Role of autoimmunity in organ allograft rejection: a focus on immunity to type V collagen in the pathogenesis of lung transplant rejection

Tina L. Sumpter and David S. Wilkes
Departments of Medicine, Microbiology and Immunology, Indiana University School of Medicine, Richard L. Roudebush Veterans Affairs Medicine Center, Indianapolis, Indiana 46202

Sumpter, Tina L., and David S. Wilkes. Role of autoimmunity in organ allograft rejection: a focus on immunity to type V collagen in the pathogenesis of lung transplant rejection. Am J Physiol Lung Cell Mol Physiol 286: L1129–L1139, 2004; 10.1152/ajplung.00330.2003.—Lung transplantation is the only definitive treatment modality for many forms of end-stage lung disease. However, the lung is rejected more often than any other type of solid organ allograft due to chronic rejection known as bronchiolitis obliterans (BO). Indeed, BO is the primary reason why the 5- and 7-yr survival rates are worse for the lung than for any other transplanted organ. Alloimmunity to donor antigens is established as the primary mechanism that mediates rejection responses. However, newer immunosuppressive regimens designed to abrogate alloimmune activation have not improved survival. Therefore, these data suggest that other antigens, unrelated to donor transplantation antigens, are involved in rejection. Utilizing human and rodent studies of lung transplantation, our laboratory has documented that a native collagen, type V collagen [col(V)], is a target of the rejection response. Col(V) is highly conserved; therefore, these data indicate that transplant rejection involves both alloimmune and autoimmune responses. The role of col(V) in lung transplant rejection is described in this review article. In addition, the potential role of regulatory T cells that are crucial to modulating autoimmunity and alloimmunity is also discussed.

bronchiolitis obliterans; regulatory T cell; major histocompatibility complex

LUNG TRANSPLANTATION is the only definitive treatment for many forms of end-stage lung disease such as emphysema, idiopathic pulmonary fibrosis, and cystic fibrosis. The first lung transplants were performed nearly 40 years ago, and currently, over 1,400 lung transplants are performed annually (44). Survival of the transplant recipients is limited by the development of chronic rejection known as bronchiolitis obliterans (BO), the leading cause of death in lung allograft recipients (44). Indeed, BO is the primary reason why the 5- and 7-yr survival rates of lung allograft recipients are <50 and 35%, respectively, post-transplantation. Survival statistics following lung transplant are the worst of all recipients of solid organ allografts (Fig. 1). The poor survival statistics take on a new importance when considered in the context of advancements of surgical techniques, immunosuppression, and other supportive measures developed for the care of these patients over the last 20 years. The current sophistication in treatment regimens has not translated into improved survival of lung transplant recipients.

Repeated acute rejection episodes have been suggested to be the main risk factor for the development of BO (52). BO also arises as a severe pulmonary complication postbone marrow transplantation. BO has also been described following inhalation injury and after viral infections and may be idiopathic, occurring after nonspecific pulmonary injury (56, 92, 103).

Although there are other examples (10), a common theme emerges, suggesting that BO following lung transplant may actually result from a local response to tissue remodeling that occurs postlung injury and not an immune response to alloantigens. Patients at risk for BO have higher local levels of soluble mediators of repair, such as IL-6, IL-8, monocyte chemoattractant protein-1, and other factors, implicating their role in BO pathogenesis (85). The link between acute rejection and BO may be the sustained injury and tissue remodeling that occurs during the rejection response. Halloran et al. (34) suggested that the “injury response” is the cause of chronic rejection of solid organs. In accord with this, a cycle of injury is initiated by either ischemia of the grafted organ or the alloimmune response and marked by release of inflammatory cytokines and other mediators promoting repair of the lung. Immunological repair and tissue remodeling marked by immune recognition of foreign and possible sequestered antigens could result in further injury.

Building on the concept that remodeling may release novel antigens into the lung, one could hypothesize that ischemia/reperfusion injury could release self-antigens that could induce the onset of BO. Models of retransplantation in which the graft is transplanted back into the original host following the onset of rejection illustrate this point (50, 97). If recognition of allogeneic major histocompatibility complexes (MHCs) mediated the rejection response, then chronic rejection may cease or diminish after the graft is retransplanted into a syngeneic host. For example, Izutani et al. (50) studied the vasculopathy of chronic cardiac rejection in mice. In this report, chronic rejec-
tion did not abate once cardiac allografts were retransplanted into syngeneic hosts. This suggests that chronic allograft rejection could proceed in the absence of allogeneic donor antigens, which could indicate that chronic rejection may involve mechanisms other than alloimmunity. A non-MHC antigen, found in both donor and recipient, exposed during the original rejection response could have a role in the pathogenesis of chronic rejection. In support of this, myosin and heat shock proteins, which are nonpolymorphic among individuals, mediate rejection in experimental models of cardiac and skin graft rejection, respectively, adding an autoimmune component to the rejection response (8, 15, 16).

