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Type I Interferons Regulate Cytolytic Activity of Memory CD8$^+$ T Cells in the Lung Airways during Respiratory Virus Challenge

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SUMMARY

Memory CD8$^+$ T cells in the lung airways provide protection from secondary respiratory virus challenge by limiting early viral replication. Here, we demonstrate that although airway-resident memory CD8$^+$ T cells were poorly cytolytic, memory CD8$^+$ T cells recruited to the airways early during a recall response showed markedly enhanced cytolytic ability. This enhanced lytic activity did not require cognate antigen stimulation, but rather was dependent on STAT1 transcription factor signaling through the interferon-α receptor (Ifnar1), resulting in the antigen-independent expression of granzyme B protein in both murine and human virus-specific T cells. Signaling through Ifnar1 was required for the enhanced lytic activity and control of early viral replication by memory CD8$^+$ T cells in the lung airways. These findings demonstrate that innate inflammatory signals act directly on memory T cells, enabling them to rapidly destroy infected host cells once they enter infected tissues.

INTRODUCTION

The resolution of acute viral infections results in the generation of pathogen-specific humoral and cellular immunity that persists for the life of the host. Memory T cells generated after infection or vaccination are maintained in both peripheral and lymphoid tissues and can provide protection during a secondary pathogen encounter (Wakim et al., 2008; Woodland and Kohlmeier, 2009). Memory T cells localized to peripheral tissues can provide immediate effector responses to secondary infections, whereas cells in lymphoid tissues provide a reservoir of antigen-specific precursors for the rapid expansion of the secondary response. In the case of T cell memory generated after respiratory virus infection, cells in the lung airways are uniquely positioned to recognize and respond to viruses, such as influenza and parainfluenza viruses, which infect and replicate within epithelial cells lining the respiratory tract. Indeed, studies in both mice and humans have shown that memory T cells specific for respiratory viruses are preferentially localized to the lung, and the gradual loss of these cells over time correlates with decreased cell-mediated protection from secondary challenge (de Bree et al., 2005; Hogan et al., 2001a; Liang et al., 1994; Turner et al., 2003).

Recent studies have shown that the recall of memory CD8$^+$ T cells during a respiratory virus challenge is a complex and dynamic process (Kohlmeier and Woodland, 2009). Importantly, there is a substantial increase in the number of virus-specific CD8$^+$ T cells in the lung airways after challenge prior to the appearance of secondary effector T cells (Chen et al., 2001; Ely et al., 2003; Heidema et al., 2008; Topham et al., 2001). This increase could not be accounted for by local proliferation of memory T cells within the airways, as indicated by the fact that the cells had not recently divided and the increase in virus-specific T cell number did not require cognate antigen stimulation. Rather, these studies have shown that the increased number of memory CD8$^+$ T cells in the airways during the early stages of a respiratory virus challenge is due to the nonspecific recruitment of circulating cells to the lung airways in response to localized inflammation. We have shown that the recruitment of memory CD8$^+$ T cells to the inflamed airways is dependent on the chemokine receptor CCR5 and that this enhanced recruitment serves to limit early viral replication (Kohlmeier et al., 2008). Several days after the recruitment of circulating memory T cells to the airways, secondary effector T cells that had expanded in the draining lymphoid tissues migrate to the lung and eradicate the infection. Importantly, it is the combination of the early recruitment of circulating memory T cells, followed by the expansion and recruitment of secondary effector T cells, that results in the enhanced speed and magnitude of the recall response to respiratory viral infection.

Although the presence of memory T cells in the lung airways plays a key role in the early control of viral replication, we do not understand the mechanisms that underlie this protection. Effector CD8$^+$ T cells can employ diverse mechanisms to eliminate infected host cells, including the engagement of surface molecules such as Fas and TRAIL-R2 and the delivery of perforin and granzymes into the cytoplasm of target cells (Brincks et al., 2008; Topham et al., 1997). However, lung airway-resident memory T cells in resting mice are poorly cytolytic compared to effector T cells (Vallbracht et al., 2006), and this decreased lytic activity correlated with reduced granzyme protein. Currently, it is not known whether the inflammatory conditions present in the lung after secondary challenge can enhance the cytolytic ability of airway-resident memory CD8$^+$ T cells. Furthermore, the cytolytic potential of circulating memory CD8$^+$ T cells recruited to the airways after infection has not been addressed.

Several studies have shown that virus-specific memory CD8+ T cells continue to express cytolytic transcripts for perforin and granzymes after viral clearance, albeit at lower amounts than effector T cells (Jenkins et al., 2007; Johnson et al., 2003). Despite the continual production of cytolytic transcripts, however, little or no cytolytic protein is expressed. Only in cases where localized antigen was present were memory CD8+ T cells able to maintain a high amount of cytolytic transcripts and to produce cytolytic protein (Mintern et al., 2007). However, studies of NK cell cytotoxicity have shown that cytokine-induced activation was sufficient to induce the translation of pre-existing granzyme transcripts (Fehniger et al., 2007; Reading et al., 2007). These studies raise the possibility that inflammatory cytokines produced after innate recognition of pathogen infection may act directly on memory CD8+ T cells to induce expression of cytolytic proteins from pre-existing transcripts, thereby "priming" these cells to rapidly destroy infected targets.

In the present study, we investigated the contributions of inflammation and antigen stimulation on memory CD8+ T cell lytic activity in the lung airways during the early stages of a respiratory virus challenge. We demonstrate that memory CD8+ T cells recruited to the airways during the early stages of a viral challenge had markedly enhanced lytic activity compared to memory CD8+ T cells from the airways of resting mice, and this correlated with increased expression of the cytolytic protein granzyme B (gzmB). Challenge with an unrelated virus or TLR ligands was sufficient to upregulate expression of gzmB protein, demonstrating that this process is not dependent on antigen. Rather, signaling through the IFN-α (Ifnar1) receptor on memory CD8+ T cells induced gzmB protein expression, resulting in enhanced lytic activity in the lung airways and substantially decreased viral loads during the early stages of a secondary respiratory virus challenge. Thus, type I interferons act directly on circulating memory CD8+ T cells to enhance their ability to kill infected target cells prior to their recruitment to infected tissues.

