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Induction of Obliterative Airway Disease in Murine Tracheal Allografts by CD8+ CTLs Recognizing a Single Minor Histocompatibility Antigen

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The role of minor histocompatibility Ag (mHAg)-specific CD8+ CTLs in the pathogenesis of chronic lung allograft rejection (bronchiolitis obliterans syndrome) remains to be elucidated. Thus, the goal of this study was to define the role of a single mHAg mismatch at the polymorphic H13 allele in the development of obliterative airway disease (OAD) after murine heterotopic tracheal transplantation. The H13a and H13b alleles encode for the SSVGVWYL (SVL9) and SSVIGVWYL (SIL9) mHAgs, respectively, presented in the context of the H2Db MHC class I molecule. Toward this, C56BL/10SnJ (H13b) tracheal allografts were transplanted into congenic B10.CE-H13a A*(30Nx)/Sn (H13a) recipients. The allografts were harvested at 30, 60, and 90 days after transplantation, and OAD lesions (epithelial damage, cellular infiltration, and luminal fibrosis) were confirmed histologically. Selected groups of mice were immunized (s.c.) or tolerized (i.v.) with the SVL9 peptide before transplantation. This single mHAg mismatch induced the development of OAD within 90 days. SVL9 immunization significantly accelerated the kinetics of the OAD lesions. In contrast, SVL9 tolerization completely abrogated the development of OAD. This was correlated with a complete abrogation of H13a-specific CD8+ CTL responses with a significant reduction in the frequency of IFN-γ-producing CTLs and the activation of TGF-β-producing CD8+ T cells. In conclusion, a single mHAg mismatch can induce the development of OAD. These data also suggest that mHAg-reactive CD8+ CTLs may play an important role in the pathogenesis of chronic lung allograft rejection in humans. The Journal of Immunology, 2005, 174: 1871–1878.

Several animal and clinical studies have indicated that acute allograft rejection is predominantly mediated through the direct allorecognition pathway due to the significant numbers of "passenger" donor-derived APCs present in the graft. The succeeding decline in the number of intragraft donor-derived APCs suggests that the direct allorecognition pathway may not play an important role in the process of chronic allograft rejection (1). Overall, the mechanisms associated with the development of chronic allograft rejection are not well understood. This is particularly true for bronchiolitis obliterans syndrome (BOS) (2), which is considered to represent chronic lung allograft rejection (3). A growing body of evidence indicates that development of BOS after lung transplantation is mainly caused by the allogeneic response against donor HLA class I and II molecules (2, 4). In this regard, recent studies from our laboratory have shown a significant correlation between indirect allorecognition of mismatched donor HLA class I and II Ags by CD4+ T cells and development of BOS (5–7). These studies strongly indicated that CD4+ T cell alloreactivity against donor HLA Ags play an important role in the pathogenesis of BOS. In contrast, the role of CD8+ CTLs in BOS pathogenesis remains to be elucidated (8).

Several recent studies have shown that the development of obliterative airway disease (OAD) in heterotopic murine tracheal allografts is a suitable model for the analysis of the immunopathogenesis of BOS (9–11). Previous studies from our laboratory have shown that MHC-mismatched tracheal allografts transplanted into normal, CD8-deficient, or Ab-deficient mice show normal development of OAD at 30 days after transplantation (12). In contrast, MHC-mismatched tracheal allografts transplanted into CD8-deficient mice show similar histopathological signs of OAD only after 60 days after transplantation (12). These studies strongly indicated that alloreactive CD4+ T cells play a predominant role in the pathogenesis of OAD in MHC-mismatched allografts. Interestingly, a recent study by Richards et al. (13) has also demonstrated that indirect presentation of a transgenic minor histocompatibility Ag (mHAg) OVA leads to the clonal expansion of OVA-reactive CD4+ T cells and development of OAD in murine tracheal allografts.

