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Evidence for Immune Regulation in the Induction of Transplantation Tolerance: A Conditional but Limited Role for IL-4

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Most experimental models of allograft tolerance depend on manipulation of immune responses at the time of transplant. In such systems, the graft itself probably plays an important role in the induction of unresponsiveness but as a consequence may suffer immune mediated damage. Ideally, recipients would be made specifically unresponsive before transplant such that the graft is protected from the outset. In this report, we demonstrate that CBA mice pretreated with donor-specific transfusion plus anti-CD4 Ab 28 days before transplant accept cardiac allografts indefinitely without further intervention. Adoptive transfer of spleen cells from mice with long term surviving grafts results in donor-specific graft acceptance in naive secondary recipients, indicating that tolerance in this system involves immunoregulation. Regulation develops as a result of the pretreatment protocol alone, since transfer of cells from pretreated but untransplanted mice to naive recipients also leads to prolonged allograft survival without additional therapy. Neutralizing IL-4 at the time of tolerance induction had no effect on graft outcome in primary recipients. However, removal of IL-4 from the adoptive transfer donors at the time of tolerance induction prevented long term engraftment in the majority of secondary recipients. Our data demonstrate that pretreatment of transplant recipients can establish immune regulation powerful enough to override the responses of an intact immune repertoire and that under stringent conditions at least, development of this regulatory population may in part be dependent on IL-4. The Journal of Immunology, 1999, 162: 1359–1366.

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ergulation of immune responses to peripheral Ags in vivo has been demonstrated in a number of models of auto-immunity (1–4) and parasitic infection (5–9). The observation that in many models of transplantation tolerance regulatory or suppressive T cells can be transferred from animals bearing long term grafts to naïve syngeneic secondary recipients (10–13) has led to the concept that T cell regulation may be a fundamental component of the mechanisms responsible for inducing and maintaining tolerance to alloantigen. The fact that, once engaged, this type of regulation appears to be self-sustaining in vivo (13, 14) suggests that if the mechanisms of regulation can be understood, protocols might be developed which could deliver true transplantation tolerance in humans.

We have established a protocol in which pretreatment of recipient mice with donor-specific transfusion (DST) under the cover of a brief course of depleting or nondepleting anti-CD4 Ab leads to specific and selective unresponsiveness (15–17). This protocol combines the powerful immunosuppressive effects of anti-CD4 Ab with the specificity of donor Ag challenge and leads to the indefinite survival of subsequent primary cardiac allografts and prolonged acceptance of secondary donor-specific skin grafts. We have previously provided indirect evidence that unresponsiveness in this system depends on a population of regulatory CD4+ T cells that develop as a consequence of the pretreatment protocol and are thus present at or before the time of transplantation (18). This implies that the graft may thus have a degree of protection from the time of the transplant itself which may have important implications for the prevention of both acute and chronic rejection. The purpose of the present study was to examine the development of this regulatory population and determine whether it could be explained in terms of the Th1/Th2 paradigm.

Materials and Methods

Mice

CBA/Ca (H-2k); C57BL/10 (B.10, H-2b) and BALB/c (H-2d) were obtained from Harlan Olac, Bicester, U.K., and housed under partial barrier conditions. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

Antigens

YTA 3.1.2 (19) (rat anti-mouse IgG2b anti-CD4) was purified from ascites as described (18). Ab purity as determined by SDS-PAGE and scanning densitometry was between 76 and 85%. 11B11 (20) (rat anti-mouse IgG1 anti-IL-4) was purified from tissue culture supernatant by DEAE ion exchange chromatography. Purity was 87%. 145-2C11 (21) (hamster anti-mouse IgG anti-CD3) was purified from tissue culture supernatant by HPLC. Purity was >90%.

All Abs used in vivo were screened for endotoxin by the Therapeutic Ab Centre, Oxford, U.K. In all cases endotoxin concentrations were <1 endotoxin unit/ml.

