CD8$^+$ T Cell–mediated Injury In Vivo Progresses in the Absence of Effector T Cells

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Abstract

Tissue injury is a common sequela of acute virus infection localized to a specific organ such as the lung. Tissue injury is an immediate consequence of infection with lytic viruses. It can also result from the direct destruction of infected cells by effector CD8$^+$ T lymphocytes and indirectly through the action of the T cell–derived proinflammatory cytokines and recruited inflammatory cells on infected and uninfected tissue. We have examined CD8$^+$ T cell–mediated pulmonary injury in a transgenic model in which adoptively transferred, virus-specific cytotoxic T lymphocytes (CTLs) produce lethal, progressive pulmonary injury in recipient mice expressing the viral target transgene exclusively in the lungs. We have found that over the 4–5 day course of the development of lethal pulmonary injury, the effector CTLs, while necessary for the induction of injury, are present only transiently (24–48 h) in the lung. We provide evidence that the target of the antiviral CD8$^+$ T cells, the transgene expressing type II alveolar cells, are not immediately destroyed by the effector T cells. Rather, after T cell–target interaction, the type II alveolar cells are stimulated to produce the chemokine monocyte chemoattractant protein 1. These results reinforce the concept that, in vivo, the cellular targets of specific CTLs may participate directly in the development of progressive tissue injury by activating in response to interaction with the T cells and producing proinflammatory mediators without sustained in vivo activation of CD8$^+$ T cell effectors.

Key words: CD8$^+$ T cell • pulmonary injury • target cell • inflammatory mediators • MCP-1

Introduction

CD8$^+$ T lymphocytes are well recognized as important contributors to the adaptive immune response to virus infection leading to virus clearance and recovery. The mechanism employed by these cells in clearing intracellular pathogens include both cytolytic and noncytolytic activities (1). CD8$^+$ T lymphocytes likewise contribute to the development of tissue injury during the host adaptive immune response both in systemic viral infection and in virus infection localized to a particular organ or tissue (2–4). For example, in experimental murine influenza infection, immunodeficient mice were observed to have a more protracted time to death than immunocompetant mice after lethal intranasal infection (5–7). The relative contribution of lytic virus infection and the virus-specific adaptive CD8$^+$ T lymphocyte response to that infection (in an organ such as the lung) to cell death, tissue injury/inflammation, and the development of organ dysfunction is, however, unclear.

Virus-specific CD8$^+$ T lymphocytes normally circulate in the blood or reside in secondary lymphoid tissues (e.g., lymph nodes, spleens, etc.; reference 8). After infection of a tissue or organ like the lung, virus-infected APCs (primarily dendritic cells) are believed to activate and migrate to lymphoid tissues where they stimulate naive, resting CD8$^+$ T cells to undergo antigen-specific activation and differentiation into effector T cells. These effector CD8$^+$ T cells then migrate from the lymphoid tissue to the site of infection. The recent development of technologies to enumerate antigen-specific CD8$^+$ T cells have provided detailed kinetic information of the development and resolution of CD8$^+$ T cell response during local virus infection (9). Results from

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the application of these techniques to identify and quantitate antigen-specific $CD8^+$ T cells suggest that naive $CD8^+$ precursors activate and proliferate in response to virus infection in lymphoid tissues and accumulate in large numbers as activated, fully-differentiated antigen-specific CTLs at the primary site of infection (e.g., the lungs; references 10 and 11). The life span of these activated CTL effectors in infected tissue appears to be very short. With the resolution of infection, these $CD8^+$ effector T cells decrease dramatically in frequency at the site of infection (11–13).

To assess the impact of antigen-specific $CD8^+$ effector CTLs on lung inflammation and injury in the absence of virus replication or the contribution of the early host innate immune response to infection, we developed a transgenic model of $CD8^+$ T cell–mediated injury (14). In this model, a viral transgene, the influenza HA, is selectively expressed in pulmonary type II alveolar cells of transgene positive mice using the lung-specific human surfactant-C promoter. Injury is induced selectively in the lungs of transgene-positive mice by the adoptive transfer of cloned populations of HA-specific $CD8^+$ CTLs with defined effector activities. Previous reports have established the HA specificity of the injury induced, the characteristics of the lung inflammatory infiltration and the contribution of specific CTL effector mechanisms (e.g., cell-mediated cytolytic and specific T cell cytokines) to the process (15, 16). These analyses indicated that $CD8^+$ T cell–induced injury in vivo does not depend on the expression of the perforin or FasL lytic mechanisms by the transferred effector $CD8^+$ T cells (16). Recent evidence in this model further suggests that chemoattractants produced by transgene positive alveolar type II cells after $CD8^+$ T cell transfer may contribute substantially to the development of lung inflammation (17).

This study was undertaken using this model: (i) to define the kinetics of expression of inflammatory mediators derived from the transferred T cells, the host inflammatory cells, and the host type II target cells during the evolution of pulmonary injury; (ii) to determine the fate of the transferred effector CTLs in the lungs after transfer; and (iii) to evaluate the impact of the CTLs on the fate and function of the HA-expressing type II alveolar cell targets in vivo. The results of this analysis reveal that effector CTL are rapidly and selectively eliminated from lungs of transgene-positive mice (within 24–48 h after adoptive transfer) before full development of lung injury and inflammation. In addition, while the expression of the T cell–specific inflammatory mediator, IFN-γ, falls concomitantly with effector CTL elimination from the lungs, the expression of other mediators (e.g., TNF-α and monocyte chemoattractant protein [MCP]-1) is sustained throughout the evolution of lung injury. Finally, we provide evidence that the transgene-expressing type II alveolar cells are not destroyed in vivo as a result of the initial encounter with the effector T cells. Rather, these cells appear to be activated by the encounter with the CTLs, and remain functional to express MCP-1 for several days after the disappearance of the CTLs. These observations support the hypothesis that alveolar target cells may actively participate in the development of lung injury and inflammation triggered by $CD8^+$ T cell recognition. Furthermore, these findings reinforce the view that immediate destruction of the antigen-bearing target cells is not the necessary outcome of effector CTL encounter with target cells in vivo, and that the progression of tissue-specific injury in response to CTL recognition does not require the continued presence of CTLs and the sustained expression of CTL effector activity in that tissue.

Materials and Methods

Mice. $H2^d$ haplotype HA-transgenic mice expressing the A/Japan/57 influenza HA under the transcriptional control of the surfactant protein C (SPC)* promoter were produced and characterized as described (14). Severe combined immunodeficient (SCID) $H2^d$ mice on the BALB/c background were initially purchased from the Jackson ImmunoResearch Laboratories. The HA transgene was transferred from conventional (immunocompetent) mice into the SCID background by standard breeding. Transgene-negative litter mates of immunocompetent and SCID transgene-positive animals served as control recipients for these experiments.

$CD8^+$ T Lymphocytes. The $CD8^+$ T lymphocyte clone, D4, specific for the HA210–219 epitope of the A /Japan/57 HA was used in the adoptive transfer studies described in this report. The clone was isolated by limiting dilution and propagated in vitro using irradiated splenocytes infected with A/Japan/57 (or A/Guiyang/57/var17) virus as described previously (18). On day 5 after in vitro stimulation of the cloned $CD8^+$ CTL, activated T cells were separated from irradiated splenocyte stimulators by density gradient separation. In some experiments the isolated cloned $CD8^+$ T cells were labeled with the vital dye CFDA-SE (Molecular Probes) as described previously (18) before adoptive transfer into mice.

