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Emergence of CD8+ T Cells Expressing NK Cell Receptors in Influenza A Virus-Infected Mice

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Both innate and adaptive immune responses play an important role in the recovery of the host from viral infections. In the present report, a subset of cells coexpressing CD8 and NKR-P1C (NK1.1) was found in the lungs of mice infected with influenza A virus. These cells were detected at low numbers in the lungs of uninfected mice, but represented up to 10% of the total CD8+ T cell population at day 10 postinfection. Almost all of the CD8+^NK1.1+ cells were CD8αβ+CD3+TCRαβ+ and a proportion of these cells also expressed the NK cell-associated Ly49 receptors. Interestingly, up to 30% of these cells were virus-specific T cells as determined by MHC class I tetramer staining and by intracellular staining of IFN-γ after viral peptide stimulation. Moreover, these cells were distinct from conventional NKT cells as they were also found at increased numbers in influenza-infected CD1−/− mice. These results demonstrate that a significant proportion of CD8+ T cells acquire NK1.1 and other NK cell-associated molecules, and suggests that these receptors may possibly regulate CD8+ T cell effector functions during viral infection.

Materials and Methods

Mice

C57BL/6 (B6) mice (8–12 wk old) were obtained from the Microbiology and Tumor Biology Center, Karolinska Institutet (Stockholm, Sweden). CD1−/− mice were generated as described previously (10) and bred in-house at the Karolinska Institutet. Animal care was in accordance with institutional guidelines.

Cells and reagents

Madin Darby canine kidney (MDCK)3 cells (American Type Culture Collection, Manassas, VA) were grown in MEM containing Earle’s salts, 10 mM HEPES buffer, 5% FCS, and antibiotics (Life Technologies, Rockville, MD). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. The influenza virus nucleoprotein-derived peptide (NP87–192), ASNNMDAM, was purchased from Research Genetics (Huntsville, AL). All Abs used were purchased from PharMingen (Stockholm, Sweden). The murine β2-microglobulin sequence encoding amino acids 1–99 cloned into pET8c plasmid (cloned by P. Robinson, Birkbeck College, London, U.K.) was a kind gift from P. J. Travers (Birkbeck College, London, U.K.). The pET3c plasmid construct encoding the extracellular domain of the H-2Dβ heavy chain modified with the substrate sequence for BirA biotinylation was a kind gift from T. N. M. Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

Preparation of cells from lungs of influenza-infected mice

Mice were infected intranasally with influenza virus A/Japan/305/57 in 50 ml of PBS at a dose of 1.5 × 105 MDCK infectious units (~30 PFU), corresponding to 1/10 LD50. At specified days postinfection, both infected and control mice were sacrificed by cervical dislocation and their lungs were removed. The lungs were minced into small pieces and incubated in RPMI 1640 (10% FCS) with collagenase type IV (250 U/ml) for 90 min at 37°C. The erythrocytes were lysed, and single-cell suspensions were prepared from the digested organs. Finally, adherent cells were removed from the cellular suspensions by incubating the cells on tissue culture petri dishes for 90 min at 37°C, and the remaining cells were used for further analysis.

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2 Address correspondence and reprint requests to Dr. Hans-Gustaf Ljunggren, Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, S-171 77, Sweden. E-mail address: hans-gustaf.ljunggren@mtc.ki.se

3 Abbreviations used in this paper: MDCK, Madin Darby canine kidney; NP, nucleoprotein-derived peptide.

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Construction of MHC class I tetramers

