was made with IL-18 and IL-12 (**Fig. 4e**). Neither of these cytokines alone, even at 1ng/ml, could activate purified *i*NKT cells to secrete significant levels of

IFNγ. In contrast, NK cells activated by IL-18 or IL-12 alone produced high levels of IFNγ, even at doses as low as 100 pg/ml and 10 pg/ml, respectively (**Fig. 4e**). To attempt and exclude a potential contribution of anti-NK1.1 mAb treatment on 'priming' NK cell activation during the purification process, several of these experiments were repeated using DX5+CD3- cells isolated from spleens, and similar results were obtained (data not shown). Additionally, NK and *i*NKT cells were purified by cell sorting from BALB/c mice using similar antibody staining panels to discriminate them (+/− TCRβ expression). As we observed for C57BL/6 mice, BALB/c NK cells produced IFNγ when single innate cell-derived cytokines were added to cultures, while *i*NKT remained unresponsive even at high doses of either IL-12 or IL-18 alone (data not shown).

We also determined if *i*NKT cells secrete IFNγ in response to IFNβ and IL-18. Purified *i*NKT and NK cells were cultured with 10pg/ml of IL-18, a dose that synergizes with IL-12 to induce their robust activation (**Fig. 4d**), together with varying amounts of IFNβ. Interestingly, *i*NKT cells were not activated in response to the combination of IL-18 + IFNβ, while NK cells were markedly stimulated (**Fig. 4c**). IFNγ secretion by NK and *i*NKT cells has been associated with the ability of IL-12 to induce STAT4 activation (29, 30). It has been previously demonstrated that NK cells express a high basal level of STAT4 bound to the IFN-I receptor, and upon exposure to IFN-I, STAT4 is displaced leading to IFNγ secretion (29). In agreement with these findings, when NK cells were exposed to high doses of IFNβ, IL-12, or IL-18, STAT4 phosphorylation was detectable within 1 hr (Supplementary Fig. 3a). Meanwhile, STAT4 phosphorylation in *i*NKT cells was also detectable at high doses of IL-12 (1 ng/ml), giving a bimodal pattern seen in an earlier study (30). However, this was absent at lower IL-12 concentrations (10pg/ml), which still robustly activated STAT4 phosphorylation in NK cells, and no STAT4 phosphorylation was observed in *i*NKT cells cultured with high doses of IL-18 or IFNβ alone (Supplementary Fig. 3b). However, STAT4 phosphorylation was detectable in *i*NKT cells at low IL-12 concentrations when combined with IL-18 or IFNβ. Consequently, the pattern of STAT4 phosphorylation in these two cell types reflects their requirements for the induction of IFNγ synthesis. Taken together, these results indicate *i*NKT cells and NK cells have different cytokine requirements to activate their production of IFNγ. These results also explain the inability of MCMV infected GM-CSF BMDCs to activate *i*NKT cells, despite high levels of virus-induced IFNβ and IL-18 production, as IL-12 production was absent.

Differential sensitivity of NK and *i*NKT cells to IL-12 in vivo

The amounts of IL-12 and IL-18 induced at ~36h after MCMV infection in the sera are in the ng/ml range (31), far above the limiting amounts used to stimulate purified *i*NKT and NK cells in our in vitro experiments shown in figure 4. IFNβ also reaches high levels in the serum at 36h, but also exhibits a much earlier peak at 6-8h in response to MCMV infection of splenic stromal cells (32). Because purified NK cells produced IFNγ in response to treatment with any one of the single cytokines at ng/ml doses (**Fig. 4e**), we postulated that in mice genetically deficient for only one of the cytokines, when compared to *i*NKT cells, the NK cells might show enhanced activation upon MCMV infection *in vivo*. Consistent with previous results (3, 10), in MCMV infected mice *i*NKT cell IFNγ synthesis was strictly dependent on IL-12 when measured by intracellular cytokine staining on cells analyzed directly ex vivo. Although the NK cell response was decreased in the absence of IL-12, these cells still produced measurable levels of IFNγ in the spleen following MCMV infection (**Fig. 5a, b**). In contrast, spleen NK cells were unable to produce IFNγ in the absence of IL-18, while *i*NKT cell IFNγ synthesis was not diminished **(Fig. 5)**. Therefore, the cytokine requirements for the activation of NK cells to secrete IFNγ in vivo may be more stringent than when these cells are purified and cultured with cytokine. Interestingly, NK cells isolated from the livers of infected mice retained the ability to secrete IFNγ in the

absence of IL-18, in agreement with previous studies (31), although the response was reduced compared to WT controls (data not shown).