Analysis of Pulmonary Cytokine, Chemokine, and Surfactant Gene Expression. At the indicated times after transfer of cloned D4 CTLs into HA^+ and HA^- recipient mice, animals were killed and lungs harvested. Total lung RNA was purified from homogenized lung tissues by extraction with Trizol (Life Technologies). Lung RNA from individual mice was prepared and analyzed separately. Cytokine and chemokine mRNA were quantitated by a RNase protection method using the RiboQuant multiprobe RNase protection assay (RPA) system (BD PharMingen) according to the manufacturer's instructions. Briefly, mRNA-specific RNA probes labeled with ^{32}[P] UTP were synthesized using multi-probe template sets designed for the indicated murine chemokine and cytokine genes. Each probe set was hybridized separately with pulmonary mRNA from transgene positive or negative recipient mice. Surfactant protein B (SP-B) gene expression was quantitated in a similar manner using a 330 base pair complementary RNA probe for the murine SP-B mRNA generated from a cloned SP-B cDNA (provided by Jeffrey Whittset, Cincinnati Children's Hospital). Hybridizations were performed overnight at 56°C before RNase treatment and electrophoresis of the protected RNA fragments on 5% polyacrylamide sequencing gels.

Identification and Enumeration of Transferred CD8 T Cells In Vivo. At the indicated times after adoptive transfer into the tail veins of recipient mice, animals were killed and the chest and ab-

Abbreviations used in this paper: HBV, hepatitis B virus; IP, inducible protein; Jak, Janus kinase; MCP, monocyte chemoattractant protein; RPA, RNAse protection assay; RANTES, regulated upon activation, normal T cell expressed and secreted; SPC, surfactant protein C; SP-B, surfactant protein B; SCID, severe combined immunodeficient.

dominal cavities were exposed. After transection of the abdominal aorta, 3 mL of cold heparinized saline was introduced into the right ventricle in order to perfuse the pulmonary vasculature and remove any residual transferred T cells remaining within the pulmonary circulation. Lungs were then excised, minced, and extruded through a wire mesh screen to produce a cell suspension. Preliminary experiments in which intact lungs or lung fragments were digested with collagenase, dyspase, and/or DNase before or after extrusion of the lung tissue through the wire mesh screen showed no difference in the yields of transferred T cells isolated from the lung parenchyma.

The $CD8^+$ T cells isolated from the lung parenchyma were enumerated by flow cytometry using monoclonal anti-CD8 antibody 53–6.7 (BD PharMingen) and H2-K^d tetramers containing the synthetic HA210–219 peptide epitope or a control H2-K^d tetramer loaded with a K^d binding peptide from the murine Janus kinase (Jak)-1 protein. The preparation of these tetramers and their use in the identification of the HA210–219 specific CTL clone, D4, have been described previously (18). $CD8^+$ T cells present in the lung parenchyma which produced IFN-γ in response to short-term in vitro stimulation with the HA210–219 or control HA204–212 peptides were identified and enumerated by intracellular cytokine staining using fluorescent antibody (anti-IFN-γ, XMG1.2; BD PharMingen). Flow cytometry analysis of $CD8^+$ T cells present in the lungs which were dye-labeled and stained against intracellular cytokine or with tetramer was performed using a FACSCaliber™ analytical flow cytometer (Becton Dickinson). Data acquisition and analysis was performed using the CELLQuest™ software package.

Morphological Analysis of Lung Tissue. At the indicated time after adoptive transfer, transgene-positive mice were killed. For light microscopy and immunohistochemistry, lungs of mice were inflated with air before fixation in 10% neutral buffered formalin. Sections were cut from fixed, paraffin-embedded tissue, and stained with H&E. For immunohistochemistry, formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections and dried onto poly-L-lysine-coated slides. The tissue sections were incubated in a steamer containing 1X target retrieval solution (DAKO) for 20 min and then deparaffinized with xylenes. Next, the deparaffinized sections were incubated with peroxide block, avidin block, biotin block, and powerblock solutions (all supplied by Biogenex). T cells were detected using anti-CD5 mAb (2.5 μg/ml) and macrophages were labeled with Mac-3 mAb (2.0 μg/ml) (BD PharMingen). The FITC-conjugated primary antibodies were labeled with biotinylated anti-FITC antibody (5 μg/ml) (Molecular Probes), followed by HRP-conjugated streptavidin (BioGenex). The slides were developed using 3,3′-diaminobenzidine tetrahydrochloride chromogen solution, counterstained with Mayer's hematoxylin, and covered with Supermount mounting medium (all reagents from BioGenex). For in situ hybridization, lungs were perfused through the trachea with 10% neutral buffered formalin and fixed. Tritiated riboprobes for MCP-1 and influenza transgene mRNA were prepared as described previously (17). In situ hybridization was performed for a 2–4-wk period before analysis.

Results

Bystander Lymphocyte Involvement in Pulmonary Injury. To evaluate $CD8^+$ T lymphocyte-mediated injury to the pulmonary parenchyma in the absence of any other inflammatory stimuli, we developed a transgenic mouse model (SPC-HA transgenic mice) in which the gene encoding the type A influenza HA (A/JAPAN/305/57, H2/N2) is expressed under the control of the SPC promoter resulting in specific expression of the HA protein in type II alveolar pneumocytes (14). We reported previously that adoptive transfer of pure clonal populations of influenza HA-specific $CD8^+$ effector CTLs into SPC-HA mice results in significant pulmonary injury, leading to progressive decrements in pulmonary function, considerable morbidity, and ultimately (at higher doses of transferred cells) mortality (references 14 and 15; Fig. 1). This $CD8^+$ T cell–mediated injury is characterized by the progressive accumulation of inflammatory cells in the alveolar walls and alveolar spaces (references 15 and 16; Fig. 2). Initially after T cell transfer (days 1–2), histologic changes in recipient lungs are subtle with evidence of extravasation of erythrocytes, a mild neutrophil infiltration, and morphologic evidence of increased alveolar capillary permeability. Over time (days 3–5), there is a massive influx of mononuclear cells into the alveolar walls and surrounding the small airways, and this massive mononuclear infiltration is temporarily associated with the progressive decrements in pulmonary function, with morbidity, and ultimately, with death. Using the same experimental model, Zhao et al. reported that the majority of the cells in this late infiltrate expressed macrophage markers although there were also appreciable numbers of $CD8^+$ and $CD4^+$ T cells evident (17).

Although the progressive injury observed in this model was assumed to be due to the specific recognition and rapid destruction of the transgene-expressing alveolar type II cells by the transferred $CD8^+$ CTLs (with a secondary

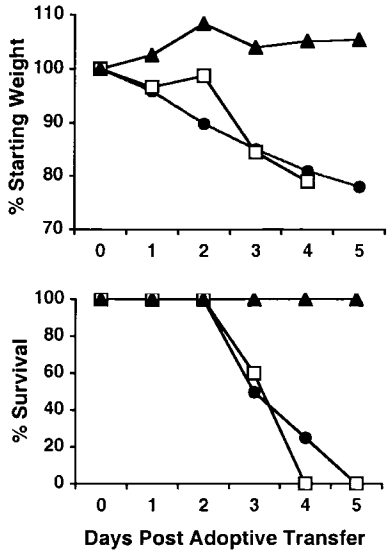

Figure 1. Morbidity and mortality of SPC-HA^+ and SPC-HA^- SCID mice after CTL T cell transfer. (A) Kinetics of weight loss (as percentage of starting weight) in HA^+ SCID mice (□), HA^+ wild-type mice (●) and HA-SCID mice (▲) after adoptive transfer of 10×10^6 cloned D4 CTLs. (B) Time course of survival of mice in A. Weight values are means of four recipient animals per group and are representative of three independent experiments.