The role of MHC allorecognition in the process of chronic allograft rejection has received a large body of interest (5–7, 14–18). In contrast, the role of mHAgs recognized by CD8+ T cells in the process of chronic allograft rejection remains unknown (19–21). The mHAgs characterized so far are peptides derived from the products of polymorphic genes (22, 23), peptides derived from donor tissues carrying new gene products absent in the recipient (24), or peptides with a differential biochemical modification such as cysteinylation (25). mHAgs can be presented by MHC class I or II molecules and recognized by CD8+ and CD4+ T cells, respectively (21, 23, 26–28). mHAgs were originally recognized by their
ability to induce the rejection of skin grafts and tumors exchanged between MHC-matched mice (29–31). Recent studies by Chai et al. (32) have demonstrated the importance of mHAgs recognized by both CD4+ and CD8+ T cells in the process of chronic rejection as well as in the development of tolerance in a HY-mismatched (male-to-female) murine skin transplantation model. In addition, the clinical relevance of human mHAgs has been established in the development of graft-vs-host disease in patients who received MHC-matched bone marrow cells (33–35). Moreover, an elegant study by Cai et al. (36) has recently demonstrated the coexistence of effector and regulatory HA-1-reactive CD8+ T cells in patients with long-term kidney allografts mismatched at the HA-1 mHAg.

The goal of this study was to define the role of mHAg-specific CD8+ CTLs in the pathogenesis of OAD. Toward this, we performed heterotopic tracheal allograft transplantation between two congenic mouse strains that differ only at the polymorphic H13 locus on chromosome 2 (23, 30, 37). The H13a allele encodes for a protein that yields the naturally processed nonapeptide SSVGVVWYL (SVL9) as the mHAg bound to the H2Db MHC class I molecule. The H13a SVL9 peptides differs from its H13b homolog SSVGGVWYL (SIL9) by a single methylene (-CH2-) group deletion (23). The H13b-derived SIL9 peptide also binds the H2Db molecule and both SVL9 and SIL9 mHAgs are detected as dominant Ags in reciprocal anti-H13a or anti-H13b immunizations (23). We show herein that MHC-matched H13a tracheal allografts transplanted into H13b recipients develop OAD with delayed kinetics compared with MHC-mismatched allografts. We also show that this process is mediated by SVL9-specific CD8+ CTLs.

Materials and Methods

Mice

C57BL/10SnJ (H13a, H2Db) and B10.CE-H13bAw(30NX)/Sn (H13b, H2Kb) mice were purchased from The Jackson Laboratory. BALB/c (H2Db) mice were purchased from the National Cancer Institute Research Resources (Bethesda, MD). All mice were housed at the Washington University School of Medicine in a pathogen-free environment. All animal procedures were performed according to the guidelines of the animal care committee of Washington University School of Medicine.

Peptides

The SSVGGVWYL (SVL9, H13a) and the SSVGGVWYWL (SIL9, H13b) peptides were synthesized by Research Genetics. The purity of peptides was determined by HPLC and mass spectrometry.

Heterotopic tracheal transplantation

Tracheal grafts were extracted from H13a, H13b (syngeneic control), and BALB/c (allogeneic control) mice and transplanted heterotopically into H13b mice. Briefly, donor mice were anesthetized and sacrificed with i.p. ketamine/xylazine (80/16 mg/kg). Mice were exsanguinated, and the trachea was exposed via an anterior midline incision. The trachea was excised and flushed with 2 ml of ice-cold Euro-Collins solution (Mediatech) with electrolyte additive (Mediatech). The graft was then placed in a small s.c. pocket on the dorsum, between the scapulae of the anesthetized recipient mice (ketamine/xylazine: 80/16 mg/kg). The wound was closed with prolene sutures. Different sets of tracheal grafts were harvested at 30, 60, and 90 days after transplantation. At the time of graft removal, the mice were anesthetized as mentioned above.

Skin transplantation

Skin grafts were extracted from H13a, H13b (syngeneic control), and BALB/c (allogeneic control) mice and transplanted into H13b mice. Briefly, recipient mice were anesthetized as described above. Tail skin grafts were harvested from euthanized donor mice, placed on a petri dish with moisturized gauze, and cut into 0.5-cm diameter pieces. Then, the skin grafts were placed on a graft bed prepared over the chest of the recipient and secured with a bandage. The grafts were examined daily, and the graft status was recorded. The grafts were considered rejected when >90% of necrosis was detected.

