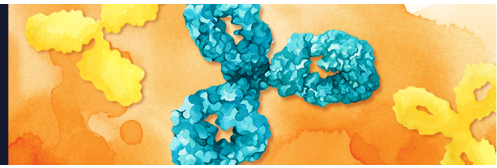


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Cutting Edge: Engagement of NKG2A on CD8⁺ Effector T Cells Limits Immunopathology in Influenza Pneumonia¹

Jing Zhou,^{*} Mitsuo Matsuoka,^{2*} Harvey Cantor,⁵ Robert Homer,^{†‡} and Richard I. Enelow^{3*‡}

Influenza pneumonia results in considerable lung injury, a significant component of which is mediated by CD8⁺ T cell Ag recognition in the distal airways and alveoli. TNF- α produced by Ag-specific CD8⁺ T cells appears primarily responsible for this immunopathology, and we have examined the negative regulation of CD8⁺ TNF production by CD94/NKG2A engagement with its receptor, Qa-1b. TNF production by antiviral CD8⁺ T cells was significantly enhanced by NKG2A blockade in vitro, and mice deficient in the NKG2A ligand, Qa-1b, manifested significantly greater pulmonary pathology upon CD8⁺ T cell-mediated clearance in influenza pneumonia. Furthermore, blockade of NKG2A ligation resulted in the enhancement of lung injury induced by CD8⁺ effector cell recognition of alveolar Ag in vivo in the absence of infectious virus. These data demonstrate that CD94/NKG2A transduces a biologically important signal in vivo to activated CD8⁺ T cells that limits immunopathology in severe influenza infection. The Journal of Immunology, 2008, 180: 25–29.

Infection with highly pathogenic strains of influenza may result in considerable lung injury and respiratory dysfunction, and the host T cell response may be an important contributor to this (1, 2). Important factors that may influence immunopathology include the distal (alveolar) distribution of infection, the overall viral load, and the magnitude of the CD8⁺ T cell response (3–5). The degree to which the individual CD8⁺ T cell effector activities that contribute to viral clearance overlap with those responsible for tissue damage is difficult to dissect, though there is evidence that TNF- α may be disproportionately responsible for injury and possibly dispensable for antiviral activity (6, 7). In addition, there is evidence that T cell effector activities are not necessarily coordinately regulated and may have distinct thresholds for activation (8, 9).

The interaction of inhibitory NK cell receptors on T cells with MHC class I ligands on APCs has been proposed to fine tune TCR-mediated activation of CD8⁺ T cells (10). One fam-

ily member, NKG2A, heterodimerizes with CD94 and engages the nonclassical MHC class I molecule Qa-1b (HLA-E in humans). CD94/NKG2A ligation on CD8⁺ T cells may result in dampened in vitro effector activity (e.g., cytotoxicity and cytokine production) (11, 12), although this has not been consistently observed (13–15). It has been shown, for example, that in mice infected with polyomavirus, CD94/NKG2A heterodimer expression was rapidly induced on antiviral CD8⁺ T cells and that these T cells showed reduced Ag-specific cytotoxicity in vitro; a blockade of CD94/NKG2A binding to its ligand, Qa-1b/Qdm, relieved this inhibition (11). Because murine polyoma infection is not cleared, it was speculated that the reduction in cytotoxic activity might serve to limit immunopathology (16). A similar effect been observed on ex vivo cytotoxicity exhibited by CD8⁺ T cells isolated from neurons of HSV-infected mice, and it was similarly proposed that this might limit the pathologic loss of neurons (12). Notwithstanding the speculation that CD94/NKG2A ligation on CD8⁺ effector cells may limit immunopathology in viral infection (and the conflicting in vitro data), this effect has not been demonstrated in vivo. We show that infection with type A influenza results in a progressive accumulation of CD8⁺ T cells in the lung that express NKG2A and that a blockade of Qa-1b/NKG2A ligation in vivo on committed CD8⁺ effector cells significantly abrogates immunopathologic lung injury.

