Hierarchical Contributions of Allorecognition Pathways in Chronic Lung Rejection

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The role of allorecognition in initiating lung graft rejection is not clearly defined. Using the heterotopic tracheal transplantation model, we examined the contributions of the indirect and direct allorecognition pathways in chronic airway rejection. Fully mismatched, wild-type grafts were transplanted into major histocompatibility complex (MHC) II$^{-/-}$, class II-like accessory molecule (H2-DMα)$^{-/-}$ using MHC I$^{-/-}$ and wild-type allorecipients as control subjects. Similarly, MHC I$^{-/-}$, MHC II$^{-/-}$, or MHC I/II$^{-/-}$ allografts were transplanted into wild-type mice with appropriate control subjects. Grafts from nonimmunosuppressed recipients were evaluated at Weeks 2, 4, and 6. Grafts transplanted into MHC II$^{-/-}$ and H2-DM$\alpha^{-/-}$ allorecipients showed a more intact epithelium and reduced lumen obliteration compared with grafts transplanted into wild-type or MHC I$^{-/-}$ allorecipients ($p < 0.05$ for each). These grafts exhibited abundant CD4+ and CD8+ cell infiltrates similar to control allografts. MHC I$^{-/-}$ and MHC I/II$^{-/-}$ but not MHC II$^{-/-}$ allografts placed in wild-type animals demonstrated less severe rejection compared with allograft control subjects ($p < 0.05$ for each). Although the indirect allorecognition pathway has the strongest influence on rejection, the direct pathway is sufficient to ultimately cause chronic airway rejection. In addition, these results suggest that MHC class I molecules are the principal alloantigens in the mouse heterotopic tracheal model of obliterative bronchiolitis.

Keywords: allorecognition; alloantigen; lung transplant; trachea transplant model; obliterative bronchiolitis

Lung transplantation has become a successful clinical therapy for individuals with diverse end-stage pulmonary diseases. Despite advances in several aspects of pulmonary transplant medicine, chronic lung allograft rejection, manifested as progressive airway obstruction, namely obliterative bronchiolitis (OB), remains the leading cause of morbidity and mortality among long-term survivors of lung transplantation (1). The treatment of OB is ineffective, mainly because of our lack of knowledge of the pathogenesis of this disease. In the mouse heterotopic tracheal transplant model, allografts, but not isografts, develop a defined, predictable succession of airway inflammation, epithelial injury and denudation, and lumen fibroproliferation, resulting in airway obliteration replicating the pathology of human OB (2). Studies have validated the use of the mouse heterotopic tracheal transplant model and yielded important initial observations showing that alloimmune injury that leads to OB (3–8).

The initial and central event that ultimately leads to graft rejection is allorecognition. Allospecific T cells may be activated by two distinct pathways (9–13). The direct pathway is defined by the allorestricted activation of recipient T cells by donor antigen-presenting cells, whereas the indirect pathway refers to activation of recipient T cells through the interaction with preprocessed allopeptides presented by recipient antigen-presenting cells in a self major histocompatibility complex (MHC) II–restricted manner. Although the mechanism for indirect allorecognition is similar to the physiologic mechanism of host defense, the mechanism of direct allorecognition pathway is less well characterized. Overall, the relative contributions of the indirect and direct allorecognition pathways to organ graft rejection remain largely unknown and have not been well studied in the tracheal transplant model.

Genetically engineered mice have been generated and used to examine the biologic function of components of the antigen processing pathway. The MHC class II molecules are heterodimers consisting of α and β chains, which are assembled in the endoplasmic reticulum, associated with the invariant chain and transported into the endosomal compartment where the invariant chain is proteolytically degraded into class II-associated invariant chain peptide (CLIP). The class II-like accessory molecule HLA-DM or its murine equivalent, H2-DM, catalyzes the dissociation of CLIP and assists in the loading of the antigenic peptides, thereby playing an important role in the orderly trafficking of MHC II-peptide complexes to the cell surface. Mice deficient in MHC class II expression have been generated by deleting the A_β gene, resulting in undetectable MHC class II expression (14, 15). Mice deficient in MHC class I expression have also been generated by disruption of the gene encoding the β2-microglobulin, a polypeptide required for proper assembly and cell surface expression of MHC class I molecules (16, 17). In addition, mice deficient in H2-DM have been generated by disruption of the H2-DMα gene, which encodes the α subunit for H2-DM. In the absence of H2-DMα, CLIP cannot be dissociated from and exogenous antigens cannot be complexed with newly synthesized MHC class II molecules (18–21). H2-DMα deficiency on the C57BL/6 background results in an almost complete blockade of alloantigen processing and presentation by MHC class II molecules (21–23).

Previous studies using MHC II-deficient (skin) allografts (i.e., absence of donor MHC II-bearing antigen-presenting cells) to eliminate the direct allorecognition pathway have concluded that indirect allorecognition is sufficient to cause rejection (24, 25). However, recent studies have indicated that allograft antigen-presenting cell cells can directly activate CD8+ T cells through MHC class I molecules and in

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the absence of CD4+ T cell "help" (26–28), resulting in rejection (26, 27). Therefore, the use of MHC II-deficient grafts cannot entirely exclude direct (MHC I-dependent) allorecognition (24, 25). In the experiments described herein, we pursued an alternative and comprehensive approach to evaluate the role and contribution of each allorecognition pathway and donor MHC molecules in lung transplant rejection. First, we transplanted wild-type tracheal allografts into MHC $II^{-/-}$ or H2-DM$\alpha^{-/-}$ mice, which have different disruptions in the class II-dependent antigen presentation process that, in effect, eliminates indirect allorecognition. Second, we transplanted MHC I-, II-, and I/II-deficient allografts into wild-type recipients to assess the role of MHC I and MHC II-mediated direct allorecognition and MHC molecules as alloantigens.

