Differentiation-Dependent Differences in Murine T Cell Susceptibility to Negative Regulation by the Lung

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A large number of viral infections are contracted via the respiratory route. Thus, an effective immune response at this site is of vital importance. Past studies in murine models analyzing a number of viruses have reported that CD8⁺ effector T cells entering the lung after respiratory infection exhibit significant functional inactivation. The impaired function in these cells has been proposed to be the result of infection-induced changes in the lung; however, we have found that loss of function can occur in effector CD8⁺ T cells present in the lung, even in the absence of infection. This functional inactivation takes place within 48 hours of entry into the lung, and is seen only in effector cells residing in the lung parenchyma, and not the airway. In this study, we have extended our findings to show that functional impairment of these effector cells is not initiated by bone marrow-derived cells, and is independent of proliferation in the lung tissue. Of critical importance, we have also determined that the susceptibility to functional inactivation is a common property shared by most effector cells. Finally, we show that the susceptibility to loss of function is actively regulated throughout differentiation. Although naive CD8⁺ T cells, like effector cells, are negatively regulated as a result of residence in the lung, memory cells exhibit profound resistance to functional inactivation. The selective resistance of CD8⁺ memory cells may allow the host to limit damage during the effector phase while retaining a protective response that can effectively limit subsequent infection.

Keywords: T cell regulation; lung; T cell differentiation; memory

The presence of functional CD8⁺ effector T cells is crucial for clearing virus from the lung tissue. These cells, upon reaching the lung, exert potent effector functions, including the release of IFN- γ and TNF- α , as well as the ability to lyse infected cells. Past studies, including those with respiratory syncytial virus, mouse pneumovirus, and simian virus (SV) 5, have reported that virus-specific CD8⁺ effector T cells entering the lung as a result of respiratory infection undergo significant loss of function (1–5). These cells have not altered their cytokine profile from typical Tc1 cells, as they produce neither IL-4 nor IL-10 (Refs. 1, 5, and our unpublished data). The impaired function in these cells had been proposed to be the consequence of infection-induced changes in the lung or of direct action by a viral protein (1, 2).

Our research efforts have provided data supporting a third model, wherein the basal lung environment harbors a signal that can “turn off” the ability of CD8⁺ T cells to respond (6). This phenomenon has since been observed in other studies (5). This nonresponsiveness encompasses all effector functions assessed (i.e., IFN- γ and TNF- α production), as well as secretion of lytic granules (1). Interestingly, in our studies, functional inactivation

CLINICAL RELEVANCE

CD8⁺ effector T cells are critical for the clearance of respiratory pathogens. However, negative regulation of their function occurs after entry into the lung, which likely has profound effects on their efficacy. In contrast, the resistance exhibited by memory T cells to functional inactivation in the lung may limit tissue damage while retaining a protective response.

was restricted to effector cells residing in the lung parenchyma and not the airway space (6).

Here, we extend our findings with a number of pivotal observations that bring us closer to identifying the mechanism by which CD8⁺ T cell responses are regulated in the lung. We have observed that the susceptibility to loss of function appears to be a general property of CD8⁺ effector cells (i.e., there is no evidence for a subpopulation that is resistant to negative regulation in the lung). Importantly, we demonstrate that the functional impairment of these effector cells is not initiated by bone marrow-derived cells, suggesting that the signal originates from a parenchymal-specific cell type either through direct cellular contact or a localized, secreted molecule. Surprisingly, although effector cells appear generally susceptible to functional inactivation, this is not the case for all T cell differentiation states, as memory CD8⁺ T cells are resistant to the regulatory effect of the lung. We propose that this differentiation state-specific susceptibility may be a mechanism by which the host can limit damage to the vulnerable tissue of the lung, yet still maintain the ability to respond to future pathogen exposure.

MATERIALS AND METHODS

Mice and Infections

Female BALB/c mice were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Thy1.1⁺ CL4 transgenic mice (7) were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in our facility. Mice were anesthetized with Avertin (2,2,2-tribromoethanol) by intraperitoneal injection. Anesthetized mice were then infected intranasally with the indicated dose of virus in 50 μ l of PBS. All research performed on mice in this study complied with federal and institutional guidelines set forth by the Wake Forest University (Winston-Salem, NC) Animal Care and Use Committee.

Generation and Maintenance of CTL Lines

Spleen cells (5×10^5 cells/well) from mice previously infected with SV5 were cocultured in complete media in the presence of 10^{-7} M M_{285–293} peptide-pulsed, irradiated BALB/c spleen cells (5×10^6 cells/well), and 10% T-stim (BD Biosciences, Franklin Lakes, NJ) as a source of IL-2. Cells from these cultures were restimulated weekly to maintain the line.