Expression of cytokine receptors by NK and *i* NKT

One possible explanation for the differing sensitivity of NK cells and *i*NKT cells to combinations of IL-12, IL-18 and IFN-I could be varying levels of cytokine receptor expression. Quantitative PCR analysis of cytokine receptor mRNA levels, as well as protein expression for those receptors where antibodies are available, was performed in these two cell populations. When compared to NK cells, both *il-12rb1* and *il-12rb2* expression levels were higher in freshly purified *i*NKT cells (~2 and 3 fold, respectively) (**Fig. 6a**), while *il-18rb1* and *il-18rap* expression was approximately three-fold lower (**Fig. 6b**). Expression of *ifnar1* was approximately two-fold higher in NK cells compared to *i*NKT cells, although the two cell populations expressed similar levels of *ifnar2* (**Fig. 6c**). To verify if the increase in mRNA expression correlated with protein, IL-12Rβ2 and IL-18Rα expression was analyzed by flow cytometry on *i*NKT and NK cells (**Fig. 6d**). While IL-12Rβ2 was readily detectable on tetramer$^-$ /CD3$^-$ NK cells, the highest level of expression was on tetramer$^+$ / CD3$^+$ *i*NKT cells. IL-12Rβ1 receptor expression was not detectable on MHC class I-reactive CD8$^+$ αβ or γδ T cells. Expression of the IL-18Rα chain was detectable on NK cells, but this was not the case for either *i*NKT cells, conventional αβ T cells or γδ T cells in the spleen (**Fig 6d**) (30, 33). Analysis of IL-12 and IL-18 receptor chain expression in iNKT and NK cells isolated from BALB/c mice revealed identical results to those seen in C57BL/6, suggesting that the differential sensitivity of these two cell populations to these innate cytokines may be a general character (data not shown). These trends in the amounts of cytokine receptor expression are consistent with the increased sensitivity of *i*NKT cells to IL-12 in the presence of IL-18, and with the enhanced ability of NK cells to respond to IL-18 + IFN-I. However, the results do not exclude the contribution of additional factors to the differential cytokine sensitivity of the two lymphocyte populations.

i NKT cells contribute to anti-viral innate defense

Previous reports have indicated that mice genetically deficient for *i*NKT cells (Jα18$^{-/-}$) can control MCMV replication normally (10, 34). However, this has only been examined in B6 mice, which have very robust NK cell-mediated MCMV defenses due to expression of the Ly49H activating receptor that interacts with the viral m157 protein (35, 36). In contrast, NK cell-mediated defense against MCMV in BALB/c mice differs significantly from that in B6 mice due to a different expression pattern of activating and inhibitory Ly49 family receptors (37), including a lack of Ly49H. Such differences, including the lack of LY49H, also pertain to many other inbred strains and wild mice as well (38). Consequently, we tested if *i*NKT cells contribute to MCMV innate defense in BALB/c mice lacking Ly49H by analyzing viral replication in the spleen and liver of *Jα18*$^{-/-}$ mice and WT littermate controls. We found that *Jα18*$^{-/-}$ mice had approximately 3-5 fold higher levels of MCMV replication in both these organs, indicating that *i*NKT cells do play a role in the early control of MCMV replication in some mouse strains (**Fig. 7a, b**). In separate experiments, we compared MCMV replication in *Jα18*$^{-/-}$ BALB/c mice that were depleted of NK cells using anti-asialo-GM1 antisera. Depleting NK cells in mice that lack *i*NKT cells resulted in a further enhancement of viral replication, which was more dramatic in the liver than the spleen (**Fig. 7c, d**). Since anti-asialo-GM1 antisera can potentially deplete activated CD8$^+$ T cells, we verified that MCMV replication was unaltered in BALB/c mice at day 4 following CD8 depletion using CD8-specific Abs (Supplementary Fig. 4). These data establish that *i*NKT cells contribute significantly, and non-redundantly, to early or innate defense to MCMV in BALB/c mice.

DISCUSSION

The importance of NK cells for innate immune responses to viral infections is well established, but while *i*NKT cells also can be activated after exposure to viruses, in the absence of synthetic glycolipid antigen activation, there are relatively few examples in which these responses are protective. Consistent with an important anti-viral role for *i*NKT cells, however, is the uncontrolled infection in response to the Varicella-zoster vaccine in rare individuals lacking *i*NKT cells or CD1d and the finding that several viruses down regulate CD1d expression, suggesting evolutionary pressure for an immune evasion mechanism targeting *i*NKT cells (39-45). Here we show that NK cells and *i*NKT cells exhibit cytokine-induced activation in response to infection with MCMV, but that their cytokine requirements for activation differ, and that both cell types contribute to host protection. These data not only definitively show that *i*NKT and NK cells are differentially activated in the context of pathogen infection, perhaps in response to distinct APC subsets or other influences, but indicate they may play distinct roles in the control of infection.

*i*NKT cells express an invariant TCR that recognizes glycolipids, raising the issue as to how they participate in the immune response to viruses. However, several additional modes of activation allow *i*NKT cells to be activated by microbes in the absence of a microbial glycolipid presented by CD1d. First, activation can result from the presentation of self-glycolipid antigens by CD1d. The relevant self antigens may including phospholipids and various types of glycosphingolipids (46), but this response also requires IL-12 production by activated APC. *i*NKT cells activated in this way produce predominantly IFNγ, in contrast to the diverse Th1 and Th2 cytokines that are elicited by strong glycolipid agonists for the TCR (e.g. αGalCer) (6, 27). An exception to the requirement for IL-12 is one study in which APC were activated with CpG ODN; in this case IFN-I was required as the co-factor for *i*NKT cell activation mediated by self antigen (8).

Second, an additional pathway for *i*NKT cell activation is purely cytokine driven, and does not require concomitant TCR engagement with any antigen presented by CD1d. In studying MCMV infection, we have shown that activation by TLR9-mediated signaling leads to IL-12 release by APC, resulting in *i*NKT cell production of IFNγ by ~36h after infection. CD1d expression was not required and IL-18 had a minimal role (3). Wesley et al. reported similar results, but they observed a contribution of IFN-I for optimal *i*NKT cell activation during MCMV infection. In addition, we have previously seen that *i*NKT cells increase expression of the activation marker CD69 early as 12h after MCMV infection, which is strictly dependent upon IFN-I produced by MCMV-infected splenic stromal cells (47). The activated *i*NKT cells do not produce IFNγ at this early time, however, at least as assessed by intracellular cytokine staining directly ex vivo, although it remains to be determined if lower amounts of IFNγ are produced or if other effector functions are acquired. Interestingly, this ‘initial’ IFN-I produced by stromal cells at 8-12h of MCMV infection is sufficient to promote IFNγ production by some NK cells (47), consistent with our results that IFNβ alone can induce IFNγ-production by purified NK cells but not iNKT cells (Fig. 4).