TYPE V COLLAGEN AND IMMUNITY DURING LUNG ALLOGRAFT REJECTION

Our laboratory has determined that immunity during lung allograft rejection involves an immune response to another self-antigen, type V collagen [col(V)] (37, 65, 114, 115). All collagen molecules are triple helices composed of α-chains (101). Col(V) is a 116-kDa heterodimer composed of α1- and α2-chains (57, 101). In the lung, col(V) is considered a minor collagen located within the perivascular and peribronchiolar connective tissues, which are sites of rejection activity (57, 63, 64). Data showing that col(V) is a target of the immune response during lung allograft rejection (37, 65, 114, 115) and that recognition of polymorphisms in donor MHC antigens stimulate rejection activity suggested that col(V) may have partial sequence homology to MHC proteins. Interestingly, the immune response to col(V) in lung transplantation appears to be directed against α1-chain of col(V) [α1(V)] and α2(V). α1(V) is nearly 80% homologous to the α2-chain of type XI collagen [α2(XI)] (14), and the gene for α2(XI) maps within the MHC class II loci in humans and mice (36). Although these data suggest col(V) peptides may have sequence homology to MHC antigens, analysis of amino acid sequences did not reveal any primary homology between col(V) and MHC molecules. However, primary sequence homology to alloantigens alone may not be required to induce alloimmunity. For example, Luz et al. (60) recently reported a single amino acid substitution in a peptide bound to MHC molecules altered the affinity of the MHC-peptide complex to the T cell receptors (TCRs). This single amino acid substitution may determine the difference between autoreactivity or alloreactivity. Alternatively, prior infections or other host factors could trigger or perpetuate immune responses to both autoantigens and alloantigens (1).

These data suggest that secondary or tertiary characteristics of the peptide, the peptide-TCR affinity, or other host factors may explain the phenomenon of col(V)-induced immunity during lung allograft rejection.

IMMUNE RESPONSE TO COL(V) CONTRIBUTE TO THE REJECTION RESPONSE

The first evidence showing that col(V) was involved in local immune responses to lung alloantigens was obtained from our murine model of acute rejection. In this model, repeated intrapulmonary instillations of allogeneic lung macrophages and dendritic cells reproduce the immunology and pathology analogous to acute rejection in recipient lungs (108). After four weekly instillations of allogeneic lung cells, recipient mice develop lymphocytic perivascular and peribronchiolar infiltrates analogous to grade 1–2 acute rejection and IgG2a antibody deposits in perivasculard and peribronchiolar tissues (108). Our ongoing studies in human lung allograft recipients undergoing rejection show similar antibody deposits in the transplanted lung. The antigen recognized by these antibodies is col(V) (D. S. Wilkes and W. Burlingham, manuscript in preparation).

During ontogeny of the immune system, negative selection in the thymus deletes autoreactive T cells, i.e., cells that express TCRs with high affinity for self-antigens (88). However, under normal conditions T cells with low affinity for self-antigens circulate in the periphery or reside in various organs (88). It is unlikely that autoreactive T cells will become activated unless there are perturbations involving immune homeostasis or exposure of sequestered self-antigens. During lung allograft rejection, rare self-antigens may be exposed resulting in the development of autoreactive T cells.

Col(V) is located beneath the basement membrane within bronchiolar and vascular tissues in the lung and possibly intercalated with type 1 collagen, the major collagen in the lung (63, 64). The inflammatory responses and architectural remodeling that occurs in these tissues during the rejection response may expose graft-infiltrating lymphocytes to fragments of col(V). Indeed, we reported that lung allograft rejection is associated with the release of col(V) fragments in bronchoalveolar lavage fluid (BAL) (37). Matrix metalloproteinases (MMPs) may degrade collagen molecules (121). MMP-2 and MMP-9 are capable of degrading col(V), and Trello et al. (96) reported activity of MMP-2 and MMP-9 in lungs of human transplant recipients during rejection. Figure 2 shows that MMP-2 and MMP-9 are active in rat lung allografts during acute rejection, thus supporting the role of MMPs in the release of col(V) fragments during the rejection response. These data support the theory that inflammation and remodeling occurring during the rejection response may lead to the release of potentially antigenic col(V) peptides.

