Coordinate Expression of Cytokines and Chemokines by NK Cells during Murine Cytomegalovirus Infection


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Coordinate Expression of Cytokines and Chemokines by NK Cells during Murine Cytomegalovirus Infection


Cytokines and chemokines activate and direct effector cells during infection. We previously identified a functional group of five cytokines and chemokines, namely, IFN-γ, activation-induced T cell-derived and chemokine-related cytokine/lymphotoxin, macrophage-inflammatory protein 1α, macrophage-inflammatory protein 1β, and RANTES, coexpressed in individual activated NK cells, CD8+ T cells, and CD4+ Th1 cells in vitro and during in vivo infections. However, the stimuli during infection were not known. In murine CMV (MCMV) infection, the DAP12/KARAP-associated Ly49H NK cell activation receptor is crucial for resistance through recognition of MCMV-encoded m157 but NK cells also undergo in vivo nonspecific responses to uncharacterized stimuli. In this study, we show that Ly49H ligation by m157 resulted in a coordinated release of all five cytokines/chemokines from Ly49H+ NK cells. Whereas other cytokines also triggered the release of these cytokines/chemokines, stimulation was not confined to the Ly49H+ population. At the single-cell level, the production of the five mediators showed strong positive correlation with each other. Interestingly, NK cells were a major source of these five cytokines/chemokines in vitro and in vivo, whereas infected macrophages produced only limited amounts of macrophage-inflammatory protein 1α, macrophage-inflammatory protein1β, and RANTES. These findings suggest that both virus-specific and nonspecific NK cells play crucial roles in activating and directing other inflammatory cells during MCMV infection.


O
riginally, NK cells were known for their capacity to kill tumor cells without prior sensitization of the host (1). However, increasing evidence now shows that NK cells are important during the innate early phase of infections, especially certain viral infections (2–4). NK cell-deficient humans suffer from recurrent infections with herpesviruses, including CMV, varicella, and HSV (5). Similarly in mice, depletion of NK cells results in increased lethality to murine CMV (MCMV)4 and higher viral replication in the spleen and liver (6–8). Certain mouse strains are resistant to MCMV (e.g., C57BL/6), whereas other strains (e.g., BALB/c) are susceptible to the infection. Work by a number of laboratories has elucidated the mechanism that controls resistance in C57BL/6 mice. Genetic mapping data indicated that Cmv1r, the resistant allele for a genetic locus localized in the NK gene complex in C57BL/6 but not BALB/c mice controls resistance to MCMV. Subsequent analysis identified the NK activation receptor Ly49H to be encoded by Cmv1r (9–11). Ly49H, a lectin-like type II membrane protein, is expressed on ~50% of NK cells. Genetic absence of Ly49H (as in BALB/c mice) or injection of anti-Ly49H mAb in otherwise resistant C57BL/6 mice results in susceptibility to MCMV infection (9–11). These studies indicated that Ly49H is critically involved in the host response to MCMV. The studies culminated in the finding by our group and others that Ly49H recognizes the product of a MCMV-encoded open reading frame, m157, which is expressed on the surface of virus-infected cells (12, 13). Interestingly, m157 has a predicted class I-like structure. Therefore, similar to inhibitory NK receptors, activation receptors like Ly49H recognize MHC class I-like ligands to mediate viral resistance (12).

Triggering of Ly49H in vitro by m157 transfecants or MCMV-infected cells leads to prompt (4–8 h) stimulation of target cell killing and NK cell production of IFN-γ (12). However, our work has shown that early during MCMV infection in vivo there is a “nonspecific” activation of C57BL/6 NK cells around days 1–2 postinfection (p.i.) characterized by IFN-γ production and proliferation of NK cells, with no preference for the Ly49H+ subset. Later during infection (days 4–6), we observed specific proliferation of Ly49H+ NK cells that can be blocked by anti-Ly49H mAb (14). We postulated that the nonspecific early NK cell responses may be due to proinflammatory cytokines, whereas the later specific responses were due to direct triggering of Ly49H.

The NK cell production of IFN-γ plays a key role in the initial antiviral response because IFN-γ induces a number of functional effects on macrophages, namely, induction of MHC class I expression, increased Ag presentation, production of antimicrobial oxygen and nitrogen intermediates, and release of IL-12 (15). Via

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IL-12 the NK-cell produced IFN-γ contributes to the differentiation of T cells into Th1/Tc1 cells (16).

During an immune response, NK cells also produce chemokines. The chemokine family comprises four subgroups according to the arrangement of conserved cysteine residues: C, CC, CXC, and CX3C families. Inflammatory chemokines like macrophage-inflammatory protein 1α (MIP-1α, CCL3), MIP-1β (CCL4), and RANTES (CCL5), belonging to the CC subgroup, are typically induced upon infection and recruit effector cells to the site of infection (17). All three chemokines are produced by a variety of cells, among them macrophages, activated NK cells, and T cells (18) and bind to CCR5 (all three molecules) and CCR1 (MIP-1α and RANTES) (19). CCR5 is expressed on macrophages, NK cells, dendritic cells (DC), and activated Th1 cells. Through this receptor, the three CC chemokines potently attract monocytes/macrophages, NK cells, and distinct subpopulations of T cells (19–21). Activation-induced, T cell-derived and chemokine-related cytokine (ATAC, XCL1), the only member of the C family of chemokines, was cloned by us (22) and independently as lymphotactin (23) and SCM-1 (24) by others. In contrast to most classical CC and CXC chemokines, ATAC has a rather restricted expression pattern, being mainly secreted by activated CD8⁺ T cells (22) and NK cells (Ref. 25 and our unpublished results).

