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Research Article

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Structural and Functional Consequences of Alveolar Cell Recognition by CD8$^+$ T Lymphocytes in Experimental Lung Disease

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Abstract

CD8$^+$ T cells infiltrate the lung in many clinical conditions, particularly in interstitial lung disease. The role(s) that CD8$^+$ T cells might be playing in the pathogenesis of inflammatory lung disease is unclear at present, as is the direct contribution of CD8$^+$ T cell effector activities to lung injury. This report describes a transgenic model used to evaluate the impact, on respiratory structure and function, of CD8$^+$ T lymphocyte recognition of a target antigen expressed endogenously in alveolar epithelial cells. We found that adoptive transfer of cloned CD8$^+$ cytotoxic T lymphocytes (CTLs) specific for an alveolar neo-antigen (influenza hemagglutinin) leads to progressive lethal injury in transgenic mice, which dramatically affects lung structure and function. Transgenic recipients of CD8$^+$ CTLs exhibited tachypnea and progressive weight loss, becoming moribund over a period of several days. Concomitantly, the animals developed a progressive interstitial pneumonitis characterized initially by lymphocytic infiltration of alveolar walls and spaces, followed by an exuberant mononuclear cell infiltration that correlated with restrictive pulmonary mechanics and a progressive diffusion impairment. These results indicate that antigen-specific CD8$^+$ T cell recognition of an alveolar epithelial "autoantigen" is, in and of itself, sufficient to trigger an inflammatory cascade that results in the histological and physiological manifestations of interstitial pneumonia. (*J. Clin. Invest.* 1998. 102:1653–1661.) Key words: T lymphocyte • alveolar cell • interstitial pneumonia • lung injury • diffusion

Introduction

A significant number of lung diseases are presumed to be T cell mediated based in part on the observation of T cell accumulation in sites of disease activity. Most of these disorders are notably characterized by the accumulation of CD8$^+$ and CD4$^+$ T cells, in addition to neutrophils and macrophages, in the alveolar space and/or the interstitium (1–4). Though injury to alveolar epithelium is usually present, it is difficult to assess the relative contribution of each inflammatory cell type to this injury. We have developed a model that allows assessment of the specific contribution of CD8$^+$ T lymphocytes to inflammatory injury, and the quantitative functional consequences thereof.

The role(s) that CD8$^+$ T cells might be playing in the pathogenesis of inflammatory lung disease is unclear at present, as is the nature of the effector function(s) expressed by CD8$^+$ T cells at sites of inflammatory activity (as well as the contribution of these effector activities to injury). CD8$^+$ T cells have been designated as a "cytotoxic/suppressor" T cell subset, though evidence supporting a suppressor role is very limited. However, it has been clearly demonstrated that CD8$^+$ cytotoxic T lymphocytes (CTLs)[1] express potent cytotoxic activity in vitro and function in the clearance of virus during experimental infection in vivo (5). It has become evident in recent years that CD8$^+$ T cells also secrete a variety of cytokines that, under certain conditions, may also contribute to viral clearance. In vitro analyses of CD8$^+$ T cell antiviral activity clearly demonstrate that cytotoxic activity is both necessary and sufficient to achieve specific lysis of virus-infected target cells (5–9). Furthermore, CD8$^+$ T cell–mediated virus clearance during experimental influenza infection, in vivo, occurs in an exquisitely antigen-specific manner, providing additional evidence that CD8$^+$ T cells use cell-associated or very short-acting effector mechanisms in the process of viral clearance from the respiratory tract (10).

Experimental influenza infection results in significant lung injury that is evident both clinically and histologically. How much of this injury is due to the direct effects of viral replication and how much is due to the host response is uncertain, since influenza is a lytic virus intrinsically capable of producing host cell injury irrespective of the cellular immune response (11). In addition, however, influenza elicits an exuberant CD8$^+$ (and CD4$^+$) T cell response, which complicates our understanding of the relative contribution of virus and host to the injury that ensues during infection. There is evidence that the host immune cell responses may contribute significantly to the damage that occurs to the lung during virus infection. Experimental influenza infection in T cell–deficient mice results in a milder, more protracted (though ultimately lethal) clinical course, with less pronounced histologic evidence of inflammatory injury than is observed in wild-type animals (11–13). Assessment of the host T cell contribution to this injury is of particular importance since it has long been hypothesized that a failure to downregulate virus-specific T cell responses during infection may lead to chronic pulmonary injury that is seen in several clinical states, such as idiopathic pulmonary fibrosis (14, 15).

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1. *Abbreviations used in this paper:* CFSE, 5-carboxyfluorescein diacetate-succinimidyl ester; CTL, cytotoxic T lymphocyte; EM, electron microscopy; HA, influenza hemagglutinin; IC, inspiratory capacity; SPC, surfactant protein C; vv, recombinant vaccinia virus.

Various models of immune-mediated lung injury have been described (16–25) and usually involve an inciting agent. The evaluation and precise definition of the contribution of the host response to injury in such systems is frequently complicated by the difficulty in separating the injury that is directly induced by the inciting agent from that mediated by the immune response. We recently developed a model system to specifically dissect the nature and consequences of the pulmonary injury mediated directly by $CD8^+$ T cell recognition of a target antigen in the lung, in the absence of any exogenous insult. In this model, the A/Japan/57 influenza hemagglutinin (HA) was expressed as a transgene in mice under the transcriptional control of the surfactant protein C (SPC) promoter in order to achieve lung-specific expression. The HA transgenic animals exhibited complete $CD8^+$ T cell tolerance to all MHC class I–restricted epitopes of HA (26). The adoptive transfer of HA-specific $CD8^+$ CTLs into these mice therefore provided a system with which to examine the specific impact of autoantigen recognition by CTLs on pulmonary structure and function. In this report, we demonstrate that $CD8^+$ T cell recognition of this lung-specific autoantigen leads to profound and lethal lung injury, characterized by restrictive changes in lung mechanics, impaired gas exchange, and marked alveolitis.

Methods

Recipient mice. SPC-HA transgenic (tg^+) mice in the H-2^d haplotype, expressing the A/Japan/305/57 HA under the transcriptional control of the SPC promoter were used in these studies (26). The transgenic animals were originally developed in the FVB strain (H-2q), and subsequently backcrossed three times with BALB/c (H-2d) mice. To exclude the possible contribution of minor histocompatibility between cell donors and recipients in our studies, all experiments included matched transgene-negative (tg^-) littermates as control recipients. Animals used in adoptive transfers were 10–12 wk of age (18–22 g).