However, the aforementioned data are indirect evidence that immune responses to col(V) are involved in the pathogenesis of lung allograft rejection. As T cells mediate rejection, col(V)-specific cellular immune activity during the rejection response was examined. T cells isolated from the lungs of mice that received instillations of allogeneic antigen-presenting cells (APCs) proliferated in response to col(V), but not col(II), a collagen found in cartilage but not the lung (37, 114, 115). Similarly, rats develop strong delayed type hypersensitivity
amounts of T helper (Th) type 1 cytokines, interferon (IFN)-
proliferate strongly in response to col(V), produce copious
rats undergoing acute and chronic rejection (37). These T cells
Moreover, col(V)-speci
not other collagens during lung allograft rejection (114, 115).

detected in normal lungs (data not shown).
92-kDa (MMP-9) and 72-kDa (MMP-2) bands in 3 individual lung allografts.
into Wistar-Kyoto (WKY) recipients. Zymography shows the presence of
MMP-9 activity in Fischer 344 (F344) lung allografts 2 wk posttransplantation
Fig. 2. Zymographic detection of matrix metalloproteinase (MMP)-2 and
MMP-9 activity in Fischer 344 (F344) lung allografts 2 wk posttransplantation
and tumor necrosis factor (TNF)-
cytic expression of speci
ogy in isograft lungs (37). The disparity between the ability of
these cells to induce disease in normal lungs compared with
isograft lungs (37). The disparity between the ability of
these cells to induce disease in normal lungs compared with
isograft lungs is likely due to ischemia-reperfusion injury that
occurs during transplantation. Our studies confirm that harvest and
transplant of isograft lungs, a process that involves ischemia-reperfusion, are associated with disruption of the perivas-
cular and peribronchioriolar tissues. We hypothesized that this
type of injury exposes col(V) to immune cells infiltrating the
graft. This hypothesis is supported by data showing the release of
col(V) fragments in BAL from isografts comparable to that
observed in allograft lungs (37).

Alloimmune responses may occur directly (via direct al-
lore cognition) or indirectly (via indirect allorecognition) (39,
42, 84, 93). The direct pathway involves presentation of
allogenic MHC class I and II antigens expressed on donor
APCs, such as dendritic cells, in the transplant lung to
recipient T cells. The indirect pathway involves processing and
presentation of donor MHC antigens by recipient dendritic
cells to recipient T cells. The direct pathway is believed to be
the primary mechanism of allorecognition in the early trans-
plant period, a time when the transplanted lung is rich in donor
APCs. Conversely, indirect allorecognition is believed to be
the major pathway for alloimmunity later in the posttrans-
plant period, coincident with the replacement of the majority of
donor APCs by those of the recipient. The indirect pathway is
classically described as a pathway for the presentation of
alloantigens. The indirect pathway also presents autoantigens
involved in rejection. Furthermore, although the direct pathway
may prime alloreactive T cells, epitope spreading that occurs
during alloimmune responses can lead to indirect recognition of
self-antigens during rejection (7, 99, 100). For example,
direct allorecognition is the mechanism by which allogeneic
APCs induce rejection-like responses when instilled into lungs of
normal mice (41). In contrast, col(V)-pulsed autologous
APCs do not induce immunological or histological alterations
when instilled into the lungs of normal mice (65). However,
intrapulmonary instillation of col(V)-pulsed autologous APCs
into alloantigen-primed lungs perpetuates the immunology and
pathology of the rejection response (65). The contribution of
indirect allorecognition to col(V) reactivity during lung trans-
plantation is exemplified by the rejection response that
results from transplanting lungs into recipients mismatched at
MHC class I but matched at MHC class II loci. For example,
transplantation of Fischer 344 (F344) rat lungs (RT11v1) into
Wistar-Kyoto (WKY) rats (RT11), a strain combination matched
at MHC class II but mismatched at MHC class I, results in
CD4+ col(V)-specific T cells (37). Because the MHC
mismatch occurs at the class I locus, and class I presents
antigens to CD8+ T cells, then the development of CD4+
col(V)-specific T cells in this model must occur via indirect
allorecognition. Similar findings were reported by Valujskikh
and colleagues (99) examining mechanisms of allorecognition
in skin graft rejection and Fedoseyeva et al. (15) investigating
cardiac allograft rejection. Collectively, these data show that
the direct pathway may initiate the rejection response and that
the indirect pathway has a key role in autoimmunity triggered
by alloimmune responses.

USE OF COL(V) TO INDUCE IMMUNE TOLERANCE
TO LUNG ALLOGRAFTS

In contrast to its role in the pathogenesis of rejection,
indirect allorecognition may be utilized to induce immune
tolerance to organ allografts (48). Nonpharmacologically
induced immune tolerance to solid organ allografts may result
from different techniques. These include injecting donor-de-
derived MHC peptides into the thymus of the recipient before
transplantation of the allograft or feeding donor-derived MHC
antigens to the host before transplantation (oral tolerance). In
either setting, donor antigens are thought to be presented
indirectly by immature dendritic cells to recipient T cells.
Depending on the dose of antigen used, these techniques
induce anergy in alloreactive T cells, eliminate alloreactive
T cells by clonal deletion, or induce activity of regulatory
T cells that actively suppress alloimmune responses (21, 105,
106, 118).