Previous studies have indicated that IFN-γ and MIP-1α control MCMV infection in vivo. First, control of MCMV replication in the liver, but not in the spleen is predominantly dependent on IFN-γ. Conversely, perforin is more important for controlling MCMV replication in the spleen compared with liver (26). Second, IL-12 and STAT4 were critical for NK cell IFN-γ production (27). Third, MIP-1α is induced in the liver through type 1 IFNs and promotes NK cell inflammation (28). This is underlined by the observation that mice deficient in MIP-1α have a decreased resistance to MCMV and a dramatically reduced NK cell accumulation as well as decreased IFN-γ production in the liver (29). These data raise the hypothesis that NK cell production of IFN-γ and chemokines may be coordinated to control MCMV and that NK cells may be triggered through different mechanisms during their responses to infection. However, the different mechanisms that lead to cytotoxic chemokine release by NK cells have not been systematically analyzed during MCMV infection. Also, the contribution of NK cell-produced cytokines/chemokines in the context of other immune cells has not been evaluated.

During Listeria infection, we recently demonstrated that IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES are coexpressed at the single-cell level in individual activated NK cells, CD8⁺ T cells, and CD4⁺ Th1 cells in vitro and in vivo. Moreover, since the five mediators were also functionally synergistic with respect to stimulating cytokine release and effector functions, we therefore termed the group “type 1 cytokines” (30). However, in the bacterial model, the specific effector cell stimuli had not been identified. Furthermore, it was not clear whether the single-cell coexpression of the five cytokines was an isolated phenomenon or common to various infectious responses. In the current work, we therefore analyzed this group of cytokines/chemokines during MCMV infection in vitro and in vivo and examined the induction pathway(s) as well as the cellular source of the five mediators. Our results show that the five mediators are indeed coordinately produced by NK cells during MCMV infection either after specific triggering of Ly49H or via innate cytokines. Surprisingly, NK cells seemed to be a major source of the five cytokines/chemokines whereas infected macrophages and other cells contributed only marginally to the release of MIP-1α, MIP-1β, and RANTES. These findings suggest that both virus-specific and virus-nonspecific NK cells play essential roles in recruiting inflammatory effector cells during MCMV infection and have the capacity to organize and shape the ongoing adaptive immune response.

Materials and Methods

Mice

C57BL/6 mice and C57BL/6-SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME), maintained under specific pathogen-free conditions, and used at 8–12 wk of age. Animal studies were approved by the Institutional Review Board at Washington University (St. Louis, MO).

Virus stocks

Smith strain MCMV was gratefully obtained from S. Virgin (Washington University) and green fluorescent protein (GFP)-MCMV was generously provided by J. D. Hamilton (Duke University, Durham, NC; Ref. 31). For in vitro experiments, MCMV was propagated on NIH 3T12 cells and culture supernatants were harvested when essentially all of the cells displayed a rounded, enlarged shape consistent with infection. MCMV was isolated from the culture supernatants using standard methods (9). Viral suspensions were frozen in aliquots at 70°C and the titer was determined by standard plaque assay (9). For in vivo experiments, a salivary gland stock of MCMV was prepared from BALB/c mice that had been infected with 10⁶ PFU MCMV and the titer was determined by standard plaque assay (32).

MCMV infection

For infection of bone marrow macrophages (BMM) in vitro, the cells were infected with multiplicities of infection (moi) ranging from 0.004 to 1 (moi = 0.004: 0.5% of cells infected; moi = 0.0156: 1% infected; moi = 0.0625: 9% infected; moi = 0.25: 33% infected, moi = 1: 70–85% infected according to number of GFP⁺ cells) as indicated in the text. Cells were infected by overlaying with virus in a minimal volume of RPMI 1640 medium containing 10% FCS medium to cover the culture surface and rocked every 10 min for 60 min. Twenty-four hours later, the BMM were washed, collected, with versene-EDTA (Invitrogen, Carlsbad, CA), and used for coculture experiments. For in vivo experiments, C57BL/6 mice were infected with 5 × 10⁶ PFU salivary gland MCMV i.p. At different time points after infection, mice were sacrificed and spleenocytes and liver leukocytes were prepared using standard procedures (14). In experiments where the cellular source of cytokines was analyzed ex vivo (Fig. 7), it was essential to minimize loss of adherent cells (macrophages, DCs). In these experiments, the tissues were homogenized using a collagenase digest before erythrocyte lysis as described previously (33). Briefly, the tissues were torn into small pieces and the fragmented tissues were incubated with 1 mg/ml collagenase P and 10 µg/ml DNase I (both from Roche Diagnostics, Basel, Switzerland) in HBSS containing 2% FCS for 30 min at 37°C with gentle shaking. For the last 5 min, 10 mM EDTA was added and the tissue fragments were minced through a 70-µm cell strainer (BD Biosciences, San Jose, CA). Erythrocytes in the single-cell suspension of the spleen were then lysed with ammonium chloride. The liver cell suspension was first purified over a 35% Ficoll gradient followed by erythrocyte lysis as described elsewhere (34). Throughout the whole preparation period, 5 µg/ml brefeldin A (BFA: Sigma-Aldrich, St. Louis, MO) were present. Additionally, the single-cell suspensions of spleen and liver were incubated for 3 h with 5 µg/ml BFA, thoroughly collected with versene-EDTA, and processed for intracellular flow cytometry.