influx of macrophages as a result of acute injury), the presence of a significant lymphocyte infiltrate in the lung parenchyma at late times after CTL transfer raised the possibility that lymphoid cells of recipient origin could contribute to the injury, possibly as a result of bystander cell recruitment and activation in the inflamed lungs. Therefore, we wanted to first determine whether there was any contribution of lymphocytes of recipient origin to the pulmonary injury induced by the transferred HA-specific CTLs. To examine this issue, we performed adoptive transfer of HA-specific $CD8^+$ effector CTL into a panel of SPC-HA^+ immunodeficient SCID mice and, in parallel, into a panel of conventional SPC-HA^+ mice [as well as congenic SCID mice lacking the transgene (SCID-HA^-)]. As Fig. 1 depicts, adoptive transfer of the effector CTL into SCID-HA^- mice had no effect on the survival of these animals (up to 21 d after transfer) and did not result in morbidity as measured by weight loss. This result is consistent with our earlier finding that the development of pulmonary injury and mortality in this model is dependent upon the expression of the HA transgene in the lungs of recipient mice (14). By contrast, both conventional SPC-HA^+ mice and the immunodeficient SCID-HA^+ mice exhibited significant weight loss by day 3 after CTL transfer (Fig. 1 A), with loss of 20% of body weight by days 4–5 after transfer. Lethal injury occurred in both HA^+ groups, and the death of all recipients was observed in both groups by days 4–5 after adoptive transfer (Fig. 1 B). This result indicated that recipient lymphocytes do not contribute significantly to the development of this $CD8^+$ T cell–mediated pulmonary injury and inflammation and suggest that recipient mononuclear cells of monocyte/macrophage origin are sufficient to orchestrate the inflammatory response in the lungs of SPC-HA^+ mice after T cell transfer.

Histologic confirmation was provided by harvesting the lungs of SCID-HA^+ recipients at day 3–4 after CTL transfer, a time at which the mice were preterminal (Fig. 2). As observed previously in conventional SPC-HA^+ recipients (14), the SCID-HA^+ lung histology was characterized by a dense infiltration of alveolar walls with inflammatory cells consisting largely of mononuclear cells with few polymorphonuclear cells scattered throughout the lung parenchyma (Fig. 2 A). Immunoperoxidase staining of fixed tissue revealed that this mononuclear cell infiltrate consisted primarily of Mac-3 antibody-staining cells, reflecting their monocyte/macrophage origin (Fig. 2 B). Parallel staining for cells of T lymphocyte origin (using anti-CD5 antibody) revealed only occasional $CD5^+$ cells presumably representing the adoptively transferred cloned CTLs (Fig. 2 C). No inflammation was evident in the lungs of SCID-HA^- mice after T cell transfer (data not shown).

Inflammatory Mediator Expression in the Lungs during the Evolution of Pulmonary Injury. The requirement for specific antigen recognition by the transferred $CD8^+$ T cells to induce pulmonary injury suggests that one or more of the antigen-dependent effector functions of the T cells (i.e., direct cytolysis and/or proinflammatory mediator release) are needed to be expressed for the development of injury. Of course, this does not preclude a contribution of nonlymphoid, innate inflammatory cells, or lung parenchymal cells of recipient origin to the progression of injury, though their recruitment and participation is also dependent upon specific recognition of antigen by transferred $CD8^+$ T cells.

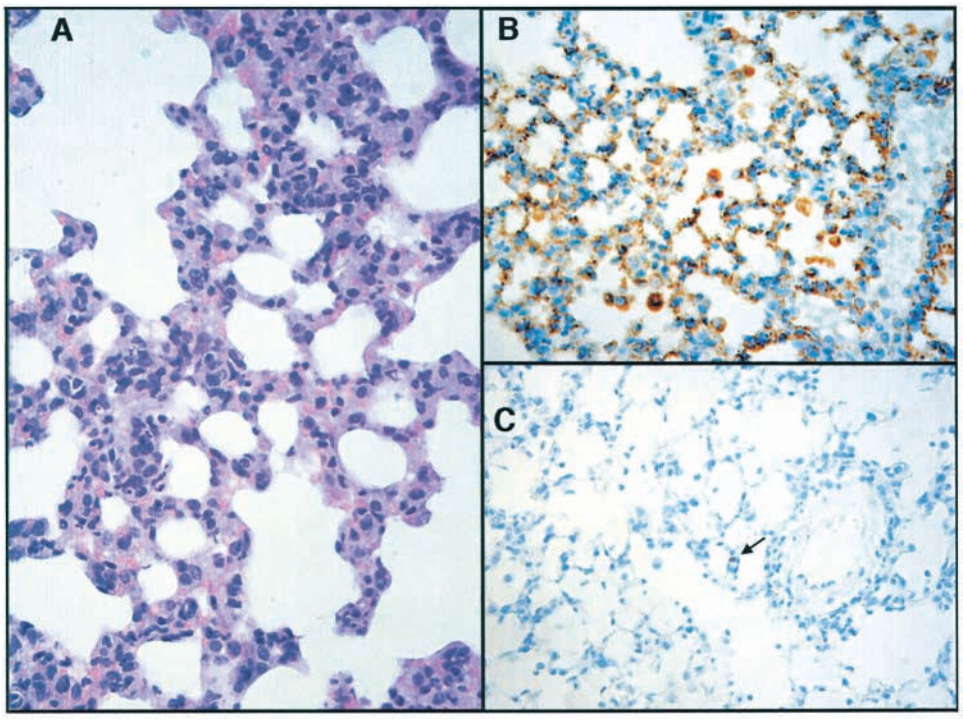

Figure 2. Histologic evaluation of lung morphology of SPC-HA^+ SCID mice. (A) Representative H&E section of lungs SPC-HA^+ SCID mice at day 3 after CTL transfer showing dense mononuclear infiltrate in alveolar walls. (B) Immunoperoxidase staining of fixed lung section with anti–MAC-3 antibody demonstrating MAC-3^+ cells in alveolar walls and alveolar spaces. (C) Immunoperoxidase staining of serial section of lung tissue examined in B with anti-CD5 antibody. Arrow indicates single $CD5^+$ cell detected in this section. Original magnification: ×250.

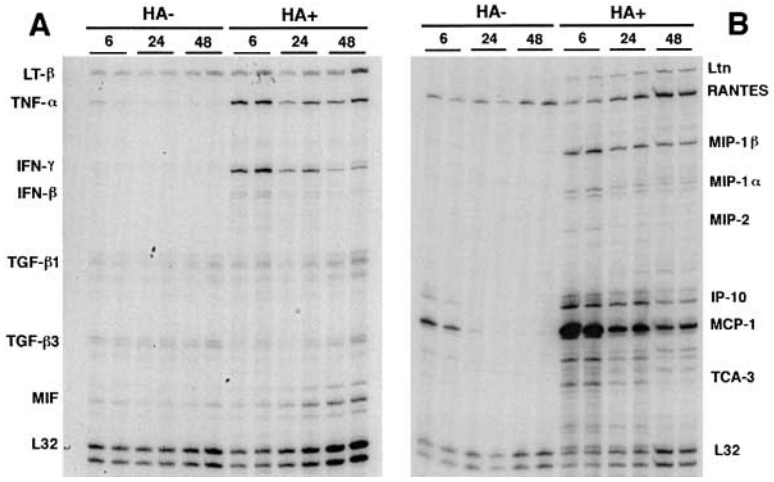

Figure 3. Early kinetics of cytokine and chemokine gene expression in SPC-HA$^+$ and SPC-HA$^-$ mice. At the indicated time (in h) after transfer of 8–10 $\times$ 10^6 cloned D4 CTLs, lungs of HA$^+$ and HA$^-$ recipients were excised, and RNA was extracted. (A) Time course of expression of the indicated cytokine genes and housekeeping genes by RPA. (B) RPA analysis of selected chemokine gene expression kinetics using the same RNA samples analyzed in A. Data displayed represent lung RNA from two transgene-positive (HA$^+$) and transgene-negative (HA$^-$), conventional (immunocompetent) recipient mice at each time point, and are representative of three independent RPA analyses of gene expression.