Data from our studies showing col(V) is an antigen during
lung allograft rejection and that col(V)-reactive T cells perpet-
uate the rejection response suggested that col(V) could be
utilized as a tolerogen to prevent lung allograft rejection. To
examine this possibility, we utilized a model of col(V)-induced
oral tolerance to determine its affect on acute and chronic lung
allograft rejection. WKY (RT11v1) rats were fed several doses
of col(V) before transplantation of lung allografts from F344
rats (RT11). In the absence of any immunosuppression, feeding

Fig. 2. Zymographic detection of matrix metalloproteinase (MMP)-2 and
MMP-9 activity in Fischer 344 (F344) lung allografts 2 wk posttransplantation
into Wistar-Kyoto (WKY) recipients. Zymography shows the presence of
92-kDa (MMP-9) and 72-kDa (MMP-2) bands in 3 individual lung allografts.
Each lane represents zymographic activity in tissue supernatants from an
individual lung allograft undergoing rejection. No zymographic activity was
detected in normal lungs (data not shown).
col(V) prevented the onset of acute lung allograft rejection (Fig. 3) and, most importantly, abrogated the development of BO (Fig. 4). The ability of col(V)-induced tolerance to prevent rejection was not haplotype specific, in that feeding col(V) was effective in suppressing acute rejection in another unrelated rat strain combination undergoing lung transplantation (114). Importantly, col(V)-induced tolerance did not induce global immune hyporesponsiveness, as cellular immune responses to nominal antigens were not suppressed in col(V)-tolerant recipients made tolerant to col(V) (114).

APC-induced immune activation of T cells is dependent on bidirectional signaling between APCs and T cells. Oral tolerance could affect both T cell and APC function. Therefore, defective antigen presentation could have contributed to the inability of T cells from tolerant rats to respond to alloantigens. However, data showing that APCs isolated from tolerant allograft recipients were comparable to APCs from normal rats in stimulating proliferation in donor-derived T cells indicate that col(V)-induced oral tolerance affected the function of T cells and not the function of APCs (114).

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Fig. 3. Top: gross anatomy of control isograft lungs (A), control allograft lungs (B), and collagen type V [col(V)]-fed allograft lungs (C) 2 wk posttransplantation (posterior view). The left (L) lung is the transplanted lung, and the right (R) is the native lung in each panel. The control allograft lung (L in B) was dark brown in color and shrunk compared with the native lung. However, the col(V)-fed allograft lung (L in C) had the appearance of the isograft lung (L in A). Control isograft lungs (A) show no pathological lesions and are identical to normal WKY lungs (data not shown). Photographs representative of 5 rats in each group. Bottom: histology of control isografts (D), control allografts (E), and col(V)-fed allografts (F) 2 wk posttransplantation. Control isografts show normal airway and vascular structures (D). Control allografts show extensive perivascular, peribronchial, and alveolar mononuclear cell infiltrates consistent with severe rejection, grade A4 (E). In contrast, allografts from rats fed col(V) before transplantation show only mild to moderate perivascular and peribronchial mononuclear cell infiltrates, grade A1–A2 (F). Photomicrographs representative of 5 rats in each group (×100 magnification). [Adapted from Yasufuku et al. (114).]

Fig. 4. Top: gross anatomy of control allograft lungs (A) and col(V)-fed allograft lungs (B) 10 wk posttransplantation (posterior view). The left lung is the transplanted lung, and the right is the native lung in each panel. The control allograft lung (L in A) was dark brown, shrunk, and firm. However, the col(V)-fed allograft lung (L in B) had a nearly normal appearance. Photographs representative of 5 rats in each group. Bottom: histology of control allografts (C) and col(V)-fed allografts (D) 10 wk posttransplantation. Control allografts show extensive interstitial mononuclear cell infiltrates, fibrosis, and obliteration of small airways by granulation tissue (BO). In contrast, allografts from rats fed col(V) before transplantation show only mild alveolar infiltrates consistent with mild acute rejection (grade A2) (D). Photomicrographs representative of 5 rats in each group (×100 magnification). [Adapted from Yasufuku et al. (115).]
Presentation of alloantigens is critical for the development of col(V)-induced oral tolerance. This is exemplified by data showing that tolerance could be adaptively transferred by T cells isolated from lung allograft recipients made tolerant by feeding col(V) but not by T cells isolated from rats fed col(V) that did not receive lung allografts. Furthermore, the overlap of autoreactivity with allostereactivity was also shown by experiments in which adoptive transfer of col(V)-specific T cells abrogated col(V)-induced immune tolerance to lung allografts (37). Examination of the immune mechanisms that mediated suppression of allostereactivity revealed that feeding col(V) followed by lung transplantation resulted in elevated systemic activity of transforming growth factor (TGF)-β that suppressed alloimmune responses during acute and chronic rejection (114). Neutralizing TGF-β recovered activity of allosreactive T cells (114). Therefore, allosreactive T cells were not lost through clonal deletion or through activation-induced cell death; allosreactive T cells were actively suppressed. These data suggested that regulatory T cells (Tregs) that produce TGF-β may have a key role in col(V)-induced oral tolerance. Indeed, data showing that tolerance to lung allografts may be adaptively transferred to naive rats (67) confirm a role for Tregs in col(V)-induced tolerance.