RESULTS

Memory CD8+ T Cells from the Airways Have Enhanced Lytic Activity after Infection with an Unrelated Virus

Early responses to secondary respiratory virus infections are mediated by both memory T cells present in the lung airways at the time of infection and circulating memory T cells that are recruited to the airways by inflammatory signals during the first few days after infection. Together, the airway-resident and recently recruited memory CD8+ T cells mediate substantial control of viral loads. However, the underlying mechanisms of viral control are unclear. Although memory CD8+ T cells in the airways of resting mice have been reported to be poorly cytolytic (Vallbracht et al., 2006), it is unknown whether the inflammatory conditions present in the airways after virus challenge can influence the cytolytic function of memory CD8+ T cells at this site. To address this question, we analyzed the cytolytic ability of Sendai-specific memory CD8+ T cells in the airways after infection with an unrelated virus, thereby allowing us to determine the impact of inflammation in the absence of cross-reactive antigen. Thus, Sendai-immune mice (45 days after Sendai infection) were administered PBS or x31 influenza virus and the lytic activity of memory CD8+ T cells from the airways was assessed 3 days later. Lytic activity was assessed by incubating CD8+ T cells sorted from the airways with congenic peptide-pulsed target cells, and the ratio of live target cells pulsed with a relevant (SenNP) or irrelevant (γHV p79) antigen was assessed by flow cytometry (Figure 1A). Memory CD8+ T cells from the airways of mice that had received an influenza infection showed a significantly enhanced ability to kill target cells expressing the SenNP antigen compared to the PBS control (Figure 1B). Although memory CD8+ T cells from the airways of resting mice did eventually gain cytolytic ability after prolonged culture with target cells, the amount of specific lysis was still significantly less than that observed with memory CD8+ T cells from the airways of influenza-challenged mice (Figure S1 available online). Thus,

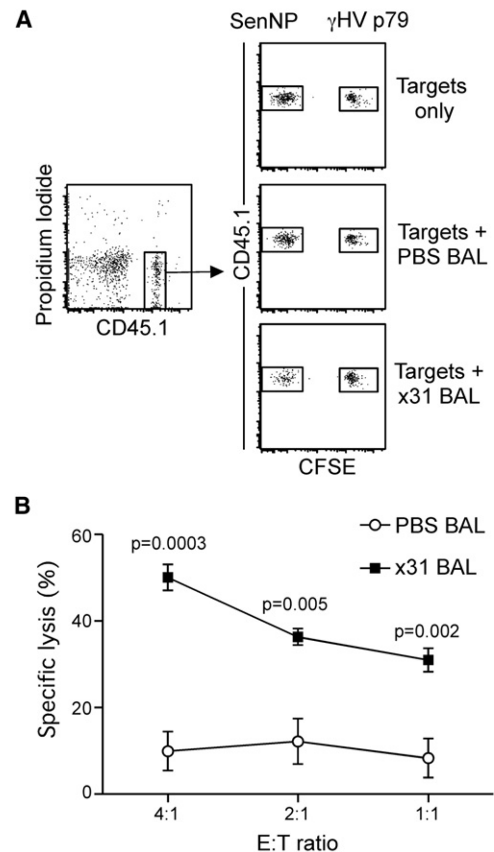

Figure 1. Memory CD8+ T Cells Recruited to the Airways after Virus Infection Have Enhanced Lytic Activity

C57Bl/6 mice were infected with Sendai virus and rested for 45 days. Mice were then challenged with PBS or x31 influenza virus, and CD8+ T cells from the BAL were purified by cell sorting 3 days later.

(A) Sorted CD8+ T cells from the BAL were incubated with congenic (CD45.1+) target cells pulsed with $SenNP_{324-332}$ ($CFSE^{lo}$) or γHV $p79_{524-531}$ ($CFSE^{hi}$) for 5 hr. Live target cells (CD45.1+ and propidium iodide−) were then analyzed by flow cytometry and the ratio of SenNP targets to γHV p79 targets was used to calculate specific lysis. Representative flow cytometry plots for each treatment are shown.

(B) The percent specific lysis (mean ± SD) of SenNP-pulsed targets after incubation with CD8+ BAL T cells from PBS-treated (open circles) or x31-infected (closed squares) mice at various effector to target (E:T) ratios. The data are representative of five independent experiments with five to eight replicate wells per group.

in addition to confirming that memory T cells in the airways of resting mice are poorly cytolytic, these data show that memory $CD8^+$ T cells in the airways after infection with an unrelated respiratory virus are primed to rapidly eliminate specific target cells.

Antigen-Independent Expression of Granzyme B Protein in Memory $CD8^+$ T Cells

$CD8^+$ T cells can employ several different mechanisms to induce the apoptosis of infected host cells, including the engagement of surface molecules such as Fas and TRAIL-R2 and the delivery of granzymes into the cytoplasm of target cells (Brincks et al., 2008; Topham et al., 1997). Because granzymes have been shown to be important for the cytolytic activity of $CD8^+$ T cells in the lung during respiratory virus infection, we investigated the expression of granzyme B (gzmB) protein in Sendai-specific memory $CD8^+$ T cells after challenge with an unrelated influenza virus. Very few resting Sendai-specific memory $CD8^+$ T cells in the airways (BAL), lung parenchyma, and spleen expressed gzmB protein (Figures 2A and 2B). However, beginning on day 2 after influenza challenge, there is a significant increase in the frequency of Sendai-specific memory $CD8^+$ T cells expressing gzmB protein in all tissues. To confirm that this expression was not due to unexpected antigen cross-reactivity, we assessed gzmB expression in the airways after intranasal administration of TLR ligands. As shown in Figure 2C, TLR ligands are also able to induce gzmB expression in memory $CD8^+$ T cells in the airways, demonstrating that inflammation alone is sufficient to increase gzmB protein expression in memory $CD8^+$ T cells. To assess the importance of gzmB for the lytic activity of memory $CD8^+$ T cells in the airways, we investigated the ability of Sendai-specific cells to kill their specific targets in the presence of various inhibitors of cytotoxicity (Figure 2D). Blocking Fas ligand- and TRAIL-mediated pathways had no effect on the lysis of SenNP-pulsed targets. In contrast, treatment with the gzmB inhibitors z-AAD-CMK and Ac-IETD-CHO (Devadas et al., 2006) resulted in a significant decrease of target cell lysis.

After clearance of a primary respiratory infection, antigen-specific memory T cells are maintained in the airways for many months (Ely et al., 2006). During a secondary infection in the lung, circulating memory T cells are rapidly recruited to the airways, further increasing the number of antigen-specific T cells at this site (Kohlmeier and Woodland, 2009). Airway-resident and recently recruited memory $CD8^+$ T cells can be differentiated based on expression of CD11a (Kohlmeier et al., 2007), allowing us to determine whether the increased gzmB expression and cytolytic ability after viral infection was similar between these two populations. Sendai-specific memory $CD8^+$ T cells that were resident in the airways at the time of virus infection ($CD11a^{lo}$) failed to express gzmB protein, and gzmB expression was limited to the recently recruited ($CD11a^{hi}$) cells (Figures 3A and 3B). Importantly, this disparity in gzmB expression correlated with cytotoxic activity, as shown by the fact that sorted $CD11a^{hi}$ Sendai-specific memory $CD8^+$ T cells were vastly superior at killing their specific targets compared to the $CD11a^{lo}$ Sendai-specific memory $CD8^+$ T cells (Figure 3C). Together with Figure 2, these data demonstrate that the antigen-independent expression of gzmB is required for the lytic activity of memory $CD8^+$ T cells recently recruited to the lung airways.