Therefore, it was of interest to examine the expression of inflammatory mediators in the lung parenchyma after T cell transfer and the relationship of mediator expression to inflammation and injury. We examined the kinetics of expression of several important cytokine and chemokine genes in lung after T cell transfer into conventional SPC-HA$^+$ transgenic mice and control SPC-HA$^-$ recipients by RPA. The results are shown in Figs. 3 and 4. In short-term kinetic analysis, lungs were harvested from SPC-HA$^+$ and control SPC-HA$^-$ mice at 6, 24, and 48 h after T cell transfer and total lung RNA was isolated for analysis of cytokine gene expression (Fig. 3 A). In HA$^-$ control mice, expression of the T cell–specific cytokine, IFN-γ, as well as, the cytokine TNF-α (which is produced by both macrophages and the CD8$^+$ T cells) was detected at low levels at 6 h after T cell transfer, but the level of these cytokine transcripts dropped to low or undetectable levels by 24–48 h. In the lungs of HA$^+$ transgenic recipients, a high level of TNF-α expression was readily detectable at the 6 h time point and was sustained >48 h. In contrast, IFN-γ expression was initially very high at the 6 h time point. While still detectable at 24 and 48 h, IFN-γ expression progressively diminished over time. The level of expression of the cytokines LT-β, TGF-β1, TGF-β2, and migration inhibitory factor in the lungs of both HA$^+$ and HA$^-$ mice were either unchanged over time or demonstrated a minimal increase in HA$^+$ lungs.

Since pulmonary injury induced by the transferred T cells becomes progressively more severe over time, we extended this RPA analysis of TNF-α and IFN-γ expression in the lungs of SPC-HA$^+$ mice to include 72 and 96 h time points. Representative results of this analysis are shown in Fig. 4 A, and demonstrate that TNF-α expression was sustained at the 72 and 96 h time points in HA$^+$ recipients, while expression of IFN-γ was found to further decrease progressively over time to very low levels by 96 h (day 4) after cell transfer. As expected, expression of neither cytokine gene was detectable at these later time points in the lungs of HA$^-$ nontransgene recipients (data not shown).

A parallel, short-term kinetic analysis of selected chemokine gene expression in the lungs of HA$^+$ and HA$^-$ recipients early after T cell transfer revealed a pattern similar to that recently published using the same model (17). As Fig. 3 B demonstrates, there was transient, low level expression of the MCP-1 CC chemokine and the IFN-dependent inducible protein (IP)-10 CXC chemokine genes in HA$^-$ recipients at 6 h after T cell transfer which diminished to background levels by 24–48 h. In HA$^+$ recipients, there was dramatically increased expression of MCP-1 which diminished slightly over time, but was still sustained at the 48 h time point. In contrast, while the expression of IP-10 was also initially very high, IP-10 fell over 24–48 h in parallel with IFN-γ expression. Expression of Ltn, MIP-

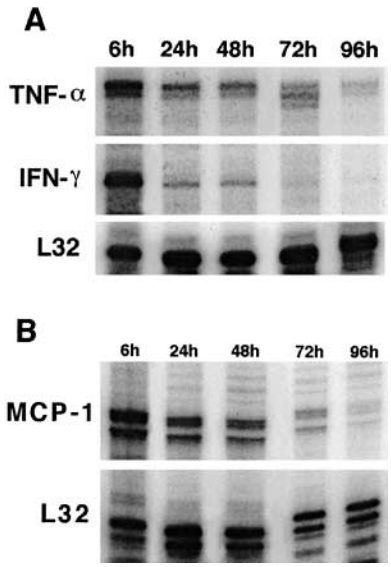

Figure 4. Extended kinetic analysis of IFN-γ, TNF-α, and MCP-1 gene expression in SPC-HA$^+$ mice. (A) Representative RPA analysis of the lung RNA at 6, 24, 48, 72, and 96 h after infection of 10 $\times$ 10^6 cloned D4 CTLs. (B) Companion analysis of MCP-1 gene expression detected lung RNA from the donor mice examined in A. The level of expression of the housekeeping gene L32 is included as a control for RNA loading. Comparable data was obtained in three independent experiments.

1α, MIP-1β, and MIP-2 was detected exclusively in the lung of the HA^+ recipients with either sustained expression or gradually decreased expression over 48 h. Expression of regulated upon activation, normal T cell expressed and secreted (RANTES) was comparable in HA^+ and HA^- recipients. Extended kinetic analysis of MCP-1 gene expression to the 72 and 96 h time points (Fig. 4 B) reveals detectable MCP-1 expression as late as 96 h after T cell transfer. So, like TNF-α, the gene expression of the chemokine MCP-1 gene (and several other chemokine genes) was sustained late in the course of injury development (days 3–4) when inflammation was most severe. The expression patterns for the other chemokines examined at 72 and 96 h in HA^+ recipients closely paralleled the tempo established over the first 48 h with MIP-1β maintaining a sustained level of expression while MIP-1α and IP-10 expression fell to undetectable levels at the later time points (data not shown). A limited analysis of the kinetics of lung cytokine and chemokine gene expression during injury development was also performed in SP-C HA^+ SCID recipients. The pattern of inflammatory mediator expression was comparable to that of conventional SP-C HA^+ mice with a sustained elevation of MCP-1 and TNF-α expression and a progressive fall in IFN-γ expression (data not shown); although in SP-C HA^+ SCID recipients, low level IFN-γ expression was still detectable out to 72 h because of the similarity in injury development and cytokine/chemokine gene expression between conventional and SCID recipients and because of the limited availability of SPC-HA^+ SCID mice, subsequent studies described below were performed in conventional SP-C HA^+ and HA^- mice.

Fate and Functional Activity of Adoptively Transferred T Cells Resident in the Lung. The cytokine IFN-γ is primarily a product of activated T lymphocytes and NK cells and is produced in response to the HA target antigen by the $CD8^+$ CTL clone used in these transfer studies (unpublished data). Since recipient T lymphocytes appear to play a minimal role in the development of pulmonary injury mediated by HA-specific CTLs, the transferred T cells were the likely source of the IFN-γ gene expression detected in the lungs of the HA^+ mice. Therefore, it was noteworthy that IFN-γ expression in the lungs decreased rapidly over time in spite of the sustained expression of TNF-α and MCP-1 and the progressive development of pulmonary inflammation. These results raised the possibility that the HA-specific $CD8^+$ T cells may not be present in the lungs (or at least may not be functioning) at later times after transfer into SPC-HA^+ mice and prompted us to examine the fate of adoptively transferred T cells resident in the lungs of HA^+ and HA^- recipients.