The engagement of activating NK receptors, such as NK1.1, CD94/NKG2C or NKG2D, provides a third, TCR-independent pathway for *i*NKT cell activation that is illustrative of their innate- or NK cell-like function (48, 49) (50). However, because productive MCMV infection inhibits cell surface expression of ligands for activating NK receptors (51, 52), it is likely this mechanism of *i*NKT cell activation would be muted or absent when directly encountering virus infected cells.

Although the critical importance of IL-12 for *i*NKT cell activation has been established, here we have characterized in detail the cytokine activation requirements for *i*NKT cells

compared to NK cells. Interestingly, IL-12 alone did induce *i*NKT cells to produce IFNγ in vitro. This is in agreement with our earlier findings that both IL-12 and IL-18 were required for *i*NKT cell stimulation in response to LPS in vitro or in vivo (9). Furthermore, activation induced arrest of *i*NKT cells in liver sinusoids was also most evident when IL-12 and IL-18 were injected together (53). By contrast, our earlier studies of the response to MCMV demonstrated the importance of IL-12, but the absence of IL-18 had only a marginal effect (3). Here we show that either IL-18 or IFN-I can synergize with IL-12 to activate *i*NKT cells in vitro. Therefore, in the context of MCMV infection, when high levels of IFN-I are produced, it is likely that IL-18 is largely redundant for *i*NKT cell activation.

We also now show that the cytokine requirements for NK cell stimulation in vitro to produce IFNγ are different, and generally less stringent, than those for *i*NKT cells. This is most noticeable in the ability of NK cells to respond to single cytokines when added at higher concentrations, as well as their responses to combinations of either IL-18 + IFNβ or IL-12 + IFNβ. The only exception to this trend was that *i*NKT cells were more responsive to IL-18 in the presence of low IL-12 levels. The differential cytokine sensitivity of these two cell types correlated with the expression of cytokine receptors, suggesting this could be a contributing factor. Furthermore, our *in vivo* data are generally consistent with the in vitro results. *i*NKT cells were unable to produce IFNγ in IL-12p40$^{-/-}$ mice, while NK cells remained partially responsive, presumably due to suboptimal activation by IL-18 and IF–I. Somewhat surprisingly, however, production of IFNγ by NK cells *in vivo* was found to be highly dependent on IL-18 in the spleen. Based on our *in vitro* experiments, we would not have predicted this result, as other cytokines from innate cells are present at the time NK cells were analyzed. However, this finding is consistent with previous work showing IL-18 is required for NK cell production of IFNγ in the spleen and for expansion of Ly49H^{+} NK cells *in vivo*, as well as for IFNγ production by NK cells co-cultured with MCMV-infected GM-CSF BMDCs (31, 54, 55). It should be noted that while the *in vitro* work defines the capacity/potential for NK or *i*NKT cells to respond to minimal cytokine concentrations, negative regulatory influences would be diminished in this set up. For example, when cytokines are added to purified NK cells, the influence of cell surface molecules expressed by APCs acting on NK cell inhibitory receptors will be minimized. Furthermore, local cytokine concentrations may not reflect serum concentrations, and the existence of such local effects is supported by the organ-specific requirement for IL-18 for MCMV-mediated NK cell activation in the spleen, but not the liver (31).

Using two common methods for generating DC populations *in vitro*, we found that GM-CSF BMDCs were capable of activating NK cells but not *i*NKT cells when infected with MCMV. The defining difference was not in the efficiency of the infection, or in their ability to generally detect virus and subsequently produce cytokines. Instead, the defect was in the absence of IL-12 in the supernatant following MCMV infection. We do not know why GM-CSF BMDCs do not produce IL-12 after MCMV infection, as they are competent to do so after exposure to CpG ODN. Regardless, as microbes evolve they are able to evade detection in numerous ways, including the blocking of production of critical cytokines from innate cells, and our results are consistent with previous work showing that productive infection of GM-CSF BMDCs with MCMV inhibits their ability to produce IL-12 in response to secondary TLR activation (56). The results from microarray analysis of gene expression suggest GM-CSF BMDCs are likely most similar to inflammatory monocytes (57). However, the phenotype of DC subsets changes rapidly after infection, and it is uncertain if there is a DC population *in vivo* after MCMV infection that truly corresponds to GM-CSF BMDCs. Nevertheless, it is certain from many studies that the cytokines produced by innate cells following infection will depend on the infecting agent and the cell subset that is activated. Therefore, extrapolating the MCMV results to other infections, it seems

reasonable to propose that circumstances exist where the balance of activated APC types and cytokines produced could favor the preferential activation of NK or *i*NKT cells.

