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Tracking the Immunoregulatory Mechanisms Active During Allograft Tolerance

Alberto Sánchez-Fueyo, Martina Weber, Christoph Domenig, Terry B. Strom, and Xin Xiao Zheng

Immunoregulatory mechanisms dependent on regulatory CD4+ T cells are believed to be critical in the maintenance of peripheral tolerance to allografts. However, a detailed characterization of the effects of these regulatory T cells has been hampered by the absence of a simple means to track and study them. In this work we provide evidence that in a murine model of islet transplantation the interactions between alloaggressive and regulatory T cells can be studied in vitro and in vivo at the single-cell level. The observations made in both an in vitro coculture system and an in vivo CFSE-based adoptive transfer model indicate that lymphocytes from tolerant allograft recipients 1) proliferate weakly to donor strain allogeneic cells but vigorously to third-party strain cells; and 2) suppress the proliferation of naive syngeneic CD4+ and CD8+ T cells to donor tissue in a cell dose- and Ag-specific manner. These effects depend on the presence of CD4+CD25+ T cells and are neutralized by anti-CTLA4 mAb or rIL-2. The principal effect of anti-CTLA4 is directed against the naive, not regulatory, T cell population. These results can be replicated in vivo by transferring lymphocyte populations into transplant recipients, proving that the graft-protecting actions of regulatory T cells are blunted by a rise in the number of allodestructive T cells (pool size model) and depend on the presence of CD4+CD25+ T cells and the integrity of the CTLA4/B7 pathway. The Journal of Immunology, 2002, 168: 2274–2281.

Immunological tolerance is defined as a state in which the immune system, in the absence of ongoing exogenous immunosuppression, does not mount a pathological response against specific Ags, while responses to other Ags are maintained (1). In several circumstances we have determined that unless lymphocyte depleting Abs are used or a state of mixed chimerism with massive central deletion is achieved, the induction of immunological tolerance to MHC-mismatched allografts requires the deletion of many alloreactive T cells (2–5). The critical role of deleterional mechanisms in the induction phase of transplantation tolerance to MHC-mismatched allografts is likely to be required, considering the remarkably large frequency of alloreactive T cells (6).

Although depletion of alloreactive clones may be critical to the induction of the tolerant state, the long-term maintenance of peripheral tolerance is believed to be dependent on self-perpetuating immunoregulatory mechanisms that actively constrain alloaggressive T cell-mediated immune responses (7–9). The presence of suppressor or regulatory CD4+ T cells (T reg) in tolerant hosts has been described by several laboratories, including our own (7–18). Regulatory CD4+CD25+ T cells, first identified as suppressor cells in 1990 (18, 19), have emerged as critical effectors in both the control of autoimmunity (20–23) and the maintenance of peripheral allograft tolerance (24, 25).

The finding in hosts mounting graft rejection of lymphocytes capable of prolonging graft survival upon adoptive transfer into naive graft recipients (15) suggests that activation of T reg is an integral component of the allograft response. In the absence of ongoing drug therapy, however, we believe that the more rapid expansion of alloaggressive T cells overrides the suppressor effect of T reg. We hypothesize, therefore, that the capacity of T reg to restrain naive lymphocytes from rejecting an allograft is not absolute and that it will fade as the numbers of potentially allodestructive T cells rise.

In this study we sought to characterize in detail, both in vivo and in vitro, the cells responsible for these immunoregulatory effects, focusing on their mechanism of action and costimulation requirements, and addressing how their impact on naive CD4+ and CD8+ T cells undergoing alloactivation fits into the predictions inherent in the pool size model of the allograft response (2).
B6AF1 recipient rendered diabetic by a single i.p. injection of streptozotocin (225 mg/kg; Sigma-Aldrich, St. Louis, MO). Allograft function was monitored by serial blood glucose measurements. Primary graft function was defined as a blood glucose level <200 mg/dl on day 3 post-transplantation, and graft rejection was defined as an elevation in blood glucose >300 mg/dl following a period of primary graft function.