Materials and Methods

Mice and experimental procedures

BALB/c and C57BL/6 mice were purchased from Taconic Farms. Qa-1b^{-/-} (17) mice and Qa-1b^{+/+} littermates were infected intranasally (i.n.)⁴ with the A/PR/8/34 virus. Activated nucleoprotein (NP)_{366–374}-specific CD8⁺ T cells were injected into infected mice 6 h postinfection. Surfactant protein C (SPC)-hemagglutinin (HA) mice (7) received i.v. transfer of activated HA_{204–212}-specific CD8⁺ T cells and anti-NKG2A/C/E mAb (clone 20d5; BD Biosciences) or isotype control via the tail vein. Mice were sacrificed 5 days after transfer. Lungs were perfused, excised, then sectioned and stained with H&E.

CD8⁺ T cell generation

A/Japan/57 HA_{204–212}-specific or A/PR/8/34 NP_{366–374}-specific CD8⁺ T cells were isolated from lungs of BALB/c or C57BL/6 mice infected with either

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⁴ Abbreviations used in this paper: i.n., intranasal(ly); BAL, bronchoalveolar lavage; BFA, brefeldin A; HA, hemagglutinin; HA204 Tet, HA_{204–212}-H-2K^d tetramer; LCMV, lymphocytic choriomeningitis virus; MLN, mediastinal lymph node; NP, nucleoprotein; SPC, surfactant protein C.

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A/Japan/57 or A/PR/8/34 virus, respectively, and restimulated in vitro with irradiated syngeneic peptide-pulsed splenocytes, as described (7).

Cell preparation from tissues

Mice were sacrificed at the indicated times after infection. The lungs, the mediastinal lymph nodes (MLN), and spleen were harvested and then minced and passed through a nylon filter (Falcon).

Flow cytometry

mAbs were purchased from BD Biosciences. Briefly, cells were stained with biotinylated anti-NKG2A/C/E, followed by PerCP-streptavidin and then with HA₂₀₄₋₂₁₂-H-2K^d tetramer (HA204 Tet) (National Institutes of Infectious and Allergic Diseases Tetramer Core Facility, Emory University, Atlanta, GA) and anti-CD8 α . For intracellular cytokine staining, cells were stimulated with peptide-pulsed P815 cells in brefeldin A (BFA) for 5 h. Cells were labeled for surface markers, permeabilized, and stained with anti-TNF- α (or isotype).

Bronchoalveolar lavage (BAL) albumin concentration

BAL was performed with 1 ml of sterile saline as described previously (18) and centrifuged at 1000 rpm for 10 min. Albumin concentration in BAL fluid was determined by ELISA (Bethyl Laboratories).

Statistics

Statistical analyses were performed with Prism (GraphPad Software) using the Student *t* test or the Mann-Whitney *U* test.

Results and Discussion

NKG2A expression is induced on Ag-specific CD8⁺ T cells in the lungs during acute influenza infection

We sought to characterize the kinetics of induction of NKG2A expression on Ag-specific CD8⁺ T cells harvested from lungs during acute sublethal infection with A/Japan/57. Although the mAb used to detect NKG2A (clone 20d5) also recognizes the NKG2 isoforms NKG2C and NKG2E, nearly all 20d5 mAb-sorted, Ag-specific CD8 T cells predominantly express NKG2A transcripts (14, 19, 20). Furthermore, NKG2A and NKG2C expression appears to be mutually exclusive and clone specific (21, 22), and CD94/NKG2C has not been shown to mediate activation of murine NK or CD8 T cells (18). Therefore, we will refer to 20d5 mAb⁺ cells as NKG2A⁺.