Tissue Preparation

For bronchoalveolar lavage (BAL), the trachea was cannulated with a syringe and 1 ml of PBS was used to collect the airway lymphocytes. Lungs were then isolated and incubated at 37°C for 1 hour in the presence of 1 mg/ml of collagenase D (Roche, Indianapolis, IN). After digestion,

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cells were passed over a ficoll gradient to isolate mononuclear cells. Mediastinal lymph nodes (MLN) and spleens were isolated and single cell suspensions made by mechanical dissociation.

Generation of Hemagglutinin Specific Memory CD8⁺ T Cells

Spleen cells from naive CL4 transgenic mice (10^6) were intravenously administered to BALB/c recipients. At 24 hours after transfer, mice were intranasally infected with 500 egg infective dose (EID) of Puerto Rico 8 (PR8) influenza virus in 50 μ l PBS. Starting at 40 days after infection, spleen and MLNs were removed and CL4 memory cells isolated by sorting for CD8⁺Thy1.1⁺ expression. In some cases, cells were labeled with 5 μ M carboxy-fluorescein diacetate, succinimidyl ester (CFSE).

Isolation and Transfer of CD8⁺IFN- γ ⁺ Cells from SV5-Infected Lungs

Single-cell suspensions of the lung tissue were stimulated for 4 hours with M₂₈₅₋₂₉₃ tetramer at 37°C. M₂₈₅₋₂₉₃ tetramer⁺ T cells producing IFN- γ were identified using the MACS mouse IFN- γ secretion assay kit (Miltenyi Biotec, Auburn, CA). Tetramer⁺CD8⁺IFN- γ ⁺ cells were sorted with the BD FACS Aria high speed cell sorter (BD Biosciences). The five sorts averaged 90% purity for the tetramer⁺CD8⁺IFN- γ ⁺ population. Recipient mice were anesthetized with Isoflurane (Hospira, Chicago, IL) by inhalation, and cells resuspended in PBS were transferred intratracheally in a volume of 50 μ l and/or intravenously in 100 μ l.

Flow Cytometric Analysis of CD8⁺ T Cells

M₂₈₅₋₂₉₃-specific CD8⁺ T cells isolated from mice after adoptive transfer were identified by staining with tetramer or CFSE, as indicated, together

with anti-CD8 α antibody (clone 53-6.7; BD Biosciences). Thy1.1⁺ CL4 memory T cells were identified by anti-Thy1.1 and anti-CD8 α antibody staining. For cytokine analysis, isolated cells were cultured for 5 hours in the presence or absence of peptide (10^{-6} M), and in the presence of Golgiplug. Peptide was added directly to isolated lung cells, as the presence of additional antigen presenting cells (APC) does not increase the percent of functional cells detected (Ref. 1; and our unpublished data). Cytokine production was assessed by intracellular cytokine staining (ICCS) using anti-IFN- γ and/or anti-TNF- α antibodies.

RESULTS

CD8⁺ T Cells Are Generally Susceptible to Loss of Function after Entry into the Lung

In our analyses of adoptively transferred established CD8⁺ effector lines or *in vivo* generated effectors, our laboratory routinely observes populations of functional as well as nonfunctional cells in the lung parenchyma (1, 6). For example, in our analyses of SV5-specific cells that enter the lung after infection, we routinely observe approximately 50% of the effector cells exhibiting function at Day 12 after infection (1, 6). Given the presence of a functional population under these circumstances, we hypothesized that effector cells may exhibit heterogeneity with regard to the susceptibility to the as-yet unidentified mediator responsible for the functional impairment, and that a subpopulation of effectors may exist that is resistant to the inactivating signal. To address this question, BALB/c mice were infected intranasally

Figure 1. Effector T cells in the lung are generally susceptible to functional inactivation. BALB/c mice were intranasally infected with 10^6 plaque forming units (PFU) of simian virus (SV) 5. On Day 12 after infection, lung tissue was isolated and M₂₈₅₋₂₉₃ tetramer⁺ IFN- γ ⁺ were sorted with the BD FACS Aria after labeling using the Miltenyi mouse IFN- γ secretion assay as described in the MATERIALS AND METHODS section. Sorted cells (7×10^4 to 1.5×10^5) were transferred intratracheally into Thy1.1⁺ recipient mice. At 2 days after transfer, recipient mice were killed, and lung tissue prepared for analysis by flow cytometry. Transferred cells were identified by CD8⁺Thy1.2⁺ surface staining and function assessed by intracellular IFN- γ staining. (A) Average percentage of IFN- γ -positive cells in the sorted population (after-sort analysis of cells isolated after IFN- γ capture assay procedure) that was used for transfer is shown in the *black bar*. IFN- γ production by adoptively transferred Thy1.2⁺ cells after restimulation in the presence (+pep) or absence (-pep) of peptide after isolation from the lung 2 days after transfer is shown. The decrease in function in the trans-

