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Trachea Allograft Class I Molecules Directly Activate and Retain CD8\(^+\) T Cells That Cause Obliterative Airways Disease\(^1\)

David M. Richards,* Stacy L. Dalheimer,* Marshall I. Hertz,† and Daniel L. Mueller\(^2\)*

Human T cells responding against transplanted allogeneic lung tissue have been implicated in late graft failure secondary to obliterative bronchiolitis. This obliterative airways disease (OAD) also develops in heterotopic murine tracheal allografts in association with graft infiltration by both CD8\(^+\) and CD4\(^+\) T cells. To date, there has been little evidence to suggest that directly alloreactive CD8\(^+\) T cells either promote chronic rejection or lead to the development of OAD following airway allotransplantation. Using L\(^d\)-specific TCR-Tg 2C CD8\(^+\) T cells adoptively transferred into wild-type B6 (H-2\(^b\)) mice and the transplantation of BALB/c (H-2\(^b\)) tracheal allografts, we now show that the direct recognition of donor-specific class I MHC molecules by host CD8\(^+\) T cells leads to their activation, clonal expansion within the graft, and differentiation to an effector phenotype with the capacity to induce airway fibrosis. In addition, these experiments demonstrate that ongoing direct alloantigen recognition within the transplanted airway tissue is necessary for the recruitment and retention of these directly alloreactive CD8\(^+\) T cells. Thus, these experiments are the first to definitively show a role for directly alloreactive CD8\(^+\) T cells in the chronic rejection that leads to OAD. The Journal of Immunology, 2003, 171: 6919–6928.

Advances in surgical technique, postoperative care, and immunosuppression have increased 1-year lung allograft recipient survival rates to >75% (1). Despite these advances, current systemic immunosuppression protocols lead to life-threatening infectious complications and fail to control the problem of chronic transplant rejection. Chronic rejection is a poorly understood process in which ongoing vascular and parenchymal inflammation leads to progressive fibroblast recruitment, extracellular matrix deposition, and organ failure secondary to tissue fibrosis (2). For the case of human lung transplantation, as well as for the murine heterotopic tracheal transplant model, chronic rejection is characterized by airway epithelial cell denudation, peribronchilar mononuclear cell infiltration, and fibrosis that eventually leads to an occlusion of the small cartilaginous airways, referred to as obliterative airways disease (OAD)\(^3\) (3–5).

The graft recipient’s adaptive immune system is responsible for graft failure secondary to chronic rejection following allogeneic organ transplantation (2, 6). A high frequency (1–10%) of T cells can directly recognize foreign MHC molecules expressed on the surface of donor graft cells; additionally, it is expected that a small, yet significant, fraction (0.001–0.01%) of any recipient’s T cells will also recognize a unique peptide derived from one of the donor’s polymorphic proteins (e.g., a donor MHC molecule) that can be presented by host MHC molecules (an indirect recognition event) (7). Historically, it has been thought that direct allo-MHC recognition plays the greater role in acute allograft rejection, whereas indirectly activated T cells play the predominant role in chronic rejection. The role of direct recognition in chronic rejection has been discounted due to a lack of experimental evidence and because the expression of foreign MHC molecules on donor-derived APC would be expected to wane as these APC are destroyed by the recipient’s immune system. Nevertheless, donor class I MHC molecules continue to be expressed on graft parenchymal cells for prolonged periods of time. This raises the possibility that host CD8\(^+\) T cells directly reactive to allogeneic class I MHC molecules would continue to participate in the chronic rejection of donor graft tissue long after transplantation.

For the case of chronic allograft rejection that leads to airway fibrosis and the development of OAD, CD8\(^+\) and CD4\(^+\) T cells have each been implicated in the tissue destruction (6, 8). Nevertheless, the specific roles of these T cells remained ambiguous. Previous work from our laboratory suggested that both donor class I MHC molecules and recipient CD8\(^+\) T cells play a role in the progression of chronic rejection in mouse heterotopic tracheal airway allografts (6). CD8\(^+\) T cells found in the allograft may have been responding directly to donor-specific MHC molecules or indirectly to unique peptide Ag expressed within the graft tissue (minor histocompatibility Ags (mHAg)), but presented by recipient APC. Alternatively, these may simply have been memory cells that can traffic through inflamed tissue or regulatory T cells that are actually protecting the graft from injury. In this study, directly alloreactive TCR-transgenic (TCR-Tg) T cells have been used to investigate the specificity, phenotype, and function of CD8\(^+\) T cells that infiltrate heterotopic tracheal allografts following transplantation. Our results demonstrate that the direct recognition of allogeneic class I MHC molecules by graft-infiltrating CD8\(^+\) T cells is continuous in airway allograft tissue and promotes the retention and persistent activation of T cells with a capacity to destroy the graft as a consequence of OAD development.

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\(^{3}\)Abbreviations used in this paper: OAD, obliterative airways disease; FSC, forward scatter; mHAg, minor histocompatibility Ags; Rag, recombinase-activating gene; TCR-Tg, TCR-transgenic.
Materials and Methods