As shown in Fig. 1, there was little expression of NKG2A on HA₂₀₄₋₂₁₂-specific CD8⁺ T cells (a dominant K^d-restricted epitope of A/Japan/57 HA) isolated from lungs on day 5 after i.n. infection (though there were few epitope-specific cells present at this time point). By day 8, the frequency of HA₂₀₄₋₂₁₂-specific CD8⁺ T cells in the lungs rose to 14% of the CD8⁺ T cell population, and >50% of epitope-specific CD8⁺ T cells expressed NKG2A. By day 12, the magnitude of the HA₂₀₄₋₂₁₂-specific response had fallen to 10% of the CD8⁺ response, while the proportion of NKG2A⁺ rose to >60%. By day 19, only 20% of epitope-specific cells in the lungs expressed NKG2A, possibly reflecting the migration of NKG2A⁺ cells out of the lung and into lymphoid organs (Figs. 1, B and C) such as the spleen (although the kinetics of epitope-specific T cell accumulation and NKG2A expression in the spleen paralleled that of T cells in the lung). Interestingly, NKG2A expression on MLN T cells was not observed at any time point, despite the large proportion that expressed CD69 on day 5 (not shown), suggesting recent Ag engagement. The kinetics of induction of NKG2A expression on epitope-specific CD8⁺ T cells in the lung correlated inversely with virus titers (not shown), with kinetics similar to that observed after experimental lymphocytic choriomeningitis virus (LCMV; Armstrong strain and clone 13) infection (13), as well as after polyomavirus infection (11). In contrast to polyoma and LCMV clone 13

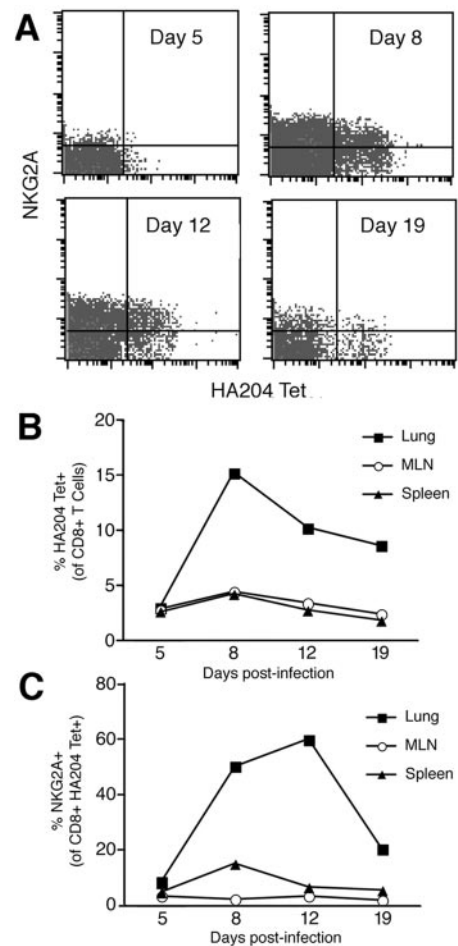


FIGURE 1. NKG2A expression on epitope-specific CD8⁺ T cells. BALB/c mice were i.n. infected with 0.5 LD₅₀ A/Japan/57 influenza. *A*, Lymphocytes were harvested from lungs at various time points and stained with anti-NKG2A, anti-CD8, and HA204 Tet. Shown are kinetics of NKG2A⁺HA204 Tet⁺ T cell accumulation in the lung (gated on CD8⁺ cells). *B* and *C*, Comparative kinetics of HA204 Tet⁺ (*B*) and NKG2A expression (*C*) in lung, MLN, and spleen at various time points after infection. Data represent three independent experiments, each with pooled tissue from 2–3 animals per time point.

(which are not cleared), clearance of influenza from the lung correlated with a decrease in the percentage of epitope-specific CD8⁺ T cells in the lung that express NKG2A. This pattern is consistent with that observed by Miller et al. (14) and is similar to that observed after infection with the LCMV Armstrong strain, a pathogen that is cleared, suggesting that persistent antigenic stimulation may be required for continued high-level expression of CD94/NKG2A. A similar pattern was observed on HA₂₀₄₋₂₁₂-specific CD8⁺ T cells after secondary A/Japan/57 infection as well (not shown). Because CD8⁺ T cell recognition of alveolar Ag may result in considerable lung injury (7, 23, 24), we hypothesized that CD94/NKG2A engagement may serve to limit CD8⁺ T cell effector activities that are particularly immunopathogenic, such as TNF- α (7).

NKG2A expression on CD8⁺ effector cells inhibits TNF- α production upon Ag engagement

The production of TNF- α by Ag-specific CD8⁺ effector cells is an important mediator of lung injury triggered by CD8⁺ T cell

Ag recognition in the distal lung parenchyma (1, 7). As previously noted, the impact of NKG2A ligation on CD8⁺ T cell activity varies depending upon the experimental system but may also vary with the specific effector function examined. In polyoma infection, for example, engagement of CD8⁺ T cell NKG2A/CD94 by Qa-1b on APC appeared to reduce cytotoxicity, whereas it enhanced the proliferative capacity and production of IL-2 by CD8⁺ effector/memory cells (19). The hypothesis that NKG2A transduces an inhibitory signal, raising the threshold for T cell activation, would not account for these effects. To examine the impact of a blockade of NKG2A/Qa-1b interaction on the production of TNF- α by influenza-specific CD8⁺ T cells in vitro, lung T cells were harvested 8 days after sublethal infection with A/Japan/57 and restimulated in vitro several times before staining for CD8 and TNF- α upon peptide stimulation (100% of the cells were NKG2A⁺ before peptide stimulation). As shown in Fig. 2A, 11.2% of CD8⁺ T cells produced TNF- α upon peptide stimulation, and this percentage was doubled in the presence of the blocking Ab to NKG2A. (A similar pattern was observed with IFN- γ production; data not shown.)

To assess the effector activity of Ag-specific T cells from influenza-infected lungs as a function of NKG2A expression, lungs were harvested 8 days after sublethal infection with A/Japan/57 and stained with anti-CD8 and HA204 Tet immediately ex vivo (Fig. 2B). Consistent with our previous observations, the HA204 Tet⁺ population at day 8 was about half NKG2A⁺. A portion of the cells was stimulated with HA₂₀₄₋₂₁₂ peptide-loaded P815 cells in the presence of BFA (which

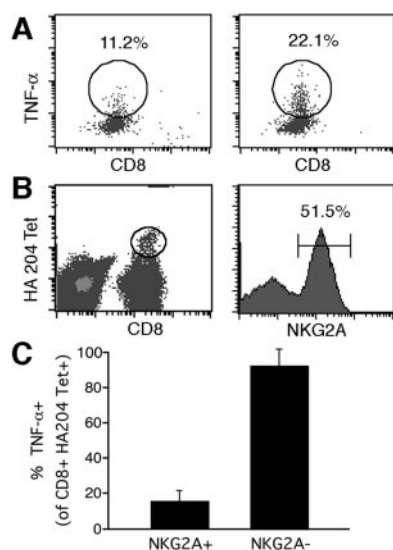


FIGURE 2. TNF- α production by CD8⁺ T cells in vitro and ex vivo. *A*, HA₂₀₄₋₂₁₂-specific CD8⁺ T cell lines (isolated from infected lungs and subjected to several rounds of in vitro restimulation) were stimulated with peptide-pulsed P815 cells for 5 h and analyzed for intracellular TNF- α in the presence of anti-NKG2A blocking Ab (*right panel*) vs IgG (*left panel*). Data represent three independent experiments with pooled tissue from 2–3 mice. *B*, Ex vivo lung lymphocytes were harvested 8 days after infection with 0.5 LD₅₀ A/Japan/57 and stained with anti-CD8 and HA204 Tet (*left panel*). Shown is NKG2A staining after gating on CD8⁺ HA204 Tet⁺ cells (*right panel*). *C*, A portion of the T cells harvested from the lungs was stimulated with peptide-pulsed P815 cells in vitro for 5 h in the presence of BFA, and the percentage of NKG2A⁺ and NKG2A⁻ cells staining for TNF- α was determined. Data represent three independent experiments (*, $p < 0.01$).

prevents de novo surface expression of NKG2A) (19) for 5 h before staining for NKG2A and intracellular TNF- α . Interestingly, the TNF- α production was almost exclusively observed in the NKG2A⁻ population (Fig. 2C), with very little produced by the NKG2A⁺ T cells. This would weigh against the idea that the NKG2A⁻ cells preferentially undergo activation-induced cell death or escape rescue therefrom, and suggests rather that NKG2A⁻ cells may be effectors of immunopathologic lung injury.

Inhibitory impact of NKG2A/Qa-1b interaction on CD8⁺ T cell-mediated pulmonary immunopathology

As noted above, there have been a variety of (conflicting) reports on the impact of NKG2A/Qa-1b interaction on T cell effector function in vitro, but there has been no demonstration of such an impact in vivo. We therefore examined its impact on T cell-mediated lung injury in experimental influenza infection. We have previously observed Qa-1b expression in gene expression arrays on RNA prepared from purified primary alveolar epithelial cells (R. Chilakamarti and R. I. Enelow, unpublished observations), suggesting a potential role in the regulation of (postpriming) T cell effector activity. Qa-1b-null mice were infected i.n. with a lethal dose of A/PR8/34 and a few hours later these animals received activated CD8⁺ T cells (a line specific for a D^d-restricted epitope of A/PR8/34 NP) via the tail vein. (After multiple rounds of in vitro stimulation, these cells were 100% NKG2A⁺; data not shown.) Five days after transfer, Qa-1b-null mice (Fig. 3B) showed significantly greater evidence of lung injury than controls (Fig. 3A). The Qa-1b-null mice also had more severe lung injury as measured by capillary leak of albumin into the BAL fluid (Fig. 3C). The interstitial and alveolar inflammatory infiltration was more intense, with significant polymorphonuclear neutrophil accumulation in the alveolar walls and more dramatic hemorrhage. Virus titers were undetectable in both groups (not shown). In the absence of T cell transfer, there was no difference in capillary leakage or histologic injury between Qa-1b-null and wild-type mice after lethal or sublethal influenza infection (data not shown). This suggests that the NK cell response to influenza infection has a limited impact on pathology (or that NKG2A blockade has limited impact on the NK cell response). Day 5 after infection is also too early to see an effect of NKG2A blockade on the primary CD8⁺ T cell response, because it was not expressed at this time point in primary infection (Fig. 1A).

To confirm the specific impact of NKG2A modulation of CD8⁺ T cell effector function on lung injury in the absence of virus effects, uninfected SPC-HA mice (expressing A/Japan/57 HA in the distal lung) (7, 23) were used as recipients of HA₂₀₄₋₂₁₂-specific, K^d-restricted CD8⁺ T cell lines (activated in vitro with influenza-infected splenocytes, 100% of which were NKG2A⁺; not shown). In addition, Ab to NKG2A (or isotype control) was coadministered at the time of T cell transfer and 5 days later immunopathology was assessed. As shown in Fig. 4, the injury was more severe in the recipients of the blocking Ab coadministered with the T cells (Fig. 4B) compared with coadministration of an isotype control (Fig. 4A), with features similar to (although somewhat less severe than) infected Qa-1b-null recipients of T cell transfer. Again, capillary leakage was significantly greater in recipients of the blocking Ab (Fig. 4C). As we have previously demonstrated (23), specific Ag recognition is required for lung injury, and no pathology was observed

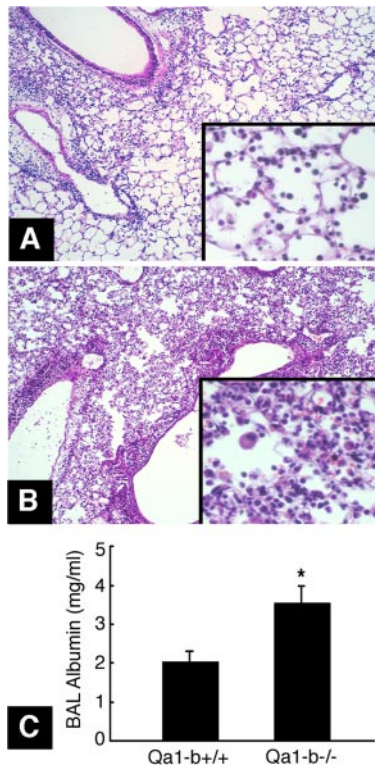


FIGURE 3. Enhanced pulmonary injury in influenza-infected Qa-1b^{-/-} mice after transfer of effector CD8⁺ T cells. Qa-1b^{+/+} and Qa-1b^{-/-} mice were infected i.n. with 10 LD₅₀ A/PR/8/34 virus and 6 h later received 5 × 10⁶ NP_{366–374}-specific CD8⁺ T cells activated in vitro. *A* and *B*, H&E-stained sections of lungs (original magnification: ×10 in large pictures, ×40 in insets) harvested 5 days after transfer from either Qa-1b^{+/+} (*A*) or Qa-1b^{-/-} (*B*) mice. *C*, ELISA for BAL albumin from Qa-1b^{+/+} and Qa-1b^{-/-} mice 5 days after infection and transfer (results expressed as the mean ± SEM of three independent experiments (three mice per group); *, *p* < 0.05)