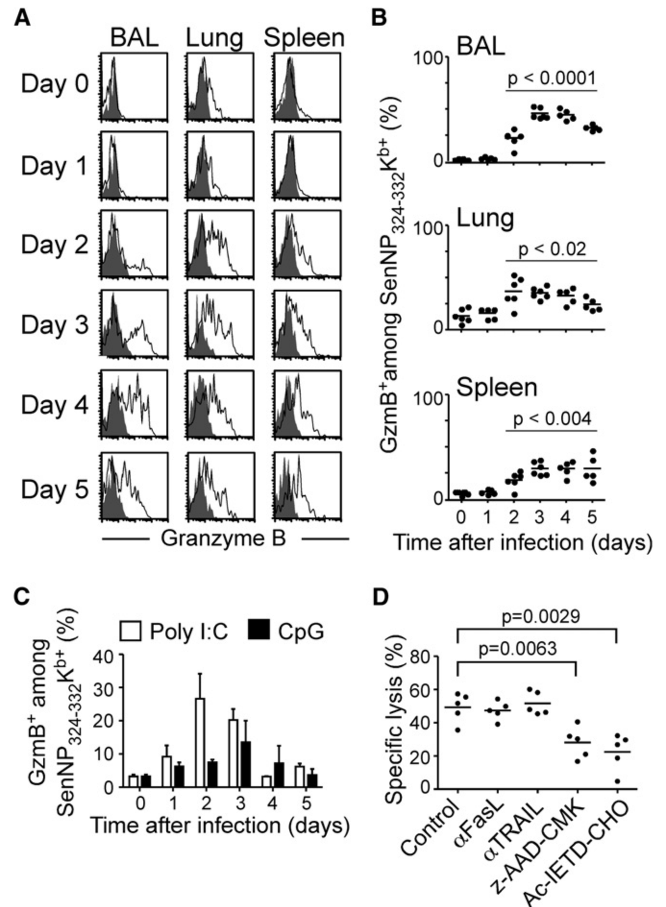

Figure 2. Granzyme B Protein Expression Is Upregulated in Memory $CD8^+$ T Cells in Response to Inflammation

C57Bl/6 mice were infected with Sendai virus and rested for 45 days prior to challenge with x31 influenza virus.

(A) Representative staining of isotype (shaded histograms) or granzyme B (open histograms) antibodies in the BAL, lung, and spleen on days 0–5 post-x31 challenge. All plots are gated on $SenNP_{324-332}K^{b+}$ cells.

(B) The frequency of $gzmB^+$ cells among total $SenNP_{324-332}K^{b+}$ cells in the BAL, lung, and spleen. Each symbol represents an individual mouse and significance was determined by comparing each time point with the values from day 0.

(C) The frequency of of $gzmB^+$ cells among total $SenNP_{324-332}K^{b+}$ cells (mean ± SD) in the BAL after intranasal administration of Poly I:C (open bars) or CpG (closed bars).

(D) Sorted $CD8^+$ T cells from the BAL of Sendai memory mice on day 3 after influenza challenge were incubated with congenic targets at a 4:1 E:T ratio with the indicated inhibitors of cytotoxicity pathways. Each symbol represents the percent specific lysis of a replicate well.

The data represent four independent experiments with at least five mice per group (A and B), three independent experiments with four mice per group (C), or two independent experiments with five wells per treatment (D).

Signaling through Ifnar1 Is Required for the Antigen-Independent Expression of Granzyme B

The finding that gzmB protein expression in memory T cells did not require cognate antigen stimulation and that gzmB expression was observed in cells located in noninfected tissues suggested that inflammatory cytokines could be playing a role in the regulation of gzmB expression. To address this question,

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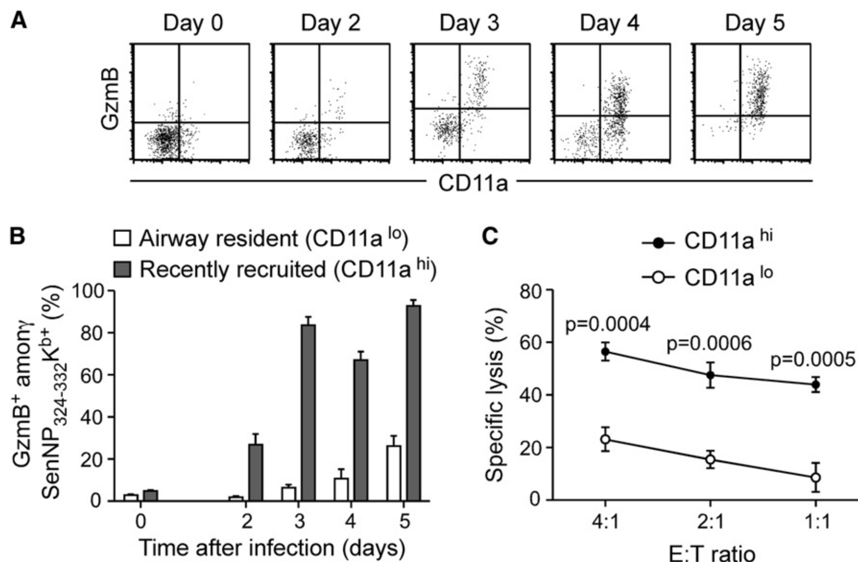

Figure 3. Granzyme B Protein Expression and Memory T Cell Lytic Activity Is Limited to Cells Recently Recruited to the Airways

(A) C57Bl/6 mice were infected with Sendai virus and rested for 45 days prior to challenge with x31 influenza virus. Plots are gated on $SenNP_{324\text{-}332}K^{b+}$ cells and show the expression of gzmB in airway resident ($CD11a^{lo}$) and recently recruited ($CD11a^{hi}$) Sendai memory $CD8^+$ T cells on days 0–5 after influenza x31 challenge. Quadrant gates were set based on isotype staining.

(B) The frequency of airway-resident (open bars) and recently recruited (shaded bars) Sendai-specific cells is graphed as the mean ± SD for days 0–5 after influenza challenge.

(C) $CD8^+$ T cells from the BAL of Sendai memory mice on day 3 after influenza challenge were sorted into airway-resident and recently recruited populations based on CD11a expression. The percent specific lysis of SenNP-pulsed targets is graphed as the mean ± SD for each population at the indicated E:T ratios.