Mice

Wild-type C57BL/6Ncr (B6; H-2b), CBA/Jc (CBA; H-2k), and BALB/cAnNcr (BALB/c; H-2k) mice, 6–8 wk old, were purchased from Charles River Breeding Laboratories (Wilmington, MA) through a contract with the National Cancer Institute animal program of the National Institutes of Health (Frederick, MD). C57BL/6-recombinase-activating gene 1 (Rag1)−/−Minc (Rag1−/−; catalogue no. 2216), B6-PL-Phy1/Cy (Thy1.1 congenic; catalogue no. 0406), B6.C-H2m12/KHeg (bm12; catalogue no. 1162), B6.C-H2m12/Bym (bm1; catalogue no. 1060), C57BL/6-Cd8a−/− (CD8−/−; catalogue no. 2065), and C57BL/6-JHfi1/mu (nu/nu; catalogue no. 0819) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). F1 (bm1 × bm12) mice were bred in our facility. 2C TCR-Tg mice were a gift from M. Mescher (University of Minnesota, Minneapolis, MN). CD8+ 2C TCR-Tg T cells (H-2b) are directly reactive to the naturally occurring octapeptide called p2Ca (LSPFPFDL) which is derived from the ubiquitous mitochondrial protein 2 oxoglutarate dehydrogenase and is present as a complex with the L chain of molecule expressed in BALB/c mice (9). The 2C TCR-Tg mice were bred to Rag1−/− and Thy1.1 and maintained in our animal facility. CD8+ T cells in these mice express both the Thy1.1 allelic marker as well as a clonotypic TCR detectable with the 1B2 mAb and are highly enriched for a naïve T cell (CD69Lhigh CD44low) (10). Experimental mice were age-matched (6–8 wk old) and sex-matched except where indicated. Animal care was provided according to National Institutes of Health guidelines and the University of Minnesota institutional animal care and use committee, and animals were housed in specific pathogen-free conditions with free access to food and water.

Abs and reagents

The following Abs and fluorochrome conjugates were purchased from BD PharMingen (San Diego, CA): FITC-, PE-, PerCP-Cy5.5- and allophycocyanin-labeled or biotinylated CD4 (RM4-5 and CD45), CD8 (53-6.7), CD69 (H1.2F3), CD69d (9C10), CD62L (MEL-14); PE- or FITC-labeled streptavidin (BD Biosciences, Franklin Lakes, NJ); PE-labeled anti-KLH (anti-keyhole limpet hemocyanin; Santa Cruz, CA), biotinylated anti-KLH (Rockland, Gilbertsville, PA), and secondary Abs conjugated to allophycocyanin, peridinin chlorophyll protein, and phycocyanin. To detect cell surface markers, Abs were diluted in blocking buffer, and tyramide reagents were applied to sections as follows: slides were slipped with Permount (Fisher Scientific, Pittsburgh, PA) at −19°C on a Leica CM1800 cryostat (Leica-Nussloch, Germany), fixed in acetone (Fisher Scientific, Fairlawn, NJ), and immediately stained with H&E (Fisher Scientific) or stored at −80°C until used for immunohistochemical staining. Slides were warmed to room temperature, and sections were hydrated with PBS for 15 min. Endogenous peroxidase activity was extinguished by incubation with 1% hydrogen peroxide (H2O2) for 30 min. Nonspecific Ab binding was blocked by incubation in anti-Fc mAb 2.4G2 for 15 min, followed by sequential 15-min incubations with avidin and biotin blocking reagents (Avidin/Biotin Blocking Kit; Vector Laboratories, Burlingame, CA). All slides were then incubated with 2 µg/ml biotinylated Abs for 30 min. For conventional immunohistochemistry, sections were incubated with HRP-conjugated streptavidin-biotin complex (ABC Elite; Vector Laboratories) for 30 min, followed by a 5-min incubation with 3,3-diaminobenzidine substrate and diaminobenzidine enhancer according to the manufacturer’s instructions, mounted, and covered-slipped with Permount (Fisher Scientific). For fluorescence immunohistochemistry, Abs were diluted in blocking buffer, and tyramide reagents were diluted in amplification diluent (PerkinElmer, Boston, MA). Primary Ab incubation, slides were washed and sequentially incubated with HRP-labeled streptavidin for 20 min and with Cy3-labeled tyramide for 5 min (PerkinElmer). All slides were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories) to preserve fluorescence. Slides were examined on an Axioplan 2 epifluorescence microscope (Carl Zeiss, Thornwood, NY), and images were captured with a SPOT camera (Diagnostic Instruments, Sterling Heights, MI). All images were imported into Photoshop 7.0 (Adobe Systems, San Jose, CA) for presentation.

Scoring and statistical analysis

H&E-stained slides were coded and examined by two blinded reviewers (M.I.H. and D.L.M.) for the presence or the absence of fibrosis as previously described (6). The p values were determined using the nonparametric χ2 test of statistical significance. Confidence intervals cited in the text reflect the SEM.

Results

Direct recognition of class I MHC alloantigens by CD8+ T cells can augment the development of OAD after tracheal allotransplantation

A strong correlation was previously shown to exist between the development of OAD after tracheal allotransplantation and infiltration of the allografts with CD8+ and, to a lesser degree, CD4+ T cells (6, 13). To test whether CD8+ cells are, in fact, necessary for the chronic rejection response that leads to airway fibrosis, BALB/c tracheal allografts were transplanted into CD8-deficient B6 mice. Perhaps surprisingly, no reduction in the development of OAD was observed in allografts transplanted into CD8−/− hosts 56 days earlier (Fig. 1A). Nevertheless, nu/nu mutant mice lacking all T cells tolerated fully allogeneic airways, whereas nu/nu mice reconstituted with spleen T cells were competent to reject these allografts (p < 0.01; Fig. 1B). This confirmed that T cells are essential for the development of OAD, but suggested that CD4+ T cells in their drinking water for 3 days beginning with the day of transplantation. No immunosuppressive agents were given to any graft recipient.

Flow cytometry

Lymph nodes draining the site of transplantation (axillary and brachial) were harvested from BALB/c mice that had been sensitized with the allografts and, previously described (6). The harvested lymph nodes were digested with collagenase and DNase and analyzed as described above. Cells were electronically gated based on forward (FSC) and side scatter properties, and at least 1000 events were collected using the FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA) software. Graft tissues were harvested at various time points, minced with sterile scissors, and stained with PE-conjugated anti-CD8+ mAb and are highly enriched for a naive T cell (CD69Lhigh CD44low) 

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cells can be sufficient to elicit a chronic rejection response when multiple MHC and mHAg differences exist.