TREGS IN TOLERANCE: AUTOIMMUNITY AND ALLOIMMUNITY

Immune tolerance inhibits injurious responses against endogenous and exogenous antigens (106). Controlling tolerance induction offers a powerful treatment modality for transplantation and autoimmunity. Tregs downregulate harmful immune responses and induce tolerance. Tregs block aberrant T cell responses against both foreign and self-antigens in the periphery, preventing proliferation and activation of both autoreactive and allosreactive T cells. The immune response during lung allograft rejection appears to be biphasic, initiating with an alloimmune response and resulting in an autoimmune response. Modulating Tregs offers a means for controlling both of these deleterious phases hypothesized to occur in the lung and cause BO. To exploit the potential of Tregs, their natural function in the lung needs to be better defined and the mechanisms whereby Tregs can be induced also need to be identified. The purpose of this section is to provide a brief overview of the phenotype and function of Tregs and their relationship to transplantation tolerance.

CLASSIFICATION OF THE REGULATORS

Tregs are commonly described as belonging to three subsets: Th3 cells, T regulatory type 1 (Tr1) cells, or thymically derived Tregs. These subsets differ in ontogeny and in mechanisms of action. Recently, Bluestone and Abbas (9) classified Tregs as two types: natural Tregs and adaptive Tregs. Natural Tregs are antigen nonspecific thymically derived Tregs that suppress T cell responses in a contact-driven, antigen-independent manner. Adaptive Tregs differentiate either from thymically derived Tregs or from CD4+ helper T cells following antigenic stimulation in the periphery. Adaptive Tregs suppress T cell responses in an antigen-specific, cytokine-driven manner. All Tregs work to maintain systemic immune homeostasis.

Tregs arise in the thymus (5) and first emerge at day 10 of neonatal development (76). Jagged-1, a Notch ligand naturally found on thymic epithelial cells, is involved in the differentiation of Tregs (116). Naturally occurring Tregs with diverse phenotypes have been isolated from the thymus, lymph nodes, spleens, and peripheral blood of rodents and humans (4). Naturally occurring Tregs with suppressive properties include γδ TCR+ cells (40), CD8+CD4− cells (17), CD4+DX5+ cells (22), and CD4−CD8−αβ TCR+ cells (19, 119). The most extensively studied Tregs constitutively express CD4 and CD25, the IL-2 receptor α-chain, in humans and in mice (82, 90, 110) and CD4 and CD45RC in rats (117). Unlike Th3 and Tr1 cells, which are clonally derived, natural Tregs are heterogeneous, evidenced by the diversity expressed in their TCR Vβ-repertoires (58). Tregs express high levels of CD5, CD54, CD38 (78), and Fas (90) and express low levels of CD62L and CD45RB (77). Tregs constitutively express the costimulatory molecules CD28, 4-1BB, glucocorticoid-induced TNF receptor (GITR), CD103, and OX-40 (62). Although Tregs express surface markers found on activated T cells, such as CD25 in mice and humans, it is important to note that naturally occurring Tregs are a unique lineage of thymically derived cells, independent from T cells activated in the periphery (49).

CD4+CD25+ Tregs suppress a plethora of autoimmune diseases. In mice, thymectomized on the third day of neonatal development causes a deficiency of CD4+CD25+ Tregs, resulting in systemic autoimmunity (5). Depleting wild-type adult mice of CD25+ cells breaks systemic tolerance, resulting in multiple autoimmune diseases (5). Multiple autoimmune diseases develop following adoptive transfer of lymph node or spleen-derived cells depleted of CD25+ cells into nude mice. Thyroiditis, gastritis, insulinitis, sialoadenitis, adrenitis, oophoritis, glomerulonephritis, polyarthritis, as well as graft-vs.-host disease have all been described in this model (82). In these model systems, naturally occurring Tregs maintain homeostasis by inhibiting autoreactive T cells.