Effector T cell populations. To obtain bulk $CD8^+$ CTL populations with a defined HA specificity, we immunized wild-type BALB/c mice with a recombinant vaccinia virus (vv) expressing an A/Japan/57 HA deletion mutant that lacks the transmembrane and cytoplasmic domain (vv[HA anchor−]). The construct retains the HA204–212 and HA210–219 epitopes recognized in association with the H-$2K^d$ MHC class I molecule by H-$2K^d$–restricted $CD8^+$ CTLs. HA-immune splenocytes from these animals, which contained $CD8^+$ memory T cells specific for these two HA epitopes, were restimulated in vitro with splenocytes infected with the A/GV/17 (H2N2) influenza strain. This virus has a single nucleotide difference in the HA gene (compared with the A/Japan/57 strain) leading to an amino acid substitution in the 204–212 epitope (N to K change at residue 207; reference 26). Consequently, this virus selectively activates and expands $CD8^+$ memory CTL precursors from the vv(HA anchor−)–primed mice that are directed to the HA210–219 epitope (common to A/GV/17 and A/Japan/57). These populations were maintained by weekly in vitro stimulation, in fresh Iscove's complete media, supplemented with 10 U/ml of IL-2. The clones were restimulated in vitro with irradiated syngeneic splenocytes that were infected with A/Japan/57 influenza. $CD8^+$ T cell clones used in these experiments were generated by limiting dilution as previously described (27), and both bulk cultures and CTL clones were derived from BALB/c mice. The clones were restimulated in vitro with irradiated syngeneic splenocytes that were infected with A/Japan/57 influenza, and placed into fresh Iscove's complete media supplemented with 10 U/ml of IL-2.

T cell cytotoxicity. 5 d after in vitro stimulation, T cells were tested for cytolytic activity using a ^{51}Cr-release assay against P815 target cells infected with A/Japan/57, or against target cells loaded with 10^{-9} M synthetic peptide representing either the 204–212 or the 210–219 epitope. In some experiments, the target cells used were MLE-15, an alveolar epithelial cell line (obtained from Dr. Jeffrey Whitsett, Children's Hospital, Cincinnati, OH) that was transfected with H-$2K^d$ (construct provided by Dr. Michael Bevan, University of Washington, Seattle, WA). Expression level was determined with flow cytometry (see below) using the anti-K^d antibody SF1.11 (American Type Culture Collection, Rockville, MD). The MLE-15 cell was derived from an animal of the FVB strain, and therefore untransfected control cells were included in cytotoxicity assays.

Flow cytometry. Bulk T cell cultures were separated from stimulator cells over isopaque-ficoll density gradient centrifugation and stained with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 (Caltag, Burlingame, CA). Flow cytometry was performed on a Becton Dickinson FACScan® using CellQuest software (Becton Dickinson, Palo Alto, CA). In some experiments, whole lungs from animals receiving fluorescent-labeled T cells (see below) were removed, minced through a tissue sieve, and inflammatory cells were separated from debris over an isopaque-ficoll density gradient before flow cytometric quantitation.

Adoptive transfer. On day 5 after stimulation, T cell clones were separated from stimulators by density gradient centrifugation and injected via the tail vein into transgenic animals. When indicated, the T cells were labeled with 1 μm CFSE (5-carboxyfluorescein diacetate-succinimidyl ester; Molecular Probes, Eugene, OR) for 10 min in serum-free media. For routine histology, the animals were killed, their trachea intubated, and their lungs inflated with 10% buffered formalin before excision. The lungs were embedded, sectioned, and stained with hematoxylin and eosin. For fluorescent localization of transferred cells, the airways were perfused with 50% OCT and snap frozen in liquid nitrogen. Frozen sections were cut and viewed without counterstain with a Zeiss fluorescent microscope. For electron microscopy (EM), lungs were inflated with 2.5% glutaraldehyde/4% paraformaldehyde and processed for routine transmission EM at the University of Virginia Cell Biology EM core facility (Charlottesville, VA). Five sections were sampled at 20 μm intervals from the pleura proximally to minimize sampling error. A single histologist, blinded to the identities of the specimens, performed the quantitative morphometric analysis.

Pulmonary function. Animals were anesthetized with ketamine/xylazine and paralyzed with pancuronium (28). Their trachea was intubated, and the animals were placed on a pressure ventilator (Kent Scientific, Litchfield, CT) at a respiratory rate of 120/min and a pressure limit of 12 cm H_2O. The inspiratory capacity (IC) of each animal was estimated by inflation with air to a pressure equivalent to 20 cm H_2O (29, 30). The lungs were then inflated with a volume equal to IC of a gas mixture containing 0.3% CO, 0.3% methane, and 20% O_2 (the balance was N). The gas was allowed to dwell for a 10-s breathhold, and then one half of the sample was drawn off and discarded as dead space while the other half drawn off as an alveolar sample (31). The alveolar sample was analyzed on a gas chromatograph (Shimadzu Instruments, Columbia, MD). Three measurements were made for each animal and the CO uptake was determined as a ratio of CO loss to methane loss (30, 32).

Statistical analysis. The paired Student's *t* test was used for all statistical analyses.

Results

Generation of HA-specific cytolytic T lymphocytes. Since SPC-HA transgenic (tg^+) mice lack A/Japan/57 HA-specific $CD8^+$ CTLs (26), we generated heterogeneous populations of HA-specific $CD8^+$ CTLs using HA-immune cells from influenza-infected wild-type (tg^-) mice and evaluated the impact of CTL recognition of the lung-specific HA autoantigen. This was accomplished by adoptive transfer of heterogeneous populations of HA-immune cells enriched for $CD8^+$ CTL effectors into tg^+ mice. To selectively activate $CD8^+$ CTL with a defined HA

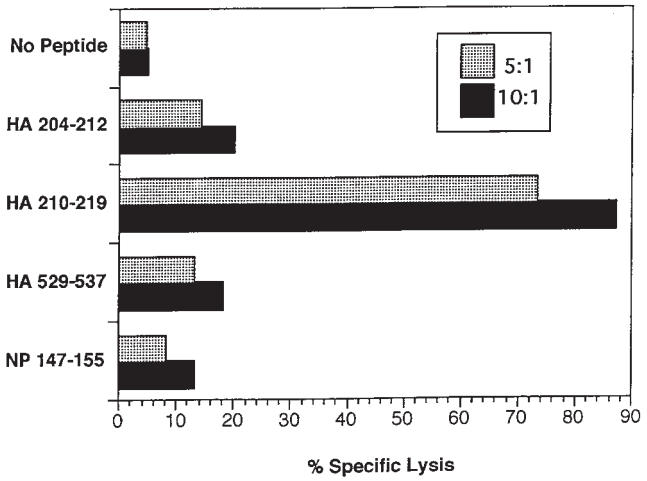

Figure 1. T cell cytolytic activity of oligoclonal CD8$^+$ bulk populations generated by immunizing normal BALB/c mice with a vv expressing the extracellular domain of the A/Japan/305/57 influenza HA (vv[HA anchor−]), and restimulating in vitro with autologous antigen presenting cells infected with the mutant A/Guyang variant 17/57 influenza. Target cells are labeled P815 cells, and were treated with 10^{-9} M synthetic peptide. Assays were harvested after 6 h. The oligoclonality of the T cell population is evident in the limited specificity for the 210–219 epitope (common to the two strains of influenza).