METHODS

Mice and Study Design

The following female mice between 8 and 12 weeks of age were used: wild-type BALB/c ($H2^d$) and C57BL/6 ($H2^b$) (Harlan Labs, Indianapolis, IN), $H2^b$ MHC class I deficient (MHC $I^{-/-}$) (17), $H2^b$ MHC class II deficient (MHC $II^{-/-}$) (14), and $H2^b$ MHC class I and II deficient (MHC $I/II^{-/-}$) (29) (Taconic Labs, Germantown, NY). In addition, H2-DM$\alpha^{-/-}$ mice were backcrossed onto the $H2^b$ background for nine generations and were bred and maintained at the University of North Carolina at Chapel Hill (UNC) (18). First, allografts from wild-type $H2^d$ mice were transplanted into fully mismatched MHC $I^{-/-}$, MHC $II^{-/-}$, H2-DM$\alpha^{-/-}$, and wild-type $H2^b$ mice. For isograft control subjects, tracheae from wild-type $H2^b$ were transplanted into wild-type $H2^b$ mice. Second, tracheal grafts were obtained from MHC $I^{-/-}$, MHC $II^{-/-}$, MHC $I/II^{-/-}$, and wild-type $H2^b$ or $H2^d$ mice and placed into wild-type $H2^d$ mice. All mice were maintained following the National Institutes of Health guidelines for the care and use of laboratory animals under specific pathogen-free conditions.

Mouse Heterotopic Tracheal Transplantation

As previously described (4–6), tracheae were transplanted into a subcutaneous pocket behind the neck of the recipient after anesthesia was achieved by injecting Domitor (5 mg/kg)/Ketamine (100 mg/kg) (Division of Laboratory Animal Medicine, UNC) intraperitoneally and then reversed with Antisedan (2.5 mg/kg) (Division of Laboratory Animal Medicine), introduced subcutaneously. No immunosuppressant was used.

Morphometry

Grafts were harvested at Weeks 2, 4, and 6. Tissue processing, staining, and image acquisition have been previously described (4–6). Detachment of the differentiated (ciliated) epithelium (epithelial injury) and lumen obliteration (fibroproliferation) were examined to determine airway rejection. The intact differentiated epithelium and total airway circumference were measured at the level of the sub-basement membrane. Quantitation of the epithelialization and fibroproliferation was performed using the Metamorph Image analysis program (West Chester, PA) by three independent, blinded reviewers and was expressed