Reagents and tolerizing protocol

B cell hybridomas were obtained from American Type Culture Collection (Manassas, VA) producing 1) a hamster mAb against mouse CD154 (MR1, IgG2a, ATCC HB11048) and 2) a rat anti-mouse CD25 (PC61.5.3, IgG1, ATCC TB222). The hybridoma cells were grown in UltraTrex culture media (BioWhittaker, Walkersville, MD), and the mAbs were affinity-purified using protein G columns. Anti-CTLA4 mAb (UC10-4F10-11) was provided by Dr. M. Sayegh (Brigham and Women’s Hospital, Boston, MA). rIL-2 was obtained from BD PharMingen (San Diego, CA), and a nonlytic IL-2/Fc fusion protein was provided by Dr. M. Sayegh (Brigham and Women’s Hospital, Boston, MA). Anti-CTLA4 mAb (UC10-4F10-11) was provided by Dr. M. Sayegh (Brigham and Women’s Hospital, Boston, MA).

In vitro MLR

Single-cell suspensions of splenic and lymph node MNLs from either naive or tolerant animals were adoptively transferred to B6AF1, irradiated transplant recipients, which were then transplanted with DBA2 (donor strain) or DBA1 (third-party strain) islet allografts. Additionally, 3.5 × 10^6 MNLs from naive or tolerant mice were CFSE-labeled and mixed together in equal proportions with unlabeled MNLs from tolerant or naive hosts, respectively. These cells were cotransferred into irradiated transplant recipients. Because cotransferred MNLs from either naive or tolerant mice could be labeled separately, this system enabled us to bidirectionally track the interactions between the lymphocytes from naive and tolerant hosts at the single-cell level.

Mice were sacrificed 8 days following transplantation, and single-cell suspensions were prepared from harvested spleens and lymph nodes. The actual size of the alloreactive T cell pool, because only the portion of the lymphocytes labeled with CFSE was included in the analysis, was ascertained by FACS analysis, was determined by FACS analysis, was >95% CD25^-. Viability, determined by trypan blue staining, exceeded 95% in all cases.

Labeling of MNLs with CFSE

Single-cell suspensions of splenic lymph node MNLs from either naive or tolerant B6AF1 mice were resuspended in HBSS at a concentration of 1×10^7cells/ml and labeled with the tracking fluorochrome CFSE (Molecular Probes, Eugene, OR). MNLs were incubated with anti-CD25 mAb-coated magnetic beads at a 1:2 bead to cell ratio for 30 min at 4°C with gentle rotation. CD25^+ T cells were isolated from the bead-cell mixture by exposure to a magnetic field using a magnetic particle concentrator (Dynal Biotech) according to the manufacturer’s instructions. The negatively selected CD25^- cells were collected, and their purity, determined by FACS analysis, was >95% CD25^-.

In vivo MLR

Single-cell suspensions of splenic lymph nodes and spleens were prepared, and RBC were removed by hypotonic shock. Magnetic beads coated with mAbs (Dynal Biotech, Oslo, Norway) were used to separate cells into CD25^+ and CD25^- subsets. Briefly, MNLs were incubated with anti-CD25 mAb-coated magnetic beads at a 1:1 head to cell ratio for 30 min at 4°C with gentle rotation. Selected CD25^+ T cells were isolated from the bead-cell mixture by exposure to a magnetic field using a magnetic particle concentrator (Dynal Biotech) according to the manufacturer’s instructions. The negatively selected CD25^- cells were collected, and their purity, determined by FACS analysis, was >95% CD25^-.

Adaptive transfer of naive and tolerant MNLs into irradiated transplant recipients

B6AF1 mice were irradiated with 1000 rad (GammaCell irradiator; Nordion, Kanata, Ontario, Canada) to effectively ablate their immune system, and various mixtures of MNLs from naive and/or tolerant hosts were injected through the tail vein. Mice were then transplanted with DBA2 or third-party strain (DBA1) islets. In the absence of lymphocyte transfer, irradiated recipients do not reject allografts and die at ~20 days of transplantation.