Anti-CD4/DST pretreatment (YTA/DST protocol)

CBA mice 8–12 wk of age were pretreated with 50 μg of purified YTA 3.1.2 i.v. on consecutive days (days –28 and –27). On day –27, the mice received a 250-μl transfusion of heparinized whole blood i.v. The animals were transplanted 28 days later (day 0) with an heterotopic abdominal cardiac allograft. Specific details of modified pretreatment protocols are given in the appropriate figure legends.
then injected iv. Transplantation was conducted 24 h after transfer. After 28 days, the mice were transplanted with a B.10 heart. Only the combined YTA/DST protocol leads to long term graft acceptance.

**Transplantation**

Heterotopic cardiac transplantation was conducted broadly as described (22). General anesthesia was provided by Hypnorm (fentanyl citrate and flumazenil; Janssen Pharmaceuticals, Piscataway, NJ) and Hypnovel (midazolam; Roche, Nutley, NJ) supplemented by Marcaín local anesthetic (bupivacaine hydrochloride; Astra Pharmaceuticals, Herts, U.K.) injected along the midline abdominal incision. Graft function was assessed by palpation, electrocardiogram (ECG), and laparotomy. Palpation scores ranged from 4 (equivalent to syngeneic graft) to 0 (lack of palpable contractions). Rejection was defined as the lack of palpable cardiac contraction or lack of electrical activity (23). ECG ratios (defined as transplant heart rate divided by native heart rate) were obtained from all long term surviving grafts at or beyond day 100.

**Histology**

Transplanted hearts were removed, embedded in Tissue-Tek (Miles Laboratories, Elkhart, IN), and snap-frozen in liquid nitrogen. Cryostat sections (7 µm) were air-dried, fixed in acetone, and stained with hematoxylin/eosin or Weigert’s elastin stain followed by Van Gieson counterstain. In this latter protocol, elastic fibers of the internal and external elastic lamina of the coronary vessels are stained black. Between five and nine sections were examined for each heart.

**Adoptive transfer**

Spleen cells from pretreated or transplanted mice were isolated by passing the tissue through a stainless steel mesh followed by depletion of RBC by osmotic shock. Cells were washed twice in RPMI supplemented with 2% FCS, resuspended in sterile saline at a concentration of 5 × 10^7/ml, and then injected iv. Transplantation was conducted 24 h after transfer.

**Bioactivity of 11B11**

The ability of our preparation of 11B11 to neutralize IL-4 was confirmed by its capacity to inhibit IL-4-dependent up-regulation of MHC class II on B cells in vitro and in vivo. For in vitro determination of activity, CBA spleen cells were incubated in RPMI 1640/10% FCS (Myocline, Life Sciences, Gaithersburg, MD) containing 100 U/ml recombinant mouse IL-4 (PharMingen, San Diego, CA) plus increasing concentrations of 11B11. After 18 h, cells were stained with TIB 120 (24) followed by mouse anti-rat FITC for class II and with rat anti-mouse phycoerythrin (Sigma, St. Louis, MO) for B cell surface Ig. To test the efficacy of our preparation of 11B11 in vivo, CBA mice were given 2 mg of 11B11 on successive days. One hour after the second dose, the mice received 50 µg of the mitogenic anti-CD3 Ab 145-2C11. After 18 h, spleen cells were isolated, and class II expression on B cells was determined by two-color FACS analysis as described above.

**Results**

**DST under anti-CD4 cover leads to indefinite graft survival**

Fig. 1 demonstrates that the combined YTA/DST pretreatment protocol previously shown to be effective in the B.10 to C3H/He strain combination also leads to operational tolerance in CBA mice in that 100% of recipients accept B.10 hearts indefinitely without further immunosuppressive treatment. As in C3H/He recipients, graft survival is entirely dependent on the combined pretreatment with anti-CD4 and DST, since administration of either DST alone or anti-CD4 alone leads only to modest graft survival (MST of 8 and 12 days, respectively).

In our laboratory, heart function is assessed by palpation, ECG, and laparotomy. At or beyond 100 days, B.10 hearts transplanted into CBA recipients in the YTA/DST model typically have palpation scores of 3 (on a scale of 0–4), have ECG ratios (transplanted vs native heart) of 95–110% (similar to syngeneic grafts analyzed at the same time point), and at laparotomy show coordinated ventricular contraction with well-perfused coronary arteries and no gross evidence of tissue necrosis. The quality of graft function in this model is confirmed by tissue histology. Fig. 2b shows a representative section taken from a B.10 heart 160 days after transplant stained with hematoxylin/eosin for overall tissue morphology and with a Weigert’s elastin/Van Gieson protocol to highlight elastin in the coronary vessels (inset). All hearts examined in this model show good myocardial preservation with little evidence of intimal proliferation characteristic of chronic rejection (25). Fig. 2a shows a section from a native B.10 heart for comparison.

**Adoptive transfer of cells from long term tolerant mice leads to tolerance in naive secondary recipients**

To explore the basis of long term allograft survival in the YTA/DST model, spleen cells were isolated from CBA recipients that had accepted B.10 hearts for >100 days and adoptively transferred into naive, unmanipulated CBA mice. The recipients were transplanted with either a specific (B.10) or third party (BALB/c) heart 24 h after cell transfer. Fig. 3 shows that whereas a third party heart was rejected by day 40, donor-specific B.10 hearts were accepted for >100 days. These hearts had palpation scores of 3 or 4 and had ECG ratios of 92, 96, and 150%. An independent histological examination indicated that although one of the three hearts showed some vasculopathy, the remaining two had normal coronary arteries with no sign of chronic rejection. A representative section from one of these hearts is shown in Fig. 2c. There is good preservation of the myocardium and elastin staining of the vessels revealed essentially normal coronary arteries (inset). The ability of cells from long term tolerant animals to attenuate the response of an intact immune system in unmanipulated recipients is consistent with the presence of a donor-specific regulatory cell population.

**Adoptive transfer of cells from pretreated-only mice leads to tolerance in naive secondary recipients**

To examine the possibility that in this model regulatory cells develop as a consequence of the YTA/DST pretreatment alone, adoptive transfer experiments were conducted in which spleen cells were transferred from pretreated-only CBA mice to naive unmanipulated CBA recipients. Transfer was conducted 28 days after pretreatment (day 0), the time when the mice would normally have been transplanted. The mice were transplanted with B.10 hearts 24 h after transfer. Fig. 4a shows that transfer of spleen cells from mice pretreated with either anti-CD4 only or DST only led to almost unmodified rejection with a median graft survival of 8 days in both groups (cf. median of 8 days in untreated CBA recipients (Fig. 1)). In contrast, the adoptive transfer of cells from YTA/DST-pretreated mice led to significantly prolonged graft survival with three of five of these otherwise unmodified recipients accepting their hearts for >100 days. ECG ratios 100 days after transplant were 72, 105, and 110%. A representative section from one of these hearts is shown in Fig. 2d. The myocardium is intact and examination of sections stained for elastin revealed little sign of intimal proliferation in the coronary vessels. Taken together, these data provide unequivocal support for the presence of a regulatory population of leukocytes in the spleen which develops before transplantation as a result of the anti-CD4/DST pretreatment alone.
Ability to transfer tolerance from pretreated-only mice is time dependent

In an analogous but distinct system, Saitovitch et al. (17) demonstrated that pretreatment with DST under the cover of a nondepleting anti-CD4 Ab led to indefinite graft survival in primary transplant recipients but that the effectiveness of the protocol depended on a critical time interval between pretreatment and transplantation. To test whether the development of regulation detected in this sensitive adoptive transfer system was also time dependent, spleen cells were transferred from YTA/DST-pretreated CBA mice 14 days rather than 28 days after pretreatment. Whereas the adoptive transfer of cells at day 0 led to significant graft prolone-
gation (Fig. 4a), transfer of cells 14 days earlier led to acute graft rejection (MST 14 days (Fig. 4b)). These data confirm that the development of unresponsiveness in this model is a dynamic process and suggest that it depends on the expansion or maturation of a regulatory population beyond a critical threshold.

Allograft survival in primary recipients is unaffected by removal of IL-4

T cells can be divided into Th1 and Th2 subsets depending on the pattern of cytokines that they produce after stimulation (26, 27), and many studies have demonstrated that at least in vitro, these two subsets have the capacity for cytokine-mediated reciprocal regulation (28–31). The fact that Th1 cytokines are often found during rejection has led to the idea that transplantation tolerance might involve deviation toward a dominant Th2-type response. Since IL-4 has been shown to play a central role in the development of Th2 cells, we asked whether this cytokine was involved in the induction of tolerance in the YTA/DST model. CBA mice were pretreated with the YTA/DST protocol in the presence or absence of the neutralizing anti-IL4 Ab 11B11. A number of factors influenced our choice of dose and timing of 11B11 administration. First, the dose was chosen to be in the range of that used previously by other groups (32–35). Secondly, the timing was chosen to coincide with a period during and following pretreatment, which we had previously shown to be critical for the induction of unresponsiveness in this model (18). We were also aware of the results of Gross et al. (34) who had demonstrated that tolerance to a soluble Ag could be abrogated with anti-IL4 Ab only when given during rather than after the period of Ag challenge. Thirdly, previous work had shown that after a single injection of 1 mg of purified 11B11, active Ab could still be recovered from mouse serum up to 7 days later, suggesting a reasonably long half-life for this Ab in vivo (36). Thus, we felt that by giving multiple 1-mg injections of 11B11 we would ensure adequate Ab availability during this critical induction period.

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Fig. 5 shows that when given from day –27, 11B11 had no discernible effect on the success of the YTA/DST protocol in that all of the mice accepted their grafts for greater than 100 days. To rule out the possibility that this lack of effect was due to a delayed neutralization of IL-4 by the Ab, a second group of mice received an additional 1 mg of 11B11 on day –28. None of the animals in any of the four groups rejected their B.10 allografts.
One trivial explanation for the lack of effect of 11B11 in our hands is that the anti-IL4 Ab used in these studies lacked bioactivity. The ability of our preparation of 11B11 to neutralize IL-4 was assessed both in vitro and in vivo using a method based on that of Flamand et al. (32) which takes advantage of the fact that IL-4 induces up-regulation of MHC class II on B cells. Fig. 6a shows that the induction of Class II expression by recombinant mouse IL-4 in vitro is inhibited by 11B11 in a dose-dependent manner, thus confirming that the preparation of 11B11 used in these experiments did indeed neutralize IL-4. To test the effectiveness of our preparation of 11B11 in vivo, CBA mice were pretreated with 11B11 followed by the anti-CD3 Ab 145-2C11. After 18 h, class II expression on B cells was determined by two-color FACS analysis (Fig. 6b). 145-2C11 is highly mitogenic in vitro (21) and in vivo results in a twofold increase in B cell class II expression (mean channel fluorescence, 1523 compared with 726 in naive mice). Administration of 11B11 reduced this up-regulation by almost 50% (mean channel fluorescence, 1067), indicating that the 11B11 preparation was capable of neutralizing IL-4 in vivo. Although this inhibition does not restore the level of Class II expression to that seen in naive cells, this is perhaps not surprising given the fact that 145-2C11 potentially activates all T cells. By comparison, estimates of the frequency of alloreactive T cells suggest that at most only 10% of the total T cell pool would be capable of responding to donor cells in the DST (37–39). In addition, because the anti-CD4 Ab used in the YTA/DST protocol induces substantial (70–90%) CD4+ T cell depletion during the period of 11B11 administration, we feel confident that the 11B11 regimen used in this system would have been capable of neutralizing most if not all of the IL-4 produced in response to alloantigen.

Evidence that IL-4 may play a role in the early induction phase of transplantation tolerance

Although neutralizing IL-4 had no effect on graft survival in primary recipients (Fig. 5), we considered it possible that a role for IL-4 might be detected only in a more sensitive assay system. To test this, CBA mice were pretreated with the YTA/DST protocol together with 1-mg doses of 11B11 or control rat IgG on days −27 to −24 as shown. On day 0, 50 × 10⁷ spleen cells were adoptively transferred to naive CBA recipients. After 24 h, these mice were transplanted with B.10 hearts. Neutralizing IL-4 during the period of tolerance induction reduces significantly the ability of these cells to transfer tolerance (p = 0.012, log-rank sum analysis).