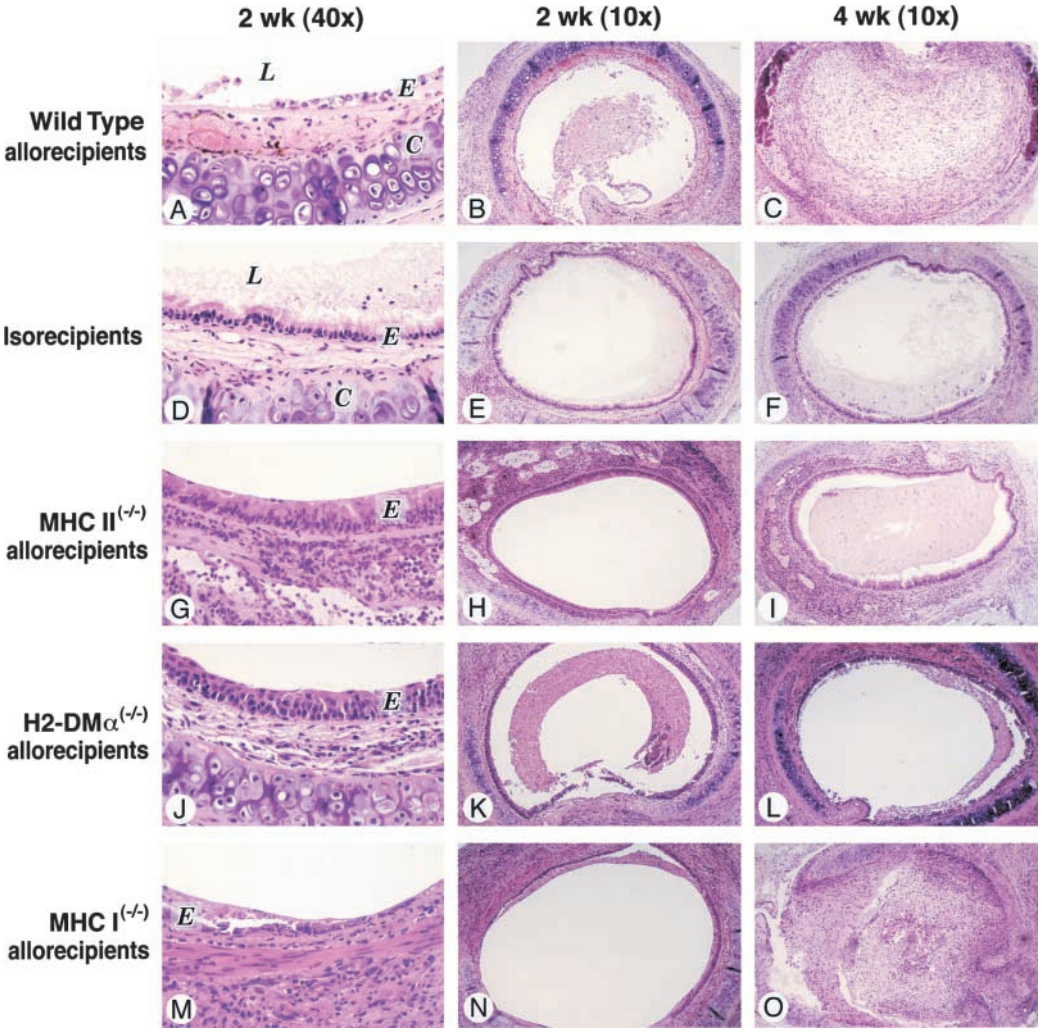

Figure 1. Histopathology of wild-type $H2^d$ tracheal grafts transplanted into wild-type, MHC-, or H2-DMα–deficient $H2^b$ allorecipients at Weeks 2 (10× and 40×) and 4 (10×) (hematoxylin and eosin). Tracheal grafts transplanted into wild-type allorecipients (*A–C*), but not wild-type isorecipients (*D–F*), showed a successive loss of intact, ciliated epithelium, and diminished lumen patency, eventuating in complete airway obliteration by Week 4. Grafts transplanted into MHC $II^{-/-}$ (*G–I*) and H2-DM$\alpha^{-/-}$ (*J–L*), but not MHC $I^{-/-}$ (*M–O*), showed significantly less severe chronic airway rejection. E = epithelium, L = airway lumen, C = tracheal cartilage.

as the fraction of total airway circumference or of the total lumen cross-sectional area, respectively (4–6).

Immunohistochemistry

Frozen sections were air dried, fixed in chilled acetone, and blocked with 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) and avidin-biotin block (Vector Labs, Burlingame, CA). The sections were incubated with monoclonal rat anti-mouse CD4+ or CD8+ antibodies, then biotinylated goat anti-rat antibody, and finally streptavidin-horseradish-peroxidase (Pharmingen, San Diego, CA). Optimal dilutions and incubation periods were determined empirically. Duplicate sections on the same slide were incubated with rat IgG2a (Jackson ImmunoResearch) as a negative isotype control. The diaminobenzidine substrate (Sigma, St. Louis, MO) was used, and sections were counterstained with Light Green (Fisher Scientific Co., Pittsburgh, PA). Cells were counted within the area outlined by an equidistant line, 10 μm outward from the sub-basement membrane. In accordance with the previously described profile of immune cellular infiltration into allografts in this model (4), grafts were examined for CD4+ and CD8+ T cells at Week 2.

Statistical Analysis

Analyses were performed using SigmaStat software (SPSS Inc., Chicago, IL). Parameters were compared at each time point using the student's *t*-test. A two-tailed α level of $p < 0.05$ was considered significant. Repeated-measures analyses of variance were used (8) to confirm the results from the *t*-test analyses.

RESULTS

Allogeneic Transplantation into Fully Mismatched, Wild-type $H2^b$ or $H2^d$ Mice

The wild-type $H2^d$ and $H2^b$ tracheal isografts showed a complete, intact, fully differentiated epithelium and patent lumen at 2, 4, and 6 weeks (n = 4, at each time point for both arms; Figures 1 and 2). Therefore, the initial ischemic insult by itself had no impact on the subsequent repair of the epithelium or the OB in this transplant model.

At 2, 4 and 6 weeks, the allografts (wild-type $H2^d$ grafts transplanted into fully mismatched, wild-type $H2^b$ recipients, n = 3 to 4, at each time point) had less intact, fully differentiated (ciliated) epithelium compared with the isografts (wild-type $H2^b$ grafts transplanted into wild-type isorecipients, n = 4, at each time point) ($p = 0.03$, $p < 0.001$, and $p < 0.001$, respectively; Figures 1 and 2A). After epithelial denudation, the lumen of the allografts displayed markedly decreased patency compared with the isografts (n = 3, $p = 0.002$ at 4 weeks, and n = 4, $p < 0.001$ at 6 weeks; Figures 1 and 2B).

Similar results were seen in wild-type $H2^b$ allografts transplanted into wild-type $H2^d$ recipients as compared with $H2^d$ allografts transplanted into wild-type $H2^b$ recipients (n = 3 to 5, each arm and time point; $p \leq 0.005$ for ciliated epithelium at 2, 4, and 6 weeks and $p < 0.005$ for lumen patency at 4 or 6 weeks; Figures 5, 6A, and 6B), which reproduced the results from previous studies (3–8). Therefore, we demonstrated a uniform profile of airway rejection across two strains of mouse recipients.

Testing the Indirect Pathway of Allorecognition

Allogeneic transplantation into fully mismatched, MHC II-deficient mice. At 2 weeks, wild-type grafts transplanted into fully mismatched MHC $II^{-/-}$ allorecipients (n = 4) had more intact, ciliated epithelium than those transplanted into wild-type allorecipients ($p = 0.01$). There was no lumen obliteration observed in any of the grafts (Figures 1, 2A, and 2B). At 4 weeks, wild-type grafts transplanted into fully mismatched MHC $II^{-/-}$ allorecipients (n = 4) had significantly more intact ciliated epithelium ($p = 0.04$) and more airway lumen patency ($p = 0.005$) than wild-type grafts transplanted into wild-type allorecipients. At 6 weeks, wild-type grafts transplanted into fully mismatched MHC $II^{-/-}$ allorecipients (n = 3) underwent epithelial denudation and lumen obliteration similar to wild-type grafts transplanted into wild-type allorecipients ($p \geq 0.2$ each; Figures 1, 2A, and 2B).