Immunization and tolerization protocols

To immunize against the SVL9 mHAg, H13a recipients were injected (s.c.) with 500 µg of the SVL9 peptide or the SIL9 peptide (control) in 200 µl of CFA at day −7 before tracheal transplantation. To tolerize against the SVL9 mHAg, H13b mice were injected (i.v.) with varying concentrations of the SVL9 peptide or the SIL9 peptide (control) in 50 µl of PBS at day −7 before tracheal transplantation. A second dose was given at day 0.

T cell isolation

Spleens were teased and passed through a cell strainer. Then, CD4+ and CD8+ T cells were negatively selected using the corresponding T cell isolation kits (Miltenyi Biotec) following the manufacturer’s instructions. Purified T cells were either >98% CD4+ or CD8+ as determined by FACS analysis (data not shown).

ELISPOT assay

MultiScreen 96-well filtration plates (Millipore) were coated for 2 h at 37°C with either IFN-γ or IL-4-specific mAbs (5.0 µg/ml; BD Biosciences) in 0.05 M carbonate-bicarbonate buffer (pH = 9.6). Next, the plates were blocked with 1% BSA for 1 h at 37°C and washed with PBS (three times). Spleen cells from H13b recipients were treated with erythrocyte lysis buffer (0.1% Metha, 155 mM ammonium chloride, 10 mM potassium bicarbonate) for 3–5 min, washed (three times), and resuspended in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with FBS (10%; HyClone), L-glutamine (2 mM), nonessential amino acids (100 mM), HEPES buffer (25 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 2-ME (50 mM). Then, the cells were plated in triplicate (3 x 10^4/well in the mHAg-coated plates. H13a (allograft donor) and H13b (syngeneic control) spleen cells were irradiated (3000 rad) and plated (6 x 10^4/well) in the wells containing responder cells. After 48 h for IFN-γ or 72 h for IL-4, the plates were washed with PBS (three times) and PBS supplemented with 0.5% Tween 20 (three times). Then, biotinylated IFN-γ or IL-4-specific mAb (2.0 µg/ml; BD Biosciences) diluted in PBS/BSA/Tween 20 was added to the corresponding plates. After 2 h at room temperature, the plates were washed with PBS/Tween 20 (three times), and HRP-labeled streptavidin (1:100; BD Biosciences) diluted in PBS/BSA/Tween 20 was added to the wells. After 2 h at room temperature, the plates were washed with PBS/Tween 20 (three times) and PBS (three times). Then, 3-amino-9-ethylcarbazole substrate reagent (BD Biosciences) was added to the wells for 2–5 min. The plates were washed with tap water to stop the reaction and air-dried. Spots were analyzed in an ImmunoSpot Series I analyzer (Cellular Technology) that was designed to detect ELISA spots with predetermined criteria for spot size, shape, and colorimetric density.

CTL assay

Spleen cells from H13b recipients were separated from erythrocytes by density gradient centrifugation using Lympholyte-M (Cedarlane Laboratories), resuspended in RPMI 1640 medium supplemented as described above, and cultured in the presence of irradiated (2000 rad) H13b spleen cells for 5 days before the functional assay. H13b spleen cells were incubated for 2 days in the presence of Con A (5.0 µg/ml) and labeled with 250 µCi of 51Cr (Na2CrO4; ICN Pharmaceuticals). After 1 h, the 51Cr-labeled H13b spleen cells (5 x 10^4/well) were plated in triplicate cultures in round-bottom 96-well plates in the presence of varying numbers of activated H13b spleen cells and incubated for 18 h at 37°C. Control wells for determining spontaneous 51Cr release contained labeled target cells alone. Maximal release was determined by adding 1% Triton X-100 to the target cells. The percentage specific lysis was calculated as follows: (experimental 51Cr release – spontaneous 51Cr release)/maximum 51Cr release x 100.