The data are representative of three independent experiments with four mice per group (A and B) or five replicate wells per group (C).

we generated mixed bone marrow (BM) chimeric mice in which one population of memory $CD8^+$ T cells lacked expression of STAT1, which is required for signaling through several cytokine receptors associated with inflammatory responses. As expected, both WT ($CD45.2^-$) and *Stat1*$^{-/-}$ ($CD45.2^+$) Sendai-specific memory T cells expressed low amounts of gzmB protein prior to influenza virus challenge (Figure 4A, top). However, whereas WT cells had increased gzmB protein expression by 3 days after challenge, *Stat1*$^{-/-}$ cells showed no increase in gzmB protein (Figure 4A, bottom). This discrepancy was true for all tissues examined, as indicated by the fact that there was a significant increase in the frequency of WT $gzmB^+$ cells in the BAL, lung, and spleen (Figure 4B). Importantly, gzmB expression in *Stat1*$^{-/-}$ effector T cells was similar to WT effector T cells (Figure S2). Thus, STAT1 signaling is required specifically for the antigen-independent expression of gzmB protein in memory $CD8^+$ T cells.

Among the proinflammatory cytokines that are produced during a respiratory virus infection, type I IFNs, IFN-γ, and IL-27 each bind to receptors that require STAT1 for signal transduction (Durbin et al., 1996; Meraz et al., 1996; Nguyen et al., 2000; Takeda et al., 2003). To determine whether these individual cytokine receptors are important for the antigen-independent regulation of gzmB protein, we generated mixed BM chimeras to compare WT and cytokine receptor-deficient cells in vivo. As shown in Figure 5, Sendai-specific memory T cells that lack Ifnar1 failed to express gzmB protein on day 3 after challenge in all tissues (Figures 5A and 5B), but there was no defect observed in cells that lacked the IFN-γ receptor (Ifngr1) (Figures 5A and 5C) or IL-27R (Figures 5A and 5D). To assess the functional importance of Ifnar1 deficiency on the lytic activity of memory T cells recruited to the lung airways, we sorted WT and *Ifnar1*$^{-/-}$ $CD8^+$ cells from the airways of mixed BM chimeras on day 3 after challenge. Both WT and *Ifnar1*$^{-/-}$ Sendai-specific cells show similar recruitment to the airways based on CD11a staining (Figure 5E). Also, the *Ifnar1*$^{-/-}$ Sendai-specific cells show greatly reduced CD69 expression, which is in agreement with previous findings (Tough et al., 1996). Importantly, when compared on a per cell basis, sorted *Ifnar1*$^{-/-}$ cells were significantly inferior to WT cells at killing their specific targets (Figure 5F). Together, these data demonstrate that signaling through Ifnar1 is required for both the antigen-independent expression of gzmB protein and the enhanced lytic activity of memory T cells in the lung airways after viral challenge.

To assess whether type I IFNs alone were sufficient to induce gzmB protein expression in memory $CD8^+$ T cells, we treated splenocytes from Sendai memory mice in vitro with IFN-α or IFN-β for 36 hr. As shown in Figures 6A and 6B, the frequency of Sendai-specific memory $CD8^+$ T cells expressing gzmB protein significantly increased after incubation with either IFN-α or IFN-β when compared to media alone. To determine whether type I IFNs had a similar effect on gzmB protein expression in human virus-specific memory $CD8^+$ T cells, we analyzed peripheral blood samples from HLA-$A2^+$ donors with known reactivity to the immunodominant influenza M1 epitope (Moss et al., 1991). As expected, FluM1-specific cells had a predominantly effector memory T cell phenotype and individual donors showed considerable variability in the expression of gzmB protein (Figures 6C and 6D). Nevertheless, culturing PBMCs in the presence of IFN-α or IFN-β resulted in a significant increase in the frequency of $gzmB^+$ FluM1-specific cells when compared to control samples from the same donor (Figures 6E and 6F). Thus, type I IFNs are sufficient to induce gzmB protein expression in both murine and human virus-specific memory $CD8^+$ T cells.

Ifnar1$^{-/-}$ Memory T Cells Provide Significantly Less Protection during Secondary Challenge

The data thus far have focused on the antigen-independent regulation of gzmB protein expression after challenge with an unrelated virus. To assess the importance of Ifnar1 signaling for the rapid expression of gzmB in memory $CD8^+$ T cells when

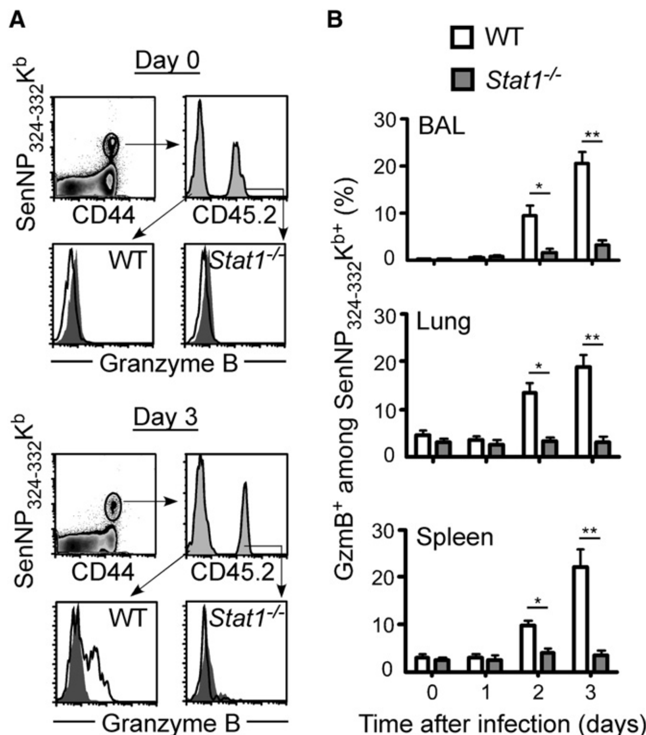

Figure 4. STAT1 Is Required for the Antigen-Independent Expression of gzmB Protein in Memory CD8⁺ T Cells

Mixed BM chimeras were generated from WT congenic ($CD45.2^{-}$) and $Stat1^{-/-}$ donors, infected with Sendai virus, and rested for 45 days prior to influenza x31 challenge.

(A) Representative staining of gzmB protein in WT and $Stat1^{-/-}$ Sendai-specific memory $CD8^{+}$ T cells in the lung prior to (day 0, top) or on day 3 after (bottom) influenza challenge. The shaded histograms are isotype staining, whereas the open histograms are gzmB staining.