We further examined the development of OAD under conditions where MHC molecule and mHAg differences were more limiting. Reactivity against mHAg differences alone was examined using male B6 grafts transplanted into female mice. The presence of male mHAg proved to be an extremely potent stimulus for the infiltration of the grafts with mononuclear cells (data not shown); however, male allografts only infrequently (but significantly more often than female isografts, p = 0.03) developed fibrosis as a consequence of OAD (Fig. 1A). Like the fully mismatched BALB/c tracheal grafts, male B6 grafts appeared to develop OAD even in CD8-deficient female B6 recipients (p = 0.03 vs female isografts) at a low rate not different from that in wild-type recipients (p = 0.71). Taken together, these genetic results were most consistent with an ability of the CD4+ T cells responding to mHAg differences to elicit OAD even in the absence of CD8+ T cells.

What, then, of the role of CD8+ T cells in the chronic destruction of airway allografts? Our previous study had demonstrated that the presence of a single K\textsuperscript{bm1} class I alloantigen on donor tracheal tissue could have an important stimulatory effect on both the recruitment of B6 CD8+ T cells into the graft tissue as well as the development of OAD when also accompanied by the I-A\textsuperscript{bm12} mutant class II MHC molecule, although the K\textsuperscript{bm1} molecule by itself proved to be a generally weak stimulus for chronic rejection (4). The inability of the K\textsuperscript{bm1} molecule in donor grafts to elicit a strong mononuclear cell infiltration in B6 recipients may be because this mutant class I molecule contains only three amino acid differences from K\textsuperscript{b} within a five-residue stretch (14); consequently, indirect presentation of a unique K\textsuperscript{bm1} peptide to a host CD4+ T cells is unlikely. This implies then that a single directly alloreactive CD8+ T cell response (such as would be targeted against an allogenic K\textsuperscript{bm1} molecule) is insufficient to elicit OAD in the absence of a simultaneous CD4+ T cell response directed against either an intact class II MHC alloantigen or an mHAg difference. Interestingly, tracheal grafts from male bm1 donor mice that express both the K\textsuperscript{bm1} molecule and X chromosome-encoded male mHAg were rejected at a high rate in female wild-type B6 recipient mice compared with MHC-matched male B6 donor grafts (p < 0.01; Fig. 1A). In addition, CD8-deficient female B6 recipients of male bm1 grafts showed a reduced rate of OAD development compared with wild-type recipients, although this difference did not reach statistical significance in our experiments (p = 0.09). Finally, experiments performed with male (bm1 x bm12)F\textsubscript{1} grafts that express single class I and II MHC differences in addition to male mHAg transplanted into female B6 recipients demonstrated a significantly higher rate of OAD development in wild-type B6 recipients than in CD8-deficient mice (p = 0.04). Thus, the direct recognition of a single allogenic class I MHC molecule by CD8+ T cells can be an important stimulus for OAD development, but is most evident when only a limited number of other class II MHC or mHAg differences coexist.

CD8+ T cells with an activated effector/memory cell phenotype demonstrate evidence of alloantigen recognition in tracheal allografts undergoing chronic airway fibrosis

To further investigate the role of directly alloreactive CD8+ T cells in the development of OAD, we used flow cytometry to phenotype the T cells recovered from rejecting tracheal graft tissues. As expected from our previous immunohistochemical analysis (6), allogenic BALB/c grafts were infiltrated with more T cells than control isografts in B6 hosts (Fig. 2A). Furthermore, T cells isolated directly from day 14 allograft tissue showed a CD8+ to CD4+ ratio of 3.19 ± 0.73. This contrasted with average CD8+ to CD4+ T cell ratios of 0.94 ± 0.08 and 0.77 ± 0.08 in the draining lymph nodes of allograft and isograft recipient animals, respectively. This enrichment of CD8+ cells specifically within the allografts was apparent by day 14 and persisted beyond day 35 (data not shown). In contrast to allografted animals, no such enrichment of CD8+ T cells was ever observed in the transplanted graft tissue of animals receiving B6 isografts (0.78 ± 0.09 ratio).

To test for evidence of alloreactivity by the CD8+ T cells recovered from transplanted airway tissues, the activation and differentiation states of the electronically gated CD8+ T cell subpopulation were examined by flow cytometry (Fig. 2B). The presence of a high level of CD44 expression on the majority of BALB/c allograft-infiltrating CD8+ T cells in B6 mice indicated an effector/memory cell phenotype and suggested that this population of CD8+ cells had arisen from a pool of Ag-experienced T cells. In contrast, the majority of the CD8+ T cells found within the draining lymph nodes of the allografted mice as well as in all tissues of the isografted control mice had a naive phenotype (CD44low). Interestingly, the CD69 activation molecule was also highly expressed on most of the allograft-infiltrating CD8+ T cells, consistent with very recent or ongoing TCR stimulation. CD8+ T cells within the draining lymph nodes of these allografted animals,
CD8$^+$ T cells were transplanted with a total of three B6 or BALB/c tracheas (as indicated), and then T cells were isolated from pooled axillary and brachial lymph nodes (top) or from the pooled trachea grafts on day 14 after transplantation (bottom; A–D). A, Total lymphoid-gated cells analyzed for CD8 and CD4 expression. B, Gated CD8$^+$ T cells examined for CD69 and CD44 expression. C, Gated CD8$^+$ T cells from BALB/c allograft recipient animals examined for binding to anti-CD69 and anti-IFN-$\gamma$ mAb (or control Ab, as indicated). D, Gated B6.PL Thy1.1$^{+/+}$ CD8$^+$ T cells examined for FSC profile and CFSE dye dilution. These B6 recipient mice had received $2 \times 10^6$ CFSE-labeled B6.PL spleen and lymph node cells 1 day before tracheal transplantation. E, Histograms comparing the FSC profile of total lymph node (gray tracing) and trachea graft-infiltrating (black tracing) CD8$^+$ T cells recovered from the isograft (left) and allograft (right) recipient mice described in D. Quadrants were established using isotype-matched irrelevant control Ab conjugates. The numbers shown in each quadrant indicate the percentage of gated cells. Duplicate animals were examined in each sample group with similar results found, and the data shown are representative of at least two independent experiments.

in contrast, demonstrated only minimal CD69 expression that did not significantly differ from that expressed by T cells in either the tracheal graft tissue or lymph nodes of isograft animals.

