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The Generation of CD25⁺CD4⁺ Regulatory T Cells That Prevent Allograft Rejection Does Not Compromise Immunity to a Viral Pathogen

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In all but a small minority of cases, continued survival of solid organ grafts after transplantation depends on lifelong, nonselective immunosuppression that, although effective, results in increased rates of infection, cancer, and vascular disease. Therapeutic strategies that engage or mimic self-tolerance may allow prolonged allograft survival without the disadvantages of nonspecific immunotherapy. Pretreatment of recipient mice with donor alloantigen combined with transient modulation of the peripheral T cell pool with anti-CD4 Ab leads to the indefinite survival of MHC-incompatible cardiac allografts without further therapy. Tolerance is dependent on CD25⁺CD4⁺ regulatory T cells that arise from naive CD25⁺ precursors and regulate rejection via both IL-10 and CTLA-4. Although these cells are clearly effective at controlling rejection, the proven ability of recently activated CD25⁺ cells to mediate bystander regulation raises the possibility that tolerized individuals might also have a reduced capacity to respond to environmental pathogens. We have examined anti-influenza responses in tolerized primary heart recipients, secondary recipients following adoptive transfer of regulatory populations, and tolerized mice in which bystander regulation has been deliberately induced. Neither virus-specific CTL activity in vitro nor the clearance of virus in vivo was significantly diminished in any of these treatment groups compared with infected unmanipulated controls. The data suggest that the induction of dominant allograft tolerance dependent on regulatory T cells does not necessarily result in attenuated responses to pathogens providing further support for the development of tolerance induction protocols in clinical transplantation. The Journal of Immunology, 2005, 174: 3290–3297.
the corresponding TCR transgenic mouse. However, more importantly, once activated, the same CD25+CD4+ population could also inhibit the proliferation of CD25+ responder cells isolated from the second TCR transgenic mouse in a manner that was both Ag and MHC independent, a phenomenon referred to as “bystander suppression”. In the transplant setting where tolerance is dependent on regulatory T cells, the presence of the graft has been shown to be essential for maintenance of the tolerant state (16) probably by promoting a continual reactivation of the regulatory T cell population. We considered it possible that reactivation of regulatory cells by the graft could lead to nonspecific bystander suppression in vivo and that although it may be possible to generate donor-reactive CD25+CD4+ T cells that regulate responses toward the allograft, an effect on responses to environmental pathogens cannot be ruled out. Therefore, the aim of this study was to use a well-defined model of transplantation tolerance where continued graft survival is known to depend on CD25+CD4+ T cells and ask whether the presence of these cells has any impact on immunity against a well-characterized pathogen, influenza virus. The hypothesis tested was that tolerant mice might show attenuated antiviral responses.

Materials and Methods

Induction of tolerance and heart transplantation

CBA (H-2k) mice were pretreated with 200 µg of the IgG2b anti-CD4 Ab YTS 177.9 (17) on days −28 and −27 and with a donor-specific transfusion (DST)5 of C57BL/10 (H-2b) blood on day −27 and day 0 (anti-CD4/DST + reboot protocol). Five days after the second DST (day +5), the tolerized mice were either transplanted with donor-specific hearts or used as cell donors for adoptive transfer experiments. Specific details are given in the relevant figure legends. Cardiac transplantation was conducted under general anesthesia according to the method outlined by Corry et al. (18).

Briefly, hearts were isolated from exsanguinated heparinized donors, and general anesthesia according to the method outlined by Corry et al. (18).

Experimental models

CBA mice were pretreated with a single DST plus the anti-CD4/DST protocol. Five days after the second DST (day +5), the tolerized mice were either transplanted with donor-specific hearts or used as cell donors for adoptive transfer experiments. Specific details are given in the relevant figure legends. Cardiac transplantation was conducted under general anesthesia according to the method outlined by Corry et al. (18).

Briefly, hearts were isolated from exsanguinated heparinized donors, and general anesthesia according to the method outlined by Corry et al. (18).

Pretreatment of CBA mice with a single DST plus the tolerogenic 177/DST protocol showed that Ag rechallenge between 3 and 5 days before transplantation increases the likelihood of detecting any effect on antiviral responses (see Fig. 2a). Preliminary results in an analogous anti-CD4/DST model showed that Ag rechallenge between 3 and 5 days before transplantation improves the quality of graft function significantly compared with the anti-CD4/single DST protocol consistent with regulatory cell reactivation (data not shown). In the second model, CBA mice pretreated and rechallenged with a second DST as above were used as adoptive transfer cell donors (see Fig. 5a).