Semi quantitative RT-PCR

CD8+ T cells were homogenized with TRIzol reagent (Invitrogen Life Technologies), and total RNA was isolated according to the manufacturer’s instructions. cDNA was synthesized by extension of primers using PowerScript reverse transcriptase (Clontech) according to manufacturer’s instructions. PCR was then performed using TaqDNA polymerase and buffer mix (Invitrogen Life Technologies) in the presence of oligonucleotide primers for IL-10 and TGF-β, and GAPDH (R&D Systems). The PCR cycling conditions were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 10 min. The amplified cDNA products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized using an UV transilluminator. The bands were analyzed using the Quantity One software (Bio-Rad), and the relative mRNA...
levels were expressed as the ratio between the density of the experimental gene band and the density of the GAPDH band.

**Histological analysis**

Cross-sectional tracheal and skin graft specimens were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned at 5-μm thickness, and stained with Mason’s trichrome. The graft specimens were examined in blinded fashion for evidence of rejection. Airways that displayed epithelial abnormalities, cellular infiltration, and connective tissue fibroproliferation with complete occlusion of the airway lumen were considered positive for OAD. An airway was considered normal if there were no epithelial abnormalities, mononuclear cellular infiltration, and lamina propria and/or luminal fibrosis.

**Immunohistochemical analysis**

Allografts were frozen, embedded in TBS Tissue Freezing Medium (Triangle Biomedical Sciences), and sectioned at 5-μm thickness. The sections were treated with 3% H2O2 for 10 min to quench endogenous peroxidase activity and blocked for 30 min with PBS supplemented with 0.5% blocking reagent (TSA Biotin System; PerkinElmer) and 2% normal goat serum (Jackson ImmunoResearch Laboratories). Then, the sections were incubated for 2 h with purified rat anti-mouse CD4, CD8, and CD11b mAbs (5.0 μg/ml; BD Pharmingen). Then, the sections were incubated for 1 h with biotin-conjugated F(ab')2 goat anti-rat IgG (1:500; Jackson ImmunoResearch Laboratories). Then, the sections were incubated for 30 min with HRP-conjugated streptavidin (1:100; TSA Biotin System; PerkinElmer). The presence of graft-infiltrating CD4+ T cells, CD8+ T cells, and CD11b+ macrophages was detected with the DAB Substrate kit (BD Pharmingen), counterstained with hematoxylin, and examined using a light microscope.

**Statistical analysis**

Differences in skin graft survival curves were assessed by means of a two-way log-rank test with the α set at p < 0.05. Differences in gene expression were assessed by means of two-tailed Student’s t test with the α set at p < 0.05.

**Results**

**Development of OAD in H13b tracheal allografts in H13b recipients**

To determine the role of a single mHAg mismatch in the pathogenesis of OAD, H13b and H13b (syngeneic) tracheal allografts were transplanted into H13b mice. As shown in Table I, 25% of the H13b allografts demonstrated mild epithelial damage and cellular infiltration at 30 days after transplantation. None of the H13b allografts (0%) showed fibroproliferative lesions and/or lumen occlusion at 30 days after transplantation (Table I). However, within 60 days after transplantation, all of the H13b tracheal allografts (100%) developed epithelial lesions as well as cellular infiltration, but fibrosis and lumen occlusion was noted only by 90 days after transplantation. None of the H13b tracheal isografts developed any OAD lesions even by 90 days after transplantation (Table I). These results clearly indicate that a single mHAg difference can induce OAD lesions in MHC-matched tracheal allografts. These results are in agreement with transplantation of other tissues such as skin allografts between mHAg-mismatched animals that have also shown delayed kinetics of the rejection process (29–31).

**Accelerated OAD development in H13b tracheal allografts in SVL9-immunized H13b recipients**

To demonstrate that recognition of the SVL9 mHAg by the H13b recipients was inducing the development of OAD in the H13b tracheal allografts, H13b allografts were transplanted into H13b mice previously immunized with the SVL9 peptide. As shown in Table II, previous sensitization with the SVL9 peptide induced an early development within 30 days of the OAD lesions in the H13b allografts. As shown in Fig. 1A, at 30 days after transplantation, H13b allografts transplanted into SVL9-immunized H13b recipients displayed complete epithelial denudation, inflammatory infiltrates, and obliteration of the lumen by fibromyxoid proliferation.