The expression of CD69 on the majority of allograft-infiltrating CD8$^+$ T cells suggested that many of these T cells were experiencing ongoing alloantigen recognition within the graft tissue itself. If true, that would imply that many of these cells were, in fact, specific for alloantigen. To further address this question, graft-infiltrating CD8$^+$ T cells were examined for the production of IFN-$\gamma$ protein by flow cytometry. Remarkably, over half of the CD69$^+$ CD8$^+$ T cells within the tracheal allograft were spontaneously producing IFN-$\gamma$ 14 days after transplantation (Fig. 2C). This effector cell activity appeared to be specifically in response to graft alloantigen recognition, as CD8$^+$ in the draining lymph nodes of the same animals did not express this lymphokine. Thus, the coordinate expression of CD69 and IFN-$\gamma$ indicated that significant numbers of graft-infiltrating CD8$^+$ T cells were specific for alloantigens expressed in the graft and were experiencing ongoing activation within the transplanted airway tissue.

**Graft-infiltrating CD8$^+$ T cells undergo blastogenesis and multiple rounds of cell division in response to tracheal allotransplantation**

The effector/memory T cell phenotype is acquired during the course of an Ag-induced proliferative response. If the high CD44 expression observed on virtually all tracheal allograft-infiltrating CD8$^+$ T cells was, in fact, a consequence of alloantigen recognition, then the cells would be expected to demonstrate evidence of cell cycle progression subsequent to the transplantation. To test this, Thy1.1$^{+/+}$ congenic B6.PL spleen and lymph node cells were labeled with CFSE and then adoptively transferred into syngeneic B6 graft-recipient mice. The following day, these animals were transplanted with either B6 syngeneic or BALB/c allogeneic tracheas. Fourteen days after BALB/c tracheal allograft placement, the majority of the Thy1.1$^{+/+}$ CD8$^+$ T cells recovered from tracheal allografts showed evidence of blastogenesis (based on an increased FSC profile) and were found with a fully diluted CFSE dye content, consistent with more than six rounds of cell division (Fig. 2D). In contrast, Thy1.1$^{+/+}$ CD8$^+$ T cells recovered from lymph nodes draining the site of tracheal allotransplantation demonstrated only a modest degree of proliferation, no different from that observed after transplantation of an isograft. Not unexpectedly, too few Thy1.1$^{+/+}$ CD8$^+$ T cells entered isografts to quantitatively analyze this population for blastogenesis or CFSE dye dilution. It should be noted that the majority of normal recipient (B6) allograft-infiltrating CD8$^+$ T cells also demonstrated evidence of blastogenesis (Fig. 2E). Taken together, the flow cytometric phenotyping of CD8$^+$ cells within tracheal allografts undergoing chronic rejection supported the hypothesis that the majority of these T cells were being stimulated through their TCR by alloantigen to undergo activation, blastogenesis, proliferation, and differentiation to an effector/memory phenotype in the days following transplantation of the tracheal allograft. Furthermore, these experiments identified IFN-$\gamma$ as one potential CD8$^+$ T effector cell lymphokine responsible for the development of OAD.
**L^d-reactive 2C TCR-Tg CD8^+ T cells are sufficient to induce OAD in BALB/c tracheal allografts**

Based on the above genetic studies using K^bm1^-bearing allografts and on the observation of a bias toward the recruitment of activated and differentiated CD8^+ T cells into fully mismatched airway allografts undergoing chronic rejection, we hypothesized that many of the allograft-infiltrating CD8^+ T cells were, in fact, directly reactive against allogeneic class I MHC molecules. To model the direct recognition of class I alloantigens within tracheal allografts, we made use of the 2C TCR-Tg mouse line bred onto the B6.PL-Rag1^-/- background (2C mice). T cells from these 2C mice are predominantly CD8^+, bear the Thy1.1 congenic marker, are highly enriched for a naive phenotype, and demonstrate uniform reactivity to L^d class I molecules in BALB/c tissues (9). To initially assess the capacity of 2C CD8^+ T cells to induce the development of OAD following transplantation with L^d-bearing tracheal grafts, 2C mice were directly transplanted with allogeneic BALB/c tracheas. Consistent with a previous demonstration that recipient 2C transgenic mice destroy heterotopic BALB/c cardiac allografts (15), transplanted BALB/c tracheal allografts became heavily infiltrated with 2C T cells and all fibroed by day 56 (data not shown). Similarly, B6 nu/nu recipient mice reconstituted with 2C spleen CD8^+ T cells and then transplanted with tracheas destroyed all BALB/c allografts, but none of the B6 isografts (p = 0.01; Fig. 1B).

Tracheal grafts transplanted into nu/nu mice were further examined by immunohistochemistry for evidence of a T cell infiltration indicative of a chronic rejection response. Syngeneic B6 grafts transplanted into 2C-reconstituted nu/nu mice demonstrated little evidence of T cell infiltration (data not shown). In contrast, 2C-reconstituted nu/nu mice heavily infiltrated BALB/c allografts with CD3^+ T cells, many of which could be shown to bind the anti-clonotypic mAb 1B2 that is specific for the 2C TCR transgene (Fig. 3, A–D). These L^d-specific T cells located within the allograft both in the submucosal areas adjacent to the tracheal cartilage as well as in the fibrotic tissue occluding the lumen of the airway. The overall number and location of T cells found in the BALB/c allografts undergoing rejection within the 2C-reconstituted nu/nu mice were similar to those observed for normal T cells in B6-reconstituted mice, except, as expected, the 1B2 clonotype was not identified in these graft recipients (Fig. 3, E and F). Thus, it appeared that a high frequency of directly alloreactive CD8^+ T cells was sufficient to induce the development of OAD in tracheal allografts as a consequence of airway infiltration.