H-2D$^b$ heavy chain and murine $\beta_2$-microglobulin protein were produced in large quantities by purifying inclusion bodies from electrocompetent Escherichia coli, BL21(DE3)pLys bacteria transformed with the relevant plasmids as described elsewhere (11, 12). The protocol described by Garboczi et al. (13) was basically followed in refolding and purification of soluble MHC class I molecules. Briefly, 12 mg of H-2D$^b$ heavy chain, 10 mg of murine $\beta_2$-microglobulin, and 4 mg of NP$\text{366-374}$ peptide were added to a 400-ml refolding solution. The solution was stirred slowly at 4°C for 72 h and concentrated to a volume of $\sim2$ ml by centrifugation filters with a cutoff of 10 kDa (Pall Gellman Laboratory, Lund, Sweden). The concentrate was placed on a fast protein liquid chromatography column (Pharmingen), and the fraction peak corresponding to the refolded MHC class I molecule was collected and biotinylated using a commercial BirA biotinylation kit (Avidity, Denver, CO). Excess biotin was removed by a size exclusion column (NAP-5) with a 5-kDa cutoff (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and eluted in PBS. The resulting biotinylated MHC monomer solution (Db-NP366) was frozen to $-80^\circ$C until further use. Before use, tetramers were generated by slowly adding streptavidin-PE or streptavidin-RED670 (Dakopatts AB, Sweden) to the MHC monomer solution at a 1:4 molar ratio.

Flow cytometry

Cells were Fc receptor blocked in 200 $\mu$l of tissue culture media containing anti-CD16/32 Ab for 30 min on ice. The cells were spun and resuspended in 50 $\mu$l of PBS containing the specified Ab (anti-CD3e-Cy, -CD8a-PE, -CD8b-FITC, -NK1.1-PE, Ly49D-FITC) at a concentration of 0.5–2 $\mu$g/ml or D$^b$-NP366 (PE or RED670) tetramer (10 $\mu$g/ml). When staining with biotinylated Abs against Ly49A, C/I, G2, TCR$\alpha\beta$, and NK1.1, an extra staining with streptavidin RED670 (1:100) for 15 min was performed. An isotype control Ab conjugated with the respective fluorescent or biotinylation tag was used for negative control staining of each specific Ab. After 30 min on ice, the cells were washed with PBS and the fluorescence intensity was measured on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Finally, the data were analyzed using CellQuest computer software (Becton Dickinson).

Determination of influenza virus lung titers

Lungs from mice infected with influenza virus were harvested into 3 ml of ice-cold PBS, minced, and centrifuged at 400 $\times$ g for 3 min. Pellet fractions were homogenized in microcentrifuge tubes using disposable
The lungs of influenza-infected mice were removed at day 10 postinfection, and the lung cells were triple stained with Ab against CD8+ and NK1.1, and permeabilized with brefeldin A. Triple fluorescence analysis using flow cytometry was performed, and the results are expressed as percentage of positive cells for IFN-γ over negative control Ab.

**Results and Discussion**

The number of cells coexpressing CD8β and NK1.1 is significantly increased in the lungs of influenza A virus-infected mice

NK cells play a vital role in protection against many virus infections and are believed to be important during early phases of viral infection (14). To determine whether NK cells could play a role in late stages of viral infection, mice were infected with influenza A virus, and the presence of NK cells in the lungs was monitored between days 7 and 14 postinfection by flow cytometry. To our surprise, an increase in NK1.1+ cell numbers was observed in the lungs of influenza-infected mice between these time points. Double fluorescence analysis revealed that these cells were not classical NK cells, since they expressed CD8β. As shown in Fig. 1A, the number of CD8β+NK1.1+ cells was markedly higher compared with controls between days 7 and 14, reaching a maximum at day 10 postinfection. Along with relative percentages, absolute numbers of CD8β+NK1.1+ cells were also significantly increased on day 10. There were ~100,000 CD8β+NK1.1+ cells/lung found on day 10 postinfection compared with 4,000 CD8β+NK1.1+ cells/lung detected in uninfected controls (data not shown). Of note, careful precautions were taken when analyzing the T cells by flow cytometry. Each experiment was performed by blocking Fc receptors with anti-CD16/32 Ab, and the cells were gated so that <1% of CD8+ T cells were stained positively for the respective isotype control Ab. Some experiments were also performed by blocking with normal mouse serum and similar results were obtained.