NK/macrophage coculture

BMM were prepared from the femora of C57BL/6 mice as previously described (12). BMM were either used uninfected or infected for 24 h with GFP-MCMV at the moi indicated in the figure legends. Freshly isolated splenocytes from C57BL/6-SCID mice were cocultured with either uninfected or GFP-MCMV-infected BMM for the indicated time points (5–8 h) at an E:T ratio of 2:1 (300,000 splenocytes and 150,000 BMM) in 96-well plates with BFA added 1 h after beginning the coculture. In a different experiment, splenocytes from C57BL/6-SCID mice were cocultured with BAF3 cells (11) either transfected with mIL7 or mock transfected (12) and BFA was added 1 h after beginning the coculture. Where indicated, the cocultures were performed in the presence of F(ab)², directed either against Ly49H or Ly49D in the final concentration of 50 µg/ml. After the indicated time points, cells were harvested with versene-EDTA and processed for intracellular flow cytometry. In some experiments, magnetically purified NK cells (85% DX5⁻) from C57BL/6 spleens were used instead of crude SCID splenocytes for setting up the cocultures. The results obtained with
Then the supernatants were collected and assayed for MIP-1α or with either noninfected or GFP-MCMV-infected BMM as indicated. NK cells and cultured in 96-well round-bottom plates for 8 h, either alone or with either noninfected or GFP-MCMV-infected BMM as indicated. Then the supernatants were collected and assayed for MIP-1α by ELISA. One representative experiment of two is shown.

these purified NK cells were essentially identical to those obtained with unfractionated SCID splenocytes (our unpublished data).

Cytokine stimulation of sorted NK cells
Splenocytes from C57BL/6 mice were magnetically sorted using anti-NK cell (DX5) microbeads according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany), resulting in a population that was usually 85% DX5+. The cells were stimulated in RPMI 1640/10% FCS medium with different recombinant cytokines (all from R&D Systems, Minneapolis, MN or PreproTech, London, U.K.) for 7 h with BFA present for the last 6 h: IL-2 (800 U/ml), IL-12 (40 ng/ml), IL-15 (80 ng/ml), IL-18 (80 ng/ml), or IFN-α (1000 U/ml). The cells were stained for NK1.1 and CD3, fixed, and counterstained for cytokines and chemokines.

Abs and flow cytometry
The following mAbs directed to cell surface Ags coupled to FITC, PE, allophycocyanin, or PerCP-Cy5.5 were used: PK136 (NK1.1), M1/70 (Mac1/CD11b), 145-2C11 (CD3; all from PharMingen, San Diego, CA) and 3D10 (Ly49H) (35). Intracellular staining of cytokines was performed with MTAC-2 (ATAC) (36), XMG1.2 (IFN-γ, BD PharMingen), and polyclonal affinity-purified antisera against MIP-1α, MIP-1β, and RANTES as described previously (36). To block unspecific binding of Abs to Fc receptors, all Abs directed to surface Ags were diluted in the presence of mAb 2.4G2 (Fcγ II/IIIIR; American Type Culture Collection, Manassas, VA). For intracellular staining of cytokines, the cells were stained for the indicated surface markers, fixed with 2% formaldehyde, and counterstained with DIGoxigenin-labeled mAbs directed against ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES followed by anti-DIGoxigenin-Cy5 or -FITC as described elsewhere (36).

Statistical analysis of intracellular cytokine coexpression
Correlation of cytokine coexpression was calculated from dot plots using the test for ϕ-correlation coefficients (37) according to the equation: ϕ = (ad - bc)/(a + b)(c + d)(a + c)(b + d)0.5, with a = percentage of cells in lower left, b = percentage of cells in lower right, c = percentage of cells in upper left, and d = percentage of cells in upper right quadrant. ϕ was considered as significant (37).

ELISA
BMM from C57BL/6 mice were either used uninfected or infected for 24 h with GFP-MCMV as indicated in the text. C57BL/6-SCID splenocytes were used as a crude source of NK cells and cocultured in 96-well round-bottom plates for 8 h either alone or with uninfected or infected BMM, respectively. The supernatants were collected and assayed for MIP-1α by a commercially available sandwich ELISA (R&D Systems).

Results
MCMV-infected macrophages stimulate fresh NK cells to produce cytokines and chemokines
To study cytokines produced by NK cells in response to MCMV infection, we incubated fresh C57BL/6 NK cells with syngeneic BMM that were infected with a virus stably expressing GFP (GFP-MCMV) (31). As a crude source of NK cells, we used C57BL/6 SCID splenocytes (deficient in T and B cells). After an 8-h coculture with infected BMM cells, large amounts of MIP-1α were released into the supernatant (Fig. 1). However, the uninfected control coculture and either cell population alone secreted approximately seven times less MIP-1α. Even infected BMM alone did not show MIP-1α secretion above background level after this brief incubation period.

In the ELISA-based experiments the cellular source of MIP-1α is not discernible. We therefore used intracellular staining and flow cytometry to show that NK1.1+CD3− cells in the coculture were a source of MIP-1α and also ATAC, IFN-γ, MIP-1β, and RANTES, the other members of our previously defined type 1 cytokine group in Listeria infection (Fig. 2). After an 8-h coculture, both Ly49H+ and Ly49H− NK1.1+ cells stained positive for these five cytokines and chemokines.