Only graft-infiltrating L^d-reactive 2C CD8^+ T cells activate, proliferate, and differentiate to effector/memory cells in response to BALB/c tracheal transplantation

To investigate the behavior and phenotype of directly alloreactive CD8^+ T cells during the course of tracheal allograft rejection and development of OAD in wild-type recipients (and to avoid the stimulatory effects of T cell adoptive transfer into a lymphopenic host), L^d-reactive 2C T cells were studied as prototypic responder CD8^+ T cells after their adoptive transfer into normal B6 graft recipient mice. Following the i.v. adoptive transfer of CFSE-labeled 2C spleen and lymph node CD8^+ T cells into these B6 mice, 0.71 ± 0.14% of draining lymph node CD8^+ cells in day 14 BALB/c (H-^2d^) allografted mice were shown to be L^d-reactive based on coexpression of the Thy1.1 congenic marker (Fig. 4A). This was little different from the frequency observed in animals receiving either a B6 isograft (0.37 ± 0.15%) or an irrelevant third-party CBA (H-^2^) allograft (0.65 ± 0.03%; Fig. 4A and data not shown). The lack of alloantigen-dependent clonal expansion of 2C TCR-Tg CD8^+ T cells in the lymph nodes draining the allograft was consistent with the observation that only a minor fraction (30 ± 19%) of the Thy1.1^+ 2C CD8^+ draining lymph node T cells appeared to have diluted their CFSE dye following BALB/c tracheal transplantation, and this again was no different from that observed for 2C cells in the lymph nodes of either B6- or CBA-grafted mice (31 ± 3 and 25 ± 8%, respectively; Fig. 5 and data not shown). The expression of CD44 and CD69 on these lymph node L^d-reactive CD8^+ T cells was also examined. Again, the expression of these molecules was similar to that on other CD8^+ T cells within the lymph nodes regardless of the strain of the tracheal graft (Figs. 4C and 5 and data not shown). It should be noted that additional experiments were performed at multiple timepoints (days 5, 6, 7, 10, 14, 28, and 35 after BALB/c allograft transplantation) and L^d-dependent 2C CD8^+ T cell clonal expansion, cell division, and differentiation were never reliably observed in the draining lymph node (data not shown). Taken together, the data suggested that most 2C CD8^+ T cells residing in the draining lymph nodes of BALB/c allografted animals are ignorant of the transplanted foreign tissue. Thus, this study could not confirm that direct allore cognition by CD8^+ T cells begins in the lymph nodes that drain the site of tissue transplantation.

In contrast to the L^d-reactive CD8^+ T cells recovered from the draining lymph nodes, 2C CD8^+ T cells infiltrating BALB/c tracheas demonstrated strong evidence of ongoing activation, cell cycle progression, and differentiation. Thy1.1^+ 2C CD8^+ T cells were, on the average, 8.6 ± 4.3% of the CD8^+ T cells recovered from the BALB/c tracheal allografts (Fig. 4, A and B), whereas 2C T cells made up <0.5% of the CD8^+ T cells found in either B6 isografts or CBA third-party allografts (Fig. 4, A and B, and data not shown). 2C CD8^+ T cells migrated into the submucosal space of BALB/c tracheal grafts adjacent to the bronchial epithelium and...
entered the fibrotic tissue that occludes the lumen in airways undergoing OAD (Fig. 6). This enrichment of the 2C clone specifically in Ld-bearing BALB/c allografts appeared to result at least in part from alloantigen-induced cell division by the 2C CD8+ T cells, given that they had entirely diluted their CFSE dye content and had a CD44high phenotype consistent with the majority of the graft-infiltrating CD8+ T cells (Fig. 5A). The uniformly high expression of CD69 on all grafts and draining lymph node CD8+ T cells as indicated. The congenic marker Thy1.1 was used to distinguish endogenous CD8+ T cells from the adoptively transferred 2C CD8+ T cells. Each bar represents the mean ± SEM of at least three animals analyzed in at least two independent experiments. Too few 2C CD8+ T cells were recovered from B6 isografts to allow for accurate phenotyping.

**Alloantigen recognition within tracheal grafts causes the specific retention and localization of alloreactive CD8+ T cells**

Our demonstration that CD8+ T cells with a specificity for the Ld class I alloantigen enrich within BALB/c allografts, but not within lymph nodes draining the graft, raised the possibility that the directly alloreactive CD8+ T cells that participate in the development of OAD are specifically recruited to the allograft or retained at that site as a consequence of Ag presentation within the graft tissue. The alternative would be that the up-regulation of ligands for effector/memory T cell homing receptors on graft endothelium as a consequence of graft inflammation is sufficient to recruit recently activated CD8+ T cells into the transplanted tissue. It has previously not been possible to investigate the effects of Ag presentation within a graft and differentiate these two possibilities, because graft-reactive T cells could not be identified and monitored. Using the Ld-specific 2C CD8+ T cell adoptive transfer system, we examined the sufficiency of graft inflammation to recruit and retain fully activated and differentiated CD8+ T cells. B6 mice that had received an adoptive transfer of 2C CD8+ T cells were transplanted with three BALB/c tracheal allografts, three CBA third-party allografts, or two grafts from each donor strain (Fig. 7). As expected, the BALB/c allografts in recipients that had received only BALB/c grafts or received both BALB/c and CBA third-party allografts, or two grafts from each donor strain demonstrated a heavy infiltration with activated (CD69high) 2C CD8+ T cells. Remarkably, mice transplanted with the combination of BALB/c and CBA allografts demonstrated a heavy infiltration with activated (CD69high) 2C CD8+ T cells. This enrichment occurred within BALB/c tracheal allografts expressing the Ld class I MHC molecule. One day before transplantation with three MHC identical tracheal grafts or with the combination of two BALB/c plus two CBA graft (as indicated), B6 recipient mice received an adoptive transfer of 2.5 × 10^6 CFSE-labeled 2C CD8+ T cells. On day 14 after transplantation, draining lymph node and graft-infiltrating cells were harvested from the mice and then analyzed by flow cytometry. A. Percentage of 2C T cells within the total CD8+ T cell population found within the draining lymph nodes (■) and tracheal allografts (□) as indicated. B. Total and 2C CD8+ T cells per graft were determined by acquiring 100% of each sample’s events and dividing by the number of grafts pooled. D. Expression of CD69 on allograft and draining lymph node CD8+ T cells as indicated. The congenic marker Thy1.1 was used to distinguish endogenous CD8+ T cells from the adoptively transferred 2C CD8+ T cells. Each bar represents the mean ± SEM of at least three animals analyzed in at least two independent experiments. Too few 2C CD8+ T cells were recovered from B6 isografts to allow for accurate phenotyping.