There are only a few examples where *i*NKT cells have been shown to affect viral clearance in the absence of pharmacologic activation by glycolipid antigen (58), and therefore a key issue is whether *i*NKT cell responses are important for limiting MCMV. We observed that viral replication was elevated in BALB/c mice that lack *i*NKT cells, especially in the liver. Depletion of NK cells from J$\alpha 18^{-/-}$ BALB/c mice led to a further increase in replication. These data indicate that NK cell activation after infection is not completely dependent on *i*NKT cells, although in some cases activation of *i*NKT cells can contribute to NK cell stimulation, and furthermore, considering the effects of NK cell depletion in *i*NKT cell deficient mice, also that the NK cell contribution to viral clearance is quantitatively quite similar to *i*NKT cell-mediated control. Therefore, NK cells and *i*NKT cells each play unique roles in antiviral defense in mice. Importantly, previous work has shown that B6 mice lacking *i*NKT cells do not show enhanced replication of MCMV, and we have also seen similar results in J$\alpha 18^{-/-}$ mice generated in this strain (data not shown). This could be due to several reasons, including the more robust, Ly49H-dependent NK cell response in B6 mice, or perhaps the differential effector cytokine production by *i*NKT cells in BALB/c mice compared to B6 mice. As HCMV infection impacts the NK cell repertoire differentially in people of varying genetic backgrounds, perhaps it is not surprising that *i*NKT cells show a differential importance in controlling MCMV replication in mouse strains, as this is certainly the case for MCMV and NK cells (59). Interestingly, as noted above, a drastic reduction in the number of *i*NKT cells and decreased CD1d expression has been associated in a few cases with disseminated Varicella infection following the administration of the vaccine strain (39, 40). Therefore we must consider the possibility that *i*NKT cell activity is particularly important in humans for control of infections by the *herpesviridae*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

BMDC	bone marrow-derived dendritic cells
APC	antigen presenting cells
Flt3L	fms-related tyrosine kinase 3 ligand
GM-CSF	granulocyte macrophage colony stimulating factor
i	invariant
ICCS	intracellular cytokine staining
IFN-I	type I interferon
MCMV	mouse cytomegalovirus

NK	natural killer
ODN	oligodeoxynucleotide
pDC	plasmacytoid DC

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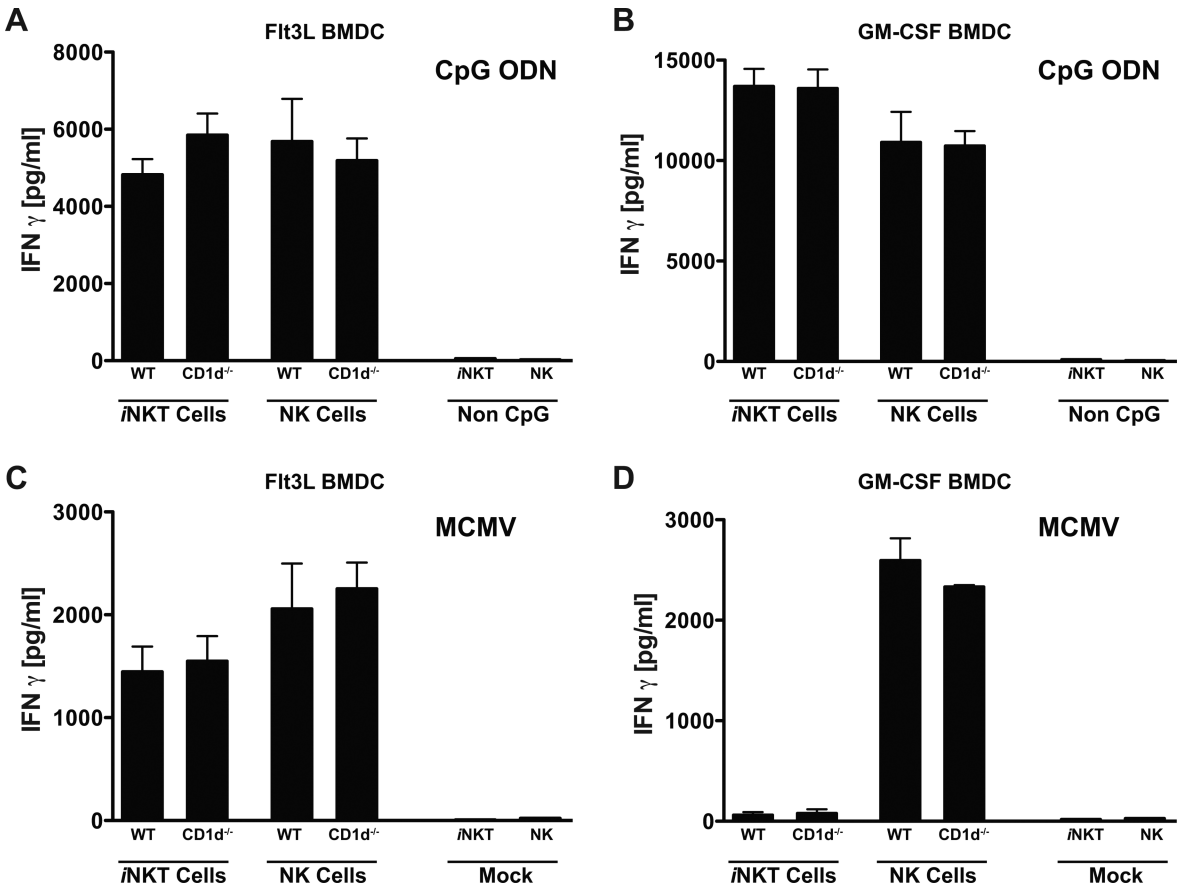

Figure 1.