**Inhibition of OAD development in H13b tracheal allografts in SVL9-tolerized H13b recipients**

It has been shown that i.v. administration of soluble Ags induces specific T cell tolerance by induction of anergy and clonal deletion as well as expansion of immunoregulatory T cells (38–40). To determine whether OAD development in H13-mismatched tracheal allografts could be abrogated by induction of tolerance against the SVL9 peptide, we administered (i.v.) different concentrations of the SVL9 peptide into H13b recipients before H13b transplanted into H13b mice.

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**Table I. Development of OAD in H13b tracheal allografts in H13b recipients**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Lumen Donor</th>
<th>Time Post-Tx (days)</th>
<th>OAD Lesions (% of Allografts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H13a</td>
<td>30</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>H13b</td>
<td>60</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>H13a</td>
<td>90</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table II. Accelerated OAD development in H13b tracheal allografts in SVL9-immunized H13b recipients**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Time Post-Tx (days)</th>
<th>Treatment (s.c.)</th>
<th>Epithelial damage</th>
<th>Cellular infiltration</th>
<th>Lumen occlusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>H13a</td>
<td>30</td>
<td>SVL9</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>H13b</td>
<td>60</td>
<td>SVL9</td>
<td>100</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>H13a</td>
<td>90</td>
<td>SVL9</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H13a</td>
<td>30</td>
<td>SIL9</td>
<td>25</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>H13b</td>
<td>60</td>
<td>SIL9</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>H13a</td>
<td>90</td>
<td>SIL9</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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*Tx, Transplantation.
tracheal transplantation. As shown in Table III, complete abrogation of OAD development was observed in H13b allografts transplanted into H13b recipients tolerized with the SVL9 peptide at concentration between 500 and 50 μg/dose. As shown in Fig. 2A, there were no epithelial damage, cellular infiltration, or fibromyxoid proliferation at 90 days after transplantation in H13b allografts transplanted into H13b mice tolerized with the SVL9 peptide. In contrast, i.v. administration of the SIL9 peptide did not prevent the development of OAD in H13b tracheal allografts transplanted into H13b recipients (Table III). As shown in Fig. 2B, at 90 days after transplantation, these allografts displayed epithelial damage, cellular infiltration, and complete lumen occlusion due to fibrosis as observed in untreated H13b recipients (Table I). Tolerization with the SVL9 peptide did not alter the course of OAD development in BALB/c tracheal allografts transplanted into H13b mice (Table III). These results suggested that tolerization with the SVL9 peptide induced an H13b-specific CD8+ CTL unresponsiveness associated with abrogation of OAD development in H13b recipients.

Induction of H13b-specific CD8+ CTL responses in H13b recipients

To determine whether H13b-specific CD8+ CTL activation occurred against the SVL9 mHAg during the process of OAD development induced by the mHAg mismatched at the polymorphic HLA-A2 allele, the frequencies of H13b-reactive CD8+ and CD4+ T cells present in spleens from SVL9-immunized or SVL9-tolerized H13b mice were determined by means of IFN-γ and IL-4 ELISpot assays. Toward this, spleen cells were harvested from recipients on day +7, CD4+ and CD8+ T cells were purified, and activated ex vivo as described above. As shown in Fig. 4A, SVL9-immunized H13b recipients demonstrated a significant increase in the frequency of IFN-γ-producing CD8+ CTLs compared with SIL9-immunized H13b recipients. In contrast, SVL9-tolerized H13b recipients did not show any levels of IFN-γ-producing CD8+ CTLs. In addition, H13b recipients tolerized with the control SIL-9 peptide demonstrated a low but detectable level of IFN-γ-producing CD8+ CTLs. No detectable levels of IL-4-producing CD8+ T cells were detected in either the immunized or the tolerantized recipients (Fig. 4A). In addition, no detectable levels of either Th1 (IFN-γ+) or Th2 (IL-4+) H13b-reactive CD4+ T cells were detected in any of the recipients (Fig. 4B). These results suggest that CD4+ T cells are not affected by the SVL9 treatments and play a secondary role in the pathogenesis of OAD in this model. However, a previous study from our laboratory has shown that indirect recognition of a transgenic HLA-A2 molecule leads to expansion of alloreactive CD4+ T cells, production of anti-HLA-A2 alloantibodies, and the development of OAD (41). Subsequent studies by Richards et al. (13) have also demonstrated that indirect recognition of a transgenic mHAg (OVA) also leads to the activation and expansion of OVA-reactive CD4+ T cells.