**FIGURE 4.** Activated 2C CD8+ T cells are specifically enriched only in tracheal allografts expressing the Ld class I MHC molecule. One day before transplantation with three MHC identical tracheal grafts or with the combination of two BALB/c plus two CBA graft (as indicated), B6 recipient mice received an adoptive transfer of 2.5 × 10^6 CFSE-labeled 2C CD8+ T cells. On day 14 after transplantation, draining lymph node and graft-infiltrating cells were harvested from the mice and then analyzed by flow cytometry. A. Percentage of 2C T cells within the total CD8+ T cell population found within the draining lymph nodes (■) and tracheal allografts (□) as indicated. B. Total and 2C CD8+ T cells per graft were determined by acquiring 100% of each sample’s events and dividing by the number of grafts pooled. D. Expression of CD69 on allograft and draining lymph node CD8+ T cells as indicated. The congenic marker Thy1.1 was used to distinguish endogenous CD8+ T cells from the adoptively transferred 2C CD8+ T cells. Each bar represents the mean ± SEM of at least three animals analyzed in at least two independent experiments. Too few 2C CD8+ T cells were recovered from B6 isografts to allow for accurate phenotyping.

**FIGURE 5.** 2C CD8+ T cells infiltrating Ld-bearing BALB/c tracheal allografts express high levels of CD44 and CD69 and demonstrate evidence of extensive prior cell division. B6 recipient mice were adoptively transferred with 2C CD8+ T cells and transplanted with either CBA or BALB/c tracheal allografts as described in Fig. 4. Thy1.1+ CD8+ draining lymph node and graft-infiltrating 2C T cells were analyzed as indicated on day 14 for CD44 expression and CFSE dye dilution (A) or for CD69 expression and CFSE dye dilution (C). CD44 and CD69 expression by endogenous lymph node (gray tracing) and trachea (black tracing) CD8+ T cells is also shown for comparison (B and D). Duplicate animals were examined within each experimental group, and all results shown are representative of at least two independent experiments. Data shown here contributed to the panels shown in Fig. 4.
The few $L^d$-reactive 2C CD8$^+$ T cells that could be isolated from the irrelevant CBA grafts in BALB/c plus CBA combination-grafted animals appeared to have a similar pattern of adhesion molecule expression (CD62L$^\text{low}$, CD44$^\text{high}$, CD11a$^\text{high}$, and CD49d$^\text{high}$), as the majority of the CD8$^+$ T cells found within these allografts (Table I). Therefore, direct alloantigen recognition within the grafts was clearly unnecessary for the continued expression of these tissue-homing molecules. Nevertheless, CD69 expression was significantly reduced ($p < 0.01$) on CBA graft-infiltrating $L^d$-specific 2C CD8$^+$ T cells compared with that either on 2C cells found within the BALB/c allografts on the opposite side of the same recipient animal or on the endogenous CD8$^+$ T cells observed within the same CBA allografts (Fig. 4C). Thus, the results suggest that prior Ag recognition may lead to phenotypic changes expected to promote the migration of the 2C CD8$^+$ T cells into inflamed graft tissue and their exclusion from secondary lymphoid tissues such as the draining lymph nodes. Nevertheless, ongoing direct recognition of allogeneic class I MHC molecules within BALB/c tracheal grafts appears essential for both continued high level expression of the CD69 activation marker and the retention of the CD8$^+$ T cells within an Ag-bearing allograft.

**IFN-γ is an effector lymphokine produced by tracheal graft-infiltrating 2C CD8$^+$ T cells in response to direct class I alloantigen recognition**

Given that allograft-infiltrating CD8$^+$ T cells can be shown to produce IFN-γ, and that OAD development is associated with the retention of directly alloreactive CD8$^+$ T cells with an effector/memory phenotype, we sought to determine whether direct class I alloantigen presentation by trachea allografts, the $L^d$-specific 2C CD8$^+$ T cells in these grafts were found to express significantly reduced amounts of IFN-γ. Like the endogenous CD8$^+$ T cells recovered from tracheal allografts, the $L^d$-specific 2C CD8$^+$ T cells in these grafts were found to express significant amounts of IFN-γ (Table I). Therefore, direct class I alloantigen recognition can be an important stimulus for the production of proinflammatory cytokines within allogeneic airway transplant tissue.

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**FIGURE 6.** Fluorescence immunohistochemistry analysis of isogenic (B6) and allogeneic (BALB/c) trachea grafts on day 56 after transplantation. A pair of MHC-identical grafts was heterotopically transplanted onto wild-type B6 mice that had been adoptively transferred with 5$x10^6$ 2C CD8$^+$ T cells 1 day before transplantation. Tracheal grafts were harvested on day 56 and snap-frozen for sectioning. The binding of biotinylated primary Ab against CD8 (A), an isotype-matched control Ab (B), or biotinylated primary Ab against Thy1.1 (C–F) was visualized on BALB/c allografts (A–C) and control B6 isografts (D and F) with Cy3-labeled streptavidin (shown in red). Slides were also counterstained with 4,6-diamidino-2-phenylindole to reveal tissue nuclei (shown in blue). The arrow identifies single T cells as shown in the magnified inset. L, lumen of the trachea grafts. These images are representative of at least two independent experiments.