Statistical analyses

The nonparametric Mann-Whitney U test was performed to compare the responding frequencies of MNLs harvested from naive and tolerant hosts and transferred into irradiated transplant recipients. MNLs were recovered from the recipients’ lymph nodes and spleen as described above. MNLs were stained for 20 min on ice with a biotinylated Ab against mouse CD4 or CD8a ( GK1.5 and 53-6.7, respectively; BD PharMingen), and then stained with streptavidin-CyChrome and PE-conjugated annexin V, anti-mouse CD69 (H1.2F3), or anti-mouse CD25 (7D4) (all from BD PharMingen). Statistical analyses were performed using a log-rank test. A value of p < 0.05 was considered statistically significant.

Results

T cells harvested from tolerant and naive recipients differ in their ability to proliferate in response to donor strain splenic stimulator cells in vitro

We used a MLR system to compare the responses of MNLs from tolerant and naive hosts to donor (DBA2) or third-party (DBA1) alloantigen. The response of lymphocytes harvested from tolerant B6AF1 hosts to donor stimulator cells was dramatically less than that of lymphocytes obtained from naive B6AF1 mice (Fig. 1). Very similar results were obtained when cultures were harvested on day 4 or when fewer responding cells were used (data not
Moreover, the proliferation of lymphocytes from naive and tolerant mice, mixed together in equal proportions, was essentially equivalent to that of lymphocytes from tolerant hosts alone, suggesting that tolerant MNLs suppress the ability of naive T cells to proliferate in response to donor MNLs in the MLR. Identical results were obtained when the responding cells were harvested from naive and tolerant C57BL/6 mice (data not shown). Interestingly, the responses of naive and tolerant lymphocytes were equivalent when third-party strain stimulator cells were used (Fig. 1). Hence, T cells from tolerant recipients are anergic to donor alloantigen but mount a normal proliferative response when confronted with other alloantigens.

MNLs harvested from tolerant hosts restrain the capacity of naive T cells to proliferate in response to donor strain splenic stimulator cells in vitro

To test the hypothesis that the decreased proliferation in response to donor strain MNLs observed when naive and tolerant MNLs were mixed was due at least in part to a suppressive effect of tolerant MNLs on naive MNLs, we performed additional MLRs in which naive and tolerant MNLs were mixed and incubated with irradiated donor (DBA2) or third-party (DBA1) stimulator cells. Cell proliferation was estimated by [³H]Tdr incorporation on day 5. Data are expressed as the mean cpm ± SEM of triplicate cultures. This experiment was repeated eight times with similar results.

FIGURE 1. Lymphocytes harvested from tolerant hosts are hyporesponsive to irradiated donor-splenic MNL stimulator cells in the MLR but react normally to third-party stimulator cells. MNLs harvested from either naive or tolerant B6AF₁ hosts were cultured together with donor (DBA2) or third-party (DBA1) irradiated stimulator cells. Cell proliferation was estimated by [³H]Tdr incorporation on day 5. Data are expressed as the mean cpm ± SEM of triplicate cultures. This experiment was repeated eight times with similar results.

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MNLs harvested from tolerant hosts inhibit the proliferation of naive T cells harvested from naive mice in a donor-specific manner in vivo

Similarly, in the in vivo CFSE adoptive transfer model the profiles of the passively transferred MNL populations revealed that 1) in
control B6AF1 mice in which no grafts are placed or that are transplanted with isogenic B6AF1 islets, lymphocytes from tolerant and naive donors exhibit similar spontaneous homeostatic proliferation (Fig. 3A); 2) the proliferative response to donor strain (DBA2) islets of CD4+ and CD8+ T cells harvested from tolerant B6AF1 mice is significantly weaker than that of CD4+ and CD8+ T cells from naive B6AF1 mice (Fig. 3, B and D; mean decrease in responding frequency, 33 and 24%, respectively); 3) with cotransfer of MNLs from naive and tolerant hosts, the ability of CD4+ and CD8+ T cells from naive mice to proliferate in response to DBA2 grafts is impaired (Fig. 3, B and D; mean decrease in responding frequency, 30 and 22%, respectively). In contrast, CD4+ and CD8+ T cells from naive and tolerant hosts mount similar proliferative responses to third-party strain (DBA1) islets (Fig. 3C).