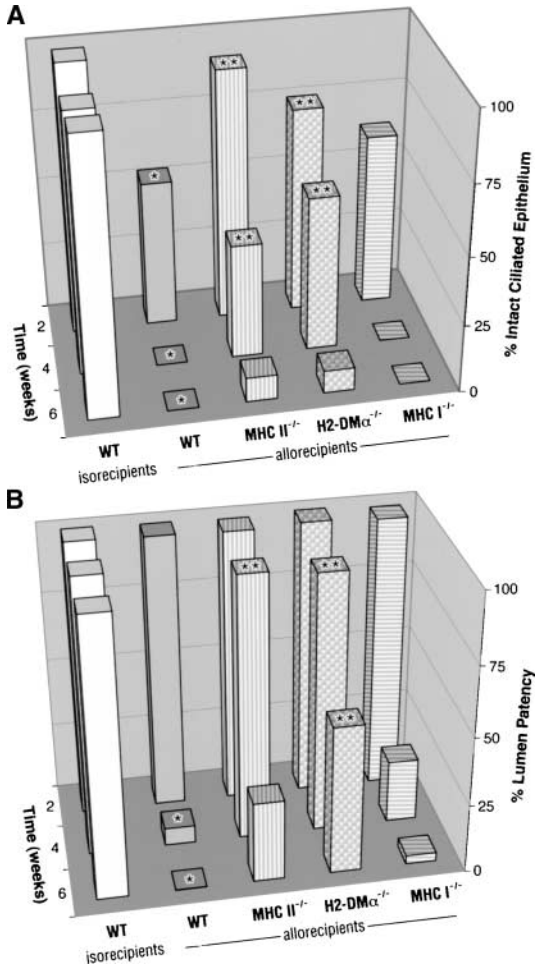

Figure 2. Airway rejection in wild-type (WT) grafts transplanted into wild-type, MHC-, or H2-DMα–deficient recipients is shown as (*A*) the mean percentage of intact ciliated airway epithelium and (*B*) the mean percentage of airway lumen patency. *Comparisons ($p < 0.05$) between the control allografts and the control isografts at the same time point. **Comparisons ($p < 0.05$) between the experimental arms and the control allografts placed in wild-type recipients at the same time point. Grafts transplanted into MHC II- and H2-DMα–deficient allorecipients, but not grafts transplanted into MHC I–deficient allorecipients, showed significantly less epithelial denudation and airway obliteration from 2 to 6 weeks when compared with grafts transplanted into wild-type recipients.

Allogeneic transplantation into fully mismatched, H2-DMα–deficient mice. At 2 weeks, wild-type grafts transplanted into H2-DM$\alpha^{-/-}$ allorecipients (n = 6) had more intact ciliated epithelium than those transplanted into wild-type allorecipients ($p = 0.007$). There was no lumen obliteration (Figures 1, 2A, and 2B). At 4 weeks, wild-type grafts transplanted H2-DM$\alpha^{-/-}$ allorecipients (n = 6) showed significantly more intact, ciliated epithelium ($p = 0.005$) and more airway lumen patency ($p = 0.001$) than grafts transplanted into wild-type allorecipients. At 6 weeks, wild-type grafts transplanted into H2-DM$\alpha^{-/-}$ allorecipients (n = 4) showed epithelial loss similar to wild-type transplanted into wild-type allorecipients ($p \geq 0.2$; Figure 2A); however, they showed significantly less lumen obliteration ($p = 0.05$; Figures 1, 2A, and 2B).