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**FIGURE 7.** Activated 2C CD8$^+$ T cells are retained only in $L^d$-bearing tracheal allografts in recipients of multiple allogeneic grafts. B6 recipient mice were adoptively transferred with 2C CD8$^+$ T cells as described in Fig. 4 and then transplanted either with three MHC identical tracheal allografts (BALB/c (A) or CBA (B)) or with the combination of two BALB/c and two CBA allografts (C), as indicated by the illustrations at the right. BALB/c tracheas are shown in white, and CBA tracheas are shown in black. Draining lymph node and graft-infiltrating CD8$^+$ T cells from these animals were analyzed on day 14 for CD69 expression. Both endogenous CD8$^+$ T cells (left panels) and Thy1.1$^+$ 2C CD8$^+$ T cells (right panels) were analyzed within the same sample tube. Note that the scale of the y-axis for each histogram shown in the right panel (Thy1.1$^+$ $L^d$-specific CD8$^+$ T cells) has been amplified 10 times over that of the corresponding left panel histogram tracing (endogenous CD8$^+$ T cells), to indicate the relative abundance of 2C CD8$^+$ T cells within the total CD8$^+$ population. Shaded profiles indicate draining lymph node samples, whereas single black lines represent cells recovered from the allografts. A total of three graft recipient animals were analyzed in each experimental group in two independent experiments, with similar results observed. The data shown in this figure contributed to the panels shown in Fig. 4.
Discourse

Previously, we had reported that both CD8⁺ and CD4⁺ T cells infiltrate trachea allograft tissues and enter the subluminal epithelial space adjacent to the cartilage as early as day 10 after transplantation (6). The experiments in this study confirm that recipient CD8⁺ T cells with direct class I MHC alloreactivity infiltrate class I disparate airway allografts, recognize donor class I molecules within the graft, and contribute to the chronic destruction of the transplanted tissue. Both endogenous graft-infiltrating CD8⁺ T cells and the TCR-Tg 2C CD8⁺ T cells with known direct class I (L²) specificity demonstrated evidence of blastogenesis, cell division, differentiation, and change in homing receptor phenotype consistent with their development of effector/memory cell function in response to transplantation of allogeneic tissue. These phenotypic changes observed with the 2C CD8⁺ T cells are consistent with results previously reported for directly alloreactive TCR-Tg CD8⁺ T cells that acutely (peak at day 7) infiltrate heart allografts (16). The finding of uniformly high CD69 expression on the L²-specific 2C CD8⁺ T cells only in those grafts that expressed the relevant donor-derived class I MHC molecule is the first direct evidence of the long-held idea that the majority of CD8⁺ CD69⁺ endogenous T cells found within allografts are also alloreactive and chronically stimulated by donor class I MHC molecules expressed there. Our immunohistochemical analysis of tracheal allografts at various times after transplantation has, in fact, indicated strong class I MHC expression on many graft parenchymal cells, including the luminal airway epithelial cells (unpublished observation). Nevertheless, the identity of the donor cells recognized by these alloreactive graft-infiltrating CD8⁺ T cells cannot be determined from this study.

The demonstration that T cell-deficient nu/nu mice reconstituted with L²-reactive 2C CD8⁺ T cells destroy BALB/c tracheal allografts as a consequence of OAD indicates that the direct stimulation of recipient CD8⁺ T cells by donor-specific class I MHC molecules expressed on airway graft cells is sufficient to induce effector functions that cause tissue fibrosis. The molecular nature of this effector activity remains unknown; however, studies using this and other transplant rejection models as well as studies of human chronic lung rejection suggest that the secretion of TNF-α and IFN-γ by CD8⁺ graft-infiltrating T cells may be an important intermediate in the process (17, 18). Our demonstration of IFN-γ expression by directly alloreactive CD8⁺ T cells within tracheal allografts undergoing OAD provides support for this idea. Both TNF-α and IFN-γ can induce a proinflammatory response by the innate immune cells that also populate the allograft and promote higher MHC and costimulatory molecule (e.g., CD86) expression on parenchymal cells and endothelium. Graft CD86 expression has, in fact, been found to be necessary for OAD development following rat airway transplantation (19). Increased MHC molecule and CD86 expression would be expected to intensify alloantigen presentation and promote aggressive T cell responses as a consequence of greater CD8⁺ T cell clonal expansion, as we have described in this report. IFN-γ is also an important stimulus for the elaboration of the chemokines called monokine induced by IFN-γ and IFN-γ-inducible protein-10 (CXCL9 and CXCL10, respectively). Monokine induced by IFN-γ and IFN-γ-inducible protein-10 production in allograft tissue have been implicated in the recruitment of T cells to allografts via binding of these chemokines to CXCXR3, leading to subsequent destruction of the transplanted tissues, including airways (20, 21).

In contrast to our findings of vigorous 2C CD8⁺ T cell responses within the tracheal allograft tissue, at no time after transplantation did we detect evidence for alloantigen recognition either by the endogenous polyclonal lymph node CD8⁺ T cells or by the L²-reactive 2C CD8⁺ T cells that continued to reside in the lymph nodes draining the grafted BALB/c tissue (up to day 35). This may not be surprising in the case of the endogenous T cells because of the relatively infrequent direct BALB/c alloantigen reactivity within the B6 CD8⁺ repertoire (expected to be ≤10%). CD8⁺ T cells residing in lymph nodes with mHAg specificity for BALB/c polymorphic gene products may be expected to be even more rare. Nonetheless, the finding that 2C TCR-Tg CD8⁺ T cells with uniform L² reactivity also demonstrated no evidence of activation within the lymph nodes draining a BALB/c allograft or in the spleen (data not shown) is perhaps unexpected and suggests that there is relatively limited or only transient movement of intact donor class I MHC-expressing cells from the tracheal graft tissue to these lymph nodes. This result contrasts with a modest clonal expansion of K²-reactive TCR-Tg CD8⁺ T cells observed in the spleen and draining lymph nodes on day 5 after heterotopic H-2b cardiac allograft transplantation (16). Whether directly reactive class I MHC-specific CD8⁺ T cells are ever primarily stimulated