To address this issue, the cloned CTLs were labeled with the cell division sensitive, intracellular vital dye, CFDA-SE (19) before adoptive transfer into HA^+ transgenic and nontransgenic mice. Lungs were removed at serial time points after cell transfer and the fate of labeled cells resident in lung homogenates was enumerated by flow cytometry. As Fig. 5 demonstrates, labeled cells were readily isolated from the lung parenchyma of both SPC-HA^+ (Fig. 5 A) and nontransgenic control recipients at 24 h after transfer (Fig. 5 B). The uniform pattern of staining intensity suggests that cell division had not occurred in the lungs of either recipient group at day 1 after transfer (20). By 48 h after cell transfer, we noted two patterns of cell accumulation in HA^+ transgene positive mice. In some recipients (Fig. 5 C), labeled cells could be detected at a low frequency and, based upon dye fluorescence intensity, had undergone several rounds of cell division. In the majority of recipients, however, dye-labeled cells were rarely identified (data not shown). In control recipients (Fig. 5 D), dye-labeled cells were detected in the lung parenchyma at 48 h and the uniformity of fluorescence intensity suggests that the transferred T cells had undergone few, if any, divisions at this time. These data are representative of four independent experiments.

By days 4–5 after T cell transfer, at a time when surviving transgene positive mice were severely ill, few if any labeled T cells were detected in lung homogenates from any HA^+ recipients (Fig. 5 E). By contrast, labeled cells were still present in the lung parenchyma of the control nontransgenic mice, and the pattern of fluorescence intensity suggested that these cells had undergone a very limited number of cell divisions (1 or 2) in the absence of antigen (Fig. 5 F).

The paucity of labeled $CD8^+$ T cells detected in the lung parenchyma by 4–5 d after transfer in HA^+ recipients was likely due to their death in situ as a result of activation-induced cell death triggered by specific antigen rec-

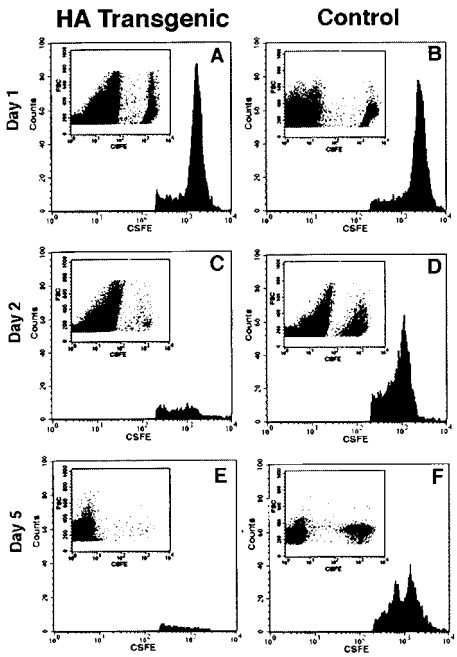

Figure 5. Kinetics of CFDA-SE–labeled CTL accumulation in the lungs. D4 CTLs were labeled with the dye CFDA-SE before adoptive transfer of 10×10^6 T cells into HA transgene-negative (control) and SPC-HA^+ (HA transgenic) recipients. On day 1 (A and B) day 2 (C and D) and day 5 (E and F), lungs were harvested; labeled T cells in the lung parenchyma were enumerated by flow cytometry. Inserts in each panel show dye fluorescence (x axis) versus forward scatter (y axis) of cells present in total lung suspensions at the indicated time points. Data are representative of four independent experiments.

ognition (21–23) or the result of the migration of these activated effector $CD8^+$ T cells out of the lungs to another site to die (e.g., the liver; references 24–26). However, the evidence suggesting extensive division of these T cells by 48 h after transfer (Fig. 5 C) raised the possibility that the cells were still present late in the progression of injury (days 4–5), but had undergone a sufficient number of divisions (and therefore CFDA-SE dye dilution) to no longer be detectable.

As an independent criterion for the presence of the transferred cells in the lungs, we made use of the fact that the D4 CTLs specifically bind MHC class I tetramers containing the HA210–219 peptide epitope (18). To detect and enumerate HA-specific T cells, dye-labeled D4 T cells were adoptively transferred into HA^+ and control HA^- mice, and lungs were harvested in order to examine infiltrating cells for HA210–219 tetramer staining and CFDA-SE dye content at days 1 and 5 after T cell transfer. At day 1, dye-labeled cells which bound HA210–219 tetramer were readily isolated from the lungs of control mice (Fig. 6 A). Specificity of tetramer staining was determined using an MHC class I tetramer loaded with a control k^d-binding peptide, derived from murine Jak-1 protein (18) (Fig. 6 B). By contrast, the majority of labeled cells isolated from the lungs of HA^+ mice at day 1 only weakly bound specific tetramer (Fig. 6 C) at a binding intensity slightly above the level of background staining observed with the control tetramer (Fig. 6 D). This result, although unexpected, was not surprising. Several recent reports have documented that engagement of the TCR on $CD8^+$ T cells in response to antigen results in the downregulation of cell surface TCR and a loss of tetramer staining (27–29). At day 5 after transfer, dye-labeled cells which bound the specific tetramer were readily enumerated in the lungs of control mice (Fig. 6 E and F), but few dye-labeled and/or tetramer staining cells were identified in the lung parenchyma of the transgene-positive mice at this time (Fig. 6 G and H).

To assess the functional status of transferred T cells present in the lungs of recipient mice late after transfer, we examined antigen-dependent IFN-γ production by $CD8^+$ T cells isolated at days 4–5 after transfer from the lungs of control and transgenic mice, in response to in vitro stimulation with the HA210–219 peptide by intracellular IFN-γ staining. As Fig. 7 A demonstrates, CFDA-SE–labeled $CD8^+$ T cells were readily identified in the lungs of con-

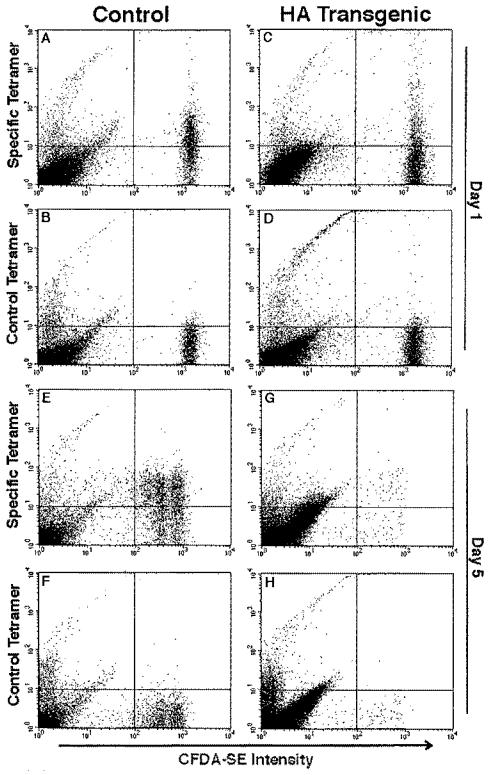

Figure 6. Tetramer-binding cells in lung parenchyma. D4 CTLs were labeled with CFDA-SE dye before transfer into SPC-HA^- (control) and SPC-HA^+ (HA transgene) recipients. At day 1 after transfer of 8–10 × 10^6 dye-labeled T cells, mononuclear cells infiltrating the lung parenchyma of recipient mice were isolated, and simultaneously examined by flow cytometry for CFDA-SE dye intensity, and for either HA210 tetramer staining (A and C), or staining by the control Jak-1 tetramer (B and D). A parallel analysis of dye intensity and HA210 tetramer staining (E and G) or Jak-1 tetramer (F and H) was performed at day 5 after transfer. Data shown are for one HA^+ and SPC-HA^- recipient at each time point and are representative of three independent experiments where the analysis of tetramer staining was performed.