Adoptive transfer of spleen cells from tolerated mice to naive syngeneic secondary recipients allows the indefinite survival of cardiac allografts without immunosuppression. Since these secondary

5 Abbreviations used in this paper: DST, donor-specific transfusion; MDCK, Madin-Darby canine kidney; HA, hemagglutinin.
recipients have a completely intact immune system modified neither by Ab therapy nor alloantigen challenge, we believe that this model represents an extremely stringent test of tolerance. Our hypothesis was that if regulation of allograft responses also resulted in attenuated antiviral responses, this second model might provide the most sensitive detection system. In both models, transplant recipients were infected with influenza 7 days posttransplant then assayed for CTL activity and viral load 11 days later at the peak of antiviral responses.

The anti-CD4/DST plus reboost protocol in primary allograft recipients leads to indefinite graft survival with only modest levels of vasculopathy

To examine the effectiveness of the anti-CD4/DST plus DST reboost protocol, pretreated CBA mice were transplanted with B.10 hearts. Control mice pretreated with anti-CD4 Ab (YTS 177) only plus a DST challenge on day 0 or with DST only plus a DST challenge on day 0 rejected their grafts with median survival times (MST) of 48 and 14 days, respectively (Fig. 1a). In contrast, mice given the combined anti-CD4/DST pretreatment plus a DST reboost on day 0 all accepted their B.10 cardiac allografts beyond 100 days with little decline in cardiac function as assessed by palpation. The integrity of these hearts was confirmed by histological evaluation ~120 days posttransplant which revealed little myocardial damage and low levels of vasculopathy (mean vascular occlusion of 28%, Fig. 1b). This compares with occlusion levels of up to 75% in transplanted mice tolerated by the anti-CD4/DST protocol then given anti-GITR or anti-CD25 Abs to perturb the function of putative regulatory cells (data not shown).

Anti-influenza CTL responses and viral clearance are unaffected by allograft tolerance in primary heart recipients

CBA mice were pretreated with the anti-CD4/DST plus DST reboost protocol, transplanted with B.10 cardiac allografts then infected with influenza virus 7 days posttransplant (Fig. 2a). This time point was chosen for influenza infection because day 7 represents a balance between recipient sensitization and possible graft adaptation after transplantation. Previous work in the mouse cardiac allograft model has shown that heart-derived donor dendritic leukocytes can be detected in the recipient spleen 1 day posttransplant and that this migration is maintained for at least 4 days (22). In untreated mice, this results in graft rejection by day 8–10. The anti-CD4/DST protocol, known to generate CD25+CD4+ regulatory cells (12), converts this into indefinite graft survival (Fig. 1a) indicating that at least 7 days posttransplant, dominant regulation in the presence of ongoing sensitization must be occurring. Indeed, in an analogous model, regulatory T cells can be generated in vivo by exposure to nominal Ag plus anti-CD4 Ab and are also capable of preventing skin allograft rejection by bystander regulation (46). These CD25+CD4+ regulatory T cells are present in the spleen as early as 1 day after Ag rechallenge and persist for at least a further 14 days (data not shown). Infection at later time points posttransplant was rejected as a strategy for looking at the effect of regulation on virus responses because of the possibility that graft adaptation rather than regulation might be responsible for continued graft survival.

Naïve CBA infected with influenza made good CTL responses with ~35% specific lysis at the highest effector to target cell ratio (Fig. 2b). Importantly, CTL responses in tolerized transplanted mice (177/DST + heart) were essentially identical to those from naïve mice indicating that the presence of dominant regulation has no detrimental effect on protective CTL immunity (Fig. 2, b and c). Interestingly, CTL responses in mice given either of the control pretreatment regimens (177 only or DST only) were considerably augmented compared with those from naïve mice possibly due to the provision of nonspecific cytokine help driven by the early stages of graft rejection (Fig. 1a).

To examine the effects of regulation on pathogen clearance from the lung, a modified ELISA assay was used to detect influenza HA expression by cell monolayers exposed to lung homogenates. This approach was validated by exposure of MDCK cells to serial dilutions of stock virus (Fig. 3a). As a positive control for clearance of the virus in vivo, naïve untransplanted mice were infected with 5 HA units of influenza PR8 then lungs were harvested 1 h and 2, 4, and 7 days later for determination of relative viral load (n = 2