### Table 1. Summary of adhesion and effector molecule expression on alloreactive CD8⁺ T cells

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<tr>
<th>Donor Graft(s)</th>
<th>Tissue</th>
<th>CD62L&lt;sup&gt;high&lt;/sup&gt; Total</th>
<th>CD62L&lt;sup&gt;high&lt;/sup&gt; L&lt;sup&gt;α&lt;/sup&gt;-specific</th>
<th>CD44&lt;sup&gt;high&lt;/sup&gt; Total</th>
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<th>CD11a&lt;sup&gt;high&lt;/sup&gt; Total</th>
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<th>INF-γ L&lt;sup&gt;α&lt;/sup&gt;-specific</th>
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<td>49 ± 6</td>
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<td>19 ± 1</td>
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<td>8 ± 3</td>
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<td>BALB/c allograft</td>
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<td>7 ± 1</td>
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<td>17 ± 3</td>
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<td>86 ± 3</td>
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<sup>a</sup> Values shown are the percentage of CD8⁺ T cells ± SEM. Data are derived from at least two independent experiments for each molecule and tracheal graft combination shown. ins, insufficient L<sup>α</sup>-specific 2C CD8⁺ T cells were recovered from isografts to analyze by flow cytometry; nd, experiment not done.
by tracheal graft-derived APC within the draining lymph node cannot be established here. Activation by donor APC may have been quickly followed by emigration of the T cells from the lymph node and their movement into the inflamed graft. Previous studies of direct CD8\(^+\) T cell alloreactivity against transplanted tumors have suggested that responder T cells are briefly stimulated within secondary lymphoid organs, but then immediately emigrate to the site of tumor cell accumulation and return to the lymphoid compartment as memory cells only when the tumor has been cleared (26). Alternatively, direct alloreactivity against transplanted airway tissue may arise from a pool of previously stimulated CD8\(^+\) effector/memory cells that already has the capacity to migrate through non-lymphoid tissue and does not rely on Ag recognition within secondary lymphoid organs for their activation (27, 28). This latter possibility seems less likely, because our 2C responder T cells are deficient for Rag1 and, therefore, would have had no previously stimulated memory cells bearing a second TCR. Furthermore, we have purified naive phenotype CD44\(^{low}\) CD8\(^{bright}\) effector cells bearing a second TCR. Additionally, we have evidence that naive CD8\(^{bright}\) CD49d\(^{+}\) memory cells bearing a second TCR are permissive for homing receptors (e.g., CD44, CD11a, and CD49d) and used them in our adoptive transfer system. These, too, demonstrate the detectable draining lymph node response to BALB/c tracheal allograft (data not shown). Regardless, these results suggest that the evaluation of CD8\(^+\) T cells within the secondary lymphoid tissues of solid organ transplant recipients cannot reveal the full potential for or severity of chronic allograft rejection by CD8\(^+\) cells that directly recognize foreign class I molecules.

The demonstration of an enrichment of CD8\(^+\) T cells with direct reactivity to a particular class I MHC molecule only within tracheal grafts that contain the relevant alloantigen supports the hypothesis that directly alloreactive CD8\(^+\) T cells are specifically recruited to or retained within rejecting grafts as a consequence of continuous direct alloantigen recognition. The retention of activated donor-specific CD8\(^+\) T cells within airway allografts may also explain their apparent absence from the secondary lymphoid organs draining the site of transplantation. The up-regulation of homing receptors (e.g., CD44, CD11a, and CD49d) permissive for trafficking through non-lymphoid peripheral tissues is probably a prerequisite for graft infiltration and retention. In addition, the loss of Cxcr2 expression by alloantigen responders would be expected to inhibit their reentry into the lymph nodes. The direct recognition of alloantigen on donor endothelial cells may promote T cell graft entry by inducing CD11a integrin clustering and increasing its avidity for CD54 coexpressed on the inflamed endothelium (29). Alternatively, ongoing TCR signaling may be essential for the continued expression of CXCR3 or some other chemokine receptor to facilitate the retention of CD8\(^+\) T cells in the allograft by chemokines that act to increase CD49d-dependent binding to CD106 or other cell-free matrix components, such as fibronectin (30–32). Thus, ongoing stimulation serves to concentrate graft-reactive CD8\(^+\) T cells as well as maintain them in a highly activated state. Alloantigen recognition within the graft may also target CD8\(^+\) effector cell activities and induce the fibrotic response responsible for the development of OAD. The findings that the K\(^{bm1}\) molecule can promote the development of OAD in B6 recipients in a CD8-dependent fashion and that L\(^{d}\)-reactive CD8\(^+\) T cells accumulate within BALB/c tracheal allografts and induce graft fibrosis in 2C Rag1\(^{-/-}\) recipient mice are both consistent with such a model.

From a clinical perspective, it is noteworthy that both the endogenous graft-infiltrating CD8\(^+\) T cells as well as the 2C Rag1\(^{-/-}\) mice with a fixed direct L\(^{d}\) reactivity demonstrated evidence of extensive cell division, indicating a robust clonal expansion during the course of the chronic rejection response. Taken together with the lack of overt allore cognition in the draining lymph nodes, this intense clonal expansion of select graft-reactive CD8\(^+\) T cells in the face of continued ignorance of the donor graft by the majority of graft-specific CD8\(^+\) T cells within the lymph nodes suggests that the induction of allograft transplantation tolerance through CD8\(^+\) T cell peripheral deletion or clonal anergy induction cannot be induced by graft alloantigens alone. Rather, the continued presence of this large pool of naive alloreactive CD8\(^+\) T cells probably predisposes to the relentless progression toward OAD that has been observed after lung allotransplantation. Thus, current immunosuppressive regimens may fail to prevent chronic transplant rejection because of their inability to fully control the proliferation of this already high frequency, directly alloreactive CD8\(^+\) T cell repertoire.

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References