Triggering of NK cells via Ly49H or innate cytokines results in production of cytokines and chemokines
On the basis of this result, we hypothesized that cytokine/chemokine production by NK cells is triggered by ligation of Ly49H with m157 expressed on infected BMM (giving rise to the signal in the Ly49H+ NK1.1+ subset) and/or through nonspecific activation of all NK subsets via innate cytokines (giving rise to the signal in...
FIGURE 3. Triggering of NK cells via Ly49H or soluble mediators results in production of ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES. A, Interaction of Ly49H with m157 induces production of ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES by fresh NK cells. Freshly isolated splenocytes from SCID mice were cocultured with BaF3 cells transfected with either m157 (BaF/m157) or mock vector (BaF/puro control) for 0.5–8 h at an E:T ratio of 2:1 in the presence of BFA. The cells were stained for NK1.1, Ly49H, and CD3, fixed, and counterstained for the indicated cytokines and chemokines. An example for the control coculture (right panels) is the result obtained for MIP-1α. Shown are the results after gating on NK1.1+CD3+ lymphocytes. B, Cytokine release by Ly49H+ NK cells can be blocked by anti-Ly49H mAb. Freshly isolated splenocytes from SCID (Figure legend continues)
both Ly49H− and Ly49H+ NK1.1+ subsets). To test these hypotheses, we triggered NK cells specifically via Ly49H by incubating fresh NK cells with a BaF transfectant expressing m157. Already after 0.5–2 h of coculture, production of ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES could be detected specifically in the Ly49H+ subset of NK cells with only little contribution by Ly49H− NK1.1+ cells (Fig. 3A, left). The peak production of cytokines and chemokines was observed at 4 h of coculture. Comitant with the production of these mediators, we observed a down-regulation of Ly49H that was not detected in the control coculture (Fig. 3A, right), although this down-regulation was variably seen. By contrast, parental BaF3 cells did not stimulate MIP-1α production or Ly49H receptor down-regulation (Fig. 3A, right). Similar data were obtained for ATAC, IFN-γ, MIP-1β, and RANTES with even lower percentages of cytokine production when NK cells were cocultured with BaF3 parental cells (data not shown; see also Fig. 3B).

Importantly, the specific cytokine production triggered in the Ly49H+ subset of NK cells after engagement of Ly49H was blocked by addition of anti-Ly49H mAb, but not by addition of irrelevant anti-Ly49D mAb, as indicated for ATAC production (Fig. 3B). This was true for the coculture of fresh NK cells with either BaF/m157 transfectants or fixed MCMV-infected macrophages. In the blocking experiments with infected macrophages, we used fixed macrophages instead of live macrophages (compare with Fig. 2) to exclude the influence of macrophage-derived cytokines. These results are very similar to those previously published by us for IFN-γ (12). Thus, Ly49H-specific stimulation by m157 on transfectants or on infected macrophages results in expression of ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES.

When we counterstained IFN-γ against ATAC, MIP-1α, MIP-1β, and RANTES at the single-cell level (Fig. 3C), we observed that virtually all IFN-γ-producing NK1.1+ cells simultaneously produced the other molecules, giving rise to a high correlation coefficient $\phi$ ranging between 0.24 and 0.39 ($\phi = 1$ represents a 100% coincidence, $\phi = 0$ is equivalent to a nonstatistical coexpression). Thus, Ly49H engagement triggers coordinate production of these five cytokines/chemokines.

Stimulation of magnetically purified NK cells with various cytokines also induced production of IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES to a variable degree (Fig. 3D). However, in contrast to m157-induced production (Fig. 3, A and B), there was no pronounced predominance for either the Ly49H+ or Ly49H− subset (data not shown). With respect to the absolute percentage of ATAC+ or IFN-γ+ NK1.1+ cells, the cytokines IL-2, IL-12, and IL-15 were about equally effective, whereas for MIP-1α and MIP-1β+ NK1.1+ cells, both IL-2 and IL-15 showed the strongest induction. RANTES production by NK cells is a special case, since RANTES is produced constitutively, as also shown previously by other groups (38, 39). These data indicate that cytokines trigger nonspecific responses from NK cells in that they do not involve direct recognition of pathogens. Taken together, these studies demonstrate two modes of stimulating cytokine and chemokine release from NK cells.

### Cellular source of cytokines and chemokines during MCMV infection in vitro

Our results so far show NK cell production of IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES as a result of the specific triggering via Ly49H or via soluble mediators, but whether macrophages could also produce these cytokines was not apparent. To address this question, we used the same coculture system and gated either on the NK1.1+ lymphocytes or on the larger, more granulated BMM identified as distinct population in the forward scatter/side scatter plot. In addition, we analyzed the cells with respect to expression of Mac1 (CD11b/CD18) often thought to be a specific macrophage marker. However, recent work in our laboratory has shown that Mac1 is also a maturation marker on NK1.1+ cells in vivo. Virtually all mature splenic NK cells express Mac1 (40), although it is expressed at a lower level than on bone fide macrophages (Fig. 4). At a moi = 0.25, ~50% of all mature NK1.1+ Mac1+ cells in the coculture produced MIP-1α, MIP-1β, and RANTES (Fig. 4A, left) and also ATAC and IFN-γ (data not shown). Surprisingly, staining of MIP-1α and MIP-1β in BMM gave only a very marginal signal (6–8% positive cells), suggesting that the BMM in the coculture produce only limited amounts of these chemokines. Yet there was clear production of RANTES by the BMM population (49% positive cells, Fig. 4A, left), indicating the functional viability of these cells. No production of endogenous IFN-γ and ATAC was detectable in the BMM population (data not shown).