**FIGURE 1.** CBA mice (H-2k) were given 200 μg of anti-CD4 Ab (YTS 177.9; days −28 and −27, i.v. + a C57BL/10 (B.10, H-2b) DST, day −27; DST alone (day −27) or YTS 177.9 alone (days −28 and −27). On day 0, all mice received a B.10 DST “reboost” and were transplanted with fully vascularized B.10 cardiac allografts 5 days later. No further treatment was given. Graft survival was assessed by palpation (a). Cardiac vasculopathy in the 177/DST + reboost group was determined histologically 100 days posttransplant and compared with that in syngeneic heart allografts 100 days posttransplant (b).
mice per time point). Viral titer reached a peak at day 4 and returned almost to background levels 7 days postinfection demonstrating that there was effective clearance of the virus (Fig. 3b). Fig. 3c shows viral load data from the tolerized transplanted and control mice shown in Fig. 2. When assayed 11 days after infection, the lungs of naive mice contained significant levels of virus but more importantly, mice tolerized by the anti-CD4/DST protocol cleared the virus as shown by the low HA signals, which were practically identical to those from uninfected mice representing essentially the background of the assay. Taken together these data demonstrate that despite the presence of CD25+ cells capable of regulating rejection responses (12) protective anti-flu responses appear to be unaffected.

Tolerance can be transferred from anti-CD4/DST-pretreated mice to naive recipients resulting in long-term allograft survival with little obstructive vasculopathy

In the primary heart model described above, long-term graft survival is dependent on the combined anti-CD4/DST pretreatment. However, pretreatment with either of the control regimens (177 only + reboost or DST only + reboost) leads to some prolongation of allograft survival (Fig. 1a) indicating that each of these components has an independent attenuating effect on rejection responses. Therefore, it could be argued that the additional effect of the regulatory cells generated by the anti-CD4/DST protocol in these primary recipients is only rather small and that, if this is the case, the model is not well suited for assessing the impact of dominant regulation on antiviral responses. To address this possibility an alternative model was used in which tolerance is transferred from pretreated mice to naive CBA secondary recipients. In this situation, regulatory cells in the transferred population are required to prevent rejection mediated by an otherwise completely intact immune repertoire. As shown in Fig. 4, adoptive transfer of spleen cells from mice pretreated with either of the control regimens resulted in acute cardiac allograft rejection, whereas transfer of cells from mice pretreated with the tolerogenic anti-CD4/DST reboost protocol resulted in long-term graft survival (Fig. 4a). The effectiveness of regulation in this system is emphasized not only by the survival of these hearts but also by the paucity of vascular lesions such that the overall vascular occlusion was <15% (Fig. 4b). One potential explanation for the reduced level of vasculopathy in these hearts compared with that seen in primary recipients (Fig. 1b) is that in the secondary recipients, adoptive transfer of regulatory cells resulted in the recruitment of naïve cells into the regulatory pool by “infectious tolerance” (23).
Protective immunity to influenza virus is unaffected by the presence of dominant regulation

CBA cell donors were pretreated with the anti-CD4/DST induction protocol then rechallenged with a second DST at day 0. Spleen cells were adoptively transferred to naive CBA recipients 5 days later, which were transplanted with B.10 cardiac allografts then infected with influenza PR8 (Fig. 5a). The mice were harvested 11 days later for CTL and viral load assays. Influenza-specific CTL activity in these adoptive transfer recipients was comparable with that from naive untransplanted mice or from naive recipients rejecting B.10 cardiac allografts (Fig. 5b). This equivalence in antiviral responses was also reflected in the viral load assay where the adoptive transfer recipients had levels of virus that were almost identical to those in control mice (Fig. 5c). Taken together these data indicate that the presence of regulation efficient enough to give the graft almost complete protection from damage (Fig. 4) does not influence immunity to influenza virus.

Recent Ag rechallenge sufficient to drive bystander regulation does not impair antiviral responses

In addition to the anti-CD4/DST protocol described above, we have also shown that CD25^+CD4^+ regulatory cells can be generated by pretreatment with unrelated “random” blood transfusion given either with (single transfusion) or without (five transfusions) anti-CD4 Ab (13). Our working hypothesis is that random transfusion generates regulatory cells that can protect skin allografts either via a mechanism of cross-reactivity or via bystander suppression. We currently favor the latter explanation because we were unable to detect an effect on viral immunity because by day 7 posttransplant the time of infection, Figs. 2 and 5) regulation had become focused due to continual reactivation of the regulatory cells by the graft itself such that regulation was relatively specific either in terms of regulatory cell function or recruitment.