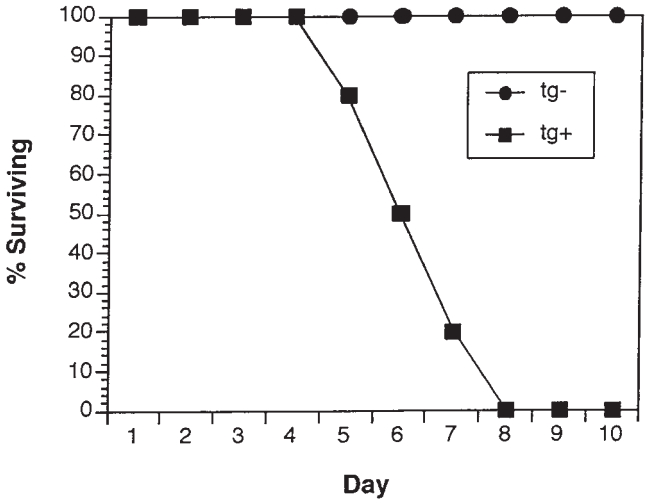

Figure 2. Survival after adoptive transfer of oligoclonal CD8$^+$ T cell bulk population. T cells were stimulated in vitro with antigen and IL-2, and 5 d later separated from the antigen presenting cells. Administration by tail vein injection of 2×10^7 T cells into tg$^+$ mice resulted in death by day 8 in 100% of the recipient animals, whereas all tg$^-$ animals survived.

specificity, we immunized wild-type BALB/c mice with a vv expressing an A/Japan/57 HA deletion mutant that encodes a protein lacking the transmembrane and cytoplasmic domain of the HA (vv[HA anchor−]). The construct retains the HA204–212 and HA210–219 epitopes recognized in association with the H-2K^d MHC class I molecule by H-2K^d–restricted CD8$^+$ CTLs. HA-immune splenocytes from these animals, which contained CD8$^+$ memory T cells specific for these two HA epitopes, were restimulated in vitro with splenocytes infected with the A/GV/17 influenza strain to selectively activate and expand CD8$^+$ CTL precursors directed to the HA210–219 epitope common to A/GV/17 and A/Japan/57. As Fig. 1 shows, in vitro restimulation of influenza specific memory CD8$^+$ T lymphocytes from the vv(HA anchor−)–infected donors leads to the selective activation of HA210-219–specific CTL effectors. Since in vitro stimulation with virus-infected APCs exclusively activates memory CD8$^+$ T lymphocytes, CTLs directed to the HA transmembrane-encoded 529–537 epitope, and the nucleocapsid protein-encoded 147–155 epitope (both shared by the A/JAPAN/57 and A/GV/17 viruses) are not induced.

Adoptive transfer of HA-specific cytolytic T cells into HA-transgenic animals. This heterogeneous population of immune splenocytes enriched for HA210-219–specific CTLs was expanded by restimulation with antigen in the presence of IL-2 to generate sufficient numbers of CTL effector cells for transfer. Flow cytometric analysis performed at the time of adoptive transfer of this heterogeneous population of immune cells enriched for HA210-219–specific CTL effectors revealed that the cells were 88% CD8$^+$ and 2% CD4$^+$ (not shown). As Fig. 2 shows, administration of 2×10^7 activated T cells into tg$^+$ mice resulted in death by day 8 in 100% of the recipient animals, whereas all tg$^-$ animals survived. Clinically, the T cell transfer resulted in the rapid development (24–48 h) of labored breathing, lethargy, and significant weight loss in tg$^+$ animals. At necropsy, gross inspection of the lungs of tg$^+$ mice revealed consolidation and patchy areas of hemorrhage. Other organs showed no gross abnormalities. Histologic examination of the lungs from tg$^+$ recipients demonstrated mild to moderate perivasculitis, bronchiolitis, and, most strikingly, extensive infiltration of the alveolar septae and airspaces with mononuclear cells and concomitant focal disruption of alveolar wall integrity and intraalveolar hemorrhage. Quantitative morphometric analysis of lung infiltrates from recipient mice by electron microscopy (at day 5 after cell transfer) revealed extensive alveolar macrophage infiltration, congestion of the vascular spaces with neutrophils and macrophages, and loss of type II epithelial cells only in tg$^+$ recipients (Fig. 3). No histologic evidence of pulmonary injury was apparent in the tg$^-$ recipients at either the light or EM level (Fig. 3). Interestingly, although lymphocytes accumulated in the lung parenchyma at 24 h (see below), by 5 d after transfer there were no lymphocytes evident in an otherwise extremely exuberant inflammatory infiltrate. The possibility of minor histoincompatibility accounting for these observations was excluded by the absence of effect of transferred T cells in the matched tg$^-$ littermate control mice. Therefore, lung injury in this system requires the expression of the HA transgene.

Homogeneous CD8$^+$ T cell populations produce lung injury. The above results suggested that within this heterogeneous population of HA-specific T lymphocytes there were effector T lymphocytes capable of inducing lethal pulmonary injury after adoptive transfer into tg$^+$ recipient mice. To directly establish that the lethal injury was mediated by HA210–219–specific CTLs within the heterogeneous immune cell population, we carried out a comparable analysis using a clonal population of CD8$^+$ HA210-219–specific CTLs derived by limiting dilution culture of a bulk population of HA-specific CTL effectors (27, 33). This CTL clone (40-2) has the same in vitro fine specifically as the heterogeneous HA210-219–specific

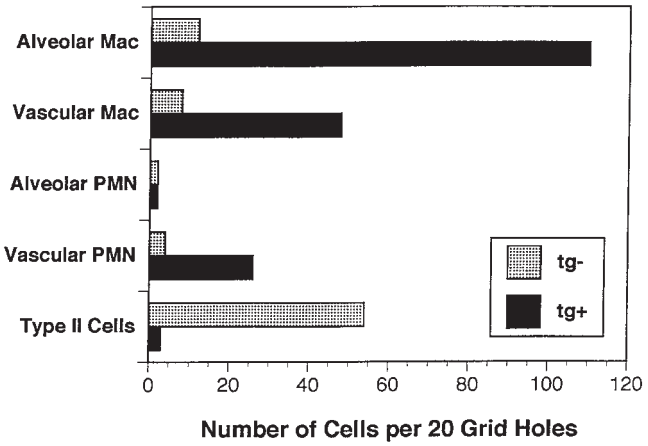

Figure 3. Quantitative morphometric analysis of lung infiltrates from recipient mice by EM (on day 5 after cell transfer of oligoclonal $CD8^+$ T cells). Five sections were sampled at 20-μm intervals from the pleura proximally, and counted in a blinded fashion. Extensive alveolar macrophage infiltration, congestion of the vascular spaces with neutrophils and macrophages, and loss of type II epithelial cells were apparent in tg^+ recipients, but not in tg^- recipients. By day 5 after T cell transfer, no lymphocytes are evident. These data are representative of three separate experiments (two animals each) with similar results.