In the respiratory and oral mucosa, maintenance of homeostasis is crucial. The mucus-lined tracts of the respiratory tract and the gut are continually assaulted with environmental antigens. Aberrant immune responses in these regions would be systemically detrimental. Tregs maintain homeostasis in these environments, with Tr1 cells predominating in the draining lymph nodes of the lung and Th3 cells predominating in the gut (3). IL-10, secreted by pulmonary dendritic cells induces differentiation of antigen-specific T cells with regulatory capabilities that differentiate from CD4+ cells. These cells, deemed Tr1 cells, secrete IL-10 and IL-4 but not TGF-β (3). Systemic administration of IL-10 increases populations of Tregs in vivo (25). In vitro, Tr1 cells generated by stimulation with exogenous IL-10 or by engagement of CD3 and complement regulatory protein in the presence of IL-2 have been described (30, 53). IL-10, produced by Tr1 cells, renders CD4+ cells anergic and unable to respond to alloantigen in mixed lymphocyte reactions (29).

Th3 cells generated in a similar manner in the gut maintain oral tolerance. After feeding with low dose of oral antigen, dendritic cells in mucosal gut-associated lymphatic tissue secrete TGF-β. TGF-β induces antigen-specific CD4+ T cells to differentiate into Th3 cells (3). Ex vivo stimulation with TGF-β generates Th3 cells from CD4+ T cells. Th3 cells, in turn, secrete large quantities of TGF-β (106). TGF-β, like IL-10 produced by Tr1 cells, is a potent immunosuppressant.
capable of blocking T cell proliferation. IL-4, also secreted by Th3 cells, acts on the Th3 cells in a positive feedback loop to increase production of TGF-β and maintain populations of Th3 cells (47). Although operating through different cytokines, both Tr1 cells and Th3 cells express markers of activated cells. CD25 has been identified on both populations (53, 120). Both populations express low levels of CD45RB (as reviewed in Ref. 29). Oligoclonality of their TCR Vβ-region indicates antigen specificity in Tr1 and Th3 cells (13, 58). Tr1 and Th3 cells arise in the periphery in response to antigen when stimulated with the appropriate cytokines.

Tr1 and Th3 cells have also been exploited in autoimmune models. After stimulation with autoantigen, Tr1 cells secreting IL-10 were isolated in a murine model of diabetes. Adoptive transfer of these Tr1 cells inhibited diabetes in secondary recipients (12). Chen et al. (13) induced Th3 cells to protect against development of autoimmune encephalomyelitis by oral administration of myelin basic protein. In our laboratory, Th3 cells from rats tolerized orally to col(V) block BO in allograft recipients (Fig. 4).

The mechanism utilized by Tregs in vivo to suppress cell-mediated immunity has been investigated extensively, but many questions remain. In vitro, Tregs in the presence of APCs block CD3-induced proliferation of CD4+ Th cells (94), CD8+ cytolytic T cells (CTLs) (76), and natural killer T cells (6) in an antigen-independent manner (95). In such a coculture system, Tregs suppress transcription of IL-2 mRNA in the Ths and CTLs, resulting in cell cycle arrest (76, 94). The interaction occurring among this cellular triad of APCs, Tregs, and responder T cells is controversial. Th3 and Tr1 cells secrete soluble mediators of immunosuppression. However, in many reports, Tregs require contact with the APC and the responder T cells to exert their suppressive function. This contact-dependent suppression may be due to membrane-bound TGF-β (4, 69) identified by expression of latency-associated peptide, which cleaves inactive TGF-β into active TGF-β (69, 73). An alternative mechanism of immune suppression may be related to elevated expression of costimulatory molecules, such as CTLA-4, on Tregs, which may compete with the responder T cells for a limited number of ligating molecules, such as B7.1 (CD80) and B7.2 (CD86) on the surface of APCs. If this were true, increasing the numbers of APCs would decrease the suppressor function of Tregs. In fact, in a coculture system with Tregs, Ths, and APCs, suppression can be enhanced by increasing the number of APCs (91). This phenomenon suggests that bidirectional signaling between APC and Tregs may augment the suppressive function of Tregs. In fact, a recent report from Grohmann et al. (28) demonstrated that CTLA-4 Ig can induce expression of indoleamine 2,3-dioxygenase (IDO) in dendritic cells. IDO is an enzyme that depletes tryptophan, resulting in suppressed T cell function. CTLA-4 is upregulated on the surface of Tregs, suggesting that Tregs may suppress other cells indirectly through interactions with APCs. Collectively, these reports suggest that there are many potential mechanisms of Treg-induced immune suppression.