(B) The frequency of WT (open bars) and $Stat1^{-/-}$ (shaded bars) Sendai-specific memory $CD8^{+}$ T cells expressing gzmB at the indicated time points is graphed as the mean ± SD (*$p < 0.05$; **$p < 0.005$). The data are representative of three independent experiments with three to five mice per time point.

antigen is present, we primed WT × $Ifnar1^{-/-}$ mixed BM chimeras with influenza PR8 to generate flu-specific cellular immunity and challenged the mice with heterosubtypic influenza x31. $FluNP_{366-374}D^{b}$-specific memory T cells that lack the Ifnar1 receptor show significantly decreased expression of gzmB on day 3 after challenge compared to WT cells, even though cognate antigen is present (Figure 7A). To investigate the relevance of antigen-independent gzmB expression for the early control of viral replication, we generated separate groups of complete chimeras reconstituted with either WT or $Ifnar1^{-/-}$ BM. In this case, chimeras were generated by transferring WT or $Ifnar1^{-/-}$ BM that had not been mixed into irradiated WT hosts. This approach was necessary to ensure that Ifnar1 was expressed on lung epithelial cells in each group (Goodman et al., 2010). Mice were then infected with influenza PR8 or Sendai virus and challenged with influenza x31. Prior to or on day 3 after challenge with influenza x31, we sacrificed mice to confirm that there were equivalent numbers of virus-specific memory $CD8^{+}$ T cells between groups (Figure S3). As expected, both WT and $Ifnar1^{-/-}$ PR8 memory mice showed significant protection compared to their Sendai memory counterparts (Figure 7B). However, PR8 memory mice reconstituted with $Ifnar1^{-/-}$ BM showed a significantly decreased ability to control viral loads (approximately 10-fold higher viral titers) compared to PR8 memory mice reconstituted with WT BM. Together, these data show that the type I interferon-dependent expression of gzmB protein enables memory $CD8^{+}$ T cells in the lung airways to more rapidly kill infected targets and contributes to early cell-mediated control of viral replication.

DISCUSSION

Although memory T cells that reside in nonlymphoid tissues have been shown to provide an immediate response to pathogen challenge, the mechanism by which memory $CD8^{+}$ T cells in the lung airways confer protection after respiratory virus challenge has not been described. In the present study, we demonstrate that inflammation induced by virus infection had a systemic effect on the circulating memory $CD8^{+}$ T cell pool, leading to the antigen-independent expression of gzmB protein. By employing mixed BM chimeric mice, we found that gzmB protein expression in these cells was dependent on STAT1 and requires signaling through Ifnar1. After the type I interferon-dependent expression of gzmB, these cells are then recruited to the airways where they display markedly enhanced lytic activity. Notably, $Ifnar1^{-/-}$ memory T cells are defective in their ability to limit early viral replication during a secondary challenge, demonstrating the importance of this process for cell-mediated immunity in the lung. Together, the data show that T cell-mediated protection to respiratory virus challenge is not merely due to increased antigen-specific cell numbers at the site of infection, but rather due to qualitative differences between airway-resident and recently recruited virus-specific memory $CD8^{+}$ T cells through the antigen-independent regulation of cytolytic protein expression.

Our data showed that the type I interferon-dependent expression of gzmB protein was required for memory T cells in the airways to rapidly destroy antigen-specific targets. Interestingly, this enhanced cytotoxicity was limited to cells recruited to the airways in response to inflammation and was not observed in cells present in the airways at the time of challenge. It is possible that the unique environment of the airways limits the ability of airway-resident cells to respond to inflammation. For example, pulmonary surfactants have been shown to inhibit T cell proliferation and suppress T cell activation (Borron et al., 1998; Wright, 2005). In contrast, circulating memory T cells unconstrained by the immunosuppressive effects of surfactants can respond to type I interferons by expressing gzmB protein prior to their arrival in the airways. Our data support this model, because the appearance of gzmB-expressing cells in the airways coincided with expression of gzmB in distant sites such as the spleen. Currently it is unclear why airway-resident memory T cells do not express gzmB after virus challenge, or how this lack of expression impacts the efficacy of the recall response. It should be noted, however, that transferring airway-resident memory T cells of the proper antigen specificity into the airways of naive mice does provide protection from a secondary challenge (Hogan et al., 2001b). In addition, other infection models have shown that nonmigratory memory T cells in peripheral tissues, defined

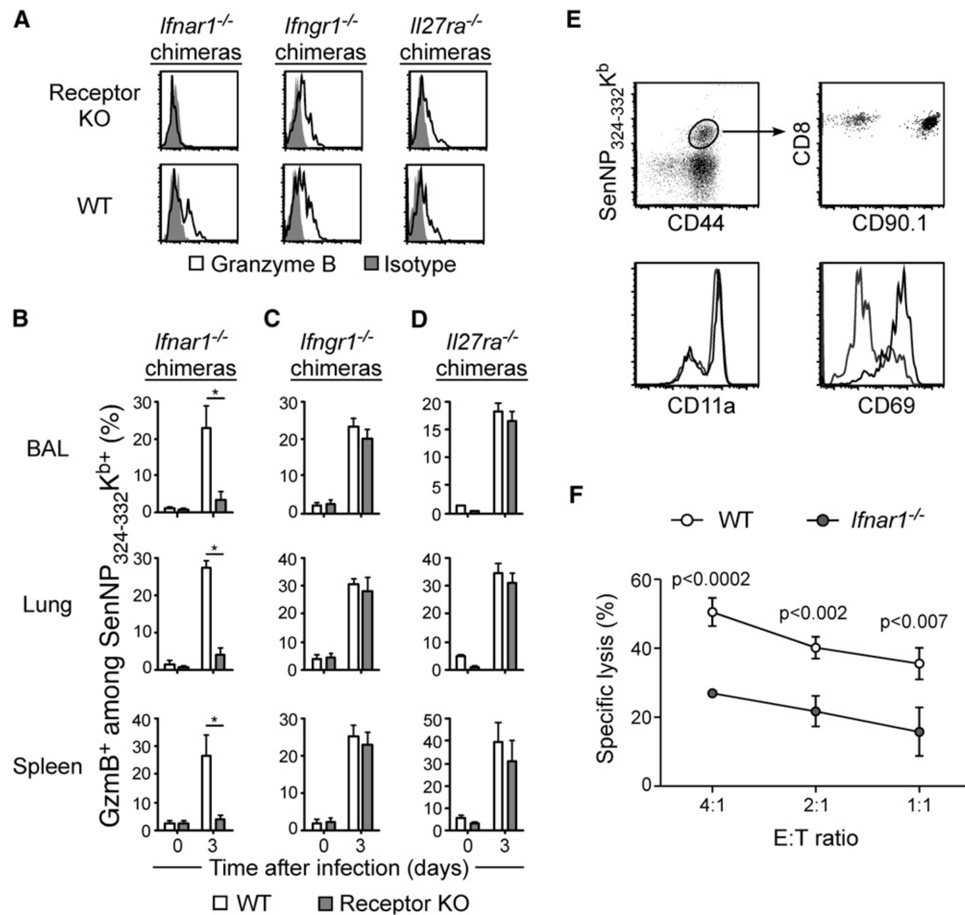

Figure 5. Signaling through Ifnar1 Is Necessary for Antigen-Independent gzmB Protein Expression and Lytic Activity of Memory CD8⁺ T Cells in the Lung Airways

Mixed BM chimeras were generated from WT and cytokine receptor-deficient donors (*Ifnar1*$^{-/-}$, *Ifngr1*$^{-/-}$, and *Il27ra*$^{-/-}$), infected with Sendai virus, and rested 45–60 days prior to influenza x31 challenge.