**Virus-specific T cells follow the same kinetics of appearance as the CD8β+NK1.1+ cells**

To relate these findings to adaptive immune responses that normally occur during this time frame (day 5–18), the presence of viral peptide-specific T cells was also monitored. To detect viral peptide-specific T cells in infected mice, H-2D+ MHC class I tetramers refolded with a D+/-binding influenza nucleoprotein peptide

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### Table I. Expression of Ly49 molecules on CD8β+ cells in influenza-infected mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Uninfected Mice</th>
<th>Influenza-Infected Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8β+</td>
<td>CD8β+NK1.1+</td>
</tr>
<tr>
<td>LY49A</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Ly49C1</td>
<td>2.4 ± 0.34</td>
<td>2.9 ± 1.2</td>
</tr>
<tr>
<td>Ly49D</td>
<td>0.8 ± 0.5</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>Ly49G2</td>
<td>1.7 ± 0.5</td>
<td>1.4 ± 0.3</td>
</tr>
</tbody>
</table>

* Lungs of influenza-infected mice (n = 3) were removed on day 10 postinoculation and pooled. Lung cells were isolated as described in Materials and Methods, stained for CD8β, NK1.1 and Ly49 molecules, and analyzed by flow cytometry. Results are expressed as mean percent positive cells ± SD of three independent experiments.

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### Table II. Viral peptide specificity of lung cells from influenza-infected mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CD8β+ NK1.1+</th>
<th>CD8β+ NK1.1+</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP366</td>
<td>11.2 ± 3.7</td>
<td>20.9 ± 1.8</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>14.5</td>
<td>28.2</td>
</tr>
</tbody>
</table>

* Lungs of normal and influenza-infected mice (n = 3) were removed on day 10 postinoculation and pooled. Lung cells were isolated as described in Materials and Methods, stained for CD8β, NK1.1, and DNP366 tetramers, and analyzed by flow cytometry. Results are expressed as mean percent positive cells ± SD of three independent experiments.

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FIGURE 4. Expression of Ly49 receptors on CD8β+NK1.1+ and CD8β+NK1.1- cell subsets in lungs of influenza virus-infected mice on day 10 postinfection. The lungs of influenza-infected mice were removed at day 10 postinfection, and the lung cells were triple stained with Ab against CD8β, NK1.1, and an Ab against the Ly49 receptor indicated at the top of each histogram plot. The cells were gated into two subsets, CD8β+NK1.1+ or CD8β+NK1.1-, and the binding of isotype control Ab or Ab against Ly49 receptors was analyzed. The number indicated in the histogram plot represents the percentage of positive cells within that region.
stained with Ab against CD8\textsuperscript{b} virus-infected mice on day 10 postinfection. A. The lungs of influenza-infected mice were removed at day 10 postinfection and the lung cells were triple stained with Ab against CD8\textsuperscript{b}, NK1.1, and D\textsuperscript{b}-NP366 tetramer. The cells were gated into two subsets, CD8\textsuperscript{b} NK1.1\textsuperscript{+} or CD8\textsuperscript{b} NK1.1\textsuperscript{−}, and the binding of D\textsuperscript{b}-NP366 tetramer was analyzed. B. Lungs cells were stimulated with ASNENMDAM peptide (1 \(\mu\)M) and IL-2 (100 U/ml) for 6 h and the cells were triple stained with Ab against CD8\textsuperscript{b}, NK1.1 and isotype control Ab or anti-IFN-\(\gamma\) Ab. The cells were gated into two subsets, CD8\textsuperscript{b} NK1.1\textsuperscript{+} or CD8\textsuperscript{b} NK1.1\textsuperscript{−}, and intracellular IFN-\(\gamma\) was analyzed. The number indicated in the histogram plot represents the percentage of positive cells within that region.

(D\textsuperscript{b}-NP366) were constructed (15, 16). CD8\textsuperscript{b}D\textsuperscript{b}-NP366\textsuperscript{+} cells were detected abundantly in the lungs of influenza-infected mice (Fig. 1B). In absolute numbers, there were ~300,000 CD8\textsuperscript{b}D\textsuperscript{b}-NP366\textsuperscript{+} cells/lung found in influenza-infected mice on day 10 postinfection. Similar to CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells, the number of these cells peaked on day 10 postinfection. Distinct CD8\textsuperscript{b}NK1.1\textsuperscript{+} and CD8\textsuperscript{b}D\textsuperscript{b}-NP366\textsuperscript{+} populations of cells were observed on day 10 in influenza-infected mice (Fig. 2, B and D, upper right quadrant) compared with normal mice (Fig. 2, A and C, upper right quadrant).