ferred cells between the time of transfer (post-sort) versus 2 days *in vivo* (gray bar) was statistically significant ($***P < 0.001$). (B) Representative data for the post-sort and reisolated populations from one of the four experiments are shown. The numbers in the upper right quadrant represent the percentages of CD8⁺ effector cells that are IFN- γ positive. Statistical analysis was performed using a two-way ANOVA. i.t., intratracheal.

with 10^6 plaque forming units (PFU) of the paramyxovirus SV5. On Day 12 after infection, lung tissue was harvested and stimulated in the presence of 10^{-6} M $M_{285-293}$ ($M_{285-293}$ is the immunodominant epitope from SV5 [8]). Functional SV5 $M_{285-293}$ tetramer⁺CD8⁺ effectors were isolated using the Miltenyi mouse IFN- γ secretion kit. This method allowed for the purification of viable, IFN- γ -producing effector cells. Isolated populations were routinely greater than 90% pure (Figure 1A after sort bar, which shows flow cytometric analysis of sorted IFN- γ ⁺ cells in the absence of further manipulation). The sorted population was intratracheally transferred into naive Thy1.1⁺ recipient mice. Of note, we have previously shown that the cells transferred by the intravenous versus intratracheal route exhibit similar loss of function (6). The intratracheal route is used here, as it promotes higher cell numbers in the lung. At 48 hours after transfer, lung tissue was isolated and cultured in the presence of peptide. Functional cells were identified by IFN- γ staining, and transferred cells identified by CD8⁺Thy1.2⁺ expression. We found that only 41% of the transferred effector cell population maintained function compared with the 96% that were functional after sorting (Figures 1A and 1B). These data suggest that the effector cell population generated *in vivo* does not contain cells that are resistant to functional inactivation in the lung.

Loss of Function Is Not Directly Associated with Known Inhibitory Receptors

Based on these results, we were interested in characterizing the features of cells that had undergone loss of function. Previous reports have identified several receptors involved/associated with negative regulation of CD8⁺ T cells, including lymphocyte-activation gene 3 (9), 2B4 (10), cytotoxic T-lymphocyte-associated protein 4 (11), paired immunoglobulin-like receptor B, GP49 (12), natural killer receptor G2a (13), B- and T-lymphocyte-associated protein (14), and programmed cell death (PD-1) (15). We questioned whether expression of these regulatory receptors may correlate with nonfunctional cells found in the lung-resident CD8⁺ T cell population. $M_{285-293}$ -specific CD8⁺ effectors were generated by intranasal infection of BALB/c mice with 10^6 PFU of SV5. On Day 12 after infection, lung tissue was harvested and prepared for analysis by flow cytometry. Lung cells were stimulated using a combination of peptide and $M_{285-293}$ tetramer. This allowed concurrent activation and labeling of $M_{285-293}$ -specific cells. Functional ($M_{285-293}$ tetramer⁺ IFN- γ ⁺) and nonfunctional ($M_{285-293}$ tetramer⁺ IFN- γ ⁻) populations were identified after peptide stimulation *ex vivo*. In analyzing the expression levels of the inhibitory receptors, we found relatively homogeneous expression of the receptors on tetramer⁺ cells regardless of function, with the exception of PD-1 (Figure 2). Interestingly, the expression of this molecule was decreased on nonfunctional cells, counter to what would be expected if PD-1 ligation were involved in functional inactivation. Together, these findings indicate that these regulatory molecules are not associated with down-regulation of effector function in lung-resident CD8⁺ T cells.

Proliferation Does Not Correlate with Nonresponsiveness

In our previous studies of CFSE-labeled, adoptively transferred cells, we had observed some proliferation of effector cells, prompting us to question if loss of function in the CD8⁺ T cell effector pool correlated with division in the lung. The mechanism responsible for the proliferation in the lung is uncertain, but may simply be residual proliferation from the *in vitro* stimulation. Alternatively, these *in vitro*-generated effectors may respond to a homeostatic cytokine signal in the lung. Regardless, this observation allowed us to determine whether proliferation