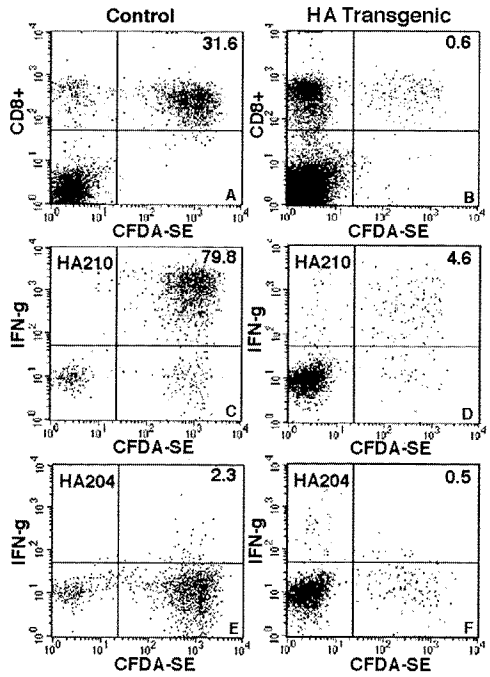

Figure 7. Ex vivo IFN-γ secretion by lung infiltration $CD8^+$ T cells. 5 d after transfer of 10 × 10^6 CFDA-SE–labeled D4 T cells into each of four SPC-HA^- (control) or SPC-HA^+ (HA transgene) recipients, pooled populations of lung-infiltrating mononuclear cells from HA^- and HA^+ recipients were examined directly ex vivo for CD8 expression and CFDA-SE dye intensity (A and B). In parallel, lung mononuclear cells were stimulated in vitro with the clone-specific HA210–219 peptide (C and D) or the control HA204–212 peptide (E and F). Antigen-dependent, intracellular IFN-γ accumulation (y axis), and CFDA-SE dye intensity (x axis) of lung mononuclear cells was determined by flow cytometry.

trol, nontransgenic mice and represented the majority of $CD8^+$ T cells isolated from the lungs (>90%). The dye-labeled cells produced IFN-γ in response to stimulation with the specific HA210–219 peptide (Fig. 7 C), but not the control HA204–212 peptide (Fig. 7 E). In SPC-HA^+ mice (Fig. 7 B), due to the exuberant inflammatory response in the lungs, $CD8^+$ T cells represent a smaller fraction of the total lung mononuclear cells than in control recipients (~10% $CD8^+$ T cells in HA^+ mice versus 30–35% $CD8^+$ T cells in HA^- control mice). As expected, <5% of the $CD8^+$ T cells isolated were dye-labeled. Few of $CD8^+$ T cells isolated from the lungs of these mice produced IFN-γ in response to specific peptide in vitro (Fig. 7 D and F) and the majority of the cells that did were positive for CFDA-SE staining (~75%).

Alveolar Type II Cell Status during Injury Progression. The above analysis suggested that the majority of $CD8^+$ T cells which enter in the lung parenchyma of HA^+ transgenic mice after adoptive transfer are eliminated from the lungs by 48 h after encounter with the target HA antigen expressed by alveolar type II cells. This loss of antigen-specific T cells over time parallels the decline in IFN-γ mRNA levels observed in the lungs after cell transfer (Fig. 4 A). However, pulmonary inflammation increased progressively over the same time frame. This implies that the sustained activity of transferred T cells is not required for injury progression and that the $CD8^+$ T cells act early in the injury process. A potential mechanism which could explain the need for antigen-specific CTLs only early in the evolution of the injury process is that these cells encounter HA-expressing type II cells in the lungs shortly after transfer and induce widespread destruction of the target cells by direct cytolysis. Inflammatory amplification might then occur both as a result of type II cell lysis and by release of proinflammatory mediators by the $CD8^+$ T cells in response to TCR engagement by antigen.

According to this hypothesis, alveolar type II cells should be rapidly eliminated from the lungs of HA^+ mice after $CD8^+$ T cell transfer. However, we recently reported that intact alveolar cells with the morphologic features of type II cells were demonstrable in situ in lung sections up to 48 h after T cell transfer into SP-C HA^+ recipients (17). Although $CD8^+$ T cell-mediated injury in this model is evident throughout the lungs (15, 16), the rate of injury progression and inflammatory cell accumulation in all regions of the lungs may not be uniform. Consequently, analysis of the number and viability of alveolar type II cells by morphologic criteria in lung sections may not provide an accurate estimate of the status of these $CD8^+$ T cell targets throughout the lung parenchyma during injury progression. Furthermore, rapid destruction of alveolar type II cells by effector T cells early in injury development (6–24 h) with compensatory regeneration and hyperplasia of these type late in injury development (16) could lead to an underestimate of the extent of alveolar type II destruction using only morphologic criteria. To assess the status of alveolar type II cells globally in the lung parenchyma during injury progression, we examined the level of murine surfactant B (SPB) mRNA in whole lung homogenates from HA^+ and HA^- T cell recipients by RPA. Since the SPB gene is constitutively expressed primarily in alveolar type II cells (30), the level of SPB message is likely to reflect the impact of the transferred $CD8^+$ T cells on type II cell integrity and numbers. As Fig. 8 demonstrates, SPB message levels in both HA^+ and HA^- recipients was unchanged over the time points sampled (6 through 96 h).

MCP-1 Gene Expression by Type II Cells In Situ. The above results suggested that alveolar type II cell numbers throughout the lungs did not fluctuate for up to 96 h after $CD8^+$ T cell transfer. This result is consistent with our previous findings that the $CD8^+$ T cells initiate injury by a perforin/FasL-independent mechanism (16) and that the interaction of the effector T cells with HA-expressing alveolar type II cells may result in the activation of these cells (17) rather than their elimination.

Since MCP-1 expression in the lungs was elevated in the lungs at 72 h after cell transfer (Fig. 4 B) when SP-B expression by alveolar type II cells was maintained, we asked if alveolar type II cells expressed MCP-1. To address this, MCP-1 gene expression was examined in the lung parenchyma by in situ hybridization. As Fig. 9 A demonstrates, the antisense probe for MCP-1 was localized to discrete sites in the alveoli of SP-C HA^+ recipient mice corresponding to the distribution of alveolar type II cells in the alveolar wall (31). The antisense probe did not bind to the lung parenchyma of HA^- nontransgenic mice at 72 h after T cell transfer (Fig. 9 B). In situ hybridization with the MCP-1 sense probe demonstrated no specific binding (data not shown). The discrete pattern of antisense MCP-1 probe localization along the alveolar wall corresponded to the distribution of HA transgene expression in alveolar type II cells of SP-C HA^+ mice using an antisense HA probe (Fig. 9 C). To further establish by morphology the presence of alveolar type II cells at 72 h after T cell transfer, we surveyed the lung parenchyma by electron microscopy. Fig. 9 D shows a representative view of an inflamed alveolus demonstrating an alveolar type II cell and an associated inflammatory cell in the alveolar wall.

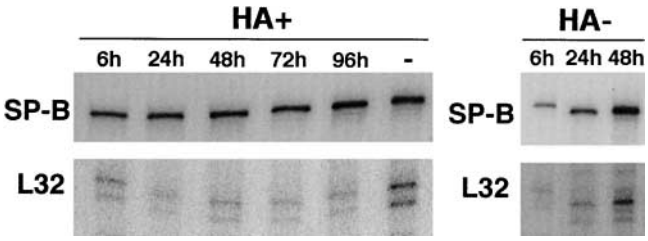

Figure 8. Kinetics of Surfactant B (SP-B) gene expression in the lungs after $CD8^+$ T cell transfer. Lungs of transgenic (HA^+) and nontransgenic (HA^-) mice were harvested at the indicated times after transfer of 10×10^6 cloned D4 CTLs per recipient and lung RNA isolated. SP-B gene expression was measured by RPA with expression of the L32 housekeeping gene included as a control for RNA loading. The level of SP-B expression in control mice not receiving D4 T cells is indicated in lane "-".