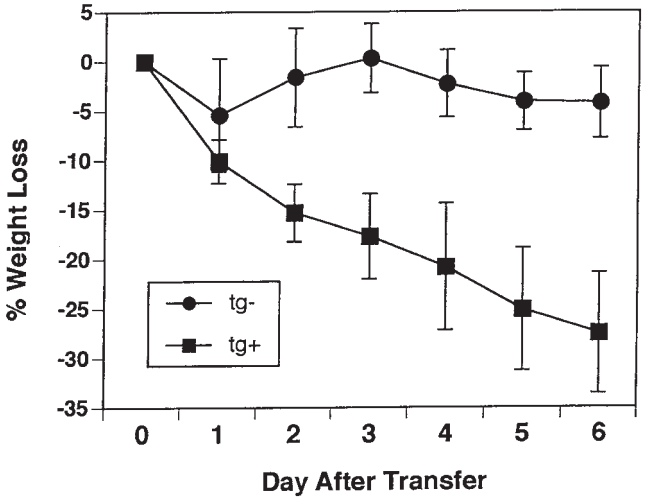

Figure 5. Weight loss in tg^+ versus tg^- mice after transfer of 5×10^6 cloned 40-2 cells. Transfer into tg^+ recipients resulted in the development of labored breathing, lethargy and progressive weight loss over a 6-d period, and was ultimately lethal.

$CD8^+$ T lymphocyte population described above (33). As Fig. 4 demonstrates clone 40-2 lyses the murine alveolar type II epithelial cell line MLE-K^d after infection with A/Japan/57 virus or after pulsing with a synthetic peptide corresponding to the H-$2K^d$–restricted HA210–219 epitope. As expected, clone 40-2 failed to recognize epithelial cell targets treated with the unrelated HA204-212 peptide epitope. The HA204-212–pulsed (and A/Japan/57-infected) MLE-K^d cells were, however, specifically lysed by CTLs directed to the HA204–212 epitope (not shown).

The $CD8^+$ HA210-219–specific CTL clone 40-2 was adoptively transferred into tg^+ and tg^- littermates. Transfer of 5×10^6 cloned 40-2 cells into tg^+ recipients resulted in the development of labored breathing, lethargy, and progressive weight loss over a 6-d period (Fig. 5). This injury was ultimately lethal in tg^+ recipients of clone 40-2. By contrast, tg^- recipients of clone 40-2 exhibited a mild transient weight loss (over the first 24–48 h) with no respiratory symptoms (Fig. 5). As Fig. 6 demonstrates, the time course of progression of lethal injury in tg^+

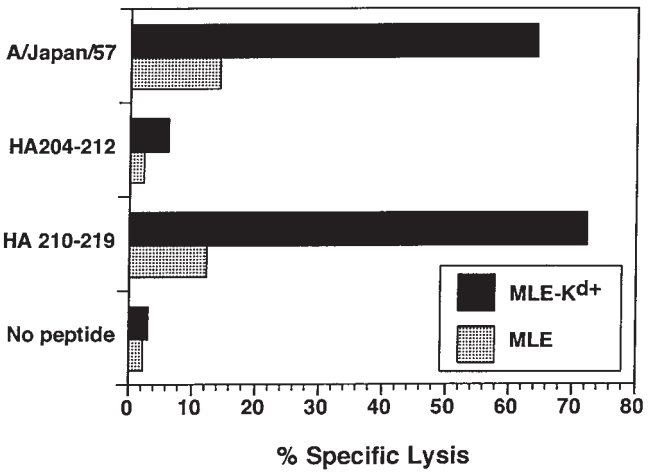

Figure 4. In vitro cytolytic activity of the $CD8^+$ T cell clone, 40-2, on labeled MLE-K^d targets (compared with untransfected MLE cells). MLE-K^d is an alveolar epithelial cell line (MLE-15) that was transfected with H-$2K^d$ (physiologic level of expression was confirmed by flow cytometry). Target cells were either infected with A/Japan/57 influenza or treated with 10^{-9} M synthetic peptide. Assays were harvested after 6 h. The specificity of the T cell cytotoxic activity is indicated by the exclusive response to the 210–219 peptide (or virus) in the context of K^d expression.

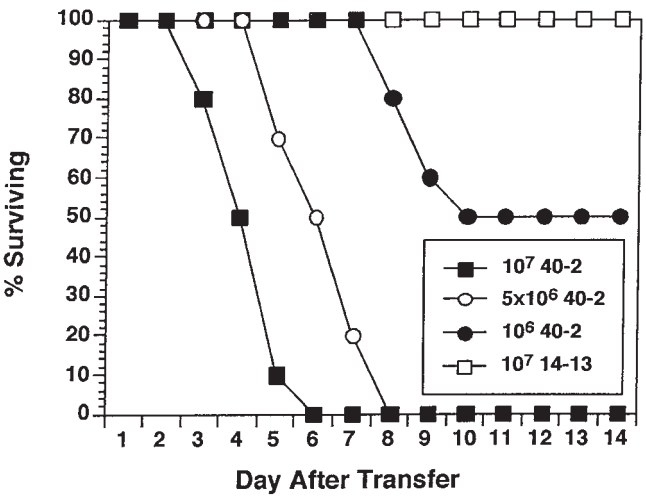

Figure 6. Survival after adoptive transfer of various doses of $CD8^+$ T cell clone, 40-2. Administration of 10^7 T cells into tg^+ mice resulted in 100% death by day 6, whereas 10^6 T cells resulted in death of 50% of recipients by day 9. Administration of 10^7 T cells specific for an irrelevant antigen (14-13, a $CD8^+$ T cell specific for the 147–155 epitope of the influenza nucleoprotein) resulted in 100% survival in tg^+ mice. There were six mice in each group. All tg^- animals that received 40-2 survived (not shown).

mice was directly dependent on the dose of clone 40-2 administered. Recipients of 10^7 cloned CTLs exhibited accelerated mortality and rapidly succumbed over a 5-6–d period. The transfer of as few as 10^6 40-2 cells produced 50% mortality, albeit over a more protracted period (Fig. 6). It is noteworthy that tg^+ mice receiving 10^7 cells of CTL clone 14-13, which is an influenza-specific CTL clone directed to the K^d-restricted influenza nucleocapsid epitope NP147–155, exhibited no morbidity or mortality (Fig. 6). This suggests that the lethal injury produced by the adoptively transferred CTL required specific antigen recognition and was not due to a nonspecific increase in susceptibility of tg^+ mice to $CD8^+$ CTL–mediated injury as a result of transgene expression in lung type II cells during development. This result is consistent with our earlier finding that tg^+ mice also show no increased acute susceptibility to influenza virus infection (26).