Figure 2. Loss of function does not correlate with the expression of inhibitory receptors. $M_{285-293}$ -specific CD8⁺ effectors were generated by intranasal infection of BALB/c mice with 10^6 PFU of SV5. On Day 12 after infection, lung cells were stimulated in the presence of peptide and $M_{285-293}$ tetramer to concurrently label and activate $M_{285-293}$ -specific cells. At the conclusion of the culture period, cells were stained with anti-CD8 and anti-IFN- γ antibodies to identify functional and nonfunctional cells, as well as antibodies to the indicated molecules. No statistically significant differences were found in the expression levels of the tested inhibitory receptors between the IFN- γ ⁺ and IFN- γ ⁻ populations, with the exception of programmed cell death 1 (PD-1). * $P < 0.05$. These data are representative of three independent experiments. Statistical analysis was performed using a one-way ANOVA.

correlated with loss of function. BALB/c mice received an intratracheal transfer of an established $M_{285-293}$ -specific CD8⁺ line that was labeled with CFSE. At 4 days after transfer, BAL cells and parenchyma-resident cells were stimulated with $M_{285-293}$ peptide for the analysis of IFN- γ production. Transferred cells were identified by CD8⁺CFSE⁺. At the time of transfer, effector cells were almost entirely functional, with greater than 90% producing IFN- γ (Figure 3A, black bar). A similar high level of function was seen in the transferred cells recovered from the BAL (Figure 3), with, on average approximately 94% of the cells producing IFN- γ regardless of whether they had undergone division. In contrast, transferred cells isolated from the lung parenchyma were, on average, only 38% functional, as determined by IFN- γ production. However, similar to what we observed in cells isolated from the BAL, the level of function was similar in nondivided versus divided cells (38 and 39% functional, respectively; Figure 3). These data indicate that the loss of function observed in the CD8⁺ effector population does not correlate with entry into division.

Loss of Function Is Observed in the Absence of Radiosensitive Cells and Is Independent of CD11c⁺ Cells

Knowing that the dampening of the CD8⁺ T cell response is restricted to the parenchyma of the lung, we turned our focus toward identifying the components of the parenchyma that may be inducing loss of function. Initially, we hypothesized that a lung-resident immune cell could be mediating this effect given the large number of immunoregulatory factors produced by these cells. To test this hypothesis, irradiation was used to eliminate the majority of bone marrow-derived cells from the lung. To establish the kinetics for the loss of hematopoietic cells from the lung, BALB/c mice were left untreated or exposed to 900 rads of γ irradiation. At 24, 48, and 72 hours after irradiation, the mice were killed, and lung tissue was prepared for analysis by flow cytometry using a panel of cell surface markers to identify distinct cell types, including CD4⁺ and CD8⁺ T cells, macrophages (F4/

Figure 3. Loss of function is independent of proliferative status in the lung. BALB/c mice received an intratracheal transfer of cultured, carboxy-fluorescein diacetate, succinimidyl ester (CFSE)-labeled $M_{285-293}$ -specific $CD8^+$ T cells (4×10^6 to 6×10^6). At time of transfer, an aliquot of the effector cells was stimulated with $M_{285-293}$ peptide and IFN- γ production was determined (Pretransfer bar). Four days post-transfer, bronchoalveolar lavage fluid and parenchymal lung tissue were isolated. Adoptively transferred (AT) cells were identified by $CD8^+CFSE^+$ staining and the functional subpopulations were determined by IFN- γ staining after peptide stimulation. (A) Averaged percentages of IFN- γ positive cells in the divided versus non-divided populations. The decrease in function between the pre-transfer and all adoptively transferred (AT) effector cells (Total, Undivided, and Divided) from the lung tissue was highly significant ($***P < 0.001$). There was no significant difference (n.s.) between lung tissue derived cells that had divided versus not. Furthermore, IFN- γ production in cells isolated by bronchoalveolar lavage was similar to that in the pretransfer population. (B) Representative data are shown for the adoptively transferred population from one of four independent experiments performed. The numbers indicate the percentage of cells per quadrant. Statistical analysis was performed using a two-way ANOVA.