The hyporesponsive/suppressor effect of lymphocytes harvested from tolerant hosts can be negated by removal of CD4+CD25+ T cells or provision of either exogenous rIL-2 or anti-CTLA4 mAb

In the MLR model removal of CD25+CD4+ T cells restores the response of MNLs harvested from B6AF1 tolerant hosts or the mixture of MNLs from naive and tolerant B6AF1 mice to donor cells (Fig. 4A). Moreover, provision of either anti-CTLA4 mAb or rIL-2 to cultures containing either lymphocytes from tolerant B6AF1 hosts or a mixture of lymphocytes from tolerant and naive B6AF1 mice disrupts the hyporesponsive state and greatly increases the proliferative response to donor spleen MNLs (Fig. 4B). Interestingly, the ability of anti-CTLA4 mAb to enhance the proliferative response of T cells from tolerant and naive mice was noted even in cultures in which CD4+CD25+ T cells were removed (Fig. 4C). Similar results were observed when responding MNLs were harvested from naive and tolerant C57BL/6 mice (data not shown). The addition of a hamster Ig isotype control did not modify the proliferation of either naive or tolerant MNLs (data not shown).

In a passive transfer model of the allograft response, MNLs harvested from tolerant hosts inhibit the capacity of T cells from naive mice to reject allogeneic islets in a donor-specific and cell dose-dependent manner

In the absence of syngeneic MNL transfer, irradiated B6AF1 transplant recipients do not reject DBA2 islet allografts and die ~20 days post-transplantation. Adoptive transfer of 10 × 10⁶ pooled lymph node and splenic MNLs from naive, but not tolerant, B6AF1 mice uniformly leads to graft rejection (mean survival time (MST), 31 days; Fig. 5A). With transfer of 15–70 × 10⁶ MNLs from naive B6AF1 mice the rejection is accelerated (MST, 22 days; Fig. 5B). In contrast, adoptive transfer of 70 × 10⁶ MNLs from tolerant B6AF1 hosts does not lead to rejection in 30% of syngeneic recipients and, while the majority of recipients experience rejection, this process occurs at a slower pace than noted with transfer of MNLs from naive mice (MST, 38; p < 0.05; Fig. 5B). In contrast, transfer of 70 × 10⁶ MNLs from C57BL/6 tolerant hosts does not elicit rejection (data not shown). These experiments highlight that T cells capable of mediating allograft rejection persist in some, but perhaps not all, tolerant hosts. Finally, MNLs from both naive and tolerant B6AF1 hosts reject third-party (DBA1) islets at same tempo (Fig. 5B).

Passive transfer of a mixture of MNLs from naive and tolerant B6AF1 hosts was undertaken to determine whether MNLs from tolerant hosts inhibit the capacity of T cells from naive mice to reject islet allografts. Using a relative excess of tolerant to naive MNLs, 55 × 10⁶ MNLs from tolerant mice plus 15 × 10⁶ from naive mice, the ability of transferred T cells from naive mice to trigger rejection was significantly delayed (36 vs 22 days; p < 0.05; Fig. 5C). The ability of MNLs from tolerant hosts to suppress allograft rejection was donor strain specific, because this suppression was not observed when a mixture of MNLs from naive and tolerant mice was passively transferred into syngeneic recipients of third-party strain (DBA1) allografts (Fig. 5C).

In accordance with our pool size hypothesis, we found that the ability of MNLs from tolerant B6AF1 hosts to constrain rejection was dose limited. For example, at a mixture of 55 × 10⁶ MNLs from tolerant hosts with 15 × 10⁶ MNLs from naive mice, rejection was delayed (Fig. 5C). When the number of MNLs from naive mice increased while the number of MNLs from tolerant hosts decreased (mixture of 35 × 10⁶ tolerant with 35 × 10⁶ naive MNLs), tolerant MNLs were unable to inhibit allograft rejection (Fig. 5C).

Insofar as the MLR experiments (Fig. 4A) indicated that CD4+CD25+ T cells are potent immunoregulatory cells, we conducted parallel experiments in the passive transfer allograft response model to determine whether the effect of MNLs from tolerant hosts upon MNLs from naive mice was dependent on the presence of CD4+CD25+ T cells among the MNL populations harvested from B6AF1 tolerant hosts. As shown in Fig. 5D, removal of this subpopulation abolished the capacity of MNLs from tolerant hosts to delay graft rejection in combixing experiments.