(A) Representative isotype (shaded histgrams) and gzmB (open histograms) staining of cytokine receptor-deficient (top) and WT (bottom) Sendai-specific memory CD8$^+$ T cells in the airways from each group of chimeras at day 3 post-x31 challenge. Histograms are gated on SenNP$_{324-332}$K^{b+} cells.

(B–D) The frequency of WT (open bars) and cytokine receptor-deficient (shaded bars) gzmB$^+$ Sendai-specific memory T cells (mean ± SD) in various tissues prior to or on day 3 after x31 challenge for (B) *Ifnar1*$^{-/-}$, (C) *Ifngr1*$^{-/-}$, and (D) *Il27ra*$^{-/-}$ mixed BM chimeras (*$p < 0.01$).

(E) Phenotypic analysis of WT (CD90.1$^+$, black) and *Ifnar1*$^{-/-}$ (CD90.1$^-$, gray) Sendai-specific memory CD8$^+$ T cells in the airways of mixed BM chimeras on day 3 after x31 challenge.

(F) WT and *Ifnar1*$^{-/-}$ CD8$^+$ T cells were sorted from the airways of mixed BM chimera Sendai memory mice on day 3 after x31 challenge. The percent specific lysis of SenNP-pulsed targets after a 5 hr incubation with sorted WT (open circles) or *Ifnar1*$^{-/-}$ (shaded circles) CD8$^+$ T cells is graphed as the mean ± SD for each population at the indicated E:T ratios.

The data are representative of three independent experiments with at least five mice per time point.

by expression of CD103, are important for limiting the viral replication, and this phenotype is similar to airway-resident memory T cells (Gebhardt et al., 2009; Kohlmeier et al., 2007). Thus, it is possible that memory T cells present in the airways at the time of challenge utilize different mechanisms, such as cytokine or chemokine production, to contribute to the recall response.

We had previously shown that the rapid recruitment of memory CD8$^+$ T cells to the airways after virus challenge was dependent on the expression of CCR5, which was required for optimal T cell-mediated protection (Kohlmeier et al., 2008). Although CCR5 was expressed on only a small frequency of resting memory CD8$^+$ T cells, viral challenge increased surface expression of CCR5 on memory T cells in the spleen and enabled these cells to migrate to CCR5 ligands. It should be noted that our data show that signaling through Ifnar1 does not impact memory T cell recruitment, as demonstrated by equivalent recruitment in mixed BM chimeras and a similar increase in CCR5 expression on WT and *Ifnar1*$^{-/-}$ memory T cells after challenge (data not shown). Rather, the importance of signaling through Ifnar1 is specific for the enhancement of memory CD8$^+$ T cell gzmB expression and lytic activity. Thus, the enhanced recruitment and augmented effector functions observed after respiratory virus challenge, although both are the result of inflammation, are controlled by independent processes.

During respiratory infections, innate recognition of virus in the lung leads to the early production of cytokines in sufficient

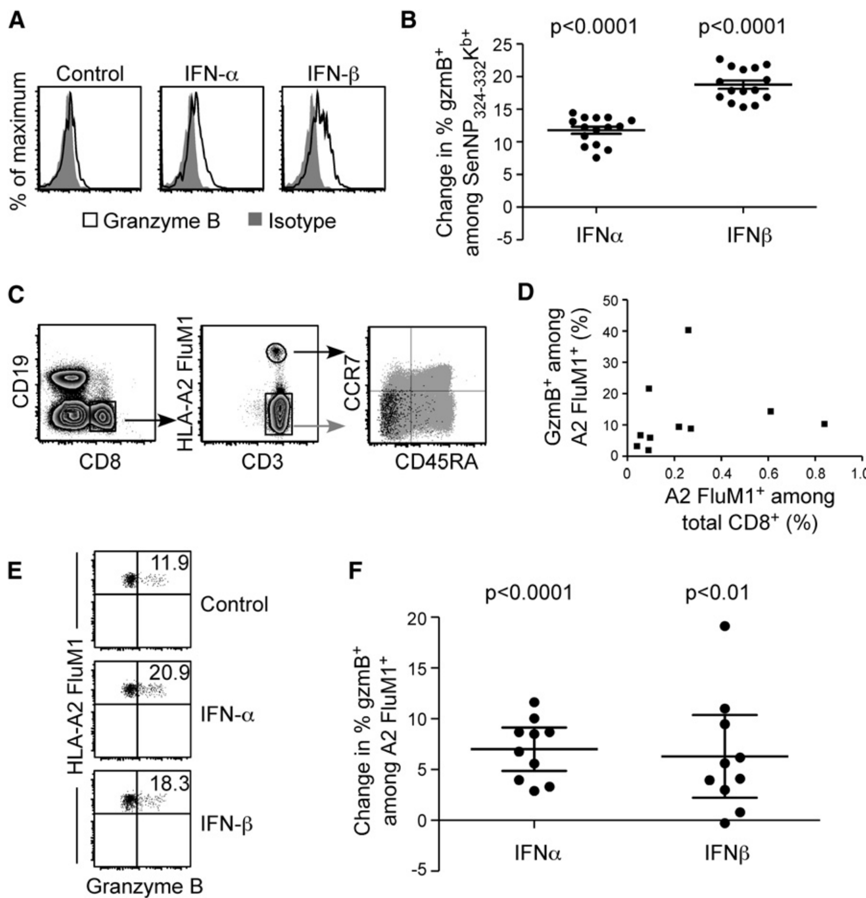

Figure 6. Type I Interferons Are Sufficient to Induce gzmB Protein Expression in Both Murine and Human Virus-Specific Memory CD8+ T Cells

(A) C57BL/6 mice were infected with Sendai virus and rested for 45 days. Splenocytes from these mice were incubated in vitro with or without IFN-α or IFN-β (2×10^4 units/ml) for 36 hr and gzmB protein expression was measured by flow cytometry. Representative staining is shown for isotype (shaded histogram) or anti-gzmB (open histogram) gated on $SenNP_{324-332}K^{b+}$ cells.