80), dendritic cells and alveolar macrophages ($CD11c$), neutrophils (Gr-1), and B cells ($CD19$). We also included $CD103$ as a marker of airway-resident dendritic cells (DC) and $CD11b$, which is expressed by parenchymal DC and macrophages. Figure 4 shows that maximal loss was observed at 24 hours after irradiation and maintained through the 72-hour time point. Knowing that maximal cell loss was apparent by 24 hours, and that there was no recovery for the next 48 hours, we chose the 24-hour time point for transfer of $CD8^+$ effector cells. BALB/c mice were exposed to 900 rads or left untreated as a control. After 24 hours, cultured $M_{285-293}$ -specific $CD8^+$ T cells were CFSE labeled and transferred by intratracheal instillation. At 2 days after transfer, the recipient mice were killed, and transferred cells in the lung tissue identified by $CD8^+CFSE^+$ staining. Function was determined by assessing IFN- γ production after restimulation with peptide. Compared with pretransfer effectors, which were 98% functional, effector cell populations isolated from the lungs of both the control and irradiated mice after adoptive transfer showed equivalent loss of function (39 and 34% functional, respectively) (Figure 5). We did note in our analyses that the lung-resident $CD11c^+$ population was partially radioresistant, with roughly half of the cells remaining in the lung environment (Figure 4B). These cells appeared not to be alveolar macrophages, as this population also expresses F4/80, and cells bearing this marker were efficiently depleted (16). To determine

whether $CD11c^+$ cells were responsible for down-regulating $CD8^+$ T cell function, these cells were purified from lung tissue and cocultured with effector cells for up to 3 days. As a control, effector cells were cultured in the absence of $CD11c^+$ cells. The $CD8^+$ T cells were then stimulated with peptide and analyzed for IFN- γ production. No statistically significant decrease in IFN- γ^+ cells was observed at any time point (Figure 5C). These data support a model wherein the loss of function seen in the $CD8^+$ effector cells is not mediated by a bone marrow-derived cell.

Naive $CD8^+$ T Cells Are Negatively Regulated as a Result of Residence in the Lung

It is clear from previous studies that T cells undergo significant changes in their expression profiles as a result of transition from naive to effector to memory cells (17). We postulated that changes that occur through the process of differentiation may affect the susceptibility of $CD8^+$ T cells to the loss of function that takes place as a result of residence in the lung. We first tested the effect of residence in the lung on naive T cells using the $Thy1.1^+$ CL4 transgenic model in which T cells express a T cell receptor (TCR) specific for the hemagglutinin (HA) peptide from influenza (7). Naive CL4 T cells were transferred into BALB/c mice by combined intratracheal and intravenous routes. This approach allowed high-level seeding of the lung, lymph nodes, and spleen.

Figure 4. Kinetics of cell loss in the lung after irradiation. BALB/c mice were left untreated or exposed to 900 rads. At 24, 48, and 72 hours after irradiation, the mice were killed, and lung tissue was prepared for analysis by flow cytometry using an array of surface marker antibodies identifying bone marrow-derived populations. (A) Total cell count of each cell population in the lung. (B) The percent of each cell type remaining in the lung when compared with the nonirradiated control group. These data are the average of three animals. Statistical analysis was performed using a two-way ANOVA.

At 2 days after transfer, the recipient mice were killed, and lung, lymph node, and spleen tissues were isolated. Cells from each tissue were stimulated with HA peptide-pulsed, LPS-activated, bone marrow-derived dendritic cells. For analysis of early responses to antigen stimulation, GolgiStop was added at the time of culture initiation, and 24 hours later TNF- α production was measured. The function in cells isolated from the MLN was set at 100%, and function in the spleen and lung calculated as a percent of the response in the MLN. Figure 6A shows the percent maximal TNF- α response of the transferred cells in the lung to be 68% of that found in cells isolated from the lymph node. The results show that there is a statistically significant decrease in the response after initial antigen contact in the naive cells recovered from the lung to the naive cells recovered from the lymph node and spleen tissue.

To assess the ability of these cells to develop effector function, cells cocultured with dendritic cells were allowed to progress for 3 days. At this time, cells were restimulated with HA peptide and IFN- γ production determined. These results show that the acquisition of effector function in cells after residence in the lung was significantly decreased (41%) compared with that in cells isolated from the lymph node and spleen (Figure 6B). Of note, analysis of proliferation indicated that there was no decrease in proliferative capacity in cells after residence in the lung (data not shown). This is similar to what we had previously reported for effector cells that had been rendered unable to exert effector function as a result of residence in the lung (6). Collectively, these data demonstrate that naive CD8⁺ T cells are negatively regulated by the residence in the lung parenchyma.