Morphologic changes in the lungs of tg^+ mice receiving 5×10^6 cloned 40-2 cells were assessed serially by light microscopy during a 5-d period after CTL transfer. By 24 h after cell transfer, the extent of pulmonary injury was surprisingly mild and consisted primarily of periarteriolar and peribronchiolar infiltration by mononuclear cells (Fig. 7, *a* and *b*). At 48 h after transfer there was intense peribronchiolar and periarteriolar cuffing by mononuclear cells with mild, and somewhat patchy, infiltration of alveolar septae by mononuclear cells resulting in slight thickening of the alveolar walls. Over the next 24 h (day 3 after cell transfer), the intensity of perivascular and peribronchiolar mononuclear cell infiltration continued to increase and was accompanied by increasing interstitial inflammation in alveolar walls and moderate mononuclear cell extravisation into alveolar spaces (Fig. 7, *c–e*). The alveolar changes were focally distributed throughout the lung and varied in intensity. 4–5 d after cell transfer, tg^+ mice exhibited progressive alveolitis with patchy intraalveolar hemorrhage, intense interstitial inflammation within alveolar septae, focal disruption of alveolar wall integrity, and marked mononuclear cell accumulation within the terminal airways and alveoli. This pattern of progressive inflammation and parenchymal injury was comparable

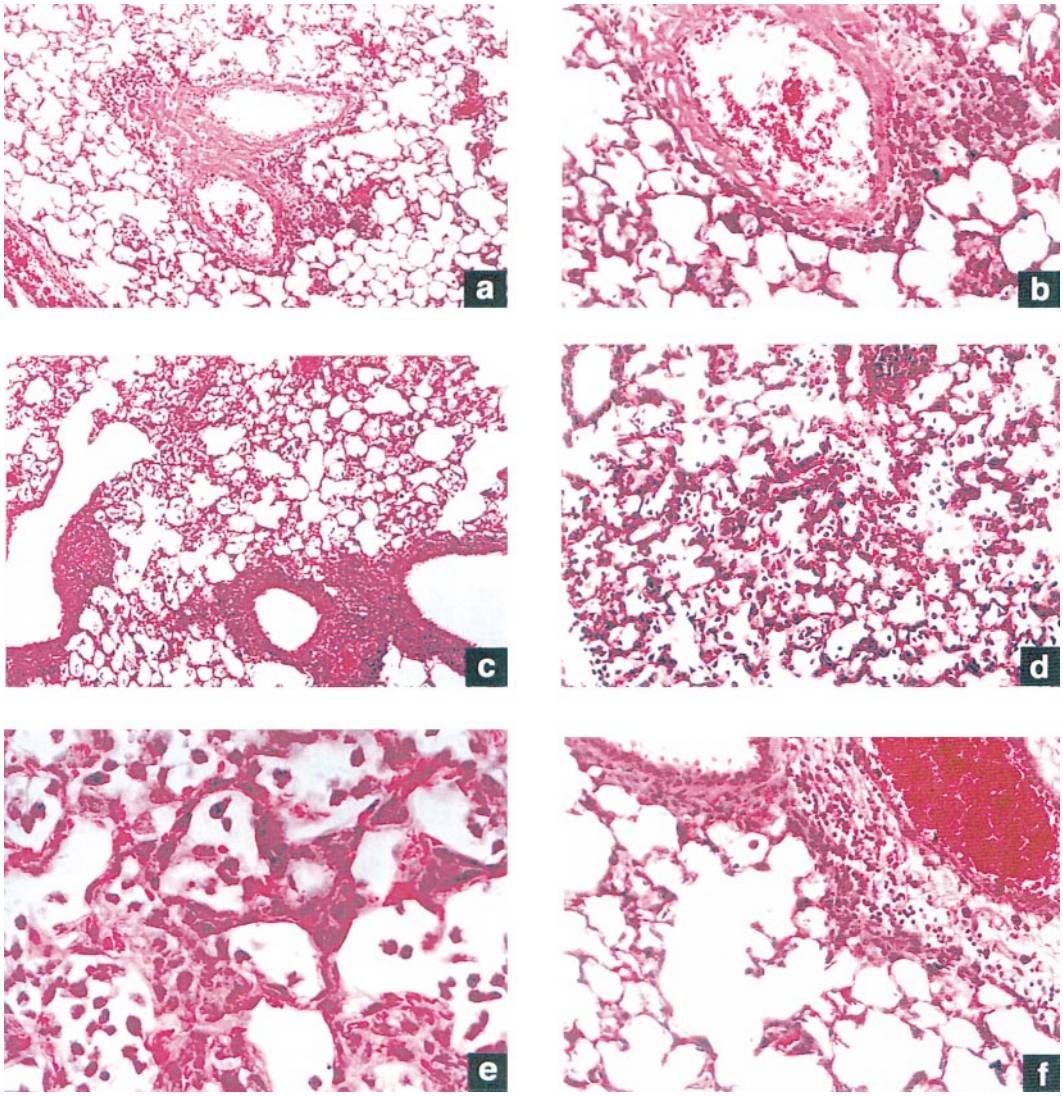

Figure 7. Morphologic changes in the lungs of tg^+ mice receiving 5×10^6 cloned 40-2 cells were assessed serially by light microscopy after CTL transfer. (*a*) Low power view of tg^+ lung 24 h after cell transfer. The extent of pulmonary injury was surprisingly mild. There is minimal interstitial infiltration with mild periarteriolar and peribronchiolar infiltration by mononuclear cells. (*b*) High power view of tg^+ lung 24 h after cell transfer. Very early septal involvement is evident. (*c*) Low power view of tg^+ lung 3 d after cell transfer. The intensity of perivascular and peribronchiolar mononuclear cell infiltration continued to increase. The alveolar changes were focally distributed throughout the lung and varied in intensity. (*d*) High power view of tg^+ lung 3 d after cell transfer. Increasing interstitial inflammation in alveolar walls and moderate mononuclear cell extravasation into alveolar spaces are apparent. (*e*) Very high power view of tg^+ lung 3 d after cell transfer. Significant alveolitis with mononuclear cell infiltration of the alveolar spaces is evident. (*f*) The lungs of tg^- mice 3 d after cell transfer. There was minimal perivascular and peribronchiolar mononuclear cell infiltration that resolved without histologic or physiologic evidence of alveolar damage.