Table III. Inhibition of OAD development in H13b tracheal allografts in SVL9-tolerized H13b recipients

<table>
<thead>
<tr>
<th>Donor</th>
<th>Time Post-Tx (days)</th>
<th>n</th>
<th>Treatment (i.v.)</th>
<th>Epithelial damage</th>
<th>Cellular infiltration</th>
<th>Lumen occlusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>H13a</td>
<td>90</td>
<td>8</td>
<td>SVL9: 500 μg</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H13a</td>
<td>90</td>
<td>5</td>
<td>SVL9: 250 μg</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H13b</td>
<td>90</td>
<td>5</td>
<td>SVL9: 12 μg</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H13b</td>
<td>90</td>
<td>5</td>
<td>SVL9: 50 μg</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>H13a</td>
<td>90</td>
<td>4</td>
<td>SVL9: 25 μg</td>
<td>100</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>H13b</td>
<td>90</td>
<td>5</td>
<td>SVL9: 12 μg</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>BALB/c</td>
<td>30</td>
<td>10</td>
<td>SVL9: 500 μg</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Tx, Transplantation.
cells and the development of OAD. Thus, to further determine whether CD4$^+$ T cells play a role in the process of OAD development in H13-mismatched tracheal allografts, we determined the levels of CD4$^+$ and CD8$^+$ T cell infiltration in H13$^a$ tracheal allografts rejected by SVL9-immunized H13$^b$ recipients. As shown in Fig. 5A, a significant infiltration by CD8$^+$ T cells was observed in rejected H13$^a$ tracheal allografts. It is noteworthy that a lower but significant infiltration by CD4$^+$ T cells was also observed in these allografts (Fig. 5B). This was an unexpected result based on the fact that no anti-H13$^a$ CD4$^+$ T cell alloreactivity was observed in H13$^b$ recipients (Fig. 4B). These data suggest that CD4$^+$ T cells may be activated by means of a “bystander” effect in the secondary lymphoid organs and induced to infiltrate the allograft by the in situ inflammatory response primarily induced by SVL9-reactive CD8$^+$ T cells. In this regard, a significant level of macrophages infiltration was observed in the rejected H13$^a$ tracheal allografts (Fig. 5C). Overall, these data suggest that CD4$^+$ T cells may play a role in the immunopathogenesis of H13$^a$ tracheal allograft rejection.

Higher TGF-β production by CD8$^+$ T cells in SVL9-tolerized H13$^b$ recipients

To determine whether SVL9 tolerance was associated with expansion of CD8$^+$ T cells producing regulatory cytokines such as IL-10 and/or TGF-β, we determined the mRNA cytokine profile of CD8$^+$ T cells from SVL9-tolerized H13$^b$ recipients. As shown in Fig. 6, CD8$^+$ T cells from SVL9-tolerant animals showed a significantly higher production of TGF-β than SIL9-tolerant (control) animals ($p = 0.0016$). In contrast, no significant differences were observed in IL-10 production between the two groups of animals. These results suggest that the SVL9 tolerance may be mediated by the expansion of regulatory CD8$^+$ T cells producing TGF-β. These results are in agreement with the results of Chai et al. (32) that showed that IL-10-producing T cells specific for the HY mHAg were not expanded in HY-tolerized animals.