To correlate the present findings to virus titers of influenza-infected lungs, the infectious activity of lung homogenates was measured at various time points after influenza virus infection. Viral activity in the lungs peaked on day 7 and dropped sharply beginning on day 10 (Fig. 3), corresponding to the peak appearance of the CD8\textsuperscript{b}D\textsuperscript{b}-NP366\textsuperscript{+} and CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells.

CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells display both T cell and NK cell phenotype

Lung cells prepared from influenza-infected and uninfected mice on day 10 were stained with Ab against CD8\textsuperscript{b}, NK1.1, and a T cell- or NK cell-associated molecule. The cells were gated into two subsets by flow cytometry, either CD8\textsuperscript{b}/NK1.1\textsuperscript{−} or CD8\textsuperscript{b}/NK1.1\textsuperscript{+}, and analyzed for other T cell- and NK cell-associated markers. More than 97% of the CD8\textsuperscript{b}NK1.1\textsuperscript{+} and CD8\textsuperscript{b}NK1.1\textsuperscript{−} cells were CD8\textsuperscript{a}TCR\textsuperscript{a}/CD3\textsuperscript{−} (data not shown). A significantly higher proportion of the NK1.1\textsuperscript{+} cells expressed the NK cell inhibitory molecules Ly49A, C/I, and G2 and the NK cell-activating molecule Ly49D compared with the NK1.1\textsuperscript{−} cells (Table I). A representative FACS plot is shown in Fig. 4. Of note, the expression levels of Ly49 molecules were equal or only slightly less than those of conventional NK cells. These data suggest that CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells are potentially regulated by both T cell and NK cell-activating and inhibitory signals.

A high percentage of CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells are influenza virus specific

To see whether CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells were virus specific, lung cells were triple stained with CD8\textsuperscript{b}, NK1.1, and D\textsuperscript{b}-NP366 tetramer. Interestingly, we found that a higher proportion of the CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells stained positive for the D\textsuperscript{b}-NP366 tetramer than the CD8\textsuperscript{b}NK1.1\textsuperscript{−} cells (Table II and Fig. 5A). Although the tetramer staining showed that the viral peptide MHC class I complex bound to the TCR of these cells, it was important to demonstrate that the TCR was functional. There was also a possibility that the tetramers bound to Ly49 receptors expressed on the CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells. To demonstrate that the CD8\textsuperscript{b}NK1.1\textsuperscript{+} cells...
cells could respond to the viral peptide. Intracellular staining of IFN-γ was performed on cells obtained from the lung. When stimulated with the viral peptide, there were two times more IFN-γ-producing cells in the CD8β⁺NK1.1⁺ subset compared with the CD8β⁺NK1.1⁻ subset (Table II and Fig. 5B). Taken together, these results strongly suggest that a large proportion of these CD8β⁺NK1.1⁺ cells were activated virus-specific T cells. In addition, we examined the proportion of CD8β⁺Dβ⁻NP366⁺ cells that expressed NK1.1. A higher proportion of CD8β⁺Dβ⁻NP366⁺ cells were NK1.1⁺ than CD8β⁻ cells that were Dβ⁻NP366 tetramer negative (Fig. 6).

**CD8β⁺NK1.1⁺ cells are not conventional NKT cells**

Conventional NKT cells which possess both TCR and NK cell-associated markers have previously been described (9). Several lines of evidence indicate that the CD8β⁺NK1.1⁺ cells in our study were different from conventional NKT cells. First, conventional NKT cells have been documented to be CD4⁻CD8⁻ or CD4⁺CD8⁻ (8, 9). Second, CD8β⁺NK1.1⁺ cells were detected in the lungs of influenza-infected CD1⁻/⁻ mice which lack NKT cells (Fig. 7). Third, conventional NKT cells are restricted to CD1, whereas the CD8β⁺NK1.1⁺ cells stained positively for H-2Db tetramers, suggesting their restriction to H-2Db and not to CD1.