Again based on a precedent in the MLR model (Fig. 4B), similar experiments were performed in which B6AF1, transplant recipients, receiving a mixed population of MNLs from naive and tolerant B6AF1 hosts, were treated with anti-CTLA4 mAb. Treatment with anti-CTLA4 mAb negated the graft-protecting effect of tolerant MNLs and actually accelerated the rate of graft rejection (Fig. 5D). By contrast, the administration of anti-CTLA4 mAb and anti-CD25 mAb (1 mg/kg for 1 mo) to B6AF1 tolerant hosts >120 days after transplantation failed to create allograft rejection or to prevent the engraftment of a second graft from the same donor (n = 2; data not shown).

Discussion

Many laboratories have shown that peripheral allograft tolerance is associated with the appearance of helper phenotype or CD4+ T cell-dependent immunoregulatory mechanisms (7–18). A detailed characterization of these immunoregulatory T cells has been hampered, however, by the absence of a simple means to track and study these cells both in vivo and in vitro.

We now provide evidence that the immunoregulatory consequences of the interaction between alloaggressive and T reg can be studied in vitro and in vivo at the single-cell level. Observations made in an MLR system indicate that 1) lymphocytes obtained from tolerant allograft hosts proliferate weakly in response to donor cells, but vigorously in response to third-party cells (Fig. 1); 2) these lymphocytes are not only hyporesponsive, but, as our experiments using the CFSE dye clearly reveal, they are also capable of suppressing the proliferation of both CD4+ and CD8+ T cells harvested from naive mice in response to donor cells (Fig. 2); 3) the anergic/suppressive effect of MNLs harvested from tolerant hosts is dependent on the presence of CD4+CD25+ T cells (Fig. 4A); and 4) the immunoregulatory effect can be ablated through provision of exogenous IL-2 or anti-CTLA4 mAb (Fig. 4B). Using the CFSE tracking dye method we were able to confirm that several of the observations made in the MLR model can be replicated in vivo. In these experiments we transferred CFSE-labeled MNLs from naive or tolerant hosts into syngeneic irradiated mice transplanted with allogeneic islets. In this model we determined that CD4+ and CD8+ T cells harvested from tolerant hosts proliferated...
IMMUNOREGULATORY MECHANISMS ACTIVE DURING ALLOGRAFT TOLERANCE

A

Responding frequency

CD4+ T cells
CD8+ T cells

Tolerant (CFSE)
Naive (CFSE)

B

Responding frequency

CD4+ T cells
CD8+ T cells

Naive (CFSE) + Naive
Tolerant (CFSE) + Naive
Naive (CFSE) + Tolerant
Tolerant (CFSE) + Tolerant

C

Responding frequency

CD4+ T cells
CD8+ T cells

Tolerant (CFSE)
Naive (CFSE)

D

Cell Divisions: 7 6 5 4 3 2 1 0

% Total cells in each division cycle

PF = 14%

Tolerant (CFSE) + Tolerant

PF = 17%

Naive (CFSE) + Tolerant

PF = 29%

Naive (CFSE) + Naive
FIGURE 3. Lymphocytes harvested from tolerant hosts are hyporesponsive to donor islets when transferred into transplant recipients, and they suppress the proliferation of naive lymphocytes in response to donor islets. CFSE-labeled MNLs (7 × 10^3) from either naive or tolerant B6AF1 mice were adoptively transferred into B6AF1 irradiated recipients, which were then transplanted with DBA2 (donor) or B6Al (third-party) islet allografts. In parallel, 3.5 × 10^7 CFSE-labeled naive or tolerant MNLs were mixed with 3.5 × 10^7 unstained tolerant or naive MNLs, respectively, and transferred into the irradiated recipients. On day 8 transplant recipients were sacrificed. In vitro (Fig. 3A) and in vivo (Fig. 3B). Indeed, tolerant MNLs appear to render naive T cells anergic to donor alloantigen, and this process is specific for donor cells. Further exploration of these experimental systems should enable a thorough dissection of the molecular basis for infectious tolerance.

