CD8$^+$ T Cells Responding to Alveolar Self-Antigen Lack CD25 Expression and Fail to Precipitate Autoimmunity

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Although the contribution of CD8$^+$ T cells to the pathogenesis of noncommunicable lung diseases has become increasingly appreciated, our knowledge about the mechanisms controlling self-reactive CD8$^+$ T cells in the respiratory tract remains largely elusive. The outcome of the encounter between pulmonary self-antigen and naive CD8$^+$ T cells, in the presence or absence of inflammation, was traced after adoptive transfer of fluorescence-labeled CD8$^+$ T cells specific for the neo–self-antigen influenza A hemagglutinin into transgenic mice expressing hemagglutinin specifically in alveolar type II epithelial cells in order: to study the outcome of alveolar antigen encounter in the steady state and under inflammatory conditions; to define the phenotype and fate of CD8$^+$ T cells primed in the respiratory tract; and, finally, to correlate these findings with the onset of autoimmunity in the lung. We found that CD8$^+$ T cells remain ignorant in the steady state, whereas transient proliferation of self-reactive CD8$^+$ T cells is induced by forced maturation or licensing of dendritic cells, increases in the antigenic threshold, and targeted release of alveolar self-antigen by epithelial injury. However, these cells fail to acquire effector functions, lack the expression of the high-affinity IL-2 receptor CD25, and do not precipitate autoimmunity in the lung. We conclude that inadvertent activation of CD8$^+$ T cells in the lung is prevented in the absence of "danger signals," whereas tissue damage after infection or noninfectious inflammation creates an environment that allows the priming of previously ignorant T cells. Failure in effector cell differentiation after abortive priming, however, precludes the establishment of self-perpetuating autoimmunity in the lung.

Keywords: autoimmunity; pulmonary inflammation; CD8$^+$ T lymphocytes; peripheral tolerance; chronic obstructive pulmonary disease

CLINICAL RELEVANCE

Compelling evidence indicates that CD8$^+$ T cells play a prominent role in the pathogenesis of a variety of noncommunicable lung diseases. In stark contrast, we know little about the mechanisms controlling self-reactive CD8$^+$ T cells in the respiratory tract. Here, we demonstrate that lung-reactive CD8$^+$ T cells are tightly controlled by the synergistic effect of peripheral tolerance mechanisms that, together, largely prevent the inadvertent priming of CD8$^+$ T cells, the differentiation of T effector cells, and the onset of autoimmunity in the lung.

Autoaggressive lymphocytes that have escaped negative selection in the thymus can cause deleterious effects when reacting against self-tissues (1). Although peripheral tolerance mechanisms, including deletion and induction of anergy, are active processes that purge autoreactive T cells from the repertoire, ignorant T cells persist in the periphery in a naive state, thereby presenting the imminent danger of becoming activated and precipitating autoimmunity (2). Whereas, in the steady state, uncontrolled expansion of self-reactive CD8$^+$ T cells is usually efficiently prevented, multifaceted immune alterations after infection or non-infectious inflammation create an environment beneficial for the priming of previously ignorant T cells, and this priming may perpetuate autoimmune disease (2).

Most of our knowledge about the mechanisms underlying the tolerance of CD8$^+$ T cells to self-antigen has been obtained in animal models of type 1 diabetes, multiple sclerosis, and colitis (3, 4). However, little is known about the mechanisms controlling self-reactive CD8$^+$ T cells in the respiratory tract, even though it has been suggested that CD8$^+$ T cells play a prominent role in the pathogenesis of a variety of lung diseases (5–8). In support of this, Enelow and colleagues (9, 10) were the first to provide direct experimental evidence that effector CD8$^+$ T cells specifically responding to alveolar self-antigen can cause lethal interstitial pneumonitis. Emerging evidence suggests that chronic obstructive pulmonary disease (COPD), the fourth leading cause of death in industrialized countries (11), may in fact be considered a T cell–mediated autoimmune disease triggered by cigarette smoking (12, 13). The proposed mechanism is the effect of noxious substances in cigarette smoke, which create an inflammatory environment in the lung resulting in the release of sequestered autoantigen and/or its immunological modification. Concomitantly, innate immune activation occurs and supports the maturation of dendritic cells (DCs), which, in turn, can prime CD8$^+$ T cells. In line with this evidence, CD8$^+$ T cells are the predominant cell type accumulating in the airways and parenchyma of patients with COPD, and have been suggested to directly cause tissue injury. These activated CD8$^+$ T cells persist for years after cessation of smoking, a finding suggesting a self-perpetuating process typical of autoimmune diseases (reviewed in Ref. 14).

To directly study the contribution of self-reactive T cells in respiratory disorders, we previously generated transgenic mice expressing the influenza hemagglutinin (HA) under the control of the surfactant protein (SP) C promoter (SPC-HA mice) specifically in alveolar epithelial cells (AECs) type II (AECIIs) (15). In the present study, we combined transgenic SPC-HA mice with an adoptive T cell transfer approach that allows direct tracking of the immunological fate of naive CD8$^+$ T cells after

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their *in vivo* encounter with alveolar self-antigen. We found that $CD8^+$ T cells remain ignorant in the steady state, whereas forced maturation or licensing of DCs, increases in the antigenic threshold, and the targeted release of alveolar self-antigen induce transient proliferation of self-reactive $CD8^+$ T cells. However, these cells failed to differentiate into effector cytotoxic T lymphocytes (CTLs) and to precipitate autoimmunity, a finding indicating the existence of powerful tolerance mechanisms that prevent ignorant T cells from provoking overt autoimmune pathology in the lung.

MATERIALS AND METHODS

Mice

Clone 4 (CL4) mice expressing a major histocompatibility complex (MHC)-I–restricted HA-specific T cell receptor (16), and SPC-HA mice expressing the influenza A/PR8/34 HA under the control of the SPC promoter, specifically in AECIIs, have been described previously (15). CL4 mice were used on the Thy1.1 congenic background to allow for tracking of HA-specific $CD8^+$ T cells after adoptive transfer into SPC-HA and BALB/c recipient mice that were used on the Thy1.2 congenic background. Transgenic mice were bred under specific pathogen-free conditions at the Helmholtz Centre for Infection Research (Braunschweig, Germany). Animal experiments were performed according to national and institutional guidelines.

Histology

Lungs were perfusion fixed with neutral buffered 4% formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin, as described previously (15). Histopathological examination was performed to evaluate the quality of and probable differences between the lung lesions of both strains. The following scoring system was established to determine the degree of severity of interstitial perivascular and peribronchial leukocyte infiltration: Grade 0, no finding; Grade 1, minimal infiltration; Grade 2, mild infiltration; Grade 3, moderate infiltration; and Grade 4, severe infiltration. The scoring was performed by board-certified pathologists (S.R.B., A.D.G.).