We speculate that the CD8β⁺NK1.1⁺ cells originated from CD8⁺ T cells and not from NK cells. Treatment of mice with anti-NK1.1 Ab, PK136, has been shown to deplete classical NK cells in mice (17). When influenza-infected mice were treated with anti-NK1.1 Ab on day 6 postinfection and lung cells were examined on day 10, CD8β⁺NK1.1⁺ cells were still detected (data not shown). In contrast, classical NK cells were absent from both the lungs and spleens of these mice. Thus, it was more conceivable that the CD8β⁺NK1.1⁺ cells developed from CD8⁺ T cells rather than from NK cells. In support of this notion, we have recently found that CD8β⁺NK1.1⁺ cells can be generated in vitro by culturing purified CD8⁺ T cells in IL-2, IL-4, and IL-15 (18). Like the cells found in the lungs of influenza-infected mice, these cells expressed NK1.1 and Ly49 molecules. Thus, we favor a scenario in which CD8⁺ T cells acquire NK1.1 and other NK cell-associated molecules upon activation. After treatment with the anti-NK1.1 Ab PK136, the observation that only a few, if any, CD8β⁺NK1.1⁺ cells were eliminated in mice may be explained by the relatively lower levels of NK1.1 expression on these cells compared with classical NK cells. This is also true for NKT cells which express lower levels of NK1.1 and are not depleted by PK136 (19).

As mentioned earlier, cytokines such as IL-2, IL-4, and IL-15 were able to induce NK1.1 and Ly49 expression on CD8⁺ T cells in vitro. The acquisition of these receptors was found to be dependent on CD122 (IL-2Rβ) (18). In line with this observation, we found that a majority of the CD8β⁺NK1.1⁺ and CD8β⁺Dβ⁻NP366⁺ cells were also CD122⁺ (data not shown). Since most of the tetramer-positive T cells expressed CD122, it was not surprising to find that a significant proportion of the CD8β⁺NK1.1⁺ cells was virus specific. Thus, the emergence of CD8β⁺NK1.1⁺ T cells in vivo may also be mediated by cytokines such as IL-2.

The presence of CD8⁺ T cells expressing NK cell-associated markers has been observed in other models. In mice, treatment with IL-2 and IL-4 induced a population of cells that was CD8α⁺TCRαβ⁺NK1.1⁺ in spleen cells (20) and NK cell-depleted splenocytes (21). In a virus model, spleens of lymphocytic choriomeningitis virus-infected mice contained CD8⁺ T cells that expressed NK1.1 (22). In the bone marrow of mice, expression of NK1.1 and Ly49 molecules have been shown to be associated with memory T cells (23). Studies performed in humans have described activated CD8⁺ T cells that possess NKR-P1C (24) or inhibitory NK cell receptors such as p58.2 (25–27), NK1-1 (27, 28), and CD94/NKG2A (25, 29, 30). Similar T cells have also been found in HIV-infected patients (31). Many of these studies propose that the presence of NK cell inhibitory molecules on T cells could either be a disadvantage to the host by inhibiting T cells that are necessary for the recovery of the host from disease or an advantage in cases of autoimmunity or graft-versus-host disease. Likewise in our system, the up-regulation of inhibitory Ly49 receptors on CD8⁺ T cells may potentially serve to dampen the immune response, e.g., by suppressing overactivated T cells that may be a potential hazard to the host. In addition, expression of Ly49 inhibitory receptors on nonspecific T cells may suppress bystander activation. In support of this notion, a previous study performed in Ly49A transgenic mice demonstrated that the expression of Ly49A inhibitory receptors on T cells can modulate activated T cell responses (32).

**Concluding remarks**

In conclusion, we demonstrate the emergence of NK cell-like CD8⁺ T cells in the lungs of influenza virus-infected B6 mice. These CD8⁺ T cells express activating and inhibitory cell surface receptors characteristic of NK cells. A large proportion of these cells bound MHC class I tetramers refolded with a viral nucleoprotein epitope and produced IFN-γ in response to peptide re-stimulation, suggesting that many of these cells were activated virus-specific CTL. The demonstration of NK cell-associated molecules on T cells reveal novel regulatory mechanisms in controlling the effector functions of T cells.

**Acknowledgments**

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**References**