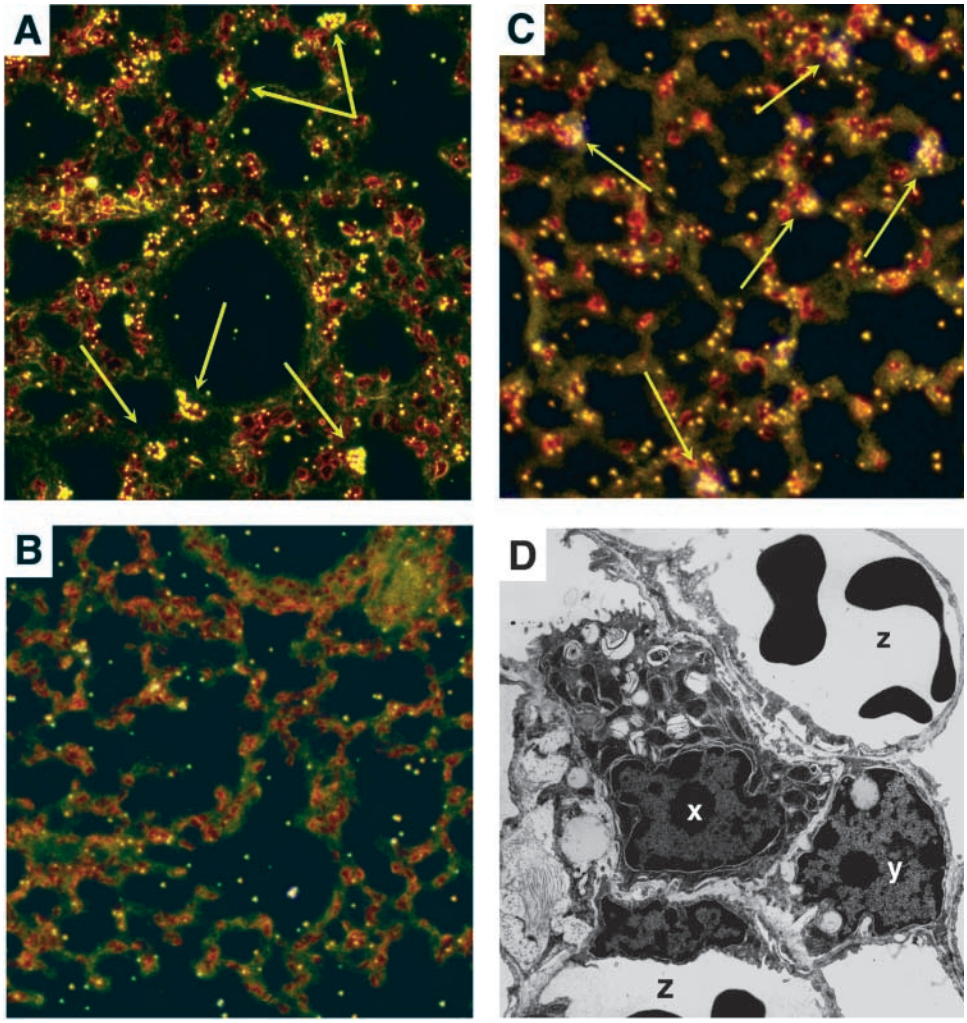

Figure 9. MCP-1 gene expression and alveolar type II cell localization. Frozen sections of lung tissue from SPC-HA$^+$ (A) and nontransgenic SPC-HA$^-$ (B) at 72 h after D4 T cell transfer were hybridized in situ with ^{3}[H]-labeled antisense and sense RNA probes specific for the murine MCP-1 gene (mRNA positive cells in dark field view: bright grain aggregates [arrows] overlying lung parenchyma with antisense probe). Original magnification: ×250. (C) In situ hybridization of SPC-HA$^+$ lung tissue with ^{3}[H]-labeled antisense probe for influenza HA mRNA. Original magnification: ×250. (D) EM section of fixed inflamed alveolar wall at 72 h after T cell transfer showing alveolar type II cell (x), infiltrating mononuclear inflammatory cell (y), and pulmonary capillary (z). Original magnification: ×8,000.

Discussion

T cell–mediated clearance of virus infection frequently involves some degree of injury to the tissue or organ involved, though the relative contributions of the immune response and the virus infection itself are difficult to determine. This study was undertaken to examine the evolution of pulmonary injury in a model of CD8$^+$ T cell–mediated lung injury, uncomplicated by virus infection, and to assess the fate of the injury-inducing T cells and the target cell population in the lung parenchyma. The results of this study indicate: (i) that lung injury is primarily due to antigen recognition by transferred CD8$^+$ T cells with little or no apparent contribution of lymphocytes of recipient origin recruited to the site of lung inflammation; (ii) that mononuclear phagocytes are the predominant inflammatory cell type recruited to the lung during the progression of this form of CD8$^+$ T cell–mediated pulmonary inflammation; (iii) that the expression of inflammatory mediators in the lung is rapid and robust—some mediators (e.g., TNF-α and MCP-1) are expressed at sustained levels throughout the course of injury development while expression of others (e.g., the T cell cytokine, IFN-γ) rapidly diminishes over time; (iv) that the great majority of injury inducing T cells are rapidly eliminated from lungs after transfer (i.e., within 48 h); and (v) that the targets of the transferred CD8$^+$ T cells (i.e., the transgene-expressing alveolar type II cells) appear not to be significantly diminished in numbers as a result of encounter with the CTLs and are induced in situ to upregulate expression of the chemokine MCP-1.

Recently, a number of reports have examined the CD8$^+$ T cell response and the development of CD8$^+$ T cell effectors during acute virus infection (8). These reports suggest that there is a rapid generation and expansion of activated CD8$^+$ CTL effectors in response to antigen. The effector CD8$^+$ T cells migrate to the site of virus infection and are then eliminated with the resolution of infection. Available evidence suggests that the effector T cells may either undergo activation-induced cell death in response to antigen (and die in situ) or leave the site of infection and migrate to the liver where the activated T cells are eliminated (24, 26). The model system described here is similar to acute infection in that cloned activated CD8$^+$ T cells after adoptive transfer reside in a site of target antigen expression (i.e., the transgene-positive lung) where the T cells encounter and

respond to the HA antigen. The fact that most of these activated effector T cells are not detected in lungs after 24–48 h is not surprising since, like activated effector cells present in lungs during acute viral infection, the transferred T cells would be eliminated after antigen encounter. In this model using whole body γ ray imaging of the distribution of radiolabeled $CD8^+$ CTLs, we have documented that the majority of transferred T cells (>80%) migrate from the lungs to the liver by 24–36 h after transfer (unpublished data). That the transferred T cells are responding to HA antigen in the lung is supported by the findings of decreased tetramer staining and diminished CFDA-SE content selectively by cells resident in the lungs of transgene-positive recipients at early times after cell transfer. Of particular interest is the fact that pulmonary inflammation and injury progress without the continual presence and sustained activity of the antigen-specific $CD8^+$ T cells in the lungs.