Antibodies and Flow Cytometry

The following antibodies were used: anti-CD8 (Ly-2, 53-6.7), anti-CD43 (1B11), and anti-Thy1.1 (OX-7), which were all obtained from BioLegend (Uithoorn, The Netherlands); anti-CD25 (7D4, pC61) and anti-CD62L (MEL-14), which were obtained from eBioscience (Frankfurt, Germany); and anti–IFN-γ (16G6) and anti–IL-2 (JES6-5H4), which were obtained from BD Biosciences (Heidelberg, Germany). The MHC-I pentamer H-$2K^d$ IYSTVASSL was purchased from Proimmune (Oxford, UK). α-CD40 (FGK-45), used for *in vivo* experiments, was purified from hybridoma supernatants by using protein G sepharose. Flow cytometry was performed on FACSCanto (BD Biosciences). Data were analyzed with Flowjo software (version 8.8.4; Olten, Switzerland). Cell sorting was performed with a MoFlo cell sorter (Cytomation, Fort Collins, CO).

Intracellular Cytokine Staining

Cranial mediastinal, caudal mediastinal, and tracheobronchal lymph nodes (hereafter summarized as bronchial lymph nodes) were collected to obtain single-cell suspensions. Lymph node cells (10^6 cells/ml) were incubated for 2 hours at 37°C with phorbol myristate acetate and ionomycin in Iscove's modified Dulbecco's medium (IMDM) with 10% FCS (all Sigma-Aldrich, Taufkirchen, Germany). Cytokine accumulation was enhanced by the addition of 5 μg/ml brefeldin A (Sigma-Aldrich), followed by incubation for an additional 2 hours. Surface staining was performed with pentamer, α-CD8, α-Thy1.1, α-CD43, or α-CD25, followed by fixation for 20 minutes with 2% paraformaldehyde, permeabilization with 0.01% Igepal CA-630 (Sigma-Aldrich), and intracellular staining for 30 minutes with α-IFN-γ or α-IL-2. Cytokine production was determined by flow cytometry.

Adoptive Transfer Experiments

CL4 splenocytes were enriched for $CD8^+$ T cells by untouched cell separation using the CD8 antibody kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified $CD8^+$ T cells were labeled with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Darmstadt, Germany) and injected into the tail veins ($3–4 \times 10^6$ antigen-specific $CD8^+$ T cells) of recipient mice. Where indicated, anesthetized mice received 6 μg LPS in PBS intranasally or 250 μg α-CD40 antibody intraperitoneally on the day of T cell transfer. Where indicated, mice received 20 μg of HA512–520 peptide mixed with 6 μg LPS in PBS intranasally 1 day before $CD8^+$ T cell transfer. When adoptive transfer was combined with infection, recipient mice were anesthetized and sublethally infected with influenza virus H1N1 (A/PR/38) or H7N7 intranasally. Unless otherwise stated, mice were killed 6 days after T cell transfer, and proliferation, activation status, and cytokine production by HA-specific $CD8^+$ T cells were assessed by flow cytometry.

In Vitro Differentiation of Cytotoxic $CD8^+$ T Cells

HA-specific $CD8^+$ T cells (5×10^5) and 5×10^6 irradiated BALB/c splenocytes were cocultured in 24-well plates in IMDM/10% FCS in the presence of 1 μg/ml HA512–520 peptide, 1 ng/ml IL-2, and 20 ng/ml IL-12. After 3 days, fresh IMDM/10% FCS supplemented with 1 ng/ml IL-2 was added to the culture. After 3 days, activated $CD8^+$ T lymphocytes were isolated by untouched magnetic-activated cell sorting separation and adoptively transferred into recipient mice (5×10^6 CTLs for single transfers; 2.5×10^6 CTLs for a transfer preceding naive $CD8^+$ T cell transfer).

IL-2 Supplementation

After adoptive transfer of naive $CD8^+$ T cells and a single intranasal LPS application (6 μg LPS in PBS), SPC-HA mice were treated twice daily intraperitoneally with 15,000 IU of recombinant mouse IL-2 (R&D Systems, Minneapolis, MN) in PBS/1% BSA. IL-2 supplementation was performed starting at Day 1 after adoptive transfer of naive $CD8^+$ T cells until Day 6, when the mice were killed.

RESULTS

$CD8^+$ T Cells Ignore Self-Antigen Expressed in the Lung

To study the specific requirements for priming of autoreactive $CD8^+$ T cells in the lung, we used the SPC-HA mouse expressing the neo–self-antigen influenza HA in the lung epithelium (15). As described previously, breading of SPC-HA with CL4 mice generating HA-specific $CD8^+$ T cells results in chronic pulmonary inflammation associated with airway obstruction and impaired lung function (17). To establish conditions allowing for directly tracking the encounters between $CD8^+$ T cells and pulmonary self-antigen *in vivo*, we performed adoptive transfer of CFSE-labeled HA-specific $CD8^+$ T cells isolated from CL4 transgenic mice into SPC-HA mice. Antigen recognition was assessed by analyzing the dilution of CFSE as an indicator of proliferation.

Unlike mice expressing HA in the intestinal epithelium, which developed severe wasting disease within a few days after $CD8^+$ T cell transfer (18), lung-specific $CD8^+$ T cells failed to proliferate in SPC-HA mice and maintained a naive phenotype ($CD43^{lo}$ and $CD62L^{hi}$, Figure 1A). Histological examination provided no evidence of antigen-specific lymphocytic infiltration in the lung (Figure 1B). Increasing the number of HA-specific $CD8^+$ T cells to 8×10^6 did not result in T cell proliferation and lung inflammation (data not shown).

Self-reactive T cells may be actively controlled by deletion or functional inactivation. Alternatively, autoreactive T cells may persist in the periphery in a naive state, called ignorance (2). To decipher which of the mechanisms prevents $CD8^+$ T cell–mediated lung inflammation, SPC-HA mice were infected with influenza virus A/PR8/34 6 days after the transfer of HA-specific T cells (Figure 1C). This virus expresses the same HA as that expressed in the alveolar epithelium of SPC-HA mice.