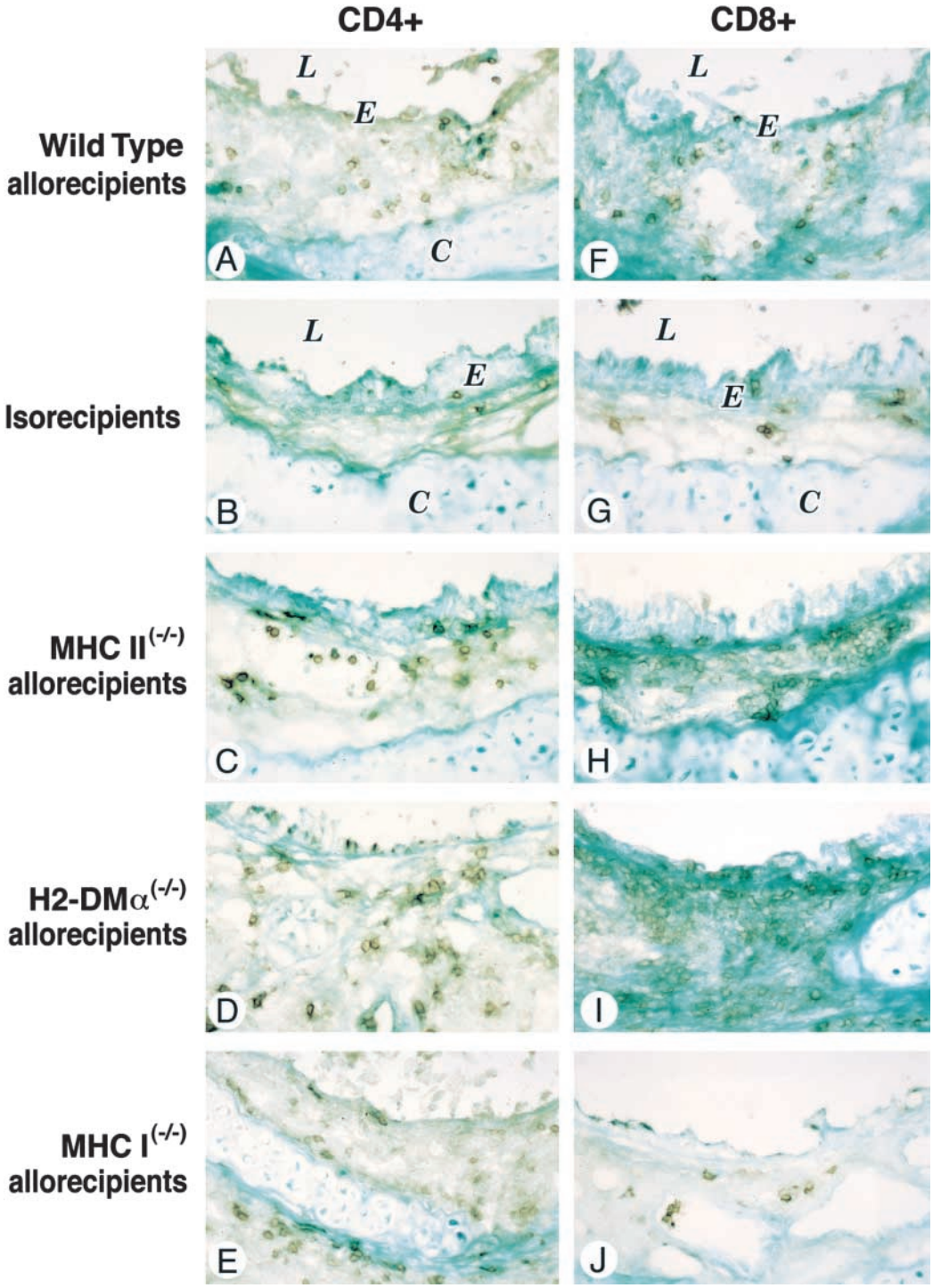

Figure 3. Immunohistochemical staining for CD4+ (*A–E*) and CD8+ (*F–J*) cells in wild-type tracheal grafts transplanted into MHC- or H2-DMα–deficient allorecipients at 2 weeks. Magnification ×40. E = epithelium, L = airway lumen, C = tracheal cartilage.

Allogeneic transplantation into fully mismatched, MHC I-deficient mice. At 2, 4, and 6 weeks (n = 4 for each), wild-type grafts transplanted into MHC $I^{-/-}$ allorecipients showed a similar amount of intact, ciliated epithelium and lumen obliteration as wild-type grafts transplanted into wild-type allorecipients ($p > 0.1$ for ciliated epithelium and lumen obliteration at each time point; Figures 1, 2A, and 2B).

Graft-infiltrating lymphocytes in wild-type grafts transplanted into fully mismatched, MHC- and H2-DMα–deficient mice. As expected, the wild-type grafts transplanted into wild-type allogeneic recipients (n = 6) had more CD4+ and CD8+ lymphocytes as compared with the wild-type grafts transplanted into isorecipients (n = 3) (CD4+: 109 ± 56.3 versus 23 ± 12.1, $p = 0.01$; CD8+: 230.7 ± 25.7 versus 16 ± 6.1, $p < 0.001$; Figures 3 and 4A). Trends toward higher mean CD4+ cell counts were found in wild-type grafts transplanted into MHC $I^{-/-}$ (n = 5), H2-DMα$^{-/-}$ (n = 4), and MHC $II^{-/-}$ allorecipients (n = 6) in comparison to wild-type grafts transplanted into wild-type allorecipients ($p = 0.07$, $p = 0.09$, and $p = 0.13$, respectively; Figures 3 and 4A). A similar mean CD8+ cell count was observed in wild-type grafts transplanted into MHC $II^{-/-}$ and H2-DMα$^{-/-}$ allorecipients when compared with wild-type grafts transplanted

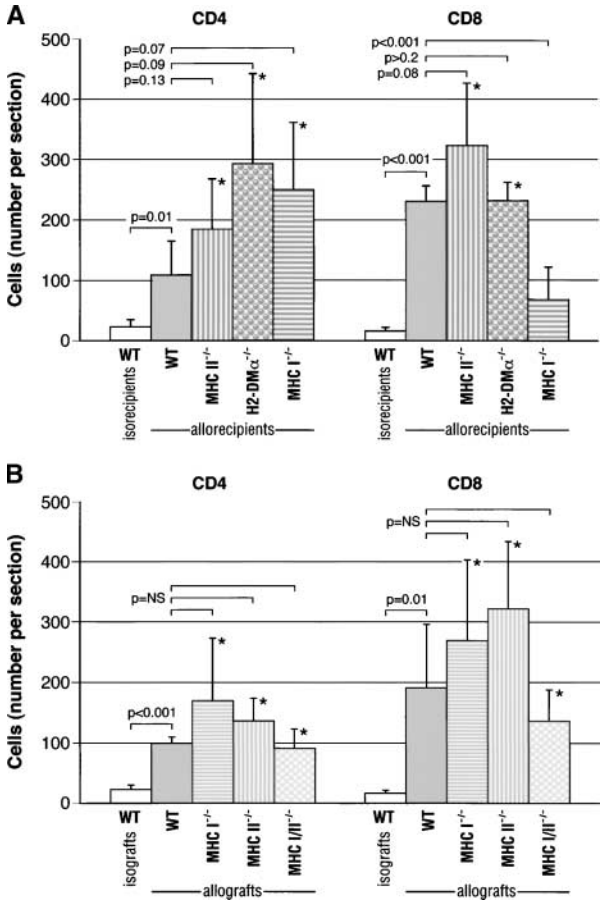

Figure 4. The mean number (± SD) of graft-infiltrating CD4+ and CD8+ lymphocytes: (*A*) wild-type grafts transplanted into MHC- or H2-DMα–deficient allorecipients. H2-DMα$^{-/-}$ and MHC II$^{-/-}$ mice showed similar numbers of allograft-infiltrating CD4+ and CD8+ lymphocytes in comparison to the control allografts ($p > 0.05$, each). A lower mean CD8+ cell count was observed in wild-type grafts transplanted into MHC I$^{-/-}$ mice ($p < 0.01$). (*B*) MHC-deficient allografts transplanted into wild-type allorecipients. MHC I$^{-/-}$, II$^{-/-}$, and I/II$^{-/-}$ allografts showed trends toward higher numbers of CD4+ and CD8+ infiltrating cells when compared with the control allografts ($p > 0.05$, each). Bracketed p values show comparisons between the experimental arms or the control isografts with the control allografts. *Comparisons ($p < 0.05$) between the experimental arms and the control isografts. WT = wild type.

into wild-type allorecipients ($p = 0.08$ and $p > 0.2$, respectively). A lower mean CD8+ cell count was observed in wild-type grafts transplanted into MHC I$^{-/-}$ mice ($p < 0.001$; Figures 3 and 4A).

Testing the Direct Pathway of Allorecognition and the Role of Donor MHC Molecules

Transplantation of MHC II$^{-/-}$ tracheal allografts into fully mismatched, wild-type mice. At each time point (n = 5 for each), the MHC II$^{-/-}$ allografts had a similar amount of ciliated epithelium and lumen obliteration as the wild-type allografts ($p > 0.2$ for ciliated epithelium or lumen obliteration at each time point; Figures 5, 6A, and 6B).

Transplantation of MHC I$^{-/-}$ tracheal allografts into fully mismatched, wild-type mice. At 2 weeks, MHC I$^{-/-}$ allografts (n = 5) had more ciliated epithelium than wild-type allografts ($p = 0.05$) and no lumen obliteration. At 4 weeks, these grafts (n = 7) showed more intact ciliated epithelium and less lumen obliteration compared with wild-type allografts ($p = 0.05$ and $p = 0.004$, respectively). By 6 weeks, MHC I$^{-/-}$ allografts (n = 5) developed lumen obliteration similar to wild-type allografts (n = 4, $p > 0.2$ for each; Figures 5, 6A, and 6B).

Transplantation of MHC I/II$^{-/-}$ tracheal allografts into fully mismatched, wild-type mice. At 2 weeks, MHC I/II$^{-/-}$ allografts (n = 4) had more intact ciliated epithelium than wild-type allografts ($p = 0.05$) and did not develop lumen obliteration. At Week 4, MHC I/II$^{-/-}$ allografts (n = 6) had more intact ciliated epithelium and less lumen obliteration compared with wild-type allografts ($p = 0.04$ and $p = 0.006$, respectively). At Week 6, although MHC I/II$^{-/-}$ allografts (n = 6) lost their intact ciliated epithelium resembling wild-type allografts ($p > 0.2$), they showed significantly less airway lumen obliteration compared with wild-type ($p = 0.04$), MHC I$^{-/-}$ ($p = 0.04$), or MHC II$^{-/-}$ ($p = 0.05$) allografts (Figures 5, 6A, and 6B).

Graft-infiltrating lymphocytes in MHC-deficient allografts. Wild-type allografts transplanted into wild-type H2^{d} mice (n = 5) showed an abundance of CD4+ and CD8+ cells compared with isografts (99 ± 10.4 versus 23 ± 7.5, $p < 0.001$, and 191 ± 104.5 versus 16 ± 5.1, $p = 0.01$, respectively; Figure 4B). MHC I$^{-/-}$, II$^{-/-}$, and I/II$^{-/-}$ allografts placed in wild-type mice had a trend toward higher CD4+ ($p = 0.14$, $p = 0.07$, and $p > 0.2$, respectively) and CD8+ ($p = 0.17$, $p = 0.06$, and $p = 0.17$, respectively) cells when compared with wild-type allografts (Figure 4B).

DISCUSSION

In this study, we report on the hierarchical importance of the indirect alloantigen recognition pathway over the direct pathway in the mouse model of chronic airway rejection. First, in the presence of the direct allorecognition pathway, we showed that the disruption of the indirect allorecognition pathway using MHC II$^{-/-}$ or H2-DMα$^{-/-}$ recipient mice, which lack cell surface MHC II molecules or have disrupted loading of peptide antigens, respectively, led to less chronic airway rejection. Because equal or greater numbers of allograft-infiltrating CD4+ and CD8+ T lymphocytes were found in wild-type allografts transplanted into H2-DMα$^{-/-}$, and to our surprise, MHC II$^{-/-}$ recipients (compared with wild-type control allografts), the attenuation of rejection was not due to the inability of the recipient's T lymphocytes to access these grafts. Second, in the presence of the indirect pathway, MHC II$^{-/-}$ allografts (and passenger leukocytes), which lack the exogenous (MHC II-mediated) direct allorecognition pathway, developed chronic rejection in the same manner as control allografts. Third, because MHC I- and MHC I/II-deficient allografts placed into wild-type animals showed diminished rejection, MHC I peptides are important alloantigens in this model of chronic lung rejection. Endogenous (MHC I-mediated) direct allorecognition was not directly tested in this model but, if present, is a less important pathway based on the finding that wild-type grafts are rejected more slowly in recipients who have impaired indirect allorecognition (but intact MHC I direct allorecognition). Finally, the fact that MHC I/II$^{-/-}$ grafts were eventually rejected suggested that non-MHC antigens (e.g., minor histocompatibility antigens) were sufficient to cause chronic rejection through the indirect pathway in this model.

The absence of indirect allorecognition pathway did not lead to indefinite survival of fully MHC-mismatched airway allografts as grafts placed into MHC II$^{-/-}$ and H2-DMα$^{-/-}$ mice but displayed a 2-week delay in epithelial denudation and lumen obliteration (Figure 2). This observation maybe explained by the fact that the direct allorecognition pathway, which triggers a less robust alloimmune response, was sufficient to result in complete airway allograft rejection at a slower pace. In addition, the equal