Discussion

Donor-specific transfusion under the cover of the depleting anti-CD4 Ab YTA 3.1.2 leads to a robust form of operational tolerance...
where mice that would normally reject B.10 hearts within about 8 days accept their grafts indefinitely without further treatment. We have demonstrated that as in some other systems, adoptive transfer of cells from animals with long term surviving grafts leads to the prolonged survival of donor-specific grafts in secondary recipients. The most straightforward interpretation of these and other data in the literature is that animals that are operationally tolerant contain a population of cells capable of regulating alloreactive immune responses. The data shown in Fig. 4 demonstrate that such a regulatory population arises in this system as a result of the anti-CD4/DST pretreatment alone. These data are particularly striking because to our knowledge this is the first time that such regulation has been demonstrated by adoptive transfer in unmanipulated naive recipients.

We have previously demonstrated that the success of this protocol depends on a small population of CD4^+ T cells that escape Ab depletion and interact with donor alloantigen during a transient period of CD4 blockade (18). During the 27-day interval between pretreatment and transplantation in this model, peripheral repopulation from the thymus occurs such that at the time of transplant the CD4^+ T cell compartment is ~40% repopulated. Although this repopulation is not complete, in mice pretreated with YTA 3.1.2 alone (where the levels of depletion and rate of repopulation are almost identical), the repopulated cells are sufficient in number to bring about the prompt rejection of B.10 hearts. Given the fact that in the YTA/DST protocol the vast majority of repopulation occurs in the absence of detectable alloantigen (donor cells are no longer detectable after 3 days as judged by immunocytochemistry and flow cytometry), we must conclude that these newly emerging CD4^+ T cells are essentially naive and as such should be quite capable of rejecting B.10 hearts at the same rate as those in mice pretreated with Ab alone. The fact that this is not the case provides compelling evidence that the success of the YTA/DST protocol depends on regulation of recently emerged donor-reactive CD4^+ cells. Our working hypothesis is that regulation is mediated initially by the small population of CD4^+ T cells which escapes depletion (since these are the only CD4^+ cells available to interact with donor Ag) but that in the longer term new thymic emigrants become part of the regulatory population by “infectious tolerance” (13).

We considered it possible that the initial encounter with alloantigen during Ab blockade might lead to Ag recognition without adequate costimulation and result in a CD4^+ T cell population with an overall Th2 bias. The production of Th2 cytokines by such a population might form the basis of T cell regulation in this system. There is abundant evidence in the literature to show that in vitro, Th1 and Th2 cells have the capacity for reciprocal regulation mediated by secreted cytokines and in vivo such regulation has been demonstrated in models of parasitic infection and autoimmune disease (1, 3, 5, 8). In experimental and clinical transplantation, rejection has often appeared to correlate with the detection of Th1 rather than Th2 cytokines (40–43). Such observations are the basis of the Th1/Th2 paradigm. In its simplest form, this model predicts that if rejection correlates with a Th1 bias then the opposite situation (tolerance) might involve a dominant Th2 response (for reviews, see Refs. 44 and 45).

Although the Th1/Th2 paradigm is attractive, the available data do not suggest such a clear distinction, and indeed, in a recent survey of 15 immune activation genes in clinical kidney transplantation, no direct evidence could be found in support of the Th1/Th2 paradigm in either rejection or stable graft function (46). A similar breakdown of the paradigm is also seen in many experimental transplant models. For example, the acute rejection of allogeneic islets in IL-2-deficient (knockout (KO)) mice (47), hearts in IFN-γ KO mice (48), and hearts in IL-2/IFN-γ double KO mice (Y. Li and T. Strom, personal communication) appears to rule out a strict requirement for either of these Th1 cytokines in graft destruction. In fact, there are data that IL-2 and IFN-γ may play an unexpected role in long term allograft survival (49, 50). As far as IL-4 is concerned, the prolonged survival of allogeneic islets (51) and hearts (52, 53) in IL-4-deficient (IL-4 KO) animals appears to rule out an absolute role for this Th2 cytokine in long term engraftment, but there are at least two possible ways in which these IL-4 KO data could be explained. Firstly there is growing evidence that there is considerable redundancy in the cytokine network. This is perhaps best illustrated by the ability in some systems for IL-15 to substitute for IL-2 because of shared receptor components and signaling pathways (44). Such redundancy might be exaggerated in KO mice in which the immune system has developed in the absence of a given cytokine. Thus, it could be argued that prolonged graft survival in IL-4-deficient mice might indeed be dependent on Th2 cells but mediated through the production of another cytokine such as IL-10 rather than IL-4 (42, 53) The second possibility (which we favor) is that IL-4 plays a significant role only in those models of tolerance that depend on T cell regulation rather than on T cell deletion, ignorance, or ambivalence.