The finding that SPB mRNA levels in HA^+ lungs was unaffected by the transferred $CD8^+$ T cell recognition is also noteworthy. The expression of the murine SP-B gene has been reported not to be influenced by inflammatory injury to the lungs (30). However, systemic T cell activation or acute pulmonary injury has been reported to lead to the downregulation of murine SP-B expression in lung tissue (32, 33). Also, in cultured alveolar cells, exposure to inflammatory cytokines (e.g., IFN-γ, TNF-α) results in suppression of SP-B gene expression (34). Furthermore, rapid destruction of alveolar type II cells by cell-associated or soluble T cell effector molecules shortly after T cell transfer could result in the compensatory hyperplasia of residual type II cells in response to injury. In using SP-B gene expression to monitor alveolar type II cell numbers and function, we wanted to survey this cell population in situ throughout the lung parenchyma to avoid any potential bias inherent in the morphological evaluation of the viability and numbers of these cells due to the sampling. By evaluating SP-B expression both at the onset of lung injury development (6 h) and as injury evolved (24–96 h), we hoped to detect any fluctuation in alveolar type II cell numbers or function during the course of injury development as a result of cell death or effects of inflammatory mediators. The absence of such fluctuations in SP-B expression in this analysis suggests that early in injury development (i.e., day 1–4), type II cells are viable and capable of producing inflammatory mediators in response to recognition by T cells. In this connection, we have previously reported morphologic evidence of alveolar type II cell loss and compensatory type II cell hyperplasia in this model by EM (15). This data was obtained at days 5–6 after cell transfer when inflammation and necrosis of inflamed alveolar walls was extensive (15). Both type II cell loss and regeneration could be occurring simultaneously in response to the massive and diffuse pulmonary injury present at this late time. Our data suggest that earlier in the evolution of injury (48–72 h), when alveolar wall necrosis is not prominent, alveolar type II cells are viable and capable of producing inflammatory mediators (Figs. 8 and 9) important in injury progression (17).

In many adoptive transfer models of T cell–mediated tissue injury (or pathogen clearance), the extent of tissue injury (or efficiency of pathogen clearance) is dependant upon the number of effector T cells transferred. We also reported a similar $CD8^+$ T cell dose dependence of injury development in this model of pulmonary inflammation and injury (14) with lower transferred cell innocula ($<5 \times 10^6$ cells) resulting in sublethal injury, minimal weight loss, moderate pulmonary inflammation, a decreased inflammatory mediator expression in the lungs, (references 15 and 16; unpublished data). The cell dose dependence of injury development is not directly compatible with a mechanism of a $CD8^+$ T cell–mediated injury where the effector T cells in the lung recycle and encounter multiple target cells before their elimination. According to this mechanism, the transfer of lower cell innoculum should also result in progressive and ultimately lethal pulmonary injury, but over a more protracted time course. The results reported here suggest a mechanism where the effector T cells initiate the injury process in the lungs and then are rapidly eliminated from the injury site. Accordingly, the extent of injury would be dependant on the number of T cells transferred.

Both the rapid elimination of the transferred effector $CD8^+$ T cells and the uniform SP-B expression over the first 96 h of injury development support the idea that the interaction of the CTL with alveolar type II target cells in situ is nonlytic and are consistent with our previous finding that the expression of cell-associated cytolytic machinery (e.g., perforin or FasL) by the transferred T cells is not required for injury development (16). There is evidence from models of virus specific $CD8^+$ T cell responses suggesting that virus clearance by $CD8^+$ CTLs in vivo can be mediated by mechanisms which do not require direct CTL-mediated lysis of virus-infected cells (1, 35). Similarly, in a transgenic model of hepatitis B virus (HBV) infection, expression of the HBV genome is suppressed through a nonlytic cytokine-mediated interaction between CTL effectors and hepatocytes in vivo (36). In this connection, it should be emphasized that the expression of a CTL target antigen as a transgene in a tissue site does not necessarily reflect the milieu in which an effector $CD8^+$ T cell encounters antigen displayed on virus-infected cells. Inflammatory mediators released by virus-infected cells and/or cells of the innate immune system in response to active infection could increase the sensitivity of target cells to direct cytolysis by CTLs. Nevertheless, our findings suggest that a nonlytic cell/cell interaction between effector T cells and target cells may result in the activation of the target cells in situ and their participation in the inflammation process (37).

The loss of IFN-γ gene expression observed in the lungs of HA^+ recipients over time after T cell transfer is readily attributable to the progressive elimination of functional HA-specific $CD8^+$ T cells early in the evolution of pulmonary injury. In contrast to the transgenic model of HBV infection (1, 36), $CD8^+$ T cell–derived IFN-γ appears not to play a critical role in vivo since HA-specific CTL effectors genetically deficient in IFN-γ production efficiently initiate the development of lethal pulmonary injury after

adoptive transfer (unpublished data). However, TNF-α, which is produced by a diverse array of cell types including activated $CD8^+$ T cells in response to antigen recognition (38, 39), is expressed at an elevated level throughout the course of injury development. TNF-α produced in an antigen-dependent fashion by the transferred T cells may be crucial in the initial phase of injury in this model (17). The persistent TNF-α gene expression evident during evolution of injury and inflammation presumably reflects the production of this cytokine by other cell types in the inflamed lung. The likely source of this TNF-α is activated mononuclear cells of monocyte/macrophage origin actively recruited into the injured lungs through the action of MCP-1 and other chemotactic factors. The definitive demonstration that recruited macrophages are the source of this TNF-α awaits more detailed analysis.

A possible link between macrophage influx into the injured lung and nonlytic recognition of alveolar type II cells by the transferred T cells comes from the analysis of the kinetics of MCP-1 expression in the lungs of HA^+ recipients. Like TNF-α, the expression of this chemokine gene in the SPC-HA^+ lungs increases extremely rapidly after adoptive transfer, and only slowly decreases throughout the evolution of pulmonary injury. MCP-1 is a potent chemoattractant of mononuclear phagocytes (37, 40). Unlike TNF-α and IFN-γ, this chemokine is not produced by activated D4 T cells or other cloned murine $CD8^+$ T cells (reference 17; unpublished data). However, MCP-1 gene expression is upregulated and MCP-1 protein is secreted by cultured alveolar type II cells in response to an antigen-specific interaction with $CD8^+$ CTL effectors in vitro (17). We show here that, 72 h after $CD8^+$ T cell transfer in SPC-HA mice, MCP-1 gene expression can be detected in situ in alveolar wall epithelial cells with the morphological and anatomic distribution of HA^+ alveolar type II cells. The sustained production of MCP-1 by the activated alveolar type II cells would contribute to the activation and recruitment of mononuclear phagocytes to the site of injury, and promote injury progression even after CTL effector cells are eliminated. Once activated, the production of inflammatory mediators by the activated, recruited macrophages would lead, in turn, to further amplification of inflammation and progressive injury to the lungs.

In conclusion, the results reported here provide insight into the process of inflammatory lung injury mediated by $CD8^+$ T cells which may be applicable both to pulmonary virus infection and to other inflammatory lung injuries where $CD8^+$ T cells have been implicated. Accordingly, effector $CD8^+$ T cells need not function continuously, but need only function transiently to initiate the development of progressive lung injury. Nonlytic interactions between effector $CD8^+$ T cells and target cells in vivo in the lung could result in the transient activation of the target cells in situ and their participation in the amplification of the injury process through the recruitment and activation of inflammatory cells. In the case of acute virus infection in the lung, the extent of virus replication in the lungs before the generation and recruitment of activated $CD8^+$ T cell effectors to the site of infection would be crucial to the ultimate outcome of infection. When virus replication is extensive, potent effector $CD8^+$ T cells (even if only transiently present and active) would trigger an inflammatory cascade of sufficient magnitude to promote extensive and potentially lethal injury—even in the face of effective viral clearance by lytic and nonlytic $CD8^+$ T cell effector mechanisms (1, 35, 39). Therefore, therapeutic control of acute virus infection may require intervention not only at the level of virus replication, but also at the level of the host immune response. A corollary implication is that rapid elimination of T cell effectors may occur in the absence of antigen clearance, as might be expected in autoimmune disease, with continued inflammation despite the absence of antigen-specific cells.

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