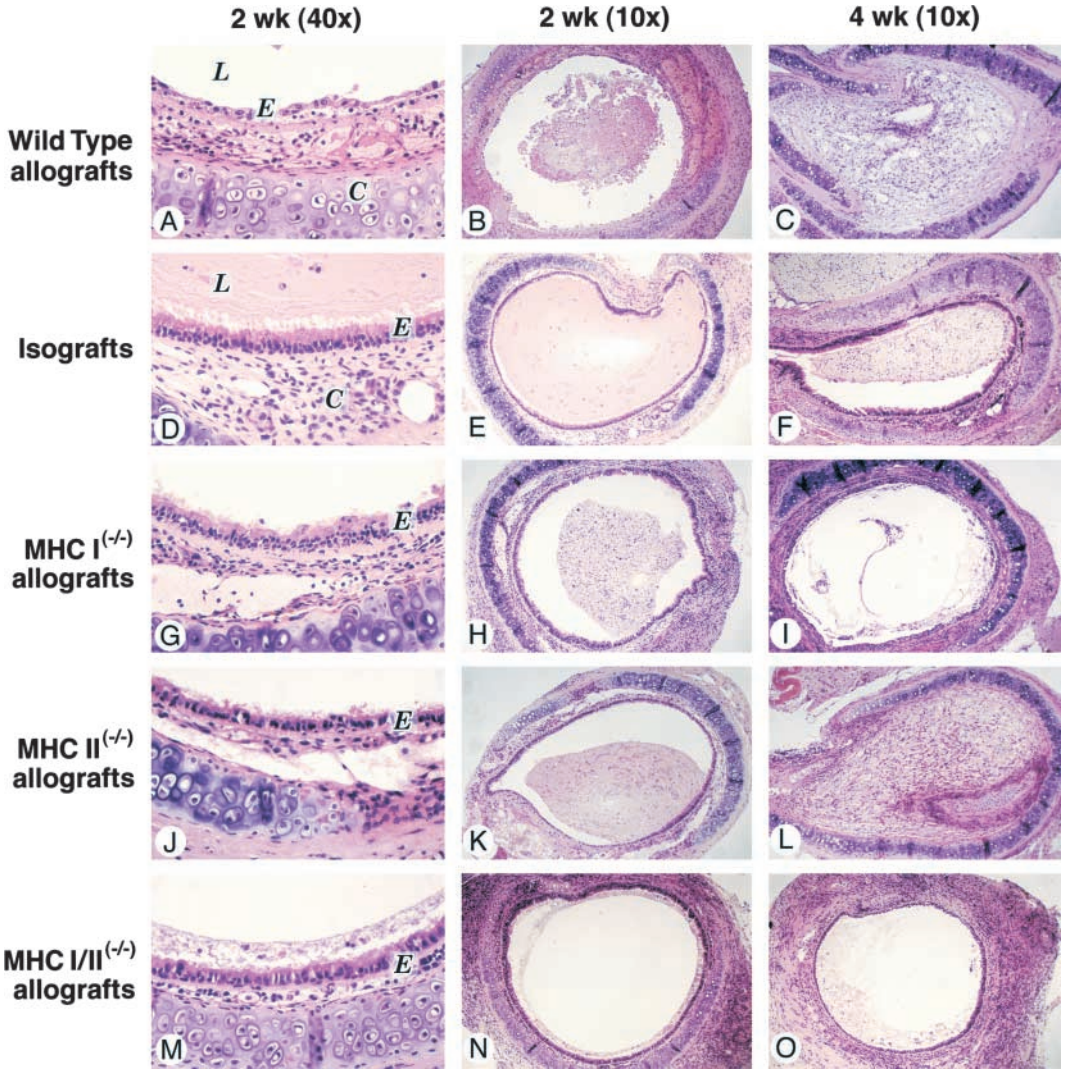

Figure 5. Histopathology of wild-type, MHC-deficient $H2^b$ tracheal allografts transplanted into wild-type $H2^d$ recipients at Weeks 2 (×10 and ×40) and 4 (×10) (hematoxylin and eosin). An identical profile of airway rejection was observed in wild-type $H2^b$ allografts transplanted into wild-type $H2^d$ recipients (*A–C*) in comparison with that in wild-type $H2^d$ allografts transplanted into wild-type $H2^b$ recipients (Figures 1A–1C). MHC I (*G–I*) and I/II$^{-/-}$ (*M–O*) but not MHC II$^{-/-}$ (*J–L*) allografts showed significantly less severe chronic airway rejection. E = epithelium, L = airway lumen, C = tracheal cartilage.

or greater numbers of infiltrating lymphocytes in these grafts compared with wild-type allografts may signify weaker donor specificities, a characteristic of direct allorecognition (30). Alternatively, cross-presentation of antigens (occurring with MHC II$^{-/-}$ and H2-DMα$^{-/-}$ antigen-presenting cells to recipient CD8+ T cells via MHC I molecules) may be responsible for this late rejection. However, this form of indirect allorecognition is almost certainly inconsequential in our experiments, as fully MHC-mismatched grafts were used. Therefore, recipient CD8+ T cells sensitized by allopeptides in the context of recipient MHC I molecules will not find such determinants on the donor grafts.

MHC II$^{-/-}$ mice, due to deficient thymic selection of CD4+ T cells during embryonic development, have limited T-cell repertoires and very low numbers of resting and stimulated CD4+ lymphocytes (14, 15). In these mice, deficient T-cell populations, rather than abnormal antigen processing, might have attenuated the rejection process that we and others (14, 15) have observed. In contrast, H2-DMα$^{-/-}$ mice have slightly reduced numbers of CD4+ lymphocytes in the lymph nodes and spleens, but express a very diverse T cell receptor repertoire, quite similar to that of wild-type animals (18–21, 31, 32). In addition, H2-DMα$^{-/-}$ CD4+ lymphocytes display an allogeneic proliferative response (18–21, 31, 33) and cytokine production (33) not different from wild-type mice. Therefore, the result of H2-DMα$^{-/-}$ mice rejecting allografts in a pattern similar to MHC II$^{-/-}$ mice confirms the importance of the indirect allorecognition and antigenic peptide loading processes in mediating airway allograft rejection. Our results are also consistent with human studies, which have reported indirect evidence for the importance of the indirect allorecognition in OB (34–37) and with animal models (38–40) and human studies (41, 42) showing the importance of the indirect pathway in other organ transplants. In addition, our findings help explain the previous contradictory results showing that either the direct (43) or indirect (44) pathway may cause chronic airway rejection in the murine tracheal transplant model by demonstrating that each pathway contributes in a different way.