Memory CD8⁺ T Cells Are Resistant to Loss of Function as a Result of Residence in the Lung Environment

We next determined whether memory cells were also susceptible to regulation in the lung. For these studies, memory cells were generated by adoptive transfer of splenocytes from Thy1.1⁺ CL4

transgenic mice into BALB/c recipient mice that were subsequently infected with the influenza virus. A minimum of 40 days after infection, spleen and MLNs were removed and the adoptively transferred HA-specific cells were isolated from the host cells by positive selection using Thy1.1 magnetic beads. Isolated cells were then transferred into naive BALB/c mice by combined intratracheal and intravenous routes. At 2 days after transfer, lung, spleen, and MLNs were prepared for analysis by flow cytometry. Transferred cells were identified by CD8⁺Thy1.1⁺ surface staining, and functional cells identified by intracellular staining for IFN- γ after peptide stimulation. In stark contrast to the results obtained with naive and effector cells, memory cells recovered from the lung did not exhibit decreased function compared with the memory cells obtained from the MLN (Figure 7A). It is important to note that CL4 cells are not inherently resistant to regulation in the lung given the effects on naive cells shown above, as well as loss of function in CL4 effectors found in the lung after adoptive transfer (our unpublished data).

Given our previous finding that cells localized to the airway did not exhibit loss of function (1, 6), it was critical to establish that transferred memory cells were capable of entering the lung parenchyma. Thus, after transfer, cells in the airway were collected by lavage and subsequently parenchymal cells were isolated from the tissue. Memory cells were recovered from both the lung tissue and the BAL. Further cells from both sites were highly functional (99%) (Figure 7B). Together, these data demonstrate that the peripheral CD8⁺ T cell memory pool is resistant to the loss of function seen in naive and effector cells that reside in the lung parenchyma.

DISCUSSION

CD8⁺ effector T cells in the lung exhibit functional impairment in many models of respiratory infection, as well as in the basal

Figure 5. Loss of function occurs in effector cells residing in the lungs after irradiation. BALB/c mice were left untreated or exposed to 900 rads. After 24 hours, CFSE-labeled M_{285–293}-specific CD8 $^{+}$ effector cells (3×10^6 to 6×10^6) were intratracheally transferred into irradiated or nonirradiated mice. Before transfer, a portion of the effector cells was stimulated with peptide to determine function at baseline (Pre-transfer bars). At 2 days after transfer, the recipient mice were killed, and lung tissue was prepared for analysis by flow cytometry. Transferred cells were identified by CD8 $^{+}$ CFSE $^{+}$ staining, and function was determined by intracellular cytokine staining (ICCS) for IFN- γ after peptide stimulation. (A) Averaged percentage of IFN- γ -positive cells in cells isolated from the lungs of control or irradiated mice from four independent experiments. There was no statistically significant difference in loss of function in cells recovered from irradiated versus nonirradiated mice. (B) Representative data from (A). The numbers represent the percentages of M_{285–293}-specific cells that produce IFN- γ . (C) CD11c $^{+}$ cells were isolated from lung tissue from nonirradiated mice and cocultured with the M_{285–293}-specific CD8 $^{+}$ T cells at a ratio of 2:1 for 24, 48, or 72 hours. Functional CD8 $^{+}$ T cells were identified by IFN- γ $^{+}$ staining 5 hours after peptide stimulation. There was no statistically significant difference in IFN- γ production between the cells cultured in the presence or absence of lung-derived CD11c $^{+}$ cells for any of the three time points. Statistical analysis was performed using a two-way ANOVA.

lung environment (1–6). Thus, the lung exerts potent immunoregulatory effects on effector T cells. The observed nonresponsiveness encompasses all effector functions assessed (i.e., IFN- γ and TNF- α production, as well as secretion of lytic granules [1–5]). Upon further observation, we discovered that the CD8 $^{+}$ T cell inactivation is unique to the parenchymal compartment of the lung, as CD8 $^{+}$ effector cells from the airway compartment maintain their functional capabilities (6).

The studies performed here build on these findings, demonstrating that the induction of nonresponsiveness in CD8 $^{+}$ T cells in the lung appears to be independent of bone marrow-derived cells. These cells (e.g., macrophages, dendritic cells, T cells, B cells, neutrophils) had been an attractive candidates given the broad array of immunoregulatory factors that they produce. The lung parenchyma consists of a number of non-bone marrow-derived cell types, including fibroblasts, smooth muscle endothelial cells, vascular endothelial cells, and pericytes (18). In addition to the variety of cells present in the interstitium, a large number of

noncellular components are present, including laminin, collagens, elastin, reticulin fibrils, fibronectin, tenascin, and proteoglycans (18, 19). The latter is a complex group of very large polysaccharide molecules that imparts a gel-like structure to the interstitium, some of which may influence T cell function (20–22). For example tenascin-C has been shown to inhibit proliferation and function in lymphocytes (20, 23), whereas matrix metalloproteinases have been shown to cleave IL-2R α (21) and activate transforming growth factor- β (22). Our findings make the important conclusion that cells and/or factors present in the lung architecture can exert potent immunoregulatory effects on CD8 $^{+}$ T cells that enter into this tissue. Determination of the cell type or factor that mediates this effect will be the focus of future studies.