(B) The percent change in gzmB expression was graphed for IFN-α or IFN-β treatment compared to control samples. Each symbol represents an individual mouse and statistical significance was determined via a paired t test (lines represent the mean ± SD).

(C) Representative staining and gating strategy to identify influenza-specific memory CD8+ T cells from the peripheral blood of healthy donors.

(D) The frequency of A2 FluM1-specific cells among total CD8+ T cells versus the frequency of gzmB+ FluM1-specific cells is graphed for each individual donor prior to in vitro stimulation.

(E) Representative staining of gzmB protein in A2 FluM1+ cells after in vitro culture with or without IFN-α or IFN-β (2×10^4 units/ml).

(F) The percent change in gzmB expression was graphed for IFN-α or IFN-β treatment compared to control samples. Each symbol represents an individual donor and statistical significance was determined via a paired t test (lines represent the mean ± SD).

The data are representative of three (A and B) or two (C–F) independent experiments.

quantities to be detected both at the site of infection and in the circulation (Hayden et al., 1998). For influenza viruses, nonstructural protein 1 (NS1) has been shown to interfere with innate detection of virus infection (Fernandez-Sesma et al., 2006; Wang et al., 2000). This interference grants the virus up to 2 days of unimpeded replication before the sudden expression of cytokines and chemokines, including type I interferons (Moltedo et al., 2009). In agreement with these findings, we first detect type I interferon-dependent gzmB protein expression in memory CD8+ on day 2 after influenza challenge. These data illustrate an important link between the innate and adaptive immune systems, whereby the initial inflammatory “danger” signals serve not only to induce a program of recruitment to the infected site, but also to prime memory T cells prior to their arrival to enable the most rapid and efficient responses.

mRNA transcripts for cytolytic molecules, namely perforin and granzymes, have been found in both quiescent NK and memory CD8+ T cells. However, the mechanisms controlling the persistence of cytolytic transcripts, and the potential importance of these transcripts for recall responses, is unclear. Recently, several studies have shown that cytokine signals received during initial T cell priming lead to chromatin remodeling of the gzmB locus that in turn allows for sustained expression (Agarwal et al., 2009; Juelich et al., 2009). Single cell analysis revealed that gzmB transcripts were not present in all memory CD8+ T cells, suggesting that the memory T cell pool is a mixture of cells that received different instructional signals during initial priming. Furthermore, the maintenance of transcripts after clearance of acute viral infection did not correlate with cytolytic protein unless the cell was subjected to chronic, intermittent antigen stimulation (Mintern et al., 2007). Our findings demonstrate that memory CD8+ T cells can respond to an inflammatory environment through the antigen-independent expression of gzmB protein. However, although gzmB protein expression is antigen independent, the ability of these cells to kill target cells is antigen dependent. These data may also explain the findings from a longitudinal study of FluM1-specific memory CD8+ T cells in humans, where a sudden increase in the frequency of FluM1-specific cells expressing gzmB was noted despite no evidence of influenza infection (Touvrey et al., 2009). It will be interesting in future studies to determine how long cytolytic transcripts are maintained in resting memory T cells, and whether this correlates with the decline of T cell-dependent protection. In addition, because previous studies have shown that cytotoxicity of virus-specific effector T cells from gzmB-deficient mice is unimpaired (Jenkins et al., 2008; Regner et al., 2009), it will be interesting to determine whether protein levels of other granzymes in memory T cells are modulated by interferon signaling.

In conclusion, we demonstrate that memory CD8+ T cells recruited to the lung airways during the early stages of a respiratory virus challenge display vastly enhanced lytic ability, even after challenge with an unrelated virus. This enhanced lytic ability

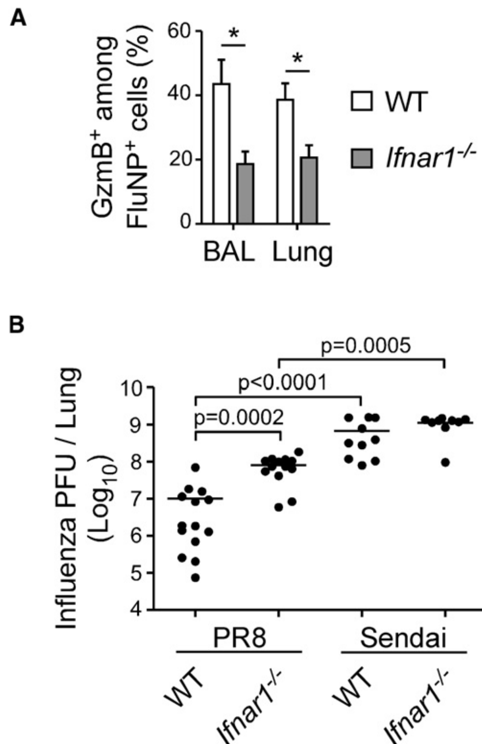

Figure 7. Ifnar1 Signaling Is Required for Maximal gzmB Protein Expression and T Cell-Mediated Protection when Cognate Antigen Is Present

WT × *Ifnar1*$^{-/-}$ mixed BM chimeras were infected with PR8 influenza and rested 45 days prior to challenge with x31 influenza.

(A) The frequency of gzmB^{+} cells among WT (open bars) or *Ifnar1*$^{-/-}$ (shaded bars) FluNP-specific T cells is graphed as the mean ± SD (*p < 0.02).

(B) Complete BM chimeras were generated with BM from either WT (C57BL/6) or *Ifnar1*$^{-/-}$ donors (BM was not mixed), infected with influenza PR8 or Sendai virus, and rested for 60 days. All mice were challenged with influenza x31 and viral titers were measured on day 3. Each symbol represents an individual mouse.

The data are representative of three (A, five mice per time point) or two (B) independent experiments.

is dependent on the antigen-independent expression of gzmB protein, which is mediated by signaling through Ifnar1 in both murine and human virus-specific memory CD8^{+} T cells. Importantly, type I interferons were critical for the rapid expression of gzmB protein even in the presence of cognate antigen, and this correlated with the efficacy of T cell-mediated protection. Thus, these data illustrate that, during a respiratory virus challenge, innate inflammatory cues act directly on memory CD8^{+} T cells to significantly enhance their lytic ability prior to arrival at the site of infection, leading to more rapid killing of antigen-specific targets and better control of early viral replication.