after the transfer of T cells specific for an unrelated Ag, with or without blocking Ab (not shown). Interestingly, in the absence of a blockade of CD94/NKG2A signaling, the degree of pathology triggered by adoptive transfer of HA_{204–212}-specific CD8⁺ T cells was still relatively modest in comparison with the very severe pathology observed after the transfer of HA_{210–219}-specific CD8⁺ T cells (7, 23). This consistent observation (R. I. Enelow and T. J. Braciale, unpublished observations) may be partly explained by the higher level of NKG2A expression that we have observed on HA_{204–212}-specific T cell lines than on HA_{210–219}-specific lines, although the reasons for this are unclear. In any event, these data demonstrate that the NKG2A/Qa-1b interaction has the impact of limiting CD8⁺ T cell-mediated lung injury in vivo. Furthermore, this effect can be clearly distinguished from the effects of the virus infection per se on lung integrity.

The abrogating effect of Qa-1b/NKG2A interaction on immunopathologic lung injury likely occurs at the effector end of the immune response, because the cells used in the in vivo studies were terminally differentiated effectors (after multiple rounds of in vitro stimulation). It remains formally possible, however, that the amelioration of injury is a consequence of the activities of Qa-1-restricted CD8⁺ regulatory T cells (17). This seems an unlikely possibility because the regulatory effects observed in the initial description of this phenomenon were not in

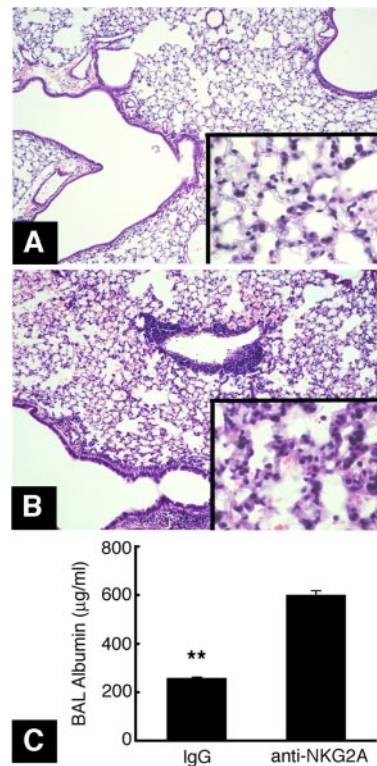


FIGURE 4. Enhanced pulmonary immunopathology in SPC-HA recipients of CD8⁺ effector T cells with coadministration of blocking Ab to NKG2A. *A* and *B*, Lungs were harvested 5 days after transfer of 5 × 10⁶ activated HA_{204–212}-specific CD8⁺ T cells into SPC-HA transgenic mice (original magnification: ×10 in large pictures, ×40 in insets) that were coadministered either isotype IgG (*A*) or Ab (*B*) to NKG2A. *C*, ELISA for albumin in cell-free BAL from SPC-HA mice 5 days after transfer of CD8⁺ T cells (results expressed as the mean ± SEM of three independent experiments (three mice per group); **, *p* < 0.01).

evidence during the primary response, but rather during the secondary response.

Highly pathogenic influenza strains are particularly predisposed to infecting the alveolar epithelium, although the damage to the lung parenchyma is likely related as much to the T cell response as to the virus itself (3). CD8⁺ T cell-mediated pulmonary immunopathology appears to be mediated in large measure by TNF-α expressed by the effector cells, and we have shown that there are intrinsic mechanisms in place to limit the vigor of this effector activity. This study demonstrates that the NKG2A/Qa-1b interaction may represent one such mechanism, and it suggests the potential importance of understanding the factors that regulate the expression of NKG2A on CD8⁺ T cells and Qa-1b on the alveolar epithelium.

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Disclosures

The authors have no financial conflict of interest.

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