To examine the impact of recent regulatory T cell activation on antiviral responses, CBA mice were pretreated with the tolerogenic anti-CD4/DST protocol then rechallenged with a second DST at day 0. These mice were then infected with influenza 1 day after rechallenge and harvested 11 days later (Fig. 6a). Day +1 was chosen for the infection interval because in an analogous model we have clear evidence that Ag rechallenge 1 day before adoptive transfer activates bystander regulation powerful enough to prevent skin graft rejection, which is Ag nonspecific. However, despite this reactivation, there was no evidence of attenuated anti-flu responses either in terms of CTL activity (Fig. 6b) or viral clearance from the lung (Fig. 6c).
great deal of attention (28) and has proven effective in a small number of patients (29). By far the greatest experience with bone marrow protocols has been obtained in the mouse where bone marrow infusion is usually combined with cytodestructive conditioning and inhibition of peripheral T cell function (28, 30–33). This approach leads to mixed chimerism and in many cases central deletion of donor-reactive T cells. However, there is evidence that even in these deletional models, regulatory T cells make an essential contribution to continued allograft survival (A. B. Adams and C. P. Larsen, unpublished observations). Thus, in models that are considered prototypic for regimens that may be introduced more widely in clinical transplantation, regulation appears to make at least a contribution to the maintenance of tolerance. Therefore, an understanding of the impact of T cell-mediated regulation on pathogen clearance is highly relevant.

The clearance of influenza virus from infected mice involves both CD4⁺ and CD8⁺ T cell responses (34), and T cells of both subsets are susceptible to regulation by CD25⁺ CD4⁺ regulatory T cells (12, 35) making influenza an appropriate pathogen for the study of the effect of alloantigen-driven regulatory T cells on viral immunity. Our experiments conducted in models in which there is a demonstrable generation of CD25⁺ CD4⁺ regulatory T cells (11, 12) revealed no adverse effect of transplantation tolerance on influenza-specific CTL or on clearance of the virus in vivo. This was something of a surprise given that regulation of rejection in these models appears to be dependent on IL-10 (12), a cytokine first identified because of its immunosuppressive properties on T cell activation and synthesis of Th1 cytokines (36). The most simple explanation for this lack of effect is that following tolerance induction and transplantation, the majority of regulatory cells migrate to and are sequestered within the graft (37) thus limiting any systemic effect. Thus, even though influenza-specific CTL were obtained from the spleens of transplanted animals (Figs. 2 and 5), a compartment we have shown to contain regulatory cells after anti-CD4⁺/DST pretreatment (11, 12), it could be argued that the presence of the graft may have selectively depleted the spleen of regulatory cells thereby allowing antiviral responses to develop relatively normally. However, this explanation cannot apply to the experiments shown in Fig. 6 where tolerized mice remained untransplanted and were infected 1 day after a second alloantigen challenge. Influenza-specific CTL with apparently normal activity were obtained from the spleens of these mice (Fig. 6b) despite the fact that alloantigen-driven regulatory cells remain in the spleen for at least 5 days after rechallenge (Fig. 4). It could also be argued that any perturbation of CTL responses caused by ongoing regulation was masked in our experiments because exogenous IL-2 was added during the in vitro expansion of CTL precursors. This is a potentially valid criticism because regulation in vitro can often be overcome by the addition of exogenous IL-2 (38–41). However, viral clearance from the lungs of infected mice, which is an entirely in vivo phenomenon involving no experimental manipulation, was also unaffected by the presence of regulatory cells (Figs. 3, 5, and 6), supporting the CTL data.

Several studies have addressed the effect that viral infection has on transplantation tolerance, and the overall view is that both prior and concurrent exposure to viral pathogens can prevent tolerance induction. Although previous viral exposure leads to memory T cells (predominantly CD8⁺) that appear to be resistant to tolerance induction (25, 26), CD8⁺ T cells driven by peri-transplant viral infection...
exposure have also been implicated in rejection of the graft itself (42, 43). As far as the converse situation is concerned, previous studies have examined the clearance of viral pathogens in mixed allogeneic chimeras (44), and although mice containing both host and donor restricted T cells may have more difficulty clearing chronic infections (45), mixed chimeras seem to clear acute viral infections at similar rates to their normal counterparts. However, much less attention has been paid to the effect that transplantation tolerance based on active T cell regulation might have on protective immunity. This is an important area because it is hoped that tolerance induction will circumvent many of the problems associated with current nonspecific immunosuppression. To our knowledge, this is the first study to examine the effects of alloantigen-driven CD25^+CD4^+ regulatory cells on responses to a viral pathogen. We have found no diminution of antiviral responses in three different situations, and although the data reflect responses to a single pathogen in one particular transplant setting, we believe our data give grounds for cautious optimism that bystander suppression may be less of a problem in transplantation than might be imagined. We are currently extending our studies to other model pathogens and transplant models to determine whether this optimism is well placed.

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Disclosures

The authors have no financial conflict of interest.

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