REGULATING THE REGULATORS

Tregs constitutively express CD25, the IL-2 receptor α-chain, suggesting that IL-2-mediated signaling may be important for Treg maintenance. In good agreement, IL-2, IL-2Rα, and IL-2Rβ knockout mice develop lethal autoimmune disorders, suggesting that IL-2 is required for the maintenance of tolerance (20, 75, 79, 89, 109). More importantly, Tregs from IL-2 knockout mice regain regulatory function in the presence of IL-2, whereas T cells from IL-2R knockout mice do not have regulatory function, suggesting that Tregs are regulated by IL-2 signaling (20). In support of this, in vitro priming of Tregs with IL-2 in the presence of TCR ligation enhances their suppressive function (94).

In vitro, Tregs do not proliferate in response to TCR ligation (91). After TCR ligation, Tregs do not synthesize IL-2, which may contribute to their anergic state. The addition of exogenous IL-2 or ligation of CD28 breaks anergy. This effect is reversible; when IL-2 or anti-CD28 are removed from the cell culture environment, Tregs return to their anergic state. Foxp3, constitutively expressed in Tregs, may maintain anergy. Foxp3 encodes scurfin, the forkhead-winged helix transcription factor that has recently come to light as a key regulator for Treg function (18, 43). Foxp3-deficient patients develop severe lymphoproliferative and autoimmune disorders (107). Overexpression of scurfin in CD4 cells inhibits proliferation and IL-2 production following ligation of the TCR (54, 86). Overexpressing Foxp3 in CD4+CD25+ T cells upregulates expression of CD25, CTLA-4, GITR, and CD103 and confers suppressive properties in these cells. Foxp3 appears to be crucial for maintaining the Treg phenotype and suppressive properties. Induction of Foxp3 may be an important event required for generation of Tregs in the periphery (18, 43).

TGF-β, a potent immunosuppressive cytokine, may also be important for the generation of Tregs in the periphery. TGF-β inhibits the function of Th1 by downregulating the IL-12 receptor and inhibits the function of Th2 cells by downregulating the expression of GATA-3 (24). Reports showing that impaired TGF-β signaling results in autoimmunity in the lung and gut indicate that this cytokine has a key role in maintaining peripheral tolerance (23, 68, 71). For example, mice expressing a dominant-negative TGF-β receptor 1 in T cells develop autoimmune disease, localized to the lung and the colon (23). Our studies have shown that TGF-β is upregulated systemically in tolerant lung allograft recipients (115). Moreover, permissiveness to TGF-β-mediated signaling may be a key feature of Tregs irrespective of the Treg phenotype. For example, we recently reported that Tregs mediating col(V)-induced tolerance to lung allografts do not express Smad7, a molecule that blocks TGF-β-mediated signaling (67). Furthermore, col(V)-induced oral tolerance to lung allografts results in Tregs that proliferate in response to TGF-β, consistent with the absence of Smad7 transcripts in these cells (67).

The importance of permissiveness to TGF-β-mediated signaling in the function of Tregs has also been shown by another group of studies in our laboratory. We reported that lung allograft rejection is associated with the development of col(V)-reactive CD4+ T cells that induce rejection “like” pathology in isograft lungs after adoptive transfer (37). These same cells, termed lung T cell-1 (LT1), also abrogated col(V)-induced oral tolerance to lung allografts (37). Interestingly, another col(V)-reactive T cell line, termed LT3, did not induce disease in isograft lungs after adoptive transfer (37). Although LT3 did not have an effect on col(V)-induced tolerance to lung allografts (37), adoptive transfer of LT3 to naive rats abrogated rejection of lung allografts, indicating that LT3 cells had
regulatory function (Fig. 5). Although these cells were phenotypically identical, examination of Smad7 profiles revealed that LT1 expressed Smad7, whereas LT3 did not express Smad7 (Fig. 6). These data are similar to our prior report of col(V)-induced tolerance to lung grafts showing that absence of Smad7 identifies the Tregs with true regulatory function (67). Interestingly, Nakao et al. (71) showed that overexpression of Smad7 in T cells is associated with a normal phenotype in mice but enhanced antigen-induced airway inflammation. Collectively, these data suggest Smad7, which determines permissiveness to TGF-β-mediated signaling, may have key roles in regulating many types of T cell responses in the lung. Smad7 expression is induced by IL-7 (45), IFN-γ (98), and TGF-β (70). The molecular mechanisms suppressing the expression of Smad7 transcripts are unknown.

Little is known about mechanisms for maintaining and expanding Tregs in the periphery. Human peripheral blood T cells with regulatory function have been expanded ex vivo in response to allogeneic stimulation in the presence of TGF-β, and these cells have regulatory activity when adoptively transferred in vivo (113, 120). Several pharmacological interventions enhance the function or quantity of Tregs. For example, intratracheal administration of Flt-3 ligand, a potent mobilizer of dendritic cells, also results in an increase in Tregs in the BAL (74). Similarly, tolerant dendritic cells generated pharmacologically with 15-deoxyspergualin or 1, 25-dihydroxyvitamin D3 and mycophenolate mofetil expand and induce Tregs in models of cardiac transplant and diabetes, respectively (2, 66). A secondary function of Tregs may be to induce tolerogenic dendritic cells, initiating a positive feedback loop for the maintenance of homeostasis (66).