Since the sparse production of MIP-1α and MIP-1β by the BMM was unexpected, we further tested the viability and functionality of our BMM preparations to exclude a technical problem. In a coculture of SCID splenocytes with BMM that had been preactivated with IFN-γ plus LPS, there was dramatic production of MIP-1α, MIP-1β, and also RANTES in the BMM (Fig. 4A, middle), whereas ATAC and IFN-γ were not detectable (data not shown). The signals were specific for the indicated chemokines, since they could be abrogated by preincubation of the staining reagents with the respective recombinant chemokines (cold blocking control, Fig. 4A, right).

Since it is possible that there could be a dose-response difference between the NK cell and BMM populations, we analyzed their responses over a range of viral inocula. In response to increasing amounts of virus, MIP-1α and MIP-1β were produced by NK cells, but only marginally by BMM in vitro. At a moi as low as 0.0156, NK cell cytokine production was readily detectable above background with minimal BMM stimulation even at a 64-fold higher viral inoculum (moi = 1.0). However, RANTES, which is constitutively expressed by NK cells, is not produced by BMM in uninfected cells or at a low moi, respectively, but is dramatically up-regulated in BMM at higher virus titers (Fig. 4B). Interestingly,
when we analyzed the expression of RANTES in infected vs uninfected macrophages (moi = 0.25), we detected RANTES almost exclusively produced by uninfected, GFP/macrophages, with only little contribution from infected GFP/macrophages (Fig. 4C). Virtually the same picture was obtained in the absence (Fig. 4C) or presence of NK cells in the culture (data not shown) and were corroborated by ELISA-based experiments for MIP-1α (Fig. 1), indicating that infected macrophages are poor producers of certain chemokines.

**FIGURE 4.** NK cells are a major source of MIP-1α, MIP-1β, and RANTES during MCMV infection in vitro. A. Predominant production of chemokines by NK cells but not macrophages during MCMV infection in vitro. BMM from C57BL/6 mice were either infected with MCMV-GFP for 24 h (moi = 0.25) or stimulated with IFN-γ plus LPS. The BMM were collected and cocultured with freshly isolated SCID splenocytes for 5 h with BFA present for the last 4 h. The cells were stained for NK1.1 and Mac1, fixed, and counterstained for the indicated chemokines. Shown are the results after differential gating on NK1.1 lymphocytes or on BMM (identified as large cells in forward scatter/side scatter diagram), respectively. For specificity control, the chemokine-Digoxigenin Abs were preincubated with the respective recombinant ligands before being adding to the cells (blocking control). The specificity control shown belongs to the IFN-γ/LPS-treated culture; identical results were obtained for the virus-treated culture. The results shown are from one representative of three experiments. B. Dose-response curve. BMM, either infected with varying doses of GFP-MCMV for 24 h (moi = 1 to moi = 0.004) or uninfected (moi = 0) were cocultured with SCID splenocytes for 6 h with BFA present for the last 5 h. The cells were collected and stained for NK1.1 and Mac1, fixed, and counterstained for MIP-1α, MIP-1β, or RANTES. Shown is the percentage of chemokine-positive NK1.1 lymphocytes (■) or Mac1 NK1.1 BMM (□) in the coculture. C, Analysis of chemokine production in uninfected vs infected macrophages. BMM were infected with GFP-MCMV for 24 h (moi = 0.25), stained for Mac1, fixed, and counterstained for chemokines. Depicted is the expression of chemokines in uninfected (GFP−) vs infected (GFP+) BMM. The results shown are from one representative of three experiments.

**NK cell production of cytokines and chemokines during MCMV infection in vivo**

When we analyzed splenocytes from infected mice immediately ex vivo (in the presence of BFA for 3 h), we found that unfractinated NK1.1 CD3− cells produce ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES early during MCMV infection in vivo concomitant with the drop of NK cell numbers around day 2 p.i. (Fig. 5A). The percentages of NK cells in the spleen for the particular experiment.
shown in Figs. 5 and 6 are on days 0 and 1 p.i., 2.2–3.5%; on day 2 p.i., 0.5–0.6%; on day 3 p.i., 0.8%; on days 4–6 p.i., 1.6–2.1%.

Whereas ATAC and IFN-γ are predominantly produced on days 1 and 2 p.i., MIP-1α and MIP-1β show a biphasic expression pattern with maximal production around day 1/day 2 p.i. and day 4 p.i. (Fig. 5B). The second phase may reflect the selective expansion of Ly49H+ NK cells due to specific proliferation (14) although there may be other mechanisms to account for a second peak in chemokine production. Interestingly, the production of ATAC, MIP-1α, and MIP-1β seemed to precede the production of IFN-γ in spleen.

**FIGURE 5.** Production of ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES by NK cells during MCMV infection in vivo. C57BL/6 mice were infected with a sublethal dose of MCMV (5 × 10^4 PFU i.p., three to four mice per time point) and sacrificed on days 1–6 p.i. As a control, uninfected mice (naive, n.i.) were analyzed in parallel. Splenocytes and liver lymphocytes were prepared, cultured for 3 h with BFA (without additional restimulation), stained for NK1.1, Ly49H, and CD3, fixed, and counterstained for the respective cytokines and chemokines. The results shown are from one representative of three experiments. A, Examples for the early time points. The FACS plots and percentages of cytokine/NK1.1/CD3 splenocytes are shown directly ex vivo for one representative animal of three to four per group. Numbers represent the percentage of total splenocytes that are NK1.1 (or NK1.1, respectively) and produce the indicated cytokines. B, Overview for the entire time course showing kinetics for spleen and liver lymphocytes. C, Positive correlation of IFN-γ with ATAC, MIP-1α, MIP-1β, and RANTES following in vivo stimulation. On day 2 p.i., splenocytes from MCMV-infected mice or uninfected mice were stained as described above; the intracellular staining was performed with IFN-γ-specific mAbs in combination with mAbs against ATAC, MIP-1α, MIP-1β, or RANTES, respectively. The correlation coefficient $\phi$ is indicated in the upper right quadrant.
As we already showed in vitro, RANTES was produced constitutively with a slight increase over the observation period (Fig. 5B).