A major finding from our studies was that all CD8 $^{+}$ effector cells appear to be susceptible to the regulation of the lung parenchyma. In our previous analyses, we always found both functional and nonfunctional populations (1, 6). This led us to speculate that some cells may be inherently resistant to the signal

Figure 6. Naive CD8⁺ T cells are susceptible to negative regulation as a result of residence in the lung. CFSE-labeled, naive CL4 T cells were transferred into BALB/c mice by combined intratracheal and intravenous routes. At 48 hours after transfer, lungs, mediastinal lymph nodes (MLN), and spleen were isolated and cells cultured in the presence of LPS matured, bone marrow-derived dendritic cells previously pulsed with 10⁻⁶ M hemagglutinin (HA) peptide. Transferred cells were identified by CFSE⁺CD8⁺ staining. (A) The percent maximal response for TNF-α production within CFSE⁺CD8⁺ cells in the spleen and lung compared with the response in the MLN at 24 hours after activation with bone marrow derived dendritic cells (BMDC) is shown. The maximum response was determined using the population obtained from the MLN and was defined as the percentage of the CL4 cells in this tissue that exhibited function after activation *in vitro*. The decrease between the lung-derived versus both the MLN and spleen-derived CL4 cells was statistically significant (****P* < 0.001). (B) The IFN-γ response at Day 3 after activation in the CFSE⁺CD8⁺ cells is shown. The decrease in IFN-γ expression between the lung and MLN effector cells was statistically significant (****P* < 0.001). These data are representative of three independent experiments. Statistical analysis was performed using a two-way ANOVA.

mediating loss of function in the lung. However, the data presented here negate this hypothesis, as functional cells isolated from the lung exhibited loss of function after transfer. Why then do we routinely observe functional cells? We would propose two possibilities: (1) functional cells are the result of recent immigrants, or (2) functional cells reside in a microenvironment in which the regulatory signal is absent. We favor the latter model, given that, in the adoptive transfer approach used in our experiments, we would predict that cells would reach the lung within a relatively short timeframe. If there are microenvironments in the lung that allow cells to retain function, our findings would suggest that, if cells leave the protective microenvironment, they would be rendered nonfunctional.

Figure 7. Memory CD8⁺ T cells do not undergo loss of function as a result of residence in the lung. Memory CL4 cells generated as a result of infection with influenza were isolated from MLN and spleen by sorting on Thy1.1 expression. Thy1.1⁺ cells (1.5 × 10⁵ to 4 × 10⁵) were transferred by combined intratracheal and intravenous routes into Thy1.2⁺ recipient mice. At 2 days after transfer, the recipient mice were killed, and lung, spleen, and MLN tissue was prepared for analysis by flow cytometry. Transferred memory cells were identified by CD8⁺Thy1.1⁺ staining. Function was assessed by IFN-γ production after *in vitro* stimulation with peptide. (A) Averaged data from four independent experiments analyzing IFN-γ production in the Thy1.1⁺CD8⁺ CL4 memory cells isolated from the MLN, lung, and spleen. Values are the percent of the response using the production from CL4 cells in the MLN as the maximum. There was no statistically significant difference in IFN-γ production between the memory cells recovered from each of the three tissues. (B) Data shown assess the function in transferred memory cells isolated from the lung parenchyma (tissue) versus the airway space (lavage). The percent maximal response (compared with the MLN) for IFN-γ expression in the Thy1.1⁺CD8⁺ cells recovered from the bronchoalveolar lavage (BAL) and the lung parenchyma is shown. Memory cells were found in both compartments, and were highly functional. These data are representative of two independent experiments, each containing multiple mice. Statistical analysis was performed using a two-way ANOVA.

Although all CD8⁺ effectors appear susceptible to the loss of function, our data demonstrate a striking difference in the susceptibility of CD8⁺ memory cells to functional inactivation in the lung. The finding that memory cells retain function was somewhat surprising, given reports that the population of virus-specific cells present in the lung after infection with respiratory syncytial virus or SV5 become increasingly nonresponsive at late times after infection (1, 2). However, it is important to note that there is a continued decrease in absolute cell number during the period in which the proportion of cells that are nonfunctional is increasing. As such, it is possible that nonfunctional CD8⁺

effectors are preferentially maintained in the parenchyma, and, as a result, become enriched over time, thus accounting for the apparent increase in nonfunctional cells as a percentage of the total. Furthermore, it is also possible that new immigrants into the lung over the first few weeks after clearance are not a random sampling of the effector/memory pool. A subset of T effector memory (T_{EM}) or less-differentiated T central memory (T_{CM}) may preferentially migrate into the lung parenchyma, seeding the lung after virus infection. These cells may exhibit increased susceptibility to inactivation. The population of cells transferred in our studies may reflect a more fully differentiated memory population that has acquired resistance after residence in the peripheral lymphoid tissue. Finally, it is possible that new lung immigrants that arrive in the lung within several months of virus clearance are not resting memory cells, but instead are activated as a result of recent antigen engagement that occurs through antigen presentation that is maintained in the draining lymph nodes, as has been reported for influenza infection (24). Thus, these cells would behave as effectors rather than memory cells.