IFNγ production by *i*NKT and NK cells in DC co-cultures. Flt3L (*A*) or GM-CSF (*B*) derived BMDC from the indicated mouse strains were treated with CpG-ODN or control treated and then cultured with purified *i*NKT cells or NK cells for 48h. Flt3L (*C*) or GM-CSF (*D*) derived BMDC from the indicated mouse strains were mock or MCMV infected and cultured with purified *i*NKT cells or NK cells for 48h. Supernatants were analyzed for IFNγ by ELISA, and shown is a representative experiment of 6 performed. ELISA results represent the mean of one experiment with three replicate cultures measured in triplicate. Error bars represent SEM (n = 9 for each set of BMDC).

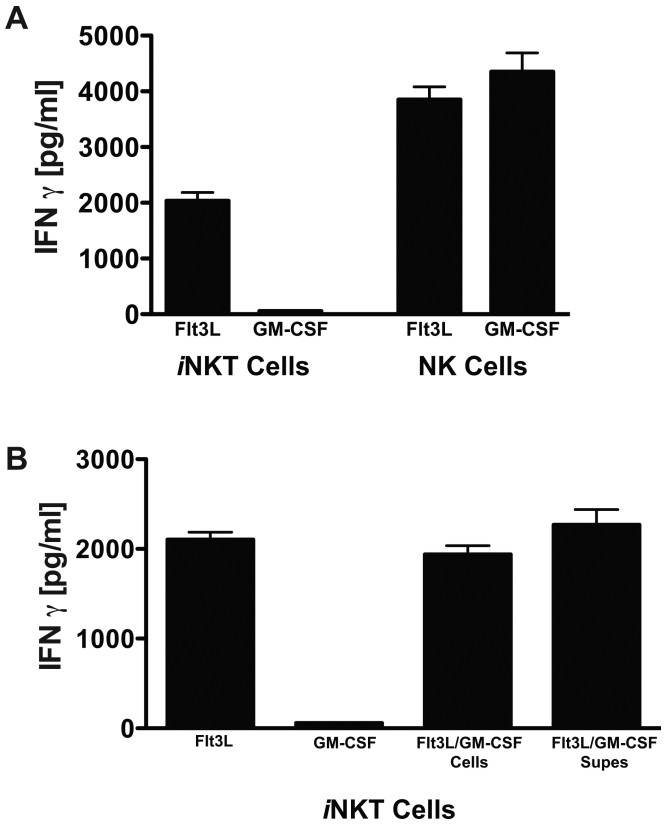

Figure 2.

Activation of *i*NKT and NK cells by MCMV-infected DC does not require cell-cell contact. (*A*), IFNγ production by *i*NKT cells or NK cells cultured for 48h with supernatants from Flt3L or GM-CSF BMDC infected with MCMV. *(B)*, *i*NKT cells were cultured with individual supernatants, a 1:1 mixture of the two APC types (Flt3L/GM-CSF cells) or with a 10:1 ratio of supernatants from MCMV-infected DC (Flt3L/GM-CSF supes). IFNγ ELISA results represent the mean of one experiment with 2 replicate cultures measured in triplicate. Error bars represent SEM (n = 6 for each culture condition). Shown is a representative experiment of 3 performed.

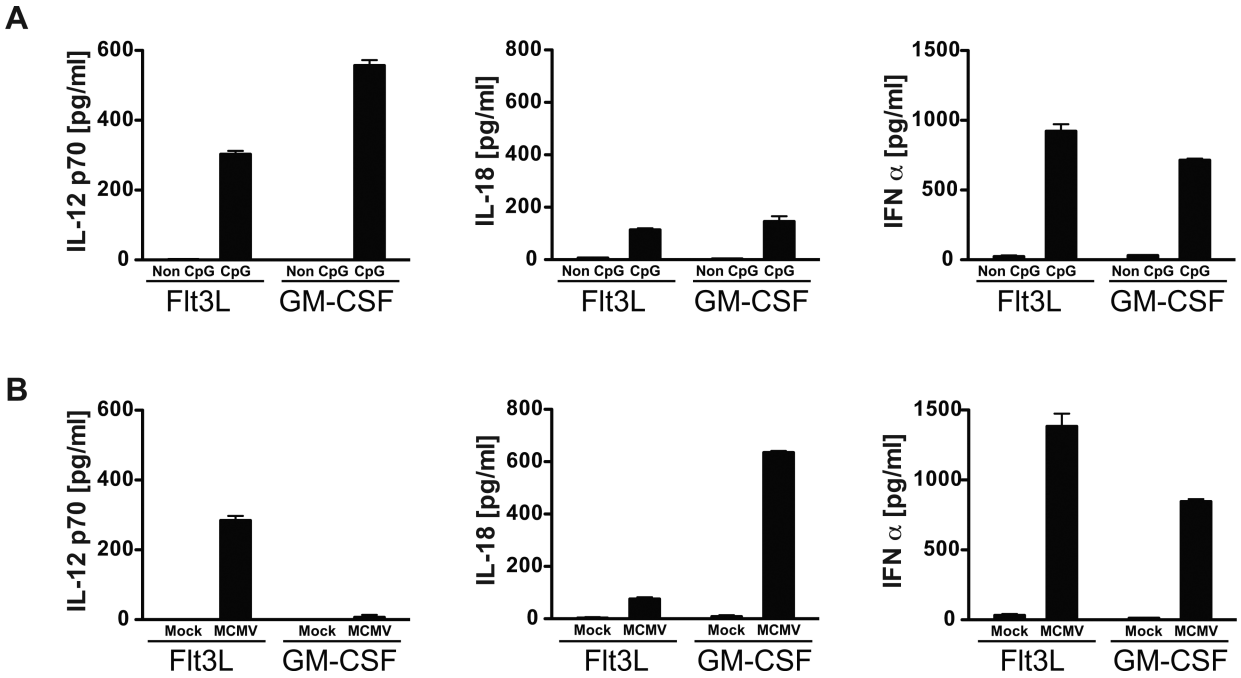

Figure 3.

Cytokine production by DCs in response to CpG or MCMV. *(A)*, Flt3L or GM-CSF BMDC were treated with CpG or control ODN (Non) for 48h, and supernatants were analyzed for IL-12 p70, IL-18 or IFNα by ELISA. *(B)*, Same as in (A), but DC subtypes were mock or MCMV infected prior to analysis of cytokine levels by ELISA. Data are representative of 4 independent experiments. Results show the mean of one experiment with 3 replicate cultures of DC measured in duplicate for ELISA. Error bars represent SEM (n = 6 for each culture condition).

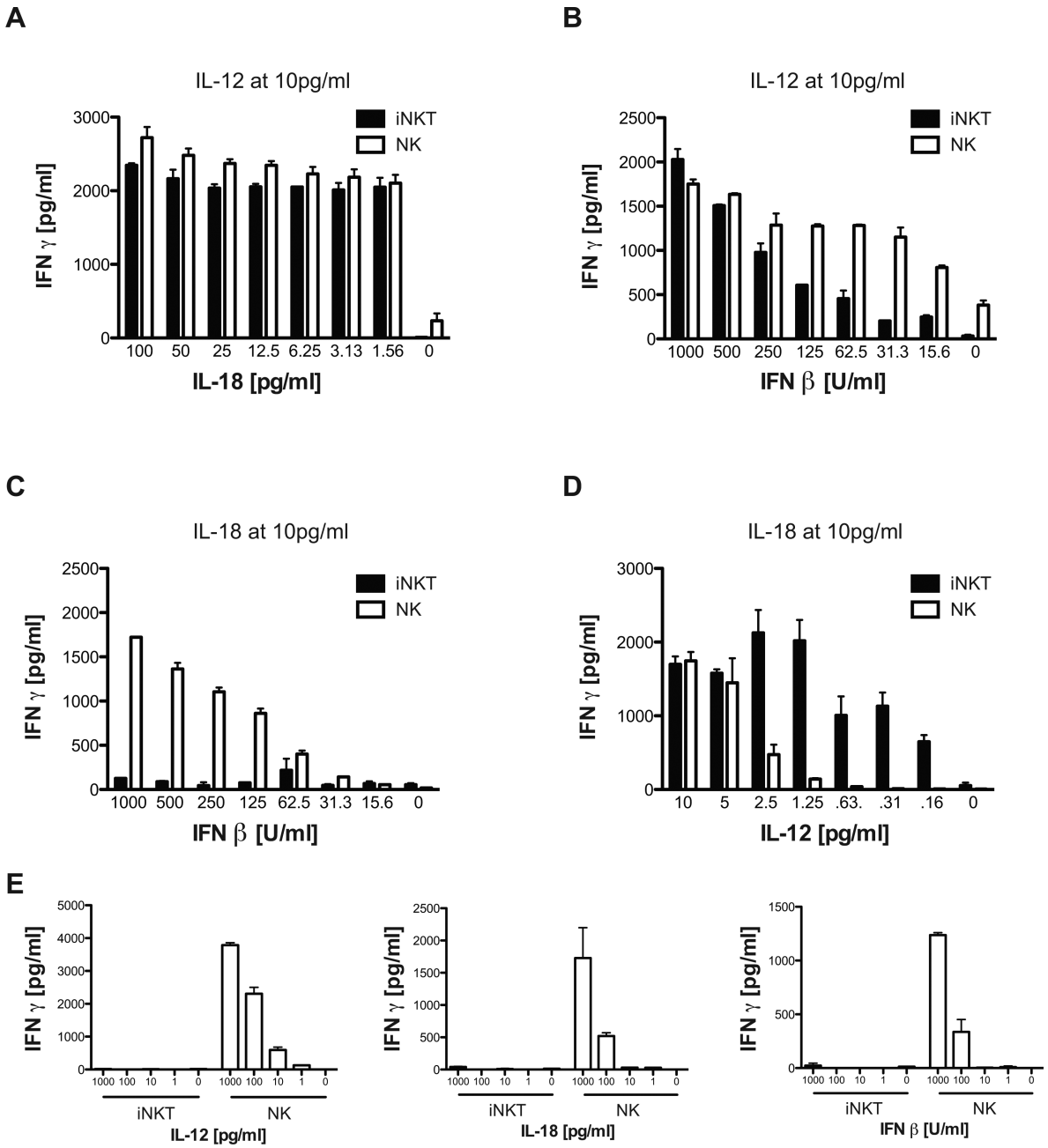

Figure 4.

Activation threshold of purified *i*NKT and NK cells to cytokines. (*A-B)*, Purified *i*NKT cells or NK cells were cultured with 10 pg/ml IL-12 and serially diluted amounts of IL-18 (*A*) or IFNβ (*B*). *C-D*, Purified *i*NKT cells or NK cells were cultured with 10 pg/ml IL-18 and serially diluted IFNβ (*C*) or IL-12 (*D*). *E*, Purified *i*NKT cells or NK cells were cultured with varying doses of IL-12, IL-18 or IFNβ. 48h later, supernatants were analyzed for IFNγ by ELISA. Data are representative of 3 independent experiments and show the mean of one experiment with 3 replicate cultures measured in triplicate for ELISA. Error bars represent SEM (n = 9 for each culture condition).

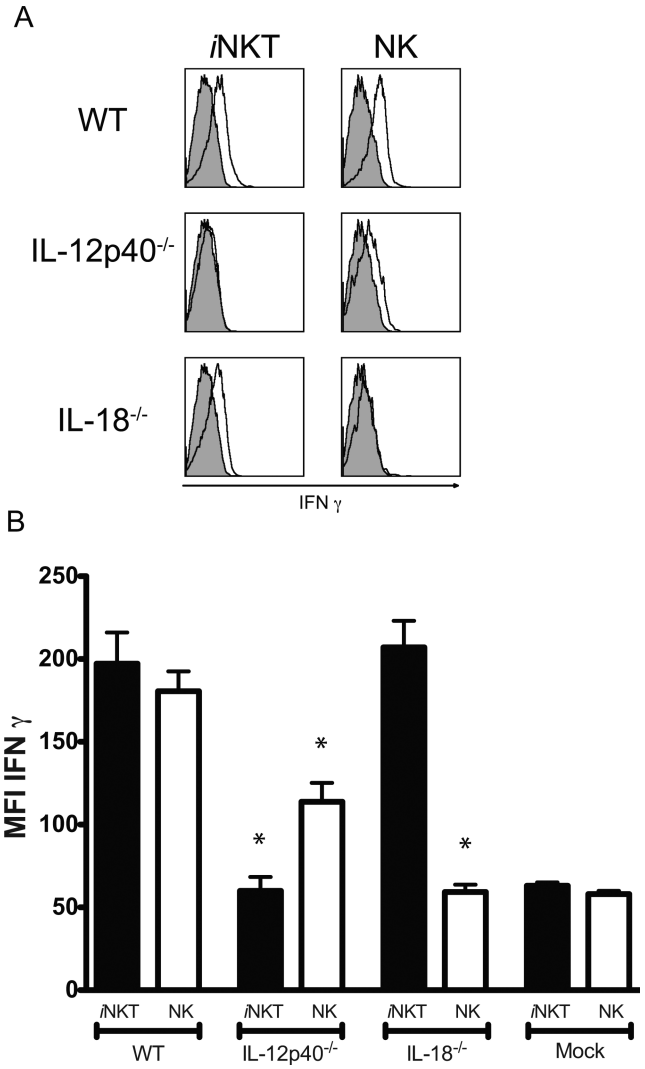

Figure 5.

*i*NKT and NK cell activation following MCMV infection in vivo. *(A)*, IFNγ expression by NK and *i*NKT cells from the spleen of indicated mouse strains 36h post-infection with MCMV (open histograms) compared with isotype controls (shaded histograms). Histograms are representative plots of three independent experiments of 3–8 mice per group. *B*, IFNγ mean fluorescence intensity (MFI) of 3–8 mice per group 36h post-infection with MCMV from a minimum of three independent experiments. Statistically significant differences using the equal variance Student *t* test are indicated with an asterisk (*, $p \leq 0.01$) comparing the responses of either WT NK or *i*NKT cells, respectively, to the corresponding responses in either of the mutant strains. Error bars represent SEM ($n = 3–8$ for each group).

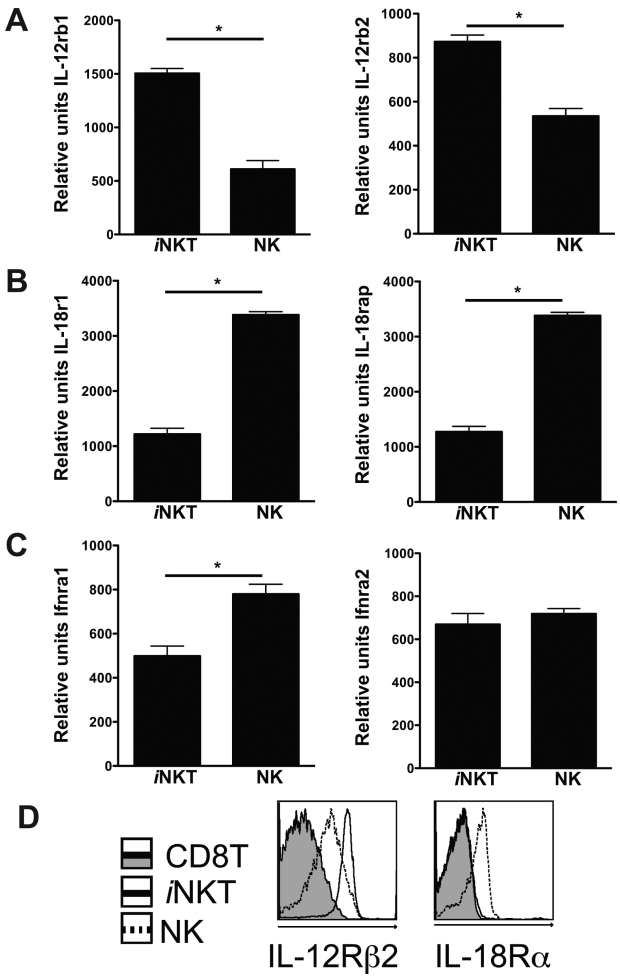

Figure 6.