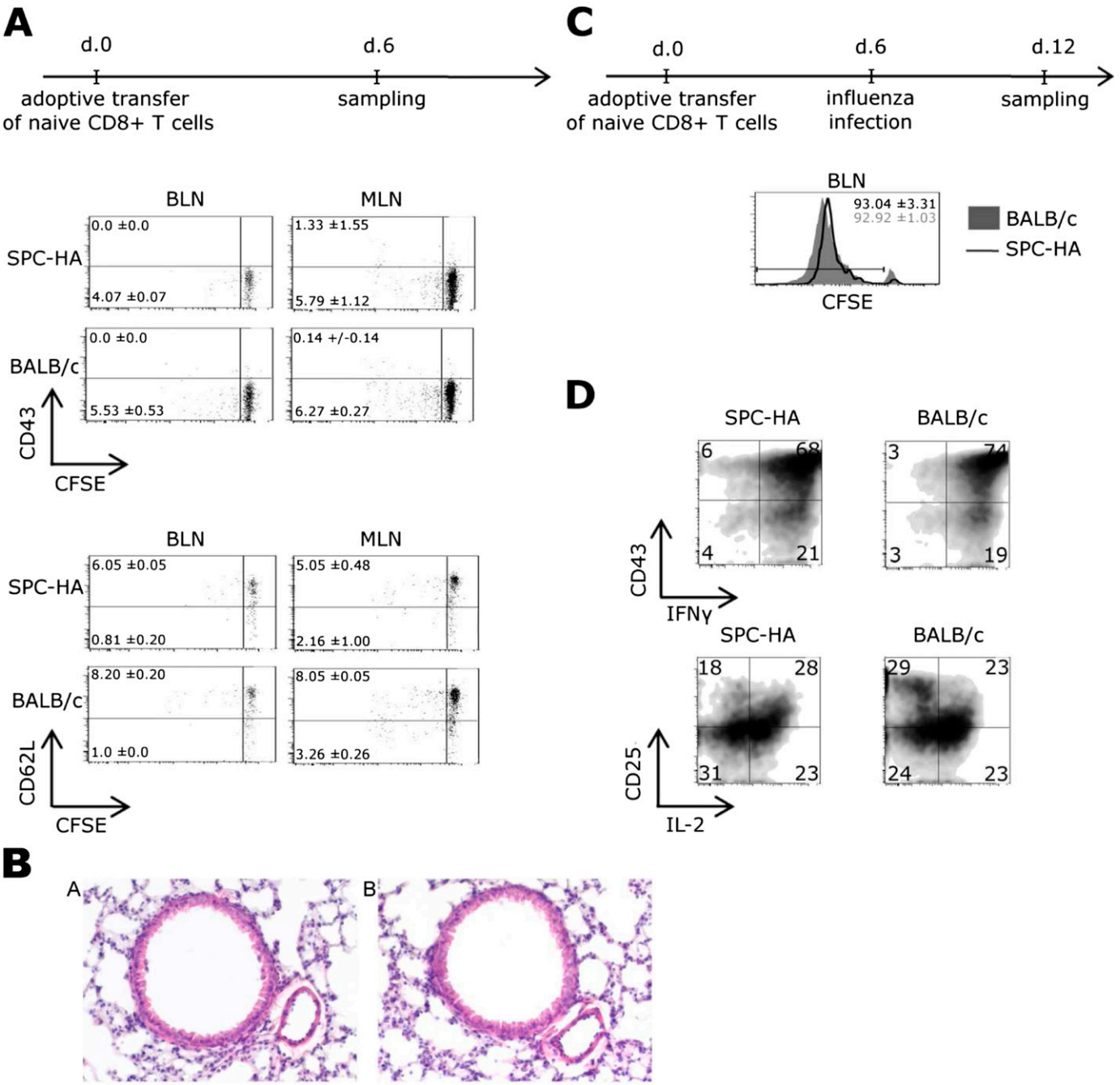


Figure 1. Naive CD8$^+$ T cells ignore alveolar self-antigen, but are fully competent to respond to viral infection. (*A*) Naive hemagglutinin (HA)-specific CD8$^+$Thy1.1$^+$ T lymphocytes isolated from the spleens of CL4 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and were then adoptively transferred into Thy1.2$^+$ surfactant protein (SP) C–HA or wild-type BALB/c mice. After 6 days, HA-specific CD8$^+$ Thy1.1$^+$ T cells recovered from lung-draining bronchial lymph nodes (BLNs) and from distant mesenteric lymph nodes (MLNs) of the recipient mice were examined for proliferation and the expression of activation-related (CD43) or homing (CD62L) markers by FACS. Data (shown as mean value [MV] ± SEM) depict the percentage of cells expressing a given marker. (*B*) Lung tissue sections from BALB/c and SPC-HA mice obtained from animals that were treated as indicated in the experimental scheme provided in *A* were stained with hematoxylin and eosin (H&E). Histological evaluation for indications of acute pulmonary inflammation revealed no abnormality in either SPC-HA mice (*A*) or BALB/c control animals (*B*). Representative photographs of samples were taken with a 20× objective and a 10× eyepiece. (*C*) At 6 days after adoptive CD8$^+$ T cell transfer, performed as described in (*A*), recipient mice were intranasally infected with the influenza virus strain, H1N1 (A/PR/38), encoding the same HA protein that is expressed as self-antigen in type II alveolar epithelial cells (AECIIs) of SPC-HA mice. Another 6 days later, proliferation of HA-specific CD8$^+$ T cells was measured by using FACS to determine loss of CFSE dye in BLNs of SPC-HA (*black line*) or BALB/c (*gray shade*) recipients. Data on histograms (obtained in two independent experiments with three mice per group analyzed individually) indicate percentages (MV ± SEM) of proliferating cells in both groups. (*D*) Proliferating (CFSE^{low}) CD8$^+$Thy1.1$^+$ T lymphocytes, depicted in (*C*), were further analyzed for the expression of activation-related surface markers (CD43 or CD25) and for the production of the cytotoxic CD8$^+$ T effector cytokines, IFN-γ and IL-2. Data represent one of two independent experiments with similar outcomes, performed with a pool of at least three mice per group.