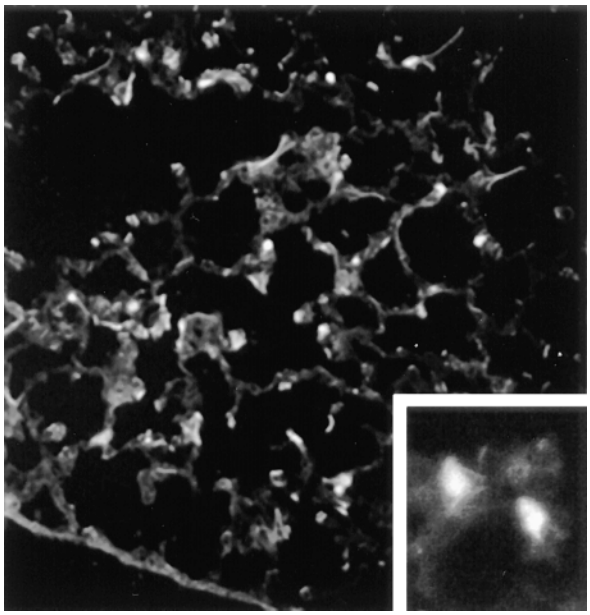

Figure 8. Histologic localization of adoptively transferred $CD8^+$ T cell clones. T cells (40-2) were labeled in vitro with CFSE and transferred into tg^+ and tg^- mice. Animals were killed and the airways perfused with 50% OCT. The lungs were removed, snap frozen, and sections were cut. Shown is a section from a tg^+ animal 24 h after T cell transfer, demonstrating the interstitial accumulation of labeled cells. These cells demonstrate the morphology of activated T lymphocytes (*inset*).

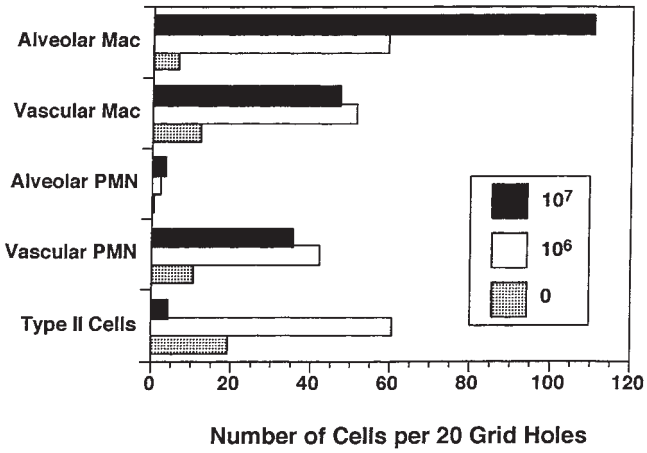

Figure 9. Quantitative morphometric analysis of lung infiltrates from recipient mice by EM (on day 5 after cell transfer of cloned 40-2 cells). Extensive alveolar macrophage infiltration and congestion of the vascular spaces with neutrophils and macrophages are apparent after transfer of 10^6 and 10^7 T cells. Loss of type II epithelial cells are apparent after transfer of 10^7 T cells. Hyperplasia of type II epithelial cells are apparent after transfer of 10^6 T cells. Again, no lymphocytes are evident in this advanced stage of the inflammatory process. These data are representative of three separate experiments with similar results.

to the changes in the lungs of tg^+ mice after transfer of uncloned bulk T lymphocyte population enriched for HA210-219–specific $CD8^+$ CTLs. The lungs of tg^- mice after HA-specific CTL transfer showed only a mild perivascular and peribronchiolar mononuclear cell infiltrate at 24 h after transfer (Fig. 7 *f*), which resolved over the next 24–48 h without evidence of alveolar damage.

To directly confirm that the observed lung injury was a direct result of $CD8^+$ T cell accumulation in the lung after adoptive transfer, 40-2 cells were labeled in vitro before adoptive transfer with an intracellular fluorescent dye, CFSE (34, 35). By 24 h after transfer, there was significant accumulation of labeled cells in the lung parenchyma, as shown in Fig. 8, and the cells had the distinctive morphology of activated T lymphocytes (*inset*). At this time point (24 h), flow cytometric analysis indicated that the labeled cells represented $\sim$ 2–3% of the total lung leukocyte population, and that this percentage was similar in the tg^+ and tg^- animals (not shown).

To directly identify and enumerate the cells infiltrating the alveolar walls and spaces, quantitative EM morphometry was carried out on lung sections of tg^+ mice at day 5 after transfer of CTL clone 40-2. As Fig. 9 shows, the mononuclear cells within alveolar walls and spaces were predominately macrophages. In spite of morphologic evidence of loss of alveolar wall integrity, PMNs were not a prominent component of the cellular infiltrate at day 5 after cell transfer. As observed after transfer of uncloned bulk CTL (Fig. 3), very few type II alveolar cells were present in the alveolar walls of tg^+ mice receiving a high dose (10^7 cells) of clone 40-2. This presumably reflects the rapid and sustained destruction of this HA-expressing epithelial target cell by HA-specific CTLs. In contrast, tg^+ recipients of a smaller 40-2 clone innoculum (10^6 cells) demonstrated a higher frequency of type II cells than tg^- mice (Fig. 7). This increased density of type II pneumocytes in tg^+ mice receiving a low CTL inoculum was reproducibly observed in several independent experiments and may reflect a compensatory regeneration of type II cells in response to the subtotal elimination of resident tg^+ type II epithelial cells mediated by the transferred HA-specific CTLs. It should be emphasized that tg^+ mice receiving as few as 10^6 cloned CTLs also succumb to lethal pulmonary injury (Fig. 6). The fact that these mice develop lethal pulmonary injury in the face of type II cell regeneration has potentially important implications for the mechanisms of pure $CD8^+$ CTL–mediated lung injury. Again, the predominant cells observed by day 5 after transfer were macrophages, with no lymphocytes apparent at this stage of the evolution of the inflammatory process.