EXPERIMENTAL PROCEDURES

Mice, Viruses, and Infections

C57BL/6, B6.SJL-*Ptprca* Pep3/BoyJ (CD45.1), B6.PL-*Thy1*^{a}/CyJ (CD90.1), and B6.129S7-*Ifngr1*^{tm1Agt}/J (*Ifngr*$^{-/-}$) mice were purchased from The Jackson Laboratory and rederived stocks were maintained at the Trudeau Institute. *Stat1*$^{-/-}$ mice on the B6 background were generously provided by J. Durbin (Columbus Children's Research Institute), *Ifnar*$^{-/-}$ mice were generously provided by J. Sprent (Garvan Institute of Medical Research) and backcrossed to B6 mice for 10 generations, and *Il27ra*$^{-/-}$ mice on the B6 background were generously provided by N. Ghilardi (Genentech) and A. Cooper (Trudeau Institute). Mixed BM chimeras were generated by transferring a 50:50 mixture of 2 × 10^7 BM cells from gene-deficient and WT congenic mice (1 × 10^7 BM cells from each donor) into lethally irradiated (950 rads) congenic recipients. In some experiments, chimeras were reconstituted with only WT or *Ifnar*$^{-/-}$ BM (2 × 10^7 cells). Chimeras were maintained on medicated food for 4 weeks and infected at 6–8 weeks postreconstitution. Sendai virus (Enders strain), Influenza x31 (H3N2), and Influenza PR8 (H1N1) were grown, stored, and titered as previously described (Hou et al., 1992). For intranasal infections, mice were anesthetized with 2,2,2-tribromoethanol (200 mg/kg) and administered 250 (Sendai), 600 (PR8), or 30,000 (x31) 50% egg infectious doses in a volume of 30 μl. All animal studies were approved by the Trudeau Institute Animal Care and Use Committee.

Human PBMCs

Cryopreserved PBMCs collected from healthy donors were purchased from Cellular Technology Ltd. Samples were prescreened by ELIspot to select for HLA-A2^{+} donors with reactivity to the immunodominant Influenza A $M1_{58\text{-}66}$ epitope (GILGFVFTL).

Reagents and In Vitro Culture

Poly I:C and CpG-B (Invivogen) were administered to mice intranasally (15 μg in 30 μl). Murine splenocytes were cultured in complete tumor medium for 36 hr with or without IFN-α or IFN-β (2 × 10^4 units/ml, PBL Interferon Source). Human PBMCs were cultured in RPMI 1640 + 10% FBS for 36 hr with or without IFN-α or IFN-β (2 × 10^4 units/ml, PBL Interferon Source).

Tissue Harvest and Flow Cytometry

Mice were sacrificed at the indicated times and cells were isolated from the lung airways by bronchoalveloar lavage (BAL), the lung parenchyma by digestion in collagenase/DNase for 1 hr at 37°C followed by percoll gradient centrifugation, and the MLN and spleen by mechanical disruption. After red blood cell lysis with ammonium buffered chloride, live cell numbers were determined by counting and trypan blue exclusion. Single cell suspensions were incubated with Fc-block (anti-CD16/32) for 15 min on ice followed by staining with $SenNP_{324\text{-}332}K^b$ or $FluNP_{366\text{-}374}D^b$ tetramers for 1 hr at room temperature. Tetramer-labeled cells were stained with antibodies to CD8, CD11a, CD44, CD45.1, CD45.2, CD69, and CD90.1 (eBiosciences). For intracellular staining, cells were fixed and permeabliized with the Cytofix Cytoperm kit (BD Biosciences) prior to staining with an antibody to granzyme B (Caltag and BD Biosciences) or appropriate isotype control. For human PBMCs, cells were stained with a Pro5 HLA-A2 Flu $M1_{58\text{-}66}$ pentamer (ProImmune) for 20 min at room temperature prior to staining with antibodies to CD3, CD8, CD19, CD45RA, and CCR7 (BD Biosciences) for 30 min on ice. Intracellular staining was performed as described above. Samples were run on FACSCalibur or FACSCanto II flow cytometers and data analyzed with FlowJo software (Tree Star). Aseptic cell sorting was performed on a FACSVantage with DiVa electronics.

In Vitro CTL Assay

Memory CD8^{+} T cells were harvested from the lung airways and purified by sorting on CD8α^{+} lymphocytes. In some experiments, airway-resident memory cells were separated from recently recruited memory cells by sorting on CD11a expression. In experiments with mixed BM chimeras, WT and *Ifnar*$^{-/-}$ memory CD8^{+} T cells were separated by sorting on CD90.1 or CD90.2. Congenic target cells (CD45.1^{+}) were prepared by pulsing splenocytes with $SenNP_{324\text{-}332}$ or γHV $p79_{524\text{-}531}$ peptides for 4 hr at 37°C. p79-pulsed targets were then labeled with 2.5 μM CFSE, and the SenNP- and p79-pulsed targets were mixed together at a 1:1 ratio. One thousand total target cells were added to a v-bottom 96-well plate, and sorted memory cells were added at an effector:target ratio of 4:1, 2:1, or 1:1. Target cells incubated alone or with memory cells of the wrong specificity (from the airways of influenza memory mice) served as negative controls. The plate was gently centrifuged (300 × g for 1 min) to bring effector and target cells into contact and incubated for 5 hr at 37°C. In some experiments, samples were incubated with 10 μg/ml anti-Fas

ligand (Biolegend), 10 μg/ml anti-TRAIL (Biolegend), 100 μM z-AAD-CMK (EMD), or 100 μM Ac-EITD-CHO (BD Bioscience) to selectively inhibit different cytolytic pathways. The ratio of live specific targets to live nonspecific targets was assessed by flow cytometry by gating on propidium iodide$^-$, CD45.1$^+$, CFSE$^{+/-}$ cells. Specific lysis was calculated with the formula: [1 − (ratio of targets only/ratio of targets + BAL cells)] × 100.

Measurement of Viral Titers

Homogenates from whole lung tissue were generated by mechanical disruption and stored at −70°C. Virus titers were measured by infecting MDCK monolayers with serial 10-fold dilutions of lung suspension in duplicate. Twenty-four hours after infection, monolayers were washed and fixed with ice-cold 80% acetone in water. Infected cell clusters were detected with biotin-labeled mouse anti-influenza A monoclonal antibody (Chemicon), followed with streptavidin-AP, and visualized with Sigma Fast BCIP/NBT substrate (Sigma). The number of plaque-forming units (PFUs) were determined microscopically, and the data are shown as the PFU/lung.

Statistics

Statistical analysis was performed with Prism 5 software (Graphpad). Unless otherwise noted in the figure legend, an unpaired two-tailed Student's t test was used to determine significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.immuni.2010.06.016.

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