Prolonged H13$^a$ skin allograft survival in tolerized H13$^b$ tracheal allograft recipients

To further determine that SVL9-tolerant H13$^b$ recipients were specifically unresponsive to H13$^a$ cells, H13$^a$ and BALB/c skin grafts were transplanted on H13$^b$ recipients at day +90 after H13$^a$ tracheal allograft transplantation. As shown in Fig. 7A, H13$^a$ skin allografts transplanted into SVL9-tolerized H13$^b$ recipients displayed a significantly longer survival time (84.3 ± 14.1 days) compared with H13$^a$ skin allografts transplanted into SVL9-immunized H13$^b$ recipients (9.7 ± 2.5 days) ($p = 0.026$). In contrast, as shown in Fig. 7B, BALB/c skin allografts were readily rejected with similar kinetics by both SVL9-immunized (9.0 ± 2.6 days) and SVL9-tolerized (8.6 ± 2.5 days) H13$^b$ recipients. H13$^b$ skin isografts survived for >100 days in SVL9-immunized or SVL9-tolerized H13$^b$ recipients (data not shown). Overall, these results clearly demonstrate specific tolerance induction to the SVL9 mHAg in the H13$^b$ recipients.

Discussion

Our laboratory has previously demonstrated a significant correlation between the development of HLA class I and II CD4$^+$ T cells indirect alloreactivity to mismatched donor HLA Ags and development of BOS after lung transplantation (5–7). These results indicate an important role for CD4$^+$ T cells in the pathogenesis of BOS. Because of the continuous supply of recipient-derived APCs into the allograft, it is thought that CD4$^+$ T cells perpetuate the rejection process by the production of inflammatory cytokines that induce macrophage activation and tissue damage/remodeling. In contrast, to this date, no correlation between CD8$^+$ CTL activation and BOS development has been observed (8). In addition, our recent animal studies have indicated that OAD development in MHC-mismatched tracheal allografts is primarily mediated by CD4$^+$ T cells activated through the indirect pathway of Ag recognition (12, 41). However, these studies also showed that alloreactive CD8$^+$ CTLs have the capacity to induce the development of OAD, albeit with delayed kinetics compared with CD4$^+$ T cells (12). Therefore, the possibility exists that, even in the case of MHC compatibility in lung transplantation, CD8$^+$ CTLs recognizing mHAg may have the ability to induce the development of BOS.

A large body of evidence has shown that mHAg play an important role in the development of graft-vs-host disease after MHC-matched allogeneic bone marrow transplantation (20). Also,
It has been shown that mHAg can induce skin allograft rejection between MHC-matched mouse strains (29–31). In related studies, the rejection process of MHC-matched rat cardiac allografts was also slower and displayed the typical histopathology of chronic rejection (42, 43). Studies from our laboratory have demonstrated that H13 mHAg mismatching can induce chronic rejection of cardiac allografts that correlated with the generation of mHAg-specific CD8$^+$ CTLs (44). Therefore, we postulated that the transplantation model reported in this communication between well-defined congenic mouse strains mismatched at a single mHAg locus may provide important new information toward understanding the pathogenesis of chronic rejection, i.e., OAD, in murine tracheal allografts.

In this study, we demonstrate that recognition of a single mHAg mismatch by CD8$^+$ CTLs can result in development of OAD in murine tracheal allografts. As to be expected, the kinetics of this rejection process was delayed. However, the SVL9-specific priming of H13$^b$ recipients before transplantation significantly accelerated the rejection process. In contrast, SVL9-specific tolerization of H13$^b$ recipients before transplantation completely abrogated the development of OAD in these allografts. These data indicate that CD8$^+$ CTL reactivity to the SVL9 mHAg was responsible for the process of OAD development in these tracheal allografts. In this regard, it has been reported that CD8$^+$ T cells mediate the destruction of vascular smooth muscle cells in allografts undergoing chronic rejection (45). In addition, Sun et al. (46) have shown that the morphologic aberrations characteristic of chronic rejection were markedly attenuated in cardiac allografts transplanted into CD8-deficient mice. A clear limitation of this study lies in the fact that the relative contribution of CD8$^+$ T cells in the pathogenesis of chronic lung allograft rejection in the clinical setting remains to be elucidated. Previous studies from our laboratory have clearly shown a correlation between development of CD4$^+$ T cell alloreactivity against mismatched MHC Ags leading to the development of chronic lung allograft rejection (5–7). However, a clear demonstration of activation and expansion of alloreactive CD8$^+$ T cells has not been shown in human lung allograft recipients with chronic lung allograft rejection (8).