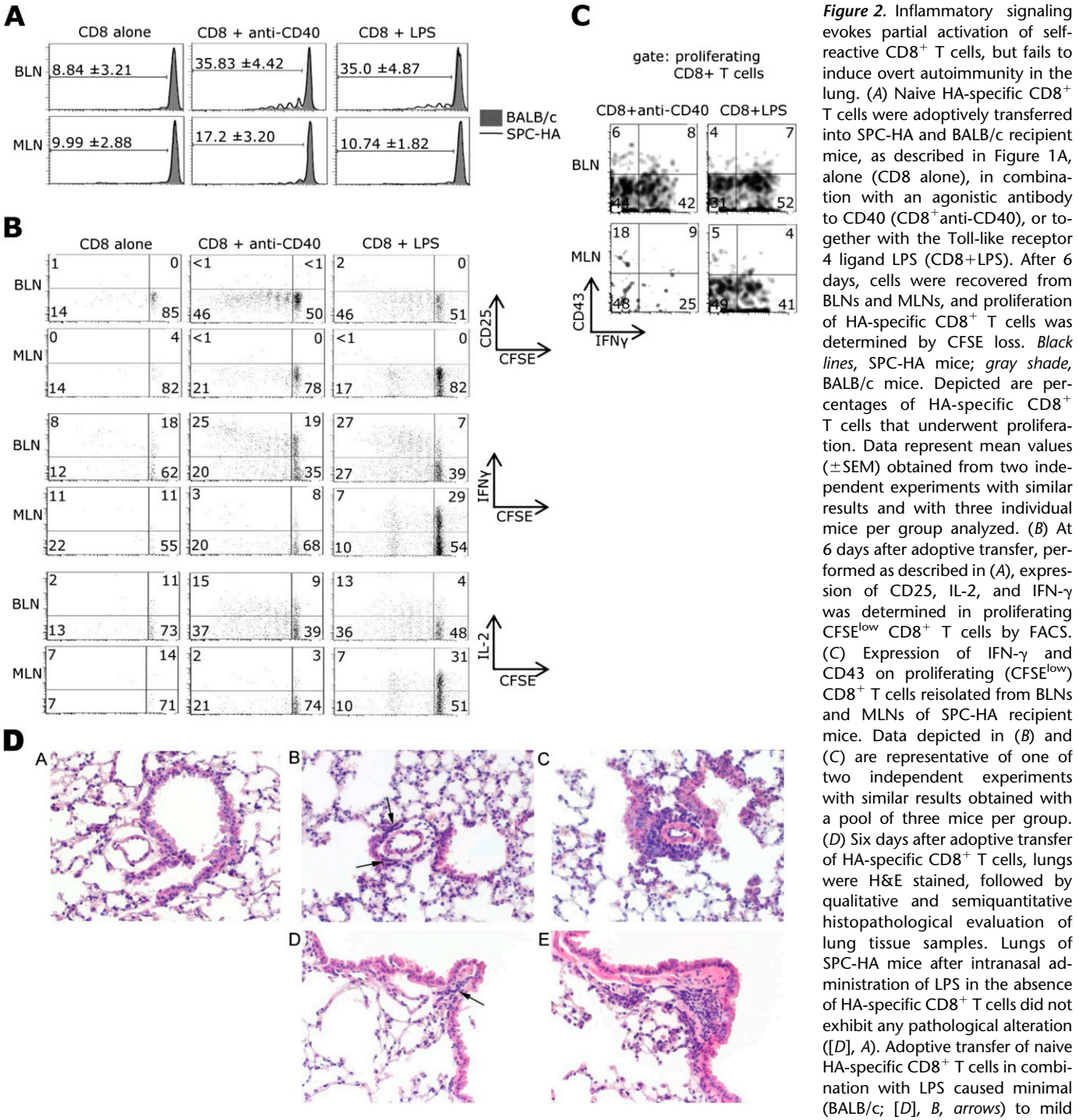


Figure 2. Inflammatory signaling evokes partial activation of self-reactive $CD8^+$ T cells, but fails to induce overt autoimmunity in the lung. (*A*) Naive HA-specific $CD8^+$ T cells were adoptively transferred into SPC-HA and BALB/c recipient mice, as described in Figure 1A, alone (CD8 alone), in combination with an agonistic antibody to CD40 ($CD8^+$anti-CD40), or together with the Toll-like receptor 4 ligand LPS (CD8+LPS). After 6 days, cells were recovered from BLNs and MLNs, and proliferation of HA-specific $CD8^+$ T cells was determined by CFSE loss. *Black lines*, SPC-HA mice; *gray shade*, BALB/c mice. Depicted are percentages of HA-specific $CD8^+$ T cells that underwent proliferation. Data represent mean values (±SEM) obtained from two independent experiments with similar results and with three individual mice per group analyzed. (*B*) At 6 days after adoptive transfer, performed as described in (*A*), expression of CD25, IL-2, and IFN-γ was determined in proliferating $CFSE^{low}$ $CD8^+$ T cells by FACS. (*C*) Expression of IFN-γ and CD43 on proliferating ($CFSE^{low}$) $CD8^+$ T cells reisolated from BLNs and MLNs of SPC-HA recipient mice. Data depicted in (*B*) and (*C*) are representative of one of two independent experiments with similar results obtained with a pool of three mice per group. (*D*) Six days after adoptive transfer of HA-specific $CD8^+$ T cells, lungs were H&E stained, followed by qualitative and semiquantitative histopathological evaluation of lung tissue samples. Lungs of SPC-HA mice after intranasal administration of LPS in the absence of HA-specific $CD8^+$ T cells did not exhibit any pathological alteration ([*D*], *A*). Adoptive transfer of naive HA-specific $CD8^+$ T cells in combination with LPS caused minimal (BALB/c; [*D*], *B*, *arrows*) to mild (SPC-HA; [*D*], *C*) perivascular and peribronchiolar lymphocytic accumulations in the lung. Similar results were obtained after adoptive transfer of $CD8^+$ T cells combined with anti-CD40 treatment in BALB/c ([*D*], *D*) and SPC-HA ([*D*], *E*) mice. Representative photographs of sections collected in three independent experiments with four to seven individual mice per group were taken with a 20× objective and a 10× eyepiece.

Therefore, ignorant, but not anergic, $CD8^+$ T cells should be able to exhibit an unrestrained response to the virus. Indeed, HA-specific $CD8^+$ T cells proliferated massively in both wild-type and SPC-HA recipient mice (Figure 1C). Most of the proliferating $CD8^+$ T cells displayed a highly differentiated effector phenotype, as indicated by elevated expression of CD43 and CD25 and secretion of IL-2 and IFN-γ (Figure 1D). Thus, alveolar-specific $CD8^+$ T cells were neither deleted nor functionally inactivated. Rather, they resided in close proximity to the antigen in an untolerized state, capable of differentiating into immune effectors.

Inflammatory Signaling Evokes Partial Activation of Self-Reactive $CD8^+$ T Cells but Fails to Induce Overt Autoimmunity in the Lung

The finding that, in the steady state, $CD8^+$ T cells ignore alveolar self-antigen while at the same time being

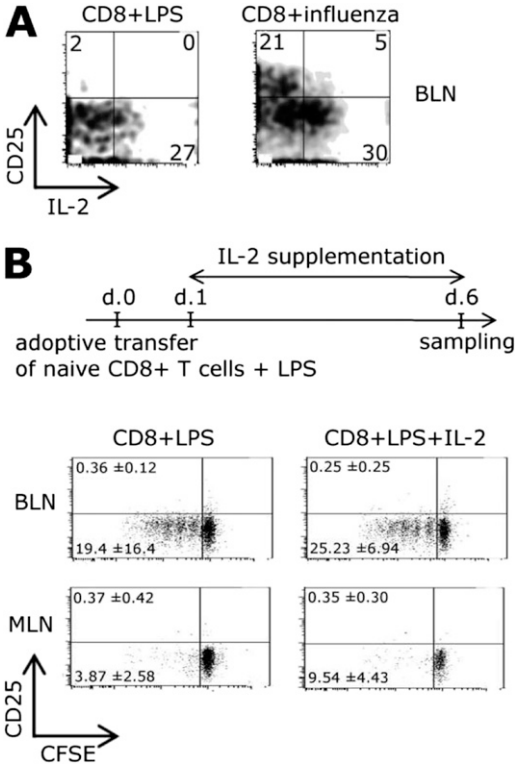


Figure 3. (*A*) CD8$^+$ T cells, expanding upon the recognition of pulmonary self-antigen, fail to up-regulate the high-affinity IL-2 receptor (CD25). HA-specific CD8$^+$ T cells were adoptively transferred into SPC-HA mice, along with intranasal application of either LPS (CD8 + LPS) or the influenza A virus H1N1 (CD8 + influenza). After 6 days, expression of CD25 and IL-2 was determined by FACS on proliferating CD8$^+$Thy1.1$^+$CFSE^{low} cells isolated from the BLNs of the recipient mice. (*B*) SPC-HA mice, which received autoreactive CD8$^+$ T cells together with intranasal application of LPS, were treated twice daily with recombinant murine IL-2. At 6 days after T cell transfer, proliferation and CD25 expression on HA-specific CD8$^+$ T cells isolated from BLNs and MLNs of IL-2–treated mice and of control mice that did not receive IL-2 were analyzed by FACS. Display data (MV ± SEM) represent one of two independent experiments with similar outcomes.