The overall picture of cytokine production by NK1.1⁺/CD3⁻/CD11002 cells appeared similar for spleen and liver lymphocytes. Notably, the production of MIP-1α and MIP-1β, especially on day 2 p.i., was increased in liver compared with spleen (Fig. 5B). In the experiment shown in Fig. 5, the production of ATAC and IFN-γ was reduced in liver compared with spleen, but in other experiments there was no gross difference between organs.

When the production of IFN-γ was correlated against ATAC, MIP-1α, MIP-1β, and RANTES on day 2 p.i. in spleen ex vivo, we again detected a strong positive correlation of the five cytokines/chemokines with each other, giving rise to correlation coefficients ϕ between 0.16 and 0.46 (Fig. 5C). This positive correlation indicates that there is a high probability that a single activated NK cell produces all five mediators simultaneously.

We also examined the contribution of the Ly49H⁺ NK cells to the overall production of cytokines and chemokines during the course of infection (Fig. 6 and Table I). It has been shown by us earlier (14) and is also evident here (Fig. 6) that the Ly49H⁻ subset increased from around 50% on day 1 p.i. to 65–80% on days 4–6 p.i. During the course of infection, the production of IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES in the Ly49H⁻ and the Ly49H⁺ subset of NK cells was maximal on day 2 p.i. At

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**Table I. Contribution of Ly49H⁺ cells to the production of cytokines and chemokines on days 1–6 p.i. after MCMV infection**

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<tr>
<td>ATAC</td>
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<td>67.1</td>
<td>12.6</td>
<td>27.8</td>
<td>6.3</td>
<td>53.9</td>
</tr>
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<td>MIP-1α</td>
<td>2.5</td>
<td>56.0</td>
<td>19.0</td>
<td>49.5</td>
<td>4.9</td>
<td>46.9</td>
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<tr>
<td>MIP-1β</td>
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<td>64.9</td>
<td>15.4</td>
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<tr>
<td>MIP-1β</td>
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<td>17.9</td>
<td>60.3</td>
<td>11.9</td>
<td>76.5</td>
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<td>38.6</td>
<td>53.4</td>
<td>48.0</td>
<td>65.6</td>
</tr>
</tbody>
</table>

*For the particular experiment shown in Fig. 6, this table shows the percentage of cytokine-producing NK cells among the total NK1.1⁺/CD3⁻ splenocyte pool (sum of upper and lower right quadrants, since only NK1.1⁺/CD3⁻ cells are shown) at different time points after infection ("total"). To obtain the contribution of the Ly49H⁺ NK cells to the production of cytokines, we used the quadrant statistics given in Fig. 6 (H⁺, percentage of cells in the upper right quadrant divided by the sum of cells in the upper right and lower right quadrants). For clarity, only one representative animal of three for each time point is shown. As can be seen by summing the upper left and right quadrants, the Ly49H⁺ subset increased from around 50% on day 1 p.i. to 65–80% on days 4–6 p.i.*
later time points, the secretion of ATAC and IFN-γ declined almost to background levels, whereas MIP-1α and MIP-1β (and also the constitutive RANTES) were produced in significant amounts. At early time points (day 1/day 2 p.i.), the contribution of Ly49H+ NK cells to the overall cytokine production ranged from ~50–70% (Table I). At later time points (days 4–6 p.i.), the contribution of the Ly49H+ NK cells to the overall cytokine production increased to up to 80–90% (especially for MIP-1α, MIP-1β, and RANTES which are significantly produced on days 4–6 p.i.). This latter finding might reflect the concomitant expansion of the Ly49H+ compartment although other mechanisms remain possible.

**Cellular source of cytokines and chemokines during MCMV infection in vivo**

Based on our in vitro results we hypothesized that NK cells, relative to other immune cells, are a major source of IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES during the early phase of a MCMV infection in vivo. To analyze this question, we infected C57BL/6 mice with a sublethal dose of MCMV i.p. and analyzed splenocytes and liver leukocytes on days 1 and 2 p.i., the early maximum of cytokine production (Fig. 7 and Table II). Electronic gating on the NK1.1+CD3− population (gate R1) showed that a significant proportion of the mature Mac1+ NK1.1+ cells in the spleen produced ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES on both days 1 and 2 (Fig. 7, upper panel). However, gating on all other cells (gate R2), excluding NK1.1+CD3− and NK1.1+CD3+, showed that their contribution to the production of our five cytokines/chemokines was surprisingly modest. There was baseline production of MIP-1α, MIP-1β, and RANTES by Mac1+ cells in gate R2 in uninfected and day 1-infected mice and this appeared to wane by day 2 (Fig. 7, upper panel) but their overall contribution was comparable to or less than the NK cell population (R1). With respect to the cellular source of cytokines and chemokines in the liver, similar results were obtained during the early phase of MCMV infection (Fig. 7, lower panel). Mac1+ NK1.1+ cells in the liver showed a more pronounced increase (as compared with spleen) in MIP-1α and MIP-1β production on day 1 p.i. that dropped to almost basal level by day 2. At the same time, the cytokine production by NK cells continued to rise and became the dominant population producing these cytokine/chemokines. Therefore, our data indicate that NK cells are a major source of IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES during early MCMV infection in vivo; this was most evident on day 2 p.i. in the liver, where the percentage of cytokine-producing NK cells was 3–12 times higher than the percentage of cytokine-producing non-NK cells (Table II and Fig. 7).