Physiological consequences of $CD8^+$ T lymphocyte–mediated pulmonary injury. In this model of $CD8^+$ CTL–mediated alveolar injury, the major histologic finding was the development of progressive alveolar wall infiltration with mononuclear cells and associated accumulation of inflammatory cells in the alveolar space. The physiologic consequences of this injury pattern might be expected to be manifest as restrictive pulmonary mechanics associated with a progressive impairment in gas exchange. Since there was also a moderately intense peribronchiolar mononuclear cell infiltrate by 48 h after cell transfer in the tg^+ mice, some degree of airflow limitation might similarly be expected as a consequence of $CD8^+$ CTL transfer into tg^+ mice. To examine the physiologic impact of $CD8^+$ CTL injury on lung function, we examined oxygen diffusion and lung volumes in tg^+ mice at serial intervals after transfer of clone 40-2. The CO uptake, a surrogate marker for O_2 diffusing capacity (32), was significantly reduced in the tg^+ animals (Fig. 10 *a*) by day 3 after transfer, and progressively declined until death. (There was also a slight diminution in CO

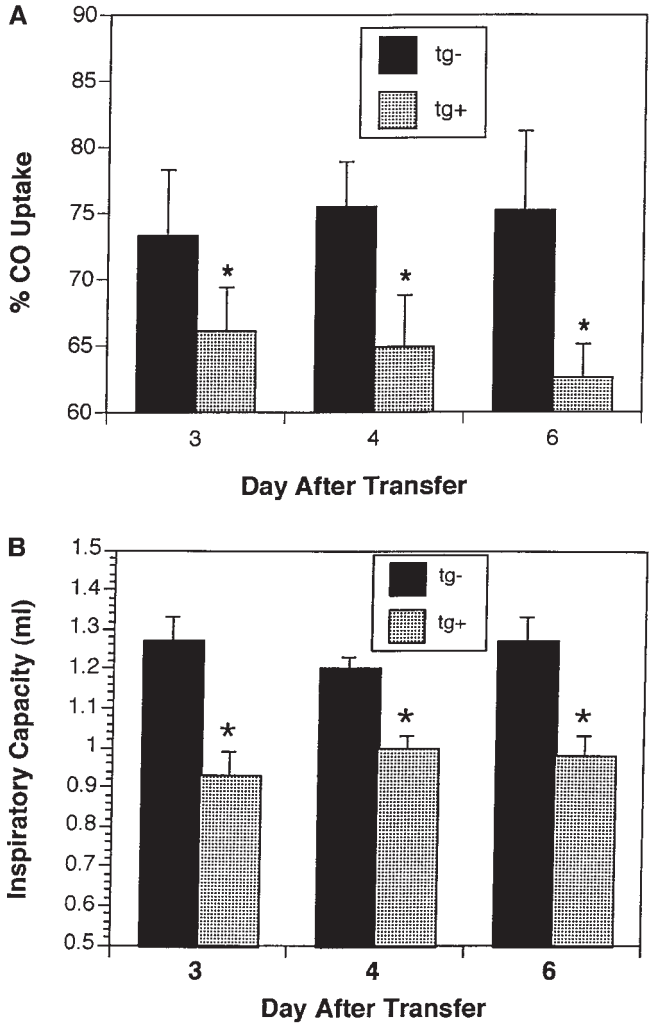

Figure 10. Physiologic analysis of tg^+ versus tg^- mice after adoptive transfer of 5×10^6 cloned 40-2 cells. Animals were anesthetized, their IC measured, and the lungs inflated with a volume of test gas equal to the IC. The test gas, containing 0.3% CO and 0.3% methane, was allowed to dwell for 10 s. One half of the sample was drawn off and discarded (as dead space), and the other half (alveolar sample) was analyzed by gas chromatography. (*a*) CO uptake measured on days 3, 4, and 6 after cell transfer. (*b*) Inspiratory capacity measured on days 3, 4, and 6 after cell transfer.

uptake in tg^+ mice at 48 h after transfer, but it was not statistically significant.) There was a marginal trend towards reduction in the CO uptake in some tg^- clone recipients at early times after transfer (24–48 h) but this was not statistically significant, and CO uptake uniformly increased over 6 d. The CO uptake of a large cohort of normal animals revealed an average uptake of 84±5% (not shown). The diffusing abnormalities evident at day 3 correlated histologically with the development of alveolar interstitial infiltrates, which were significant by day 3 after transfer (Fig. 7, *c–e*). In contrast, the periarteriolar infiltration that was prominent by 48 h after transfer did not affect CO uptake, suggesting that these vascular changes were of little physiologic consequence. The inspiratory capacity of the tg^+ was significantly reduced by day 3 after CTL transfer when compared to tg^- animals receiving T cells (Fig. 10 *b*). This reduction in inspiratory capacity also correlated temporally with the development of significant interstitial inflammation. Interestingly, unlike the progressive decline in gas exchange, inspiratory capacity did not decline with time over the course of the "disease." This suggests that while the development of interstitial inflammation was the primary determinant of both the restriction and the diffusion abnormality, other factors (see Discussion) may have contributed to the progressive decline in gas exchange. In support of this is the fact that the interstitial infiltration and associated alveolar injury was not completely uniform in distribution, despite the uniform expression of the HA transgene in type II pneumocytes throughout the lung parenchyma (26).

Discussion

This report describes a model system to evaluate the impact of $CD8^+$ T lymphocyte recognition of a target antigen expressed endogenously in a single cell type in the lung (the type II pneumocyte) on respiratory structure and function. We found that adoptive transfer of as few as 10^6 HA-specific cloned $CD8^+$ CTLs leads to the development of lethal injury in tg^+ mice. Injury was restricted to the lungs with no evidence of inflammatory infiltrates or tissue damage detected histologically in other major organs, i.e., liver, kidneys, heart, etc. This suggests that the organ-specific damage produced by the transferred effector T lymphocytes was an outcome of recognition of the HA transgene at its site of expression in the lung, i.e., the type II alveolar epithelial cell. The fact that the transfer of $CD8^+$ CTLs into tg^- mice failed to produce injury or further supports the dependence of $CD8^+$ CTL–mediated lung injury on specific antigen recognition. Likewise, the absence of injury in tg^+ mice receiving irrelevant $CD8^+$ CTL clones (directed to an epitope in the influenza nucleocapsid protein) suggests that HA transgene expression in the lung does not lead to a nonspecific increase in susceptibility to injury. Finally, since similar lethal pulmonary injury was observed in tg^+ mice after transfer of either the HA-specific CTL clone 40-2, or a heterogeneous population of HA-specific T cells, the functional and morphologic changes observed after T lymphocyte transfer were not uniquely associated with cloned CTL in long-term culture.