We have been able to demonstrate an effect of neutralizing IL-4 only when cells from pretreated mice were adoptively transferred into naive unmanipulated recipients (Fig. 7). This suggests that IL-4 plays a critical role only in the most stringent situations of transplantation tolerance where long term graft acceptance might depend on a delicate balance of several component factors. The YTA/DST protocol may achieve this balance relatively easily in primary recipients and is therefore not IL-4 dependent, but in adoptive transfer recipients we suggest that the balance is more delicate and thus easily perturbed by the removal of IL-4. In an adoptive transfer system designed to provide precisely this type of fine balance between regulation and rejection of skin grafts mismatched for multiple minor Ags, Davies et al. (54) demonstrated a significant reduction in graft survival when adoptive transfer recipients were treated with anti-IL4 Ab.

Chen et al. (14) have demonstrated that tolerance to BALB/c hearts can be induced in CBA recipients by perioperative treatment with nondepleting anti-CD4 plus anti-CD8 Abs. Tolerance could be adoptively transferred from animals bearing long term surviving grafts to naive secondary hosts in a way similar to that shown in the present study (Fig. 3). Administration of 11B11 in the perioperative period had no effect on tolerance induction in primary CBA recipients, much like that shown in Fig. 5 but in contrast to the results shown in Fig. 7, neutralizing IL-4 had no effect in their adoptive transfer system. However, a key difference that may explain this apparent inconsistency is that in the study of Chen et al. (14) the anti-IL4 Ab was given to the adoptive transfer recipients around the time of cell transfer, whereas in the present study anti-IL4 was given to the cell donors during the time of tolerance induction. Thus, the two apparently conflicting observations could be reconciled by saying that although IL-4 may play a role in the induction of regulation-based tolerance perhaps by driving the differentiation and expansion of Th2-like cells, once such a population is large enough it is no longer IL-4 dependent. Such a possibility is consistent with the observation that regulation is both a time- and dose-dependent phenomenon (17, 54, 55) and with the possibility that regulatory cells exert their effector function by competition for Ag or costimulation rather than the secretion of soluble factors (56).

Although several studies have shown that the absence of IL-4 has little effect on allograft acceptance, other data indicating that Th2 cytokines might be involved in allograft survival cannot be
ignored. For example, in a perioperative anti-CD4 model, Mottram et al. (41) have shown that long term surviving heart allografts but not rejecting grafts contained infiltrating T cells positive for IL-4 and IL-10. In addition, it has been shown recently that neutralizing either IL-4 or IL-10 abrogated long term heart allograft survival in mice treated with anti-LFA-1 plus anti-ICAM-1 Abs (42). Sirak et al. (57) have shown recently that whereas wild-type B6 mice treated with either gallium nitrate or anti-CD4 Ab show prolonged survival of DBA/2 hearts, the same protocols were much less effective in IL-4-deficient B6 recipients. In an anti-CD4 Ab model, Onodera et al. (58) have demonstrated that tolerance could be transferred from rats bearing long term heart allografts to lightly irradiated syngeneic secondary recipients. Whereas there was no evidence for the involvement of Th2 cytokines in the primary graft recipients, there was a selective up-regulation of IL-4 and IL-10 in secondary recipients following adoptive transfer and donor-specific transplantation.

The results of our adoptive transfer experiments may explain the apparently contradictory role of IL-4 in graft acceptance, because we have demonstrated that under extreme conditions removal of IL-4 can clearly interfere with tolerance that is based on T cell regulation. Although the relative importance of IL-4 might well depend on the precise system under examination, we believe that the development of allograft tolerance in humans will surely represent such an extreme situation. We therefore suggest that manipulation of cytokines such as IL-4 should still be considered as part of an overall approach to improve the long term outcome of clinical transplantation.

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by low interleukin-2 and interferon-γ mRNA levels and by strong accumulation of major histocompatibility complex transcripts in the graft. Transplantation 54: 219.