Cytokine receptor expression by *i*NKT and NK cells. *(A-C)*, Purified splenic *i*NKT or NK cells were analyzed by quantitative real-time PCR for their expression of IL-12 (*A*), IL-18 (*B*) and IFN-I (*C*) receptor mRNAs. Bar graphs show each receptor chain or receptor associated protein mRNA levels relative to the L32 ribosomal protein gene expression. Differences between groups were evaluated for statistical significance by the two-tailed unpaired Student *t* test. Data are the mean of 2 independent experiments performed in duplicate. Error bars represent SEM (n=4); =$p<0.05$. *(D)*, Flow cytometric analysis of $CD19^-$ splenocytes for IL-12Rβ2 (left panel) and IL-18Rα (right panel) expression on $tetramer^+$ *i*NKT cells (solid line), $tetramer^-$ NK cells (dashed line) and $tetramer^-$ αβ and γδ $CD8^+$ T cells (shaded histogram). Histograms are representative plots of 3 mice per group from 2 independent experiments.

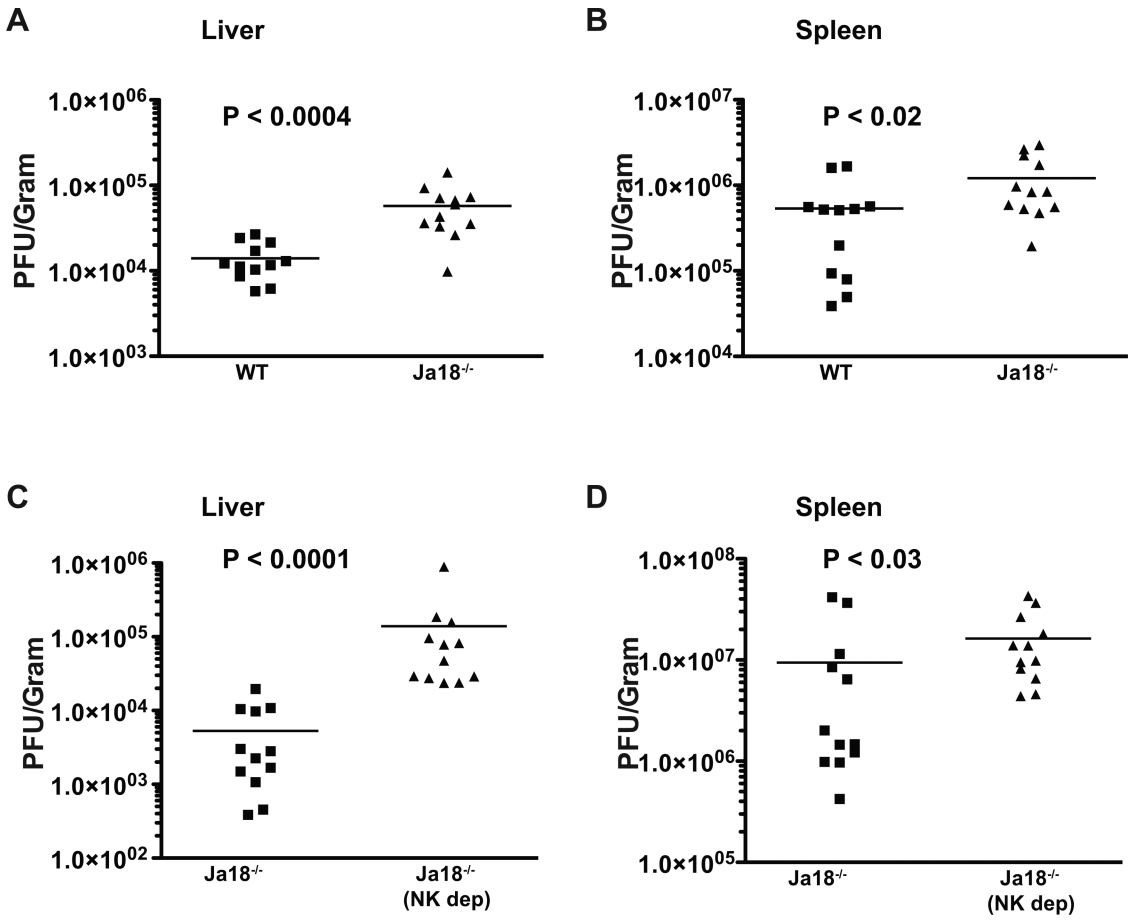

Figure 7.

*i*NKT and NK cells mediate non-redundant, innate defense against MCMV. Groups of BALB/c Jα18$^{+/+}$ *i*NKT cell-sufficient (WT) or BALB/c Jα18$^{-/-}$ *i*NKT cell-deficient littermate control mice were infected with MCMV, and viral pfu levels were determined in the liver *(A)*, and spleen *(B)* 4 days later. *(C-D)*, BALB/c Jα18$^{-/-}$ *i*NKT cell-deficient mice were depleted of NK cells (NKdep), or not, and subsequently infected with MCMV. MCMV replication levels in the liver *(C)*, and spleen *(D)* were determined 4 days later. For *(A-D)*, results are representative of 12 mice per group from a minimum of 2 independent experiments. Error bars represent SEM (n = 12 for each group). Differences between groups were evaluated for statistical significance by the two-tailed unpaired Student *t* test. Note that the data shown in *(C)* and *(D)* are from experiments carried out at separate times from those in *(A)* and *(B)*, and therefore the absolute values of pfu cannot be directly compared.