immunocompetent prompted us to investigate their potential danger to cause autoimmunity in the context of inflammatory signaling. Because DCs are the predominant antigen-presenting cells that constitutively present self-antigen, it is likely that ignorance occurs because DCs cannot acquire sufficient quantities of alveolar antigen, or because the costimulatory capability of DCs is insufficient in the steady state. To provoke the transition of DCs into an immunogenic state, we combined adoptive T cell transfer with the provision of LPS or an agonistic antibody to CD40 (DC "licensing") (19). Indeed, inflammatory signaling induced antigen-specific proliferation in a proportion of alveolar-specific CD8$^+$ T cells (Figure 2A). However, proliferating cells exhibited impaired effector differentiation, as indicated by limited release of IL-2 and IFN-γ (Figure 2B) and by a low number of CD43$^+$IFN-γ$^+$ cells among the proliferating cells (Figure 2C). Of note, although some IL-2 was produced, proliferating CD8$^+$ T cells failed to up-regulate the high-affinity IL-2Rα chain CD25 (Figure 2B). In line with abortive effector cell differentiation, only mild lymphocytic infiltration scattered in the lungs of SPC-HA recipients was detectable; this infiltration was, however, not effective in provoking overt autoimmunity (Figure 2D).

Lack of CD25 Expression on Expanding CD8$^+$ T Cells Cannot Be Restored by IL-2 Supplementation

Whereas CD8$^+$ T cell activation after influenza infection resulted in both the production of IL-2 and the expression of CD25, T cells primed by alveolar self-antigen in the absence of infection failed to express CD25 (Figure 3A). To test whether incomplete effector cell differentiation may involve failures in the positive IL-2/IL-2R feedback loop (20), we repeatedly used IL-2 to treat SPC-HA mice that previously received adoptive transfer of alveolar-specific CD8$^+$ T cells in the presence of LPS (21). However, IL-2 supplementation neither induced CD25 up-regulation on CD8$^+$ T cells nor influenced the proliferative capability of these cells (Figure 3B).

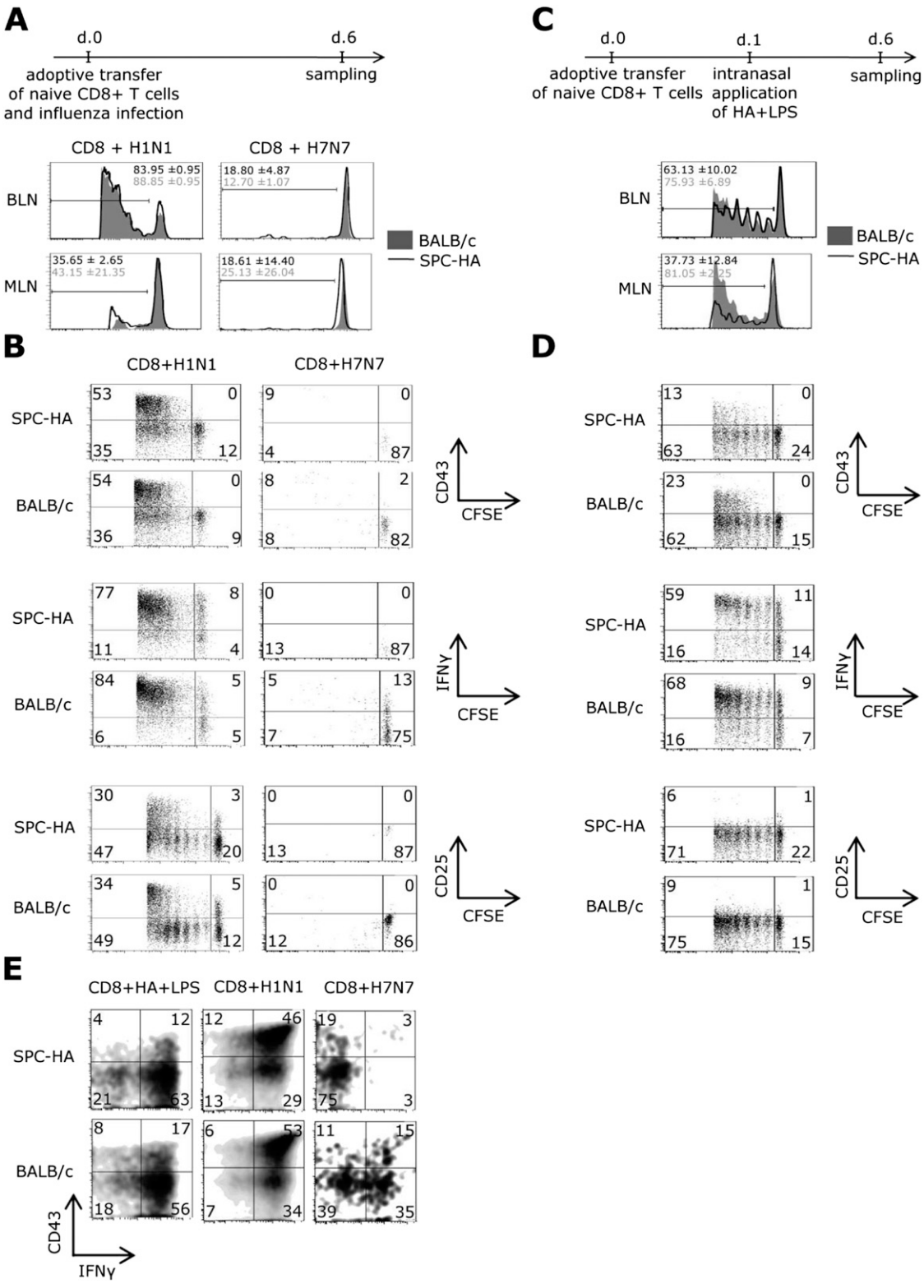
Infection-Related Alveolar Tissue Damage and Increases in the Antigenic Threshold Fail to Precipitate CD8$^+$ T Cell–Mediated Autoimmunity

Tissue damage, which is a common by-product of infection, leads to augmented release of self-antigen alongside pathogen components, thereby allowing T cells to encounter self-antigen in an immunogenic context (reviewed in Ref. 2). To investigate whether physiological epithelial damage results in functional activation of self-reactive CD8$^+$ T cells, we infected adoptively transferred mice with influenza virus H7N7 not recognized by CL4-derived CD8$^+$ T cells, or with H1N1 as a control. Weight monitoring and histology showed a similar disease course for both viruses (data not shown). Whereas CD8$^+$ T cells proliferated in H1N1-infected mice, no proliferation (Figure 4A) or T cell activation (Figure 4B) occurred after H7N7 infection, suggesting that infection-related epithelial damage does not allow the release of sufficient quantities of self-antigen to provoke a T cell response within the self-ignorant CD8$^+$ T cell subset.

Because the self-antigen level is believed to be decisive for the induction of autoimmunity (22), we questioned whether the addition of exogenous HA-peptide would increase the antigenic threshold to a level matching CD8$^+$ T cell–priming requirements. To this end, mice received intranasal application of the HA peptide together with LPS. Indeed, providing the external antigen induced massive proliferation of CD8$^+$ T cells; however, no significant differences in proliferation were observed between SPC-HA mice and BALB/c mice, indicating that proliferation can be attributed to exogenous HA (Figure 4C). Of note, despite massive expansion, proliferating CD8$^+$ T cells expressed considerably lower levels of CD43, IFN-γ, and, in particular, CD25 (Figure 4D) than did H1N1-primed CD8$^+$ T cells. Accordingly, a lower proportion of CD43$^+$IFN-γ$^+$ T effector cells was found in mice that received HA than in influenza-infected animals (Figure 4E).

Lung-Reactive CD8$^+$ T Cells Expanding after CTL-Mediated Tissue Destruction Lack Effector Cell Differentiation and CD25 Expression

Tissue damage involves the release of cytosolic components during necrotic cell death and the degradation of the extracellular matrix by apoptotic cells; together, these factors generate endogenous adjuvants that allow DCs to maintain immune stimulation (reviewed in Ref. 2). To investigate the immunogenicity of self-antigen released by CTL-mediated tissue destruction, we transferred *in vitro*–differentiated HA-specific CTLs into SPC-HA and BALB/c recipients. HA-specific CTLs responded to AECII antigen and caused overt lung inflammation in SPC-HA, but not in BALB/c mice (Figure 5A, *A* and *B*). Whereas the lungs of BALB/c mice appeared to be normal 3 weeks after transfer (Figures 5A and 5C), lung inflammation was still evident in



SPC-HA mice, although markedly alleviated (Figures 5A and 5D). To examine whether CTL-mediated tissue damage resulted in priming of $CD8^+$ T cells, and whether such immune activation could precipitate autoimmunity in the lung, SPC-HA mice were given naive HA-specific $CD8^+$ T cells 5 days after CTL injection (Figure 5B). Indeed, CTL-induced self-antigen release resulted in massive expansion of transferred $CD8^+$ T cells specifically in the bronchial lymph nodes of SPC-HA mice (Figure 5B), indicating improved cross-presentation of self-antigen. However, histological examination did not reveal any evidence of acute $CD8^+$ T cell–driven pulmonary inflammation (Figures 5A, 5E, and 5F). Again, only a minor fraction of expanding $CD8^+$ T cells produced IFN-γ or up-regulated CD43 (Figure 5C), and only a few $CD43^+$IFN-γ^+ HA-specific $CD8^+$ T effector cells were detectable in SPC-HA mice (Figure 5D) after CTL-mediated tissue damage. Of note, despite massive

Figure 4. $CD8^+$ T cell priming by virus-derived antigen, but not targeted release of endogenous self-antigen after virus-induced AEC damage, results in functional activation of $CD8^+$ T cells in the respiratory tract. (*A*) SPC-HA and BALB/c control mice were adoptively transferred with CFSE-labeled HA-specific $CD8^+$ T cells, followed by intranasal infection with a sublethal dose of either influenza A virus H1N1 (expressing the HA protein recognized by $CD8^+$ T cells derived from CL4 donor mice) or H7N7 (expressing an unrelated HA protein). After 6 days, proliferation of transferred HA-specific $CD8^+$ T cells was determined in BLNs and MLNs of recipient mice. *Black lines*, SPC-HA mice; *gray shade*, BALB/c recipient mice. Depicted are percentages of HA-specific $CD8^+$ T cells that underwent proliferation. (*B*) Expression of CD25, CD43, and IFN-γ by proliferating ($CFSE^{low}$) HA-specific $CD8^+$ T cells measured in BLNs of SPC-HA and BALB/c recipient mice treated as described in (*A*). (C) SPC-HA and BALB/c mice were adoptively transferred with CFSE-labeled HA-specific $CD8^+$ T cells (Day [d] 0), followed by intranasal application of 20 μg HA peptide together with 6 μg LPS (d1). Proliferation of transferred HA-specific $CD8^+$ T cells (d6 after T cell transfer) was measured in BLNs and MLNs of recipient mice. *Black lines*, SPC-HA mice; *gray shade*, BALB/c mice. Numbers indicate percentage of $CD8^+$ HA-specific T cells that underwent proliferation in both groups of recipient mice. (*D*) CD25, CD43, and IFN-γ expression on proliferating ($CFSE^{low}$) HA-specific $CD8^+$ T cells in BLNs of SPC-HA and BALB/c mice, treated as in (*C*), was analyzed by FACS. (*E*) Mice were treated as described in (*A*) and (*C*), respectively, and analyzed for CD43 and IFN-γ expression on proliferating $CFSE^{low}$ HA-specific $CD8^+$ T cells at Day 6 after transfer. Display data (MV ± SEM) obtained from two independent experiments with three mice per group analyzed individually (*A* and *C*), or are representative dot plots from one of two independent experiments with similar outcomes, with a pool of three mice per group analyzed (*B*, *D*, and *E*).

proliferation of $CD8^+$ T cells, these cells failed to up-regulate the expression of CD25 (Figure 5C).

DISCUSSION

Inflammation mediated by T cells is a key component of prevalent lung diseases (2), and recent findings suggest that COPD can be considered an autoimmune disease (14). Because COPD is expected to be the third leading cause of death worldwide by 2020 (11, 23), improved therapies are urgently needed, but their development will require in-depth understanding of the mechanisms controlling T cell reactivity to pulmonary self-antigen.

Our finding that, in the steady state, $CD8^+$ T cells ignore alveolar self-antigen was somewhat unexpected. We and others have shown previously that the recognition of intestinal and hepatic self-antigen by $CD8^+$ T cells results in overt autoimmunity (18, 24). Thus, the tissue in which priming takes place may in part determine the induction of autoimmunity. In addition, antigen expression levels were shown to determine tolerance outcomes, with low levels favorably being ignored and high levels resulting in cross-presentation and subsequent deletion of $CD8^+$ T cells (22). In SPC-HA mice, the amount of alveolar HA may be comparably low, because AECIIs comprise only ~4% of the alveolar surface (25). However, *in vitro*–differentiated CTLs were capable of recognizing AECII antigen, and the transfer of HA-specific $CD4^+$ T cells to SPC-HA mice resulted in acute lung inflammation (15, 26, 27), demonstrating that the HA antigen is not sequestered and is accessible for recognition by T cells. This is further supported by previous studies performed by Enelow and colleagues (9, 10), who showed that adoptive transfer of *in vitro* differentiated CTLs into transgenic animals expressing a different influenza HA in AECs resulted in extensive interstitial pneumonitis that was associated with significant morbidity and mortality.

It has been proposed that, in addition to the amount of self-antigen, the presence of an inflammatory environment is decisive for the outcome of the encounter between antigen and autoreactive T cells. Tissue damage may allow the release of self-antigen and induce DC maturation, thereby allowing provocation of a T cell response within the previously self-ignorant repertoire (2). Indeed, the provision of exogenous antigen in the presence of LPS induced massive $CD8^+$ T cell proliferation and IFN-γ release. However, effector cell differentiation remained fragmentary, and, in line with the findings of Yoon and colleagues (28), peptide antigen encounter did not induce CD25 expression in dividing $CD8^+$ T cells.