**Discussion**

In this study, we demonstrated that specific triggering of the activation receptor Ly49H on naive, primary NK cells initiates production of a group of cytokines and chemokines, namely, IFN-γ, ATAC/lymphotactin, MIP-1α, MIP-1β, and RANTES from the Ly49H+ subset of NK cells. Whereas RANTES production by NK cells is different from the other four members of the group (it is produced constitutively), we have defined a new induction pathway for IFN-γ and MIP-1α, the two most prominent members of this group of cytokines and chemokines in the context of NK cell-mediated resistance to viral infection. These data concur with those of Ortaldo et al. (38) who found that Ab cross-linking of the DAP12-coupled activation receptor Ly49D resulted in 10- to 20-fold up-regulation of ATAC, MIP-1α, and MIP-1β in DNA microarray, RT-PCR, and RNase protection studies of stimulated IL-2-activated NK cells. Indeed, our own studies of Ly49D activation via recognition of Chinese hamster ovary cells also induces specific NK cell responses resembling those of specific Ly49H activation (data not shown). Thus, stimulation of primary NK cells through their activation receptors not only leads to cytotoxicity (12) but also production of a panel of proinflammatory cytokines and chemokines that can modulate subsequent host immune responses.

During the course of MCMV infection, other cytokines are also produced that stimulate NK cell activities. IFN-γ is induced via IL-12 and MIP-1α is produced in an IFN-αβ-dependent way, respectively (27, 28). Our results indicate that other cytokines like IL-2, IL-15, and IL-18 also induced IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES from NK cells to a variable degree. However, with such stimuli there was no preference for the Ly49H+ or Ly49H− subset of NK cells. From these in vitro data, it can be anticipated that in vivo infection would lead to cytokines induced by both the activation receptor-mediated pathway and the cytokine-mediated pathway. Indeed, MCMV infection in vitro and early in vivo in results in release of all five cytokines/chemokines from both Ly49H+ and Ly49H− subsets of NK cells with no predominance for one or the other subset. Both in vitro and early in vivo (day 2 p.i.) production of our group of cytokines by NK cells is positively correlated with each other, meaning that a single activated NK cell has a high probability to coproduce all five molecules together. Later during infection in vivo (day 4 p.i.), the production of ATAC and IFN-γ ceases whereas MIP-1α and MIP-1β are produced in a second wave concomitant with the adaptive-like proliferation of Ly49H+ NK cells. During this second wave, we observed that the contribution of the Ly49H+ subset of NK cells to the overall cytokine production rises to 80–90% which might reflect the increase in the relative number of Ly49H+ cells, although other mechanisms may be operating, such as the response of Ly49H+ NK cells to other series of cytokines. Nevertheless, NK cell production of IFN-γ, ATAC, MIP-1α, MIP-1β, and RANTES is induced by nonspecific stimuli, such as IL-2, IL-12, IL-15, IL-18, and type I IFNs, as well as by specific NK cell activation through Ly49H, and both mechanisms contribute to in vivo responses of NK cells.

In our previous work, we have shown that ATAC, MIP-1α, MIP-1β, and RANTES are expressed together with IFN-γ in activated Th1 (but not Th2) cells, CD8+, and NK cells (30). Because the cosecretion of the five molecules at the single-cell level is not restricted to Th cells but is similarly relevant for NK cells during bacterial and, as we have shown here, during viral infection, we consider ATAC, MIP-1α, MIP-1β, RANTES, and IFN-γ as a tight group of type 1 cytokines (rather than Th1 cytokines), especially on the basis of their functional cooperation (30). Other cytokines like TNF-α and GM-CSF are also produced by activated NK cells, but they are not correlated with ATAC, MIP-1α, MIP-1β, RANTES, and IFN-γ at the single-cell level, at least not in Listeria infections (30). For MCMV infections, we cannot exclude the possibility that other cytokines or chemokines, either known or yet to be identified soluble mediators, are produced by NK cells and this point certainly deserves further investigation. In humans, it was previously shown that NK cells can be distinguished into distinct subsets according to their cytokine profile in vitro and in vivo (41–43). Similar to Th subsets, NK1 cells predominantly produce IFN-γ whereas NK2 cells release IL-5 and IL-13 upon stimulation. In those studies, differential expression of chemokines was not analyzed. Until now, there is no hint that NK1 and NK2 subsets also exist in mice, especially during infection. If they do exist, we would expect ATAC, MIP-1α, MIP-1β, and RANTES in the NK1 subset on the basis of the cosecretion with IFN-γ. Further work has to be done to clarify this point.
FIGURE 7. NK cells are a major source of ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES early during MCMV infection in vivo. C57BL/6 mice were used uninfected as control or infected with a sublethal dose of MCMV (5 × 10^4 PFU i.p.) and sacrificed on day 1 or 2 p.i. Spleens and livers were homogenized using a collagenase digest (see Materials and Methods), the single-cell suspensions were cultured for (Figure legend continues)
synergistically on DC with respect to IL-12 release in viral infections, this mechanism might provide a link to the specific proliferation of Ly49H+ NK cells. Very recently, Andrews et al. (50) showed that CD8α+ DC are essential for the expansion of Ly49H+ NK cells by a mechanism involving IL-12 and IL-18. In this scenario, infected DC expressing m157 could stimulate NK cell-type 1 cytokine release and this group of cytokines would then act reversibly back on DC to induce IL-12. Thus, the production of our five cytokines/chemokines might be involved in NK cell cross-talk with other cellular components of innate immunity.