How are $CD8^+$ memory cells rendered resistant to the loss of function? In regard to the microenvironment model proposed previously here, it is tempting to speculate that memory cells may localize differently in the lungs, preferentially trafficking to the microenvironment that is free of the regulatory signal. Although we have attempted these studies in our transfer model, the low number of cells spread throughout the large area of the lungs has made these analyses technically problematic. Certainly, the expression of chemokine receptors and adhesion molecules are dynamically regulated in T lymphocytes through the process of differentiation from naive to effector to memory cells, as well as in cells that enter into distinct tissues (25, 26). For example, it has been postulated that integrin expression can be altered as a result of tonic/continuous signaling due to environmental factors present in a particular tissue (26). With regard to the lung, entry into this tissue appears to induce expression of the collagen-binding integrin very late antigen-1 (27). Very late antigen-2 expression has also been associated with T cell entry into the interstitium (27). Migration within tissue is also the result of cell type-specific production of chemokines (28). As an example, in the gut, CCL9 is selectively produced by small intestinal epithelial cells promoting migration of responsive cells to this site. Thus, these or other molecules may be differentially regulated in the effector versus memory cells after entry into the lung, thereby promoting differential localization. An alternative model to differential localization is that memory cells fail to express the putative receptor necessary for triggering loss of function. As such, even when in the presence of the signal, memory cells would remain ignorant. Discrimination among these possibilities awaits further study.

To our knowledge, studies to determine whether the loss of function in $CD8^+$ lung effectors occurs in humans have not been performed. Translation of the findings to humans is severely hampered by the observation that nonfunctional cells are found in the parenchyma, whereas airway cells are highly functional (6). Thus, studies would be limited to situations where human lung tissue could be obtained. It seems likely that the inactivation of effector cells has evolved as a means to limit tissue damage in the lung. An overly robust immune response after infection is potentially fatal, suggesting a beneficial consequence from turning off some effectors in the lung after a defined period of time. Immunopathology as a result of an overly robust $CD8^+$ T cell response has been demonstrated in mouse models of influenza and respiratory syncytial virus infection (29, 30). Here, pathology is thought to be mediated by the effects of $TNF-\alpha$ on epithelial cells, which results in the release of proinflammatory cytokines (31). A similar situation is certainly possible in the case of human respiratory infection with highly permissive viruses (e.g., influenza)

that elicit a robust T cell response. It is worth noting that the loss of function in this situation likely differs from that observed in chronic infections, such as hepatitis C virus or human immunodeficiency virus (32), as we observe loss of function in T cells even in the absence of cognate antigen. Thus, the host has developed multiple approaches for protection from immune-mediated pathology.

In contrast to the loss of function in effector cells, resistance to this effect in memory cells should allow these highly efficient cells to respond quickly, thereby limiting virus spread and reducing overall virus load. This would also limit expansion of secondary effectors. Limiting viral burden early may also prevent pathology that can result from virus-mediated cytopathic effects, as well as increased production of proinflammatory cytokines by innate cells. For example, IL-1 and $TNF-\alpha$, which are produced at high levels by activated innate responders, have been shown to promote lung pathology (33, 34). Therefore keeping these responses at appropriate levels would promote clearance while limiting damage.

In summary, our findings show that $CD8^+$ effector cells are generally susceptible to inactivation by a mediator present in the lung parenchyma. The current data support a model wherein the mediator is derived from a nonhematopoietic cell. Critically, although naive $CD8^+$ T cells are also susceptible to the negative regulatory effects of the lung environment, $CD8^+$ memory cells appear resistant. Our studies presented here have significantly furthered our understanding of the regulatory effects on $CD8^+$ T cells in the lung and direct further studies to identify parenchymal components of the lung that negatively regulate effector T cells in this critical tissue. Identifying the mechanism by which $CD8^+$ T cell function is regulated within the lung could lead to the development of therapeutics that could enhance the effector T cell function in the lung when needed, or, conversely, dampen function during an overly robust immune response.

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