In a more physiological approach to the targeted release of self-antigen, alveolar damage was induced in SPC-HA mice by transfer of *in vitro*–differentiated HA-specific CTLs. Subsequently, transferred naive alveolar-specific $CD8^+$ T cells indeed massively expanded. However, proliferation was uncoupled from effector cell differentiation. Interestingly, targeted tissue destruction in SPC-HA mice infected with an H7N7 virus not recognized by autoreactive $CD8^+$ T cells did not reveal any signs of T cell activation. Respiratory epithelial cells are the primary targets of both influenza replication (29) and the host response to influenza infection (30). Thus, we expected that CTL-mediated killing of AECIIs would result in the release of the self-antigen for improved cross-presentation. However, whereas other infections precipitated autoimmunity (31–34), this was not the case in our study. One possible explanation for the observed differences is that the amount of self-antigen in our system is too low in the steady state, and that even targeted release by infection-induced epithelial damage may not be sufficient to reach the antigenic threshold required for optimal T cell activation. As mentioned previously here, HA-expressing AECIIs constitute only a minor cellular subpopulation in the lung (25), whereas, in those settings where infection resulted in breakdown of self-tolerance, the neo–self-antigen was expressed ubiquitously in MHC class I^+ cells (31) or in enterocytes that are highly abundant in the gut (33). One might speculate that a more prevalent neo–self-antigen would have caused overt $CD8^+$ T cell–mediated autoimmunity in the lung as well. On the other hand, maintenance of $CD8^+$ T cell tolerance to alveolar self-antigen, even under inflammatory conditions, may highlight the existence of exceptionally stringent tolerance mechanisms specifically in the lung. In support of this, a recent publication (23) reported that the target cell type exerts a prominent effect in determining the differentiation path of $CD8^+$ T cells, and that $CD8^+$ T cell priming by respiratory epithelial cells resulted in only partial CTL differentiation and impaired IFN-γ production. Of note, airway epithelial cells express molecules that suppress host inflammatory responses and support the conversion of activated T cells into regulatory T cells (27, 35, 36). Thus, the loss of $CD8^+$ T cell tolerance to AECII-expressed self-antigen may be prevented by negative signals delivered by the alveolar epithelium. Aside from AECs, several other immunosuppressive factors unique to the lung environment may contribute to the observed lack of $CD8^+$ effector cell differentiation. For instance, lung SPs exhibit widespread immune modulating properties (reviewed in Ref. 37), and have been demonstrated to directly interfere with T cell activation (38, 39). Of note, pulmonary surfactant components inhibit DC maturation, and thus their ability to subsequently stimulate T cell proliferation (40). Moreover, a prominent role of SPA in controlling T cell responses in the lung has recently been demonstrated in a mouse model for allergen-mediated pulmonary inflammation (41). Thus, in contrast to other tissues