Studies both in animal models and humans have shown that the expansion of regulatory T cells with different surface phenotypes can be responsible for the induction of allogeneic tolerance (47–49). Several type 2 cytokines including IL-10 and TGF-β have been shown to have regulatory functions (50, 51). It has been demonstrated that...
IL-10 is a potent inhibitor of T cell activation and has tolerizing effects on these cells (50). Further, recent evidence has indicated that TGF-β is also responsible for the regulatory capabilities of the CD4⁺CD25⁺ T cells (51). Our results suggest that SVL9 tolerization may lead to the activation and expansion of TGF-β-producing regulatory CD8⁺ T cells and are in agreement with the results of Chai et al. (32) that showed that IL-10-producing T cells specific for the tolerizing mHAg (HY) were not expanded in a murine model of skin allograft. However, additional functional studies are necessary to fully determine that this T cell subpopulation is solely responsible for the maintenance of tolerance to a mHAg in this model.

Data presented in this paper demonstrate that a single amino acid substitution resulting in a minor histocompatibility antigenic difference can result in chronic allograft rejection of solid organs such as OAD development following tracheal transplantation. Furthermore, our data demonstrate that sensitization of minor Ag can result in acceleration of the kinetics of development of OAD. Therefore, it raises the possibility of early development of BOS in sensitized individuals who are receiving lung transplantation, even if they are MHC compatible between the donor and recipient. A recent report by Choi et al. (28) has shown a limited reactivity to few immunodominant mHAg in the presence of many mHAg disparities. They demonstrated that reactivity to a single mHAg (H60) was dominant, suggesting that tolerance induction to a selected dominant mHAg may prevent rejection of allografts with multiple mHAg mismatches. Current studies in our laboratory are toward determining whether induction of tolerance to a single mHAg (H13⁺) could result in abrogation of OAD development in male H13⁺ tracheal allograft carrying multiple mHAg (H13⁺ and HY) transplanted into female H13β recipients. There is also a good possibility that induction of tolerance to mHAg may result in the induction of tolerance to MHC Ags by “linked suppression.” We are currently performing experiments to validate these possibilities using the tracheal transplantation model of OAD. It is of considerable interest to note that some of the mHAg presented in the context of HLA-A2, a high-frequency MHC Ag in the population, are well defined. Therefore, one can envision the possibility of using mHAg peptide as a tool for induction of tolerance to human allografts including in bone marrow transplantation wherein graft-vs-host disease remains a major problem due to mHAg incompatibilities.

References


![FIGURE 6](image-url) Higher TGF-β production by CD8⁺ T cells in SVL9-tolerized H13β recipients. CD8⁺ T cells from SVL9-immunized and SVL9-tolerized H13β recipients were harvested at day +7. Then, the IL-10, TGF-β, and GAPDH mRNA expression was measured by RT-PCR, and the bands were analyzed using the Quantity One software (Bio-Rad). The relative mRNA levels are expressed as the ratio between the density of the GAPDH band and the density of the GAPDH template. The results are expressed as the mean ± SD and are representative of three different experiments. IL-10 expression in SVL9-immunized vs SVL9-tolerized H13β recipients: p = 0.20. TGF-β expression in SVL9-immunized vs SVL9-tolerized H13β recipients: p = 0.0016.

![FIGURE 7](image-url) Prolonged H13β skin allograft survival in tolerized H13β tracheal allografts recipients. H13β (A) and BALB/c (B) skin grafts (n = 3) were transplanted into SVL9-immunized (●-) and SVL9-tolerized (●-) H13β recipients at day +90 after tracheal allograft transplantation. The grafts were examined each day, and rejection was defined by complete necrosis and was confirmed histologically by H&E and Masson’s trichrome stains. H13β allograft survival in SVL9-immunized vs SVL9-tolerized H13β recipients: p = 0.025. BALB/c allograft survival in SVL9-immunized vs SVL9-tolerized H13β recipients: p = 0.56.