Previous studies, using MHC-deficient grafts, have demonstrated the importance of MHC I molecules in pancreatic transplantation (45, 46), MHC II molecules in heart transplantation (47), and both MHC I and II molecules in skin transplantation (24, 29). Although the MHC molecules expressed on the graft may function as alloantigens, they may also participate in the direct allorecognition pathway as antigen-presenting molecules. Therefore, efforts to understand the importance of MHC alloantigens in previous studies using MHC$^{-/-}$ grafts only (and not using MHC$^{-/-}$ hosts) may have been confounded by the variable importance of direct allorecognition among different organ transplants and vice versa (24, 25). By using MHC-deficient mice as

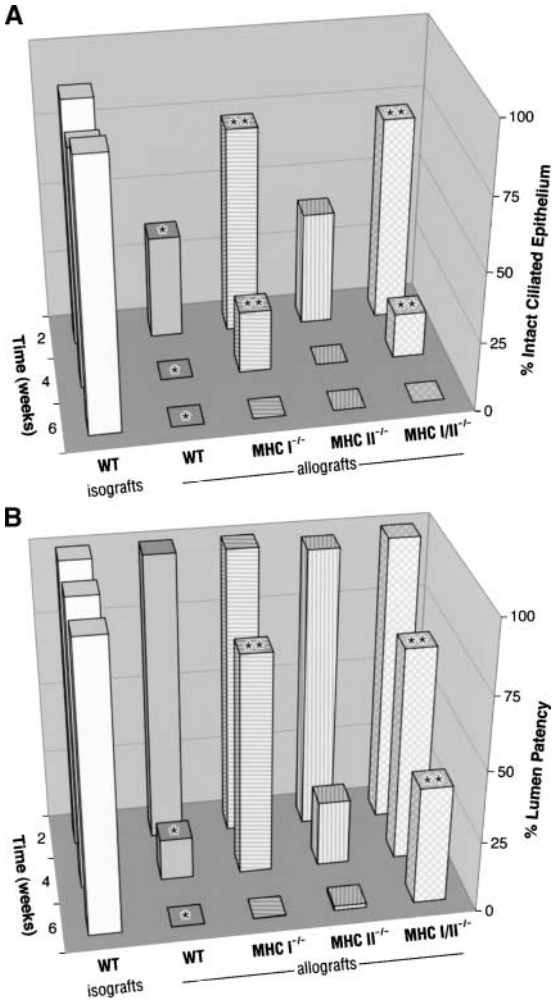

Figure 6. Airway rejection in wild-type (WT), MHC-deficient tracheal allografts transplanted into wild-type allorecipients, shown as (*A*) the mean percentage of intact ciliated airway epithelium and (*B*) the mean percentage of airway lumen patency. *Comparisons ($p < 0.05$) between the control allografts and the control isografts at the same time point. **Comparisons ($p < 0.05$) between the experimental arms and the control allografts at the same time point. MHC I- and I/II-deficient allografts, but not MHC II-deficient allografts, showed significantly less epithelial denudation and airway obliteration from 2 to 6 weeks when compared with wild-type allografts.

both donors and recipients in separate experiments, our study design is unique in its ability to assess the role of MHC molecules both as antigen-presenting molecules and as alloantigens. This has allowed us to demonstrate unquestionably that MHC I peptides are important alloantigens in this model of chronic lung rejection. This finding is consistent with clinical studies in lung transplant recipients that mismatches at HLA class I increase the risk and severity of chronic lung rejection (48–51). Taken together, these observations suggest that matching donor and recipient HLA may reduce chronic lung rejection clinically.

Some limitations are worthy of note in this study. First, although the heterotopic mouse airway model has been successfully employed to study the pathogenesis of OB (2–8, 43, 44), it does not perfectly replicate human OB due to the heterotopic position of the graft and the rapid course of rejection. Second, in MHC $I^{-/-}$ mice, low levels of MHC I heavy chains may reach the cell surface and may play a role in rejection in both the MHC $I^{-/-}$ and MHC $I/II^{-/-}$ tracheal grafts (17, 52, 53). Likewise, although H2-DM$\alpha^{-/-}$ mice express predominantly surface self CLIP-bound (and not allopeptide-bound) MHC II molecules, not all cell surface MHC II molecules are bound to CLIP (32, 54). However, the significance of non–CLIP-bound MHC II in graft rejection is not clear (18). In addition, the mechanism of H2-DM–dependent peptide loading is allele specific. The exchange of CLIP for allopeptides in H2-A^b mice that we employed is highly dependent on H2-DM expression in comparison to any other mouse strains (e.g., H2-A^k, H2-A^d) (21–23). Third, because the pretransplant and post-transplant graft-specific microenvironment may account for the different contributions of allorecognition pathways among organ transplants and models, the result in this lung transplant model may not be applicable to others and requires further investigation. Finally, the effect of immunosuppressants used in clinical transplant, which may have different influences on direct and indirect alloreacitivity, was not tested here. We are making plans to include immunosuppressants in future experiments.

In conclusion, this comprehensive study of alloantigen recognition in the mouse heterotopic tracheal transplant model demonstrated a hierarchical order of allorecognition pathways for initiating chronic airway rejection. Although the indirect allorecognition pathway is more important, the significance of both allorecognition pathways should be taken into consideration when developing strategies to protect recipients from allograft rejection. This is the first study that takes advantage of H2-DM$\alpha^{-/-}$ mice to demonstrate the importance of the allopeptide loading process in indirect recognition in lung transplant rejection. In addition, we demonstrated that MHC class I molecules are the principle alloantigens triggering rejection in the mouse model of OB, whereas MHC II and minor antigens are likely to play a less important role. Further studies in other models, as well as in the clinical arena, are essential to confirm these findings. Studies to assess the donor-directed specificities of airway graft-infiltrating lymphocytes are also necessary and are under current investigation.

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