The predominant histologic finding associated with CTL-mediated lung injury in this model was mononuclear cell infiltration of alveolar walls, which increased over time after CTL transfer. The transferred T cells trafficked into the lung parenchyma early (at 24 h), but as the injury process evolved, the interstitial inflammation was accompanied by progressive accumulation of mononuclear phagocytes in alveolar septae and spaces that became especially prominent at later times after cell transfer, i.e., days 5–6. Perivascular and peribronchiolar infiltration (cuffing) of small vessels and airways by mononuclear cells was demonstrable 24 h after CTL transfer. 24 h after transfer of T cells the degree of interstitial infiltration was mild, and it was evident around small airways and vessels of both tg^+ and tg^- mice. This cuffing did not progress over time in tg^- recipients. In contrast, the infiltration around small vessels and airways in the lungs of tg^+ mice progressively increased in intensity at day 2–3 after CTL transfer (Fig. 6) and remained prominent until the mice succumbed to lethal pulmonary injury. The more prominent and sustained infiltration observed exclusively in the tg^+ mice may reflect ongoing re-

cruitment of inflammatory cells of recipient origin into the lung parenchyma as a result of HA transgene recognition by the transferred CTLs. The temporal progression of the inflammatory infiltration from the bronchovascular bundles outward into the peribronchiolar interstitium was also observed by Curtis et al. using a different model (36). This may reflect, in part, the general traffic patterns of activated T lymphocytes into the lung parenchyma. Consistent with this idea is the fact that mild peribronchiolar/periarteriolar infiltration was evident in the tg^- mice, although the intensity of the peribronchiolar/periarteriolar infiltration was markedly greater in the tg^+ mice. We hypothesize that the intense alveolar inflammation observed in the tg^+ mice leads to the recruitment of host inflammatory cells from the vascular compartment into the interstitial spaces around nearby bronchioles, perhaps as a result of outward diffusion of soluble mediators produced in the alveolus in response to injury. Alternatively, or additionally, the mononuclear cells initially accumulating around vessels and airways may represent an effect of inflammatory cells trafficking out of the parenchyma via the peribronchiolar lymphatics, as suggested by Stein-Streilein et al. who demonstrated such an apparent trafficking pattern 7 d after histoincompatible lymph node cells were administered intratracheally into hamsters (37).

The impact of HA recognition in the lung by transferred CTLs on the clinical status (as reflected in weight loss; Fig. 5) and the pulmonary function of tg^+ mice was quite pronounced. Of particular note, the oxygen diffusing capacity (as estimated by CO uptake) of tg^+ mice was dramatically decreased by day 3 after CTL transfer and declined progressively over the next several days until the mice expired. In contrast, lung compliance (as measured by IC), which was also markedly reduced in tg^+ mice by day 3 after cell transfer, showed no further decline over time, in spite of declining clinical status and histologic evidence of progressive lung injury. The potential implications of these findings are discussed below. Overall, the pulmonary function abnormalities in this model of $CD8^+$ T cell–mediated lung injury closely mimic those seen in many of the interstitial lung diseases, particularly those which have an acute presentation, and supports the notion that the histolopathologic findings in this model correlate quantitatively with functional impairment of the lung.

An important aim of this study was to test the hypothesis that antigen-specific $CD8^+$ T cell recognition of a single epithelial "autoantigen" is, in and of itself, sufficient to trigger an inflammatory cascade that results in the manifestations of interstitial pneumonia, both histologically and physiologically. It is interesting to note that adoptive transfer of 10^7 T cells resulted in a complete loss of alveolar epithelial cells, similar to the epithelial denuding seen in disease states associated pathologically with diffuse alveolar damage. In contrast, transfer of 10^6 T cells resulted in type II epithelial hyperplasia, as is seen in more chronic inflammatory states, and likely represents normal proliferative repair mechanisms that are initiated after a subtotal loss of alveolar epithelium (2). We hypothesize that the epithelial cell loss is a result of direct cytolysis by $CD8^+$ CTLs, since the dropout occurs after T cell accumulation in the parenchyma and precedes the prominent accumulation of macrophages. Consistent with this view, we could also demonstrate direct cytotoxic activity by $CD8^+$ CTLs on the alveolar epithelial cell, MLE-K^d, in vitro. After the specific recognition and destruction of type II cells by $CD8^+$ CTLs, there is the progressive accumulation of a mononuclear inflammatory infiltrate in the lung parenchyma, which may be a consequence of chemokine production by the injured epithelial cells. Alternatively, the input T cells may secrete cytokines that lead to this mononuclear cell recruitment. In either case, though, it appears that the development of mononuclear cell inflammatory infiltrates requires specific antigenic recognition by HA-specific $CD8^+$ CTLs (since injury was not seen in tg^- animals), the severe impairment of pulmonary function more closely correlated temporally with the recruitment and accumulation of cells of the monocyte/macrophage lineage in the lungs.

The fact that the entire cascade of inflammatory events that results in interstitial pneumonia stems from a single set of known molecular interactions provides a unique opportunity to correlate histologic patterns and physiologic dysfunction. It has been suggested that gas exchange abnormalities in interstitial pneumonia may result from pulmonary capillary obliteration/thrombosis, increased septal wall thickness, and ventilation/perfusion mismatching (29, 38–40). Our results suggest that the abnormal diffusion correlates with alveolar septal infiltration. Decreased inspiratory capacity (restriction) may be a marker for septal thickening, but curiously, in this study the restriction peaked at day 3 and did not progress, even as the septal infiltration and the diffusion abnormalities progressed. This suggests that a factor other than the diminution in IC may have contributed to the progressive decrease in CO uptake. One such possibility is ventilation/perfusion mismatching, which has been proposed as a primary mechanism altering gas exchange in human interstitial lung disease, particularly at rest (39, 40). The discordance between the progressivity of the diffusion abnormality and the restrictive abnormality may, in part, reflect some nonuniformity of the injury as the process becomes more severe (41). The mechanisms underlying the nonuniformity are unclear, since the transgene is expressed throughout the lung parenchyma, but focal or patchy injury is common of many clinical and experimental autoimmune disease states. Another potential explanation for this discordance may lie in the progressive nature of the alveolar septal necrosis observed over time, and the possibility that this may result in a progressive loss of alveolar-capillary surface area without progressive loss of lung compliance or volume.

We have demonstrated that recognition of a lung "autoantigen" by $CD8^+$ T cells is sufficient to produce lung injury in the absence of an injury stimulus such as viral infection, and that this injury results histologically and physiologically in a disease pattern typical of interstitial pneumonia. Although experimental influenza virus infection results in extensive damage to respiratory epithelium, presumably due to a direct lytic effect of virus replication in these cells, the histologic characteristics of influenza pneumonitis have many features in common with this model of pure $CD8^+$ T lymphocyte–mediated injury. An intriguing hypothesis concerning the pathogenesis of idiopathic interstitial pnemonia is that a normal cytotoxic $CD8^+$ T cell response to viral infection may, in predisposed individuals, result in the inappropriate activation of a "self-reactive" $CD8^+$ CTL clone, or dysregulation of a virus-specific CTL, leading to sustained or enhanced injury (qualitatively or quantitatively). This type of injury may be mediated or amplified by inflammatory cells that are not antigen specific, such as the infiltrating cells of the monocyte/macrophage lineage observed in this report. Clinical observations lend support to the model that a disregulated antiviral $CD8^+$ T cell response may

lead to sustained alveolar injury and interstitial pneumonia, such as the commonly noted antecedent "flu-like" illness that has been observed with several forms of interstitial lung disease (14, 42).

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