A surprising result of our studies at the single-cell level was the finding that NK cells seemed to be a major source of the five examined cytokines/chemokines in vitro in a coculture system and also in vivo in the early phase of infection. Infected macrophages and other cells contributed only marginally to the release of MIP-1α, MIP-1β, and RANTES. With regard to MIP-1α, this observation is somewhat discrepant from previous studies in which macrophages were identified as a putative source of MIP-1α in the liver (28). However, the results can be reconciled if the temporal production of MIP-1α and the expression of Mac1 on NK cells are considered. In this study, we demonstrated that MIP-1α is produced by uninfected and day 1 Mac1+ “NK1.1” cells (probably macrophages and/or DC) in liver and spleen but thereafter their MIP-1α production declined. In addition, we demonstrated that MIP-1α is produced by NK cells that express Mac1, a marker of functionally mature NK cells (40) that preferentially produce cytokines. In other words, although macrophages (or APCs in general) are able to produce MIP-1α as key chemokine in a number of settings, they do not produce much MIP-1α during MCMV infection.

Our in vitro coculture experiments led to another interesting finding, the pattern of RANTES (and MIP-1α) production by macrophages vs NK cells. In response to MCMV infection, RANTES production by NK cells did not change dramatically. However, with increasing virus titer in the culture the macrophages started to produce large amounts of RANTES, a fact that we also corroborated at the mRNA level via RNase protection assay (B. G. Dorner, L. Yang, and W. M. Yokoyama, unpublished data). Importantly, the infected macrophages contributed only very marginally to the RANTES production, the major portion was produced by the uninfected macrophages in the same culture. The result hints at an active mechanism of chemokine down-regulation in infected macrophages. Three different mechanisms might account for this effect. An MCMV-encoded open reading frame could directly down-regulate chemokine production in infected cells (similar to the effect that m152 has on MHC class I (51, 52)). Alternatively, a MCMV-encoded open reading frame could encode a chemokine-binding protein, similar to chemokine-binding proteins identified in gamma-herpesvirus 68 and vaccinia virus (53–56) that can bind and neutralize the intracellularly generated chemokines. The third possibility would be a host-induced cytokine that represses chemokine production in infected cells. It has been shown for example that MCMV infection down-regulates MHC class II expression on macrophages by induction of host IL-10 in infected cells (57). Experiments in our laboratory are under way to clarify which mechanism accounts for the chemokine-down-regulation in macrophages.

Table II. Frequency of cytokine-producing NK cells and non-NK cells in spleen and liver early after MCMV infection

<table>
<thead>
<tr>
<th>% in total spleen</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
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<tbody>
<tr>
<td>NK1.1</td>
<td>3.6</td>
<td>3.1</td>
<td>1.5</td>
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<tr>
<td>NK1.1*</td>
<td>96.4</td>
<td>96.9</td>
<td>98.5</td>
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<tr>
<td>ATAC<em>NK1.1</em></td>
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<td>0.34</td>
<td>0.27</td>
</tr>
<tr>
<td>ATAC<em>NK1.1</em></td>
<td>0.05</td>
<td>0.11</td>
<td>0.15</td>
</tr>
<tr>
<td>IFN-γ’NK1.1*</td>
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<td>0.09</td>
<td>0.73</td>
</tr>
<tr>
<td>IFN-γ’NK1.1+</td>
<td>0.03</td>
<td>0.05</td>
<td>0.42</td>
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<tr>
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<td>0.80</td>
<td>0.58</td>
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<td>RANTES’NK1.1+</td>
<td>1.01</td>
<td>0.42</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table II. Frequency of cytokine-producing NK cells and non-NK cells in spleen and liver early after MCMV infection

3 h with BFA (without additional restimulation), thoroughly removed from the culture plate with 1 mM EDTA, and stained for NK1.1, Mac1, and CD3. After fixation, the cells were counterstained for the respective cytokines and chemokines. The results are depicted as dot plots after electronic gating on NK1.1 CD3+ cells (gate R1) or on all cells (gate R2) excluding NK1.1+ CD3+ NK T cells. In terms of numbers, ~10 times more cells are shown in R2 than in R1 for the spleen and ~2.5 times more cells are shown in R2 than in R1 for the liver. The result is representative for one of three experiments with three mice per group each time; one representative animal is shown.
To sum up our observations in MCMV infections, our findings support a model in which NK cells are a major source of the cytokines ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES either after specific triggering of activation receptors or after nonspecific stimulation via soluble cytokines. All other cells including macrophages and DC contribute only marginally to the production of these mediators. Our results strengthen the concept involving a role for NK cells as innate inflammatory cells that have the capacity to attract and activate target cells and shape the ongoing adaptive immune response.

Acknowledgments

Technical assistance by Liping Yang is greatly appreciated.

References

14. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to ATAC, IFN-γ, MIP-1α, MIP-1β, and RANTES after specific triggering of activation receptors or after nonspecific stimulation via soluble cytokines. All other cells including macrophages and DC contribute only marginally to the production of these mediators. Our results strengthen the concept involving a role for NK cells as innate inflammatory cells that have the capacity to attract and activate target cells and shape the ongoing adaptive immune response.

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References