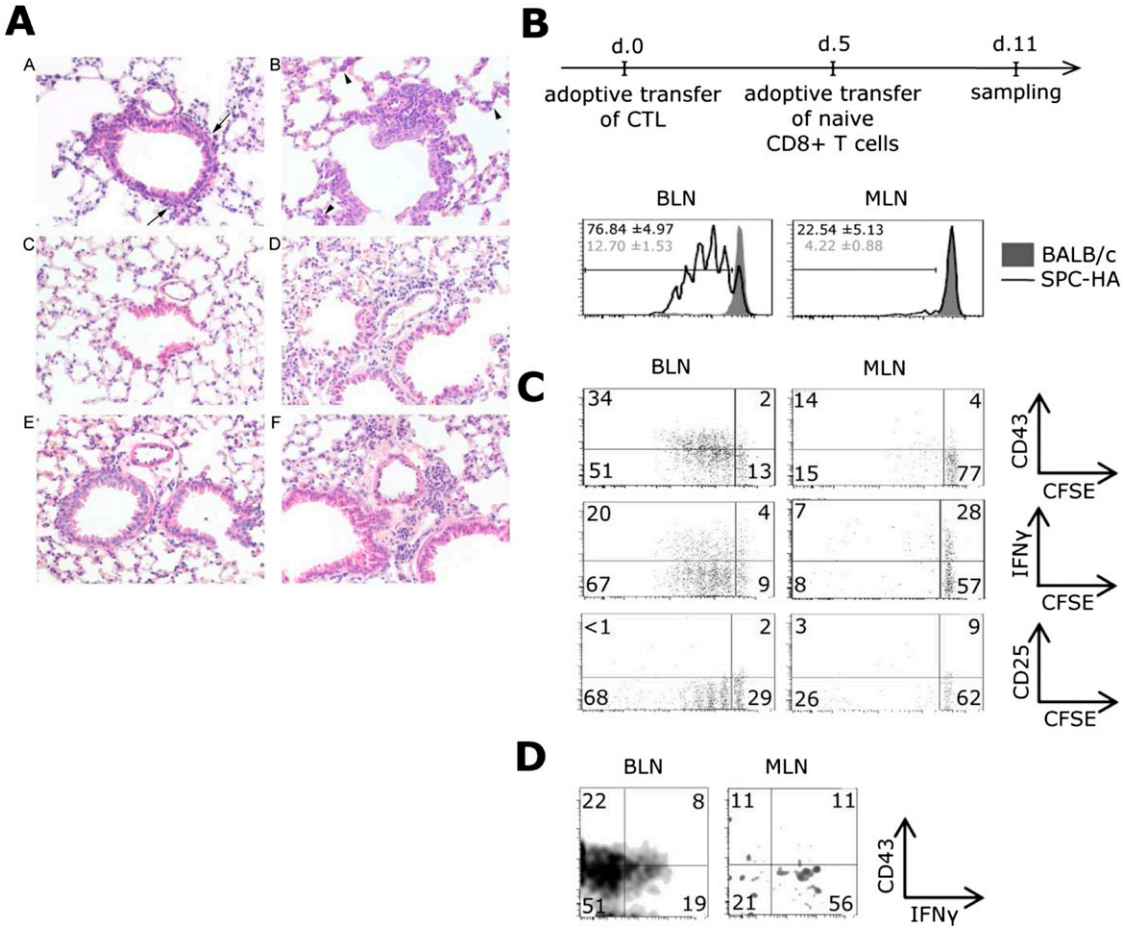


Figure 5. Tissue destruction by cytotoxic $CD8^+$ T cells (cytotoxic T lymphocyte [CTLs]) enhances the proliferation of naive $CD8^+$ T cells, but fails to provoke self-perpetuating autoimmunity. (*A*) Effector CTLs were generated *in vitro* from naive HA-specific $CD8^+$ T cells, as described in MATERIALS AND METHODS, and 5×10^6 CTLs were adoptively transferred into SPC-HA and BALB/c recipient mice. At 6 days after CTL transfer, minimal lymphocytic perivascular and peribronchial infiltrations were detected in the lungs of BALB/c mice ([*A*], *A*; *arrows*), whereas the lungs of SPC-HA mice ([*A*], *B*) exhibited moderate lymphocytic perivascular, peribronchial, and partially interstitial (*arrowheads*) lymphocytic infiltrations. At 3 weeks after CTL transfer, no histological changes were detectable any longer in the lungs of BALB/c mice ([*A*], C), whereas perivascular and peribronchiolar lymphocytic infiltration were still present in SPC-HA mice ([*A*], *D*). Lungs of BALB/c ([*A*], *E*) and SPC-HA ([*A*], *F*) mice that received 2.5×10^6 CTLs followed by transfer of 4×10^6 naive HA-specific $CD8^+$ T cells 5 days later exhibited no evidence of exacerbation of lung inflammation, unlike the lungs of mice that received CTL transfer only. Representative photographs of H&E-stained lung sections collected from two independent experiments with at least three individual mice per group were taken with a 20× objective and a 10× eyepiece. (*B*) *In vitro*–differentiated CTLs (2.5×10^6) were injected into SPC-HA and BALB/c mice. After 5 days, recipient mice were adoptively transferred with CFSE-labeled naive HA-specific $CD8^+$ T cells. At 6 days after the second transfer, proliferation of naive $CD8^+$ T cells was determined in BLNs and MLNs of the recipient mice by flow cytometry. *Black lines*, SPC-HA mice; *gray shadows*, BALB/c mice. (*C*) Proliferation and expression of CD25, CD43, and IFN-γ on HA-specific $CD8^+$ T cells that had been injected into SPC-HA mice after CTL transfer, as described in (*B*). (*D*) Phenotype of proliferating $CD8^+Thy1.1^+CFSE^{low}$ cells that were transferred into SPC-HA mice, as described in (*B*), 5 days after CTL transfer. Expression of CD43 and IFN-γ is shown. Data represent MV (±SEM) obtained from two independent experiments with three mice per group analyzed individually (*B*), or depict representative data from two independent experiments with a pool of at least three mice per group analyzed (*C* and *D*).

lacking high surfactant levels, such as the liver or the intestine, SPs being overrepresented in the lung may either directly or indirectly prevent efficient $CD8^+$ T cell priming and effector cell differentiation after alveolar self-antigen recognition in SPC-HA mice. Aside from SPs, another hallmark of the lung environment is the preponderance of macrophages within the alveoli. Like surfactant components, alveolar macrophages have been demonstrated to antagonize DC function and interfere with T cell priming in the lung (42, 43), and, thereby, may be critically involved in regulating T cell activity and immunological tolerance in the respiratory tract.

To improve priming of alveolar-specific $CD8^+$ T cells by DCs, we provoked the transition of DCs from a tolerogenic to an immunogenic state. To this end, we combined adoptive transfer of naive $CD8^+$ T cells with the provision of LPS or α-CD40 (19, 44). Indeed, manipulation of the DC maturation status induced T cell proliferation; however, these cells failed to provoke autoimmunity. One possible explanation might be that, next to signal 1 (T cell receptor ligation) and signal 2 (costimulation), the third signal (IL-12 released by activated DCs), which is crucial for effector cell development, is either lacking or too week under these conditions (45). Hernandez and colleagues (46, 47) described the uncoupling of proliferation and gain of effector function by $CD8^+$ T cells. Here, the encounter between self-antigen and pancreas-specific $CD8^+$ T cells induced only a partial activation program in which $CD8^+$ T cells acquired a limited proliferation potential and failed to up-regulate CD25 expression and to produce IFN-γ (22, 46, 48). Contrary to this finding, alveolar-specific $CD8^+$ T cell

proliferation was accompanied by the production of IFN-γ in a portion of $CD8^+$ T cells, which, however, remained $CD43^-$. Whereas, in naive $CD4^+$ T cells, the *Ifng* gene locus is epigenetically repressed, this locus is already partially remodeled toward a transcriptionally competent configuration in naive $CD8^+$ T cells, which renders them highly prone to rapid IFN-γ secretion after T cell receptor ligation (49). Of note, acquisition of cytotoxic effector cell function has been demonstrated to be regulated independently from IFN-γ secretion (50), emphasizing that IFN-γ–secreting $CD8^+$ T cells that respond to peptide or self-antigen stimulation are not necessarily effector cells. This is well in line with the observed weak expression of CD43. Although conflicting data exist regarding its immunologic functions, CD43 is known to be strongly up-regulated on antigen-specific effector $CD8^+$ T cells (51). Moreover, CD43 expression on $CD8^+$ T cells directly correlates with gain of effector function (i.e., cytotoxic activity is restricted to virus-specific $CD43^{high}$ $CD8^+$ T cells) (52). Together, low numbers of $CD43^+IFN\text{-}\gamma^+$ HA–specific T cells indicate default effector cell differentiation, which is supported by the overall absence of autoimmune tissue damage.

Whereas $CD8^+$ T cells capable of inducing intestinal (18) and liver (24) inflammation displayed high levels of CD25 expression, $CD8^+$ T cells responding to alveolar antigen were completely unable to up-regulate CD25. $CD8^+$ T cells defective in signaling via CD25 have been shown to differentiate inefficiently into CTLs (53), and prolonged CD25 expression on activated $CD8^+$ T cells supports terminal effector cell differentiation (54). Although the current dogma asserts that the expression of IL-2 and CD25 is necessary for cell cycle progression of activated T cells, IL-2 is dispensable for the initiation of $CD8^+$ T cell cycling (55). In contrast, the presence of IL-2 and signaling via CD25 play a decisive role in augmenting the growth of $CD8^+$ T cells, particularly in nonlymphoid tissues, including the lung, where the absence of CD25 expression on $CD8^+$ T cells results in impaired expansion and growth arrest (55, 56). This is in line with our observation that $CD8^+$ T cells forced to proliferate in response to alveolar self-antigen, even though they enter the cell cycle and proliferate, remain $CD25^-$ and fail to differentiate into effector cells.

Our findings suggest the existence of tolerance mechanisms that act in concert to protect the lung from the deleterious effects of inadvertently primed $CD8^+$ T cells. These effects include ignorance of self-antigen in the steady state, inefficient effector cell differentiation in inflammatory settings, and limited expansion of self-reactive $CD8^+$ T cells after abortive priming in a process that may depend on the lack of CD25 expression. Although we did not directly explore the consequences of smoke-induced airway inflammation on the extent of epithelial self-antigen release and potential activation of formerly ignorant $CD8^+$ T cells, our findings directly support the proposed three-step model for the development of COPD (14) after long-term exposure to potentially noxious substances, especially tobacco smoke. During cigarette smoking, the lung is exposed to thousands of xenobiotic compounds and free radicals that directly injure lung epithelial cells (57, 58). Continuous innate immune activation supports the maturation of DCs, which, together with epithelial damage and concomitant release of epithelial antigen, improves cross-presentation of self-antigen, thereby resulting in expansion of self-reactive T cells. In our model, we produced only short-term immune activation by single-dose LPS, α-CD40, or acute infection, which resulted in self-limiting expansion of lung-reactive $CD8^+$ T cells. However, the airways of smokers experience sustained exposure to proinflammatory substances. On the basis of our findings, we hypothesize that permanent activation of innate and adaptive immunity in the airways of smokers or persons otherwise exposed to toxic inhaled substances supports not only priming, but also terminal effector $CD8^+$ T cell differentiation, which leads to the development of self-perpetuating autoimmunity in the lung.

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