The role of CD4+CD25+ T lymphocytes as suppressor or regulatory cells is well established in several autoimmune models (20, 31). In a rat allograft model IL-2R+ T cells were identified as essential to the immunoregulatory cell process (18). In several more recent reports the CD25+ marker or the CD45RBlow phenotype have been used to identify T reg among the lymphocytes of tolerant allograft recipients (24, 30) or in a graft-vs-host disease model (25). The effector function of CD4+CD25+ T reg has been studied in vitro with respect to the sensitivity to anti-CTLA mAb. The results have not been uniform; in some settings anti-CTLA4

weakly upon encounter with donor, but not third-party, alloantigen. Moreover, MNLs from tolerant hosts restrain the proliferation of both CD4+ and CD8+ T cells from naive syngeneic mice in an Ag-specific fashion (Fig. 3, B and C).

In addition, the present study clearly shows, using an in vivo adoptive transfer model of the allograft response, that the graft-protective effect of regulatory lymphocytes is far from infinite. The clinical outcome, rejection or tolerance, resides in a fragile balance between the contingent immunoregulatory and alloaggressive T cells. A certain ratio of regulatory to alloaggressive lymphocytes is required to produce effective donor-specific suppression of allograft rejection (Fig. 5C). These findings are in agreement with our pool size model (2), which hypothesizes that depletion or inactivation of alloaggressive T cells during the period of tolerance induction is required to permit the pool size of T reg to reach the critical levels that enable them to dominate the allograft response. In accordance with results in both allograft (24, 25, 30) and autoimmune models (20, 22, 31), CD4+CD25+ T cells are required for effective immunoregulation (Fig. 5D).

In concert with the implications inherent in the model of infectious tolerance (7–9), MNLs harvested from tolerant hosts inhibit the ability of naive T cells to respond to donor alloantigen in vitro (Fig. 2A) and in vivo (Fig. 2B). Indeed, tolerant MNLs appear to render naive T cells anergic to donor alloantigen, and this process is specific for donor cells. Further exploration of these experimental systems should enable a thorough dissection of the molecular basis for infectious tolerance.

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FIGURE 4. The presence of CD4+CD25+ T 0 cells is required for T reg activity, and T reg action is neutralized by provision of exogenous rIL-2 or anti-CTLA4 mAb. A. MNLs harvested from either naive or tolerant B6AF1 hosts were cultured together with donor (DBA2) irradiated splenic stimulator cells. Identical experiments were performed after removal of the CD4+CD25+ T cell subset from the tolerant responding cells with anti-CD25-coated magnetic beads. B. MNLs harvested from either naive or tolerant B6AF1 hosts were cultured together with donor (DBA2) irradiated stimulator cells in the presence of exogenous IL-2 (100 U/ml) or anti-CTLA4 mAb (25 μg/ml). C. MNLs harvested from either naive or tolerant hosts were depleted of the CD4+CD25+ T cell subset and cultured with donor (DBA2) irradiated stimulator cells in the presence of anti-CTLA4 mAb (25 μg/ml). Cell proliferation was compared with that of unfractonated MNLs from naive and tolerant hosts. Cell proliferation was estimated by [3 H]TdR incorporation on day 5. Data are expressed as the mean cpm ± SEM of triplicate cultures of one representative experiment. These experiments were repeated three times each with similar results.
mAb blocks T reg effects (20), while the action of T reg is insensitive to anti-CTLA mAb in other models (32). Because of uncertainties regarding the consequences of anti-CTLA4 mAb for T reg function, we undertook parallel experiments in the MLR and adoptive transfer models of transplant biology.

Our data indicate that an intact B7/CTLA4 pathway is required to maintain the anergic/suppressor phenotype of tolerant lymphocytes in the MLR (Fig. 4B). In this system anti-CTLA4 mAb acts at least in part by increasing the proliferation of conventional CD25⁺ T cell subsets in response to donor Ag even in the absence of CD4⁺CD25⁺ T reg (Fig. 4C). The validity of these observations made in an in vitro model are strongly supported by the experiments summarized in Fig. 5D. While adoptive transfer of MNLs from tolerant hosts comixed with MNLs from naive hosts...
organ allografts by thymocytes: specific unresponsiveness by thymocyte transfer. J. Exp. Med. 149:1042.