**TREGS IN TRANSPLANTATION TOLERANCE**

Before the acceptance of Tregs as a true entity, Hall et al. (32) described the “CD4+ suppressor cell” in an MHC mismatch model of cardiac transplantation. In these studies, suppressor cells isolated from the spleen or the lymph nodes of cyclosporine-treated rats surviving >75 days following cardiac transplant blocked cardiac rejection in a second recipient for >100 days. The CD4+ suppressor cells isolated in these studies were CD25+ and CD45RC+ (33), matching the phenotype of what was later identified as a Treg. Following this pioneering work, Tregs with diverse phenotypes have been identified in a number of experimental transplant model models, including models of skin, cardiac, islet, and lung transplants (61, 67, 104). Tregs derived from these model systems regulate alloreactive T cells through IL-10 and CTLA-4 (64), IL-4 (11), or a cytokine-independent manner (27).

As mentioned previously, transplant tolerance can be induced by a number of protocols. These treatments include using antibodies to deplete CD4 cells or immunosuppressive agents such as cyclosporine A (CsA), followed by a regimen tolerizing the recipient to donor antigen, such as infusion with donor blood or oral administration of donor MHC (72). Other methods include blockade of costimulatory molecules (CD40, CD154) (102). These protocols result in the generation of Tregs. Many of these methods interfere with the normal function of T cells themselves and may alter Treg efficiency. For example, Kinglsey et al. (55) have demonstrated that a secondary exposure to alloantigen increases percentages of Tregs originally generated following exposure to alloantigen through immunization with donor strain blood in conjunction with an anti-CD4 antibody.

Because of the reliance on artificial regimens to generate Tregs in transplant, questions arise as to the relationship of these cells with naturally arising Tregs. Like natural Tregs, those generated using these regimens rely on the thymus. For example, removal of the thymus diminishes but does not abrogate Treg-induced tolerance following a tolerization protocol of oral donor splenocytes administration and depleting anti-CD4 antibody (72). To determine the efficacy of natural, thymically derived T cells in inhibiting graft rejection, Graca et al. (27) compared CD4+CD25+ splenocytes from mice rendered tolerant to MHC-incompatible skin grafts with naïve CD4+CD25+ splenocytes. Although both sets of regulators were able to block alloresponses, fewer regulator cells from...
mental models (46, 81). Adoptive transfer of thymocytes from recipients and increase risk of BO. In support of this theory, col(V)-induced cellular immunity in human lung allograft transcription may have the untoward effect of suppressing or function optimally (94), calcineurin inhibitors that block IL-2 key activity is the induction of IL-2. As Tregs require IL-2 to though calcineurin may induce several functions in T cells, a through a calcineurin-dependent pathway (35, 51, 59). Al-
CsA and FK506 inhibit T cell function and alloreactivity (112) suggest that calcineurin inhibitors block development of rejection response. In a recent review, Wood and Sakaguchi immunsuppressants, administered early in transplant, may block Treg development. In turn, blocking Tregs could aid the development of autoimmunity that contributes to the rejection response.

FUTURE DIRECTIONS

Data showing that col(V) is an antigen during lung allograft rejection and col(V)-induced oral tolerance prevents lung allograft rejection raise several issues. The most important issue yet to be resolved lies in identifying the epitopes of col(V) that are either tolerogenic or antigenic. This is intriguing, in that not all col(V)-reactive T cells induce pathology after adoptive transfer (37). This suggests that there may be different antigenic regions within col(V) that are recognized as antigens during the rejection response. The difference between antigenic and potentially tolerogenic peptides of col(V) could be related to their primary sequence or affinity for TCRs. Data showing that not all col(V)-reactive T cells induce disease could also be related to differential expression of costimulatory molecules on these cells, rendering them less susceptible to activation or more resistant to active suppression by Tregs. These questions are currently under investigation. Alternatively, strategies designed to block expression of Smad7 in T cells could be a novel approach used to enhance Treg function. Finally, having been demonstrated in renal (8), heart (16), and lung allografts (37), the role of autoimmunity in the rejection response is established. Because Tregs are believed to function primarily as suppressors of autoimmunity, more investigations are needed to develop immunosuppressive regimens that preserve and enhance Treg function, resulting in the blockade of alloimmunity and autoimmunity in the transplant recipient.

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Invited Review

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