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T Cell Subsets and In Vitro Immune Regulation in “Infectious” Transplantation Tolerance

Yuan Zhai,* Xiu-Da Shen,* Manfred Lehmann,† Ronald Busuttil,* Hans-Dieter Volk,‡ and Jerzy W. Kupiec-Weglinski*‡

CD4-targeted mAb therapy results in permanent acceptance of cardiac allografts in rat recipients, in conjunction with features of the infectious tolerance pathway. Although CD4+ T cells play a central role, the actual cellular and molecular tolerogenic mechanisms remain elusive. This study was designed to analyze in vitro alloimmune responses of T lymphocytes from CD4 mAb-treated engrafted hosts. Spleen, but not lymph node, cells lost proliferative response against donor alloantigen in MLR and suppressed test allograft rejection in adoptive transfer studies, suggesting compartmentalization of tolerogenic T cells in transplant recipients. A high dose of exogenous IL-2 restored the allogeneic response of tolerogenic T cells, indicating anergy as a putative mechanism. Vigorous proliferation of the tolerogenic T cells in in vivo MLR supports the existence of alloreactive lymphocytes in tolerogenic T cell repertoire and implies an active operational suppression mechanism. The tolerogenic splenocytes suppressed proliferation of naive splenocytes in vitro, consistent with their in vivo property of dominant immune regulation. Finally, Vigorous proliferation of the tolerogenic T cells in in vivo MLR supports the existence of alloreactive lymphocytes in tolerogenic T cell repertoire and implies an active operational suppression mechanism. The tolerogenic splenocytes suppressed proliferation of naive splenocytes in vitro, consistent with their in vivo property of dominant immune regulation. Finally, Vigorous proliferation of the tolerogenic T cells in in vivo MLR supports the existence of alloreactive lymphocytes in tolerogenic T cell repertoire and implies an active operational suppression mechanism. The tolerogenic splenocytes suppressed proliferation of naive splenocytes in vitro, consistent with their in vivo property of dominant immune regulation.

The infectious tolerance pathway represents one of the putative mechanisms by which the host immune system can be reprogrammed and then guided to accept a transplant or to arrest autoimmunity (1, 2). Indeed, CD4-targeted therapy can induce a form of allospecific tolerance in both mouse and rat transplant models, which can be then adoptively transferred by CD4+ regulatory T cells into new cohorts of naive test recipients (3, 4). The most prominent feature was the demonstration that once induced, tolerance could be maintained and perpetuated by what has been termed an infectious T cell-dependent regulatory mechanism. Accordingly, T cells from tolerant hosts can disable naive lymphocytes so that they too fail to trigger rejection. These, in turn, may become tolerant by themselves, developing the capacity to disable naive lymphocytes after adoptive transfer into new test recipients. Although the number of original cells from mAb-treated recipients had become diluted out, the capacity to induce tolerance is amplified over multiple cell generations in new cohorts of engrafted animals (5). Hence, the maintenance of such a tolerant state results from a regulatory mechanism that becomes dominant under the cover of initial mAb therapy. Once established, the infectious mechanism requires continuous or closely repetitive Ag stimulation and is quite robust (6, 7). This process may explain why no further immunosuppression is needed to maintain tolerance-permissive environment.

We have previously reported that 1) treatment with RIB-5/2, a CD4-nondepleting mAb, produces indefinite survival of LBNF1 cardiac allografts in presensitized Lewis (LEW) rat recipients (4); 2) donor-specific and organ nonspecific tolerance can be then transferred by spleen cells into new cohorts of test graft recipients (4); 3) regulatory CD4+ T cells are instrumental in this model, as their selective depletion prevents the acquisition of tolerance (4); 4) thymus-dependent regulatory CD4+ T cell-mediated effective memory and suppression depend on the presence of donor Ag rather than “graft adaptation” (7); and 5) selective up-regulation of IL-4 at the graft site is useful for tolerance maintenance in test rat recipients conditioned with regulatory T cells (8). Hence, both local type 2 cytokine expression pattern in the graft itself and circulating regulatory T cells influence allograft outcome in the infectious tolerance pathway (9).

Our current knowledge of the infectious tolerance mechanisms derives primarily from in vivo experiments in animal transplantation models. Indeed, tolerogenic lymphocytes in such allograft recipients have not been characterized in vitro, which hampers our appreciation of the cellular and molecular mechanisms underlying this dominant immune regulation pathway. In this study, we attempted to establish an in vitro cell culture system that would recapitulate some cardinal in vivo features of the infectious tolerance pathway. Our results demonstrate: 1) selective compartmentalization of tolerogenic IL-2-responsive T lymphocytes in the spleen of long term allograft recipients; 2) vigorous in vivo proliferation of T cells from tolerant hosts in allogeneic donors; 3) the ability of splenocytes from tolerant hosts to suppress proliferation of naive splenocytes in the coculture assay, an ultimate test for a dominant immune regulation; and 4) the CD45RC− subset in tolerogenic T cells hyporesponsive to alloantigen and able to impose the suppressive function upon normal T cells. To the best of our knowledge, these studies are the first to provide direct evidence that a nondeletional, anergy-like regulatory mechanism(s) might...
operate via a discrete T cell subset in the infectious tolerance pathway.

Materials and Methods

Animals and grafting techniques

Inbred male adult rats, weighing 200–250 g, were used (Harlan Sprague-Dawley, Indianapolis, IN). LEW (RT1b) rats served as recipients of cardiac allografts from (LEW × BNF1, LBNF1) hybrids. Brown-Norway (BN; RT1b) rats were used as skin donors, and Wistar-Furth (WF; RT1b) served as heart/cell donors for specificity studies. Full thickness skin was sutured bilaterally to appropriate defects in the chest of prospective recipients. Hearts were transplanted to the abdominal great vessels by standard microvascular techniques. Graft function was monitored daily by palpation, and rejection was defined as the day of cessation of heartbeat.

Allograft model

LEW rats were sensitized with BN skin grafts (day −7), followed 1 wk later by transplantation of LBNF1 hearts (day 0). These cardiac allografts are rejected in an accelerated manner in <36 h (acute rejection occurs in 7–8 days) (10). Animals were treated with CD4 nondepleting (RIB-5/2) mAb (5 mg/rat i.v. on days −7 (day of skin graft), −4, −1, and 0 (day of heart graft) and on days 1, 4, 7, 10, 14, and 21 thereafter). This regimen induces >100-day acceptance of cardiac allografts, with concomitant cardiac features of the infectious tolerance pathway (4, 7–9).

MLR in vitro and in vivo

Rat splenocytes or lymph node (LN) cells were prepared as single-cell suspensions. RBC were lysed by RBC lysis buffer (Sigma, St. Louis, MO). After washing twice with RPMI 1640 medium and 1% FBS, cells were resuspended in culture medium (RPMI 1640 supplemented with 20 mM HEPES, 10 mM sodium pyruvate, 2 mM l-glutamine, 50 mM 2-ME, 1× MEM-nonessential amino acid solution, 1× MEM-vitamin solution, 1× antibiotic/antimycotic solution, and 10% FBS) at 5 × 10^6/ml. One hundred microliters of LEW lymphocytes were added to a U-bottom 96-well plate (Corning Glass, Corning, NY), mixed with the same volume of gamma-irradiated (2000 rad) alloimmune donor-type (BN/LBNF1) or third-party (WF) splenocytes in the presence of 2 mM N^2-monomethyl-l-arginine, an inhibitor of the macrophage form of NO synthase. There were four replicates for each reaction combination. Con A (2 µg/ml) was used as a positive control. [3H]Thymidine (1 µCi) was added to each well in the last 16–18 h of culture. LEW cells were harvested onto filters (Skatron, Sterling, VA) with a Skatron 12-well semiautomatic cell harvester. The cpm of the filter membrane were measured in scintillation liquid (Cytoscnt; ICN Bio-medical, Costa Mesa, CA) on an LS 6000IC Beckman Coulter, Palo Alto, CA).

In vivo MLR, LEW rat lymphocytes were labeled with CFSE (Molecular Probes, Eugene, OR) at 4 nM in PBS for 15 min at 37°C. The unconjugated CFSE was eliminated by washing with cells with FBS (20%), supplemented RPMI medium. The labeled cells were resuspended in PBS at 5 × 10^6/ml, and 2 ml of these cells (1 × 10^6) were injected into gamma-irradiated (1000 rad) BN (or LEW as negative controls) rats via the tail vein. On day 3, spleens were harvested, and splenocytes were stained with anti-rat TCR αβ-PE (R73), CD4-CyChrome (OX-35, BD Pharmingen, San Diego, CA), Topro 3 (1 nM; Molecular Probes) was added as a viable dye. Four-color flow cytometry was performed on a FACS Calibur dual-laser cytometer (BD Biosciences, Mountain View, CA). Topro 3-negative (viable) cells in the lymphocyte gate stained positively for TCR αβ and CD4 were analyzed for CFSE intensities.

Adoptive cell transfer studies

To determine the presence of regulatory T cells, we performed adoptive transfer studies in which 100 × 10^6 ethrythrocyte-free spleen or LN cells were administered i.v. to lightly gamma-irradiated (450 rad) syngeneic secondary recipients. These were challenged 24 h later with donor-specific test cardiac allografts. We have used this in vivo adoptive cell transfer system previously (4, 7–9). Indeed, gamma-irradiation does not affect host alloreactivity per se, as the majority of these recipients reject their transplants in a normal acute fashion (8–10 days), elicit a brisk (4–7 days) re- jection after transfer of splenocytes from sensitized rejecting controls, yet maintain test grafts indefinitely after infusion of spleen cells from CD4 mAb-pretreated long term tolerant hosts. The cells successfully engraft after adoptive transfer, and their immune potential may be accurately determined in gamma-irradiated “test-tube” rats.

Abbreviation used in this paper: LN, lymph node.

Isolation of T cell subsets

Single-cell spleen cell suspensions (1–2 × 10^6) were enriched for T cells with a nylon wool column (10 ml; PolyScience, Warrington, PA). T cells were then incubated with CD45R/C mAb (OX-22, BD Pharmingen) at 4°C for 30 min with 1 × 10^5 cells/5 µg Abs). After washing, Dynal beads coated with anti-mouse IgG (CETEC, Leiden, Netherlands) and Dynal, (Dyna) coated mouse IgG (Dyna, Dalian, China) were added to the Ab-coated T cells to separate negatively selected CD45R/C and CD45R/C cells, according to the manufacturer’s manual. Cell subsets were resuspended at 1–2 × 10^6/ml, and cells (100 µl/well) were used for MLRs. In mixing cultures, 1 × 10^6 CD45R/C cells or 2 × 10^6 CD45R/C cells were mixed with 5 × 10^5 T cells from naive or rejecting recipients.

Results

Splenocytes, but not LN cells, from tolerant rats are hyporesponsive to alloantigen in vitro

Lymphocytes from groups of age-matched untreated LEW hosts (rejected BN skin and LBNF1 cardiac grafts), and long term (>100 days) tolerant CD4 mAb-treated cardiac graft recipients were analyzed for in vitro alloreactivity in the MLR assay. As shown in Fig. 1A, splenocytes, but not LN cells, from tolerant hosts had significantly lower proliferative responses against donor-type allograft; the magnitude of cell proliferation within the tolerant splenocyte pool was depressed to ~15–25% of that in rejecting controls. Polyclonal activation with Con A stimulated proliferation of all T cell groups, indicating no general defects in the ability of proliferation in tolerant splenic T cells. FACScan staining of CFSE-labeled lymphocytes after 4-day culture showed the CD4^+ T cells to be the dominant cell type in the allosensitive population and confirmed diminished frequency of tolerant cells that proliferated after alloantigen stimulation (Fig. 1B). Addition of Con A restored CD4^+ T cell proliferation in vitro.

Splenocytes, but not LN cells, from tolerant rats confer unresponsiveness to alloantigen in vivo

We then performed adoptive transfer experiments to correlate T cell hyporesponsiveness in vitro with their in vivo ability to suppress allograft rejection. Groups of lightly irradiated (450 rad) secondary syngeneic recipients were infused with either splenocytes or LN cells (100 × 10^6) from tolerant donors (>100 days post-transplant), followed 1 day later by cardiac engraftment. Indeed, in agreement with our previous findings (4), all test cardiac allografts survived long term (>100 days) in secondary recipients, which were injected with tolerant splenocytes (n = 6; data not shown). In contrast, test cardiac allograft survival of 8.4 ± 2.3 days (mean ± SD; n = 7) after transfer of LN cells from tolerant hosts was not different from those recorded in irradiated, otherwise unmodified recipients (10.3 ± 7.3 days; n = 12) or in those given normal naive cells (6.8 ± 0.5 days; n = 4). Collectively, these results document the compartmentalization of tolerogenic T lymphocytes in the infectious tolerance pathway with spleen, but not LN, cells showing hyporesponsiveness to alloantigen stimulation in vitro as well as operational activity in the in vivo adoptive transfer system. Therefore, to explore putative immune regulation mechanisms leading to transplantation tolerance, we focused on spleen cells from long term cardiac allograft recipients.

In vivo MLR reveals the existence of alloreactive CD4^+ T cells in tolerant splenocytes

A number of interlocked immune mechanisms may contribute to hyporesponsiveness in vitro and in vivo. The deletion of alloreactive T cells irreversibly decreases clonal size in the lymphocyte population. In addition, regulatory T cells, induced under the cover of CD4–targeted therapy, may act on otherwise alloreactive T cells to prevent their activation within a normal lymphocyte pool. In the
latter case, close cell-cell contact may be required (11). In vivo MLR provides an ideal setting to differentiate between deletional and nondeletional mechanisms. Hence, CFSE-labeled splenocytes from tolerant LEW recipients of LBNF1 heart grafts (>100 days post-transplant) were injected i.v. into gamma-irradiated (1000 rad) LEW syngeneic hosts, against irradiated (2000 rad) LEW (syngeneic, Syn) or BN (allogeneic, Allo) splenocytes. One million LEW cells were cultured with an equal number of stimulator cells or 2 μg/ml ConA (four replicates per sample) for 4 days. Cells were pulsed with [3H]Thymidine (1 μCi/well) and harvested 16–18 h later, and [3H]Thymidine incorporation was counted. The average cpm ± SD for each sample was charted. Note that splenocytes, but not LN cells, from tolerant hosts were hyporesponsive to alloantigen in vitro. Results are representative of four independent experiments. B, CFSE-labeled LEW splenocytes from rejecting (Rej) or tolerant (Tol) cardiac allograft recipients were incubated with either allogeneic BN splenocytes (2000 rad) or Con A (2 μg/ml) in bulk cultures. On day 4, cells were analyzed by FACS for staining with fluorochrome-labeled Abs. Viable CD4+ or CD8+ cells were gated, and CFSE intensities were plotted. The upper panel depicts dot blots for CD4+ and CD8+ cells, whereas the lower panel summarizes proliferation of CD4+ cells. Fewer numbers of tolerant CD4+ splenic T cells proliferated upon allostimulation, whereas Con A-stimulated vigorous proliferation of both rejecting and tolerant T cells. Results are representative of two independent experiments.

alloreactive T cells do exist in the tolerogenic splenocyte pool, and that regulatory T cells may impose immunosuppression in vitro via a close cell-cell contact. However, in the in vivo setting with regulatory and other T cells dispersed, activation and proliferation of alloreactive T cells may readily occur.

High concentration of exogenous IL-2 restores proliferation of tolerogenic splenocytes in vitro
To examine the role of IL-2 in the hyporesponsiveness of tolerogenic splenocytes against alloantigen, exogenous IL-2 was added to the in vitro MLR. As shown in Fig. 3, with increasing doses of IL-2 the magnitude of proliferation was boosted in tolerogenic splenocyte cultures. However, the dose of IL-2 required to restore normal proliferation in vitro was relatively high. Indeed, IL-2 at 10 U/ml, a dose known to recover anergic T cells (13), increased the proliferation level only to ~33% of that in the control. The allospecific proliferation indexes did not change significantly, as inclusion of IL-2 boosted syngeneic cell proliferation as well. Thus, two possibilities can be envisioned: 1) tolerogenic splenocytes may have contained an anergic T cell component; or 2) IL-2 could abrogate the regulatory T cell-driven suppression.

Activated tolerogenic splenocytes inhibit proliferation of normal splenocytes in vitro
One of the cardinal features of regulatory T cells in vivo is their ability to prevent other alloreactive T cells from rejecting the grafts (3, 4). To mimic such a regulatory function in vitro, we have established a coculture system in which equal numbers of splenocytes from different sources were mixed (1:1 ratio), and the resulting total proliferation was analyzed. Fig. 4 depicts results of a typical mixing experiment in which tolerogenic and normal splenocytes were added in concert to in vitro MLR. Interestingly, tolerogenic splenocytes not only lost alloreactive responses against alloantigen, but also suppressed the proliferation of normal splenocytes. Indeed, a half-million tolerogenic splenocytes mixed with the same number of normal splenocytes at the start of a 4-day culture reduced the proliferation of normal splenocytes to ~10% compared with that of cultured cells with alloantigen alone. Thus, spleen cells in animals tolerized in an infectious manner can disable naive lymphocytes so that they fail to proliferate in vitro.

Allo-hyporesponsiveness and suppression are restricted to CD45RC+ T cell subset in tolerant hosts
We have previously shown that a population of CD4+ T cells was responsible for immune suppression in vivo, as evidenced by prevention of test allograft rejection in an adoptive transfer system (4). To further dissect the immunoregulatory T cell network, we separated T lymphocytes based on their CD45RC levels. As shown in Fig. 5, if CD25 staining was applied to CD45RC subsets, most CD25+ cells were detected in the CD45RC− T cell fraction. Both CD45RC+ and CD45RC− T cells from rejecting recipients responded vigorously to alloantigen in in vitro MLR (Fig. 6). In contrast, although unseparated T cells from tolerant hosts revealed a lower proliferative response to donor Ag, the CD45RC− subset showed a relatively normal response. The hyporesponsiveness of tolerant T cells was restricted selectively to the CD45RC+ subset.

LEW cells consistently revealed significantly lower proliferation against third-party (WF) cells in vitro, while testing them under the conditions for LEW vs BN/LBNF1 reaction. When we compared the responses of tolerant vs rejecting T cells against WF Ag, we observed comparable levels of proliferation for both T cell types, indicating donor-specific down-regulation of alloreponses in the tolerant T cell pool (Fig. 6).
Consistent with our results from one-way MLR, CD45RC+, but not CR45RC+, T cells from tolerant recipients imposed suppressive function and significantly \( p < 0.007 \) inhibited the proliferation of normal T cells against alloantigen in the coculture assay (Fig. 7).

Discussion
The principal findings of this study, which was designed to analyze in vitro alloimmune responses of lymphocytes from tolerant rat recipients of cardiac allografts, are as follows: 1) Spleen, but not LN, cells lost proliferative response against donor alloantigen. This correlated with the ability of spleen, but not LN, cells to suppress test allograft rejection in vivo after adoptive transfer. 2) A high dose of exogenous IL-2 restored proliferation of tolerogenic cells. 3) In vivo MLR with CFSE-labeled cells has revealed a comparable proliferation profile of splenocytes from rejecting and tolerant hosts following transfer into allogeneic donor-type test recipients. 4) Tolerogenic splenocytes suppressed the proliferation of naive splenocytes in the coculture assay. 5) The CD45RC+ T cell subset in tolerogenic spleen cells was hyporesponsive to alloantigen stimulation and suppressed proliferation of normal T cells in the coculture assay. Hence, nondeletional, anergy-like active regulatory mechanisms may operate in the infectious tolerance pathway via CD4+CD45RC+ T cells within the tolerant splenocyte, but not LN, pool.

The divergent ability of lymphocytes from spleen and LN to affect in vitro and in vivo alloimmune responses illustrates a previously overlooked aspect, i.e., the compartmentalization of the putative regulatory T cells in long term tolerant graft recipients. Indeed, only splenocytes from CD4 mAb-treated tolerant rats had...
a markedly reduced allo-proliferative response in MLR. Furthermore, tolerant spleen, but not LN, cells were capable of suppressing the proliferation of naive splenocytes in the coculture assay. Others have shown that LN cells have lower proliferative responses against Con A stimulation compared with splenocytes, possibly due to the relative lack of accessory cells (14). Consistent 

with our present in vitro data, spleen, but not LN, cells adoptively transferred transplantation tolerance into new cohorts of syngeneic test animals. Thus, it is plausible that regulatory T cells preferentially accumulate in the spleen, or the frequency of regulatory T cells in the spleen is much higher compared with that in the LN compartment. Recent studies have also shown that regulatory T cells may express a memory-type phenotype (15, 16). Perhaps memory T cells fail to sequester in LN because they lack L-selectin Ags on their surface (17). Indeed, we have consistently observed much lower frequency of CD3+CD4+CD45RClow T cells in LN compared with the spleen (14 vs 38%) of tolerant rat recipients (Y. Zhai and J. W. Kupiec-Weglinski, unpublished observations). Alternatively, the development of regulatory T cells, which requires the direct alloantigen contact may occur preferentially in recipient’s spleen. Moreover, preliminary data show a very early accumulation of regulatory T cells in the graft itself, i.e., at the site of direct Ag contact (B. Sawitzki and H.-D. Volk, manuscript in preparation). Collectively, although we favor the idea that regulatory T cells recognize Ag through direct allo-presentation, we cannot exclude a possible role of indirect presentation in our experimental system. Indeed, the latter has been recently suggested to play a role in the acquisition of tolerance in a mouse skin transplant model (18).

It was initially surprising to find out that CFSE-labeled tolerogenic splenocytes proliferated vigorously in vivo following infusion into allogeneic test rats, considering their relatively low allogeneic responsiveness in vitro. However, because alloreactive T cells were not deleted in the tolerogenic splenocyte pool in our model, the in vivo MLR setting may have disrupted the conditions required for active immune regulation. As shown in some autoimmune disease models, close cell-cell contact may be required for regulatory T cells to exert immunosuppressive effects on other T cells (11). It is doubtful that regulatory T cells and other alloreactive T cells interact closely with APCs in the allograft-loaded environment of allogeneic recipients. The situation is different in adoptively transferred test allograft recipients, in which regulatory T cells are able to prevent T cell activation, because the site of Ag
contact is restricted to the graft and the draining lymph nodes, allowing a more direct cell-cell contact similar to that occurring in in vitro cultures. Alternatively, in vivo milieu (e.g., high IL-2 levels induced by gamma irradiation) may break the anergy state of alloreactive T cells or abrogate suppression of alloreactive lymphocytes by regulatory T cells (10). Consistent with the latter hypothesis, a high dose of exogenous IL-2 successfully restored allo-proliferative in vitro response of tolerogenic splenocytes in this study.

The recapitulation of in vivo tolerogenic effects of splenocytes from CD4 mAb-pretreated recipients in in vitro cell culture system provides us with a unique opportunity to dissect immune mechanisms at work in the infectious tolerance pathway. The isolation of a T cell subset that imposes suppression on other T cells is also consistent with our in vivo MLR studies, which showed persistence of alloreactive cells in the tolerant splenic T cell pool. Two major regulatory pathways in autoimmune disease models involve cytokines and cell-cell contact (19). Rat regulatory CD4+ T cells were found to be of CD45RClow phenotype and to produce IL-2 and IL-4, but not IFN-γ, upon in vitro stimulation (20). Moreover, IL-4 and TGF-β are critical in preventing autoimmunity, as neutralization of either of the two cytokines abrogated the protective effects of normal syngeneic CD4+ T cells in the adoptive transfer system (21). Our finding that the tolerant CD45RC+ population contained regulatory T cells, which were both hyporesponsive to alloantigen stimulation and able to suppress proliferation of naive T cells in vitro, indicates that cells involved in the acquisition of transplantation tolerance might represent a different regulatory T cell lineage compared with those in autoimmunity. A subset of memory CD4+ T cells, identified by the naive phenotype of CD45RC+, has been shown to derive from CD45RC− memory cells in the absence of stimulating alloantigen and to be relatively long-lived compared with CD45RC− memory T cells (22). The tolerant splenocytes in our studies were harvested from recipients bearing long term (>100 days) cardiac allografts. As donor-type APCs (direct allo-recognition) for CD4 T cells at this late time point may have been diminished, CD45RC+ regulatory T cells in this study may well represent CD45RC− ‘revertants.’

Although inhibition of the normal T cell response by tolerant CD45RC+ in the coculture assays was not that dramatic, it was statistically significant (p < 0.007). These cells went through extensive manipulation in vitro, and the culture conditions may not have been optimal to exert their immunosuppressive functions. Therefore, it is reasonable to suggest that under optimal conditions and without much in vitro stress, the tolerant CD45RC+ T cells will be much more effective in suppressing other cells in vivo. Indeed, future adoptive transfer experiments should determine whether the CD45RC+ T cell subset exerts similar allo-hypo-responsive and suppressive properties in vivo. As for the CD25 phenotype, we have observed that most of the CD25+ T cells resided within the CD45RC− subset in both tolerant and rejecting recipients’ spleens. Because the efficacy of our Dynal bead-assisted separation of CD25+ T cells was very low, we were unable to set up sufficient numbers of CD25+ T cells in MLRs. However, based on the CD25 staining profile and CD45RC subset results, we suspect that CD25+ cells in tolerant recipients will be characterized by relatively normal proliferative responses.

We have detected increased production of IL-4 and IL-10 in tolerant splenocytes even after polyclonal T cell activation (Y. Zhai and J. W. Kupiec-Weglinski, unpublished observations). Such a Th2-type deviation was associated with the tolerant state and restricted to a small population of CD4+ T cells. This correlates with the results of our adoptive transfer studies in which infusion of tolerogenic spleen cells led to selective up-regulation of Th2-type cytokines at the graft site of secondary test recipients (8), whereas depletion of CD4+ cells in the transferred inoculum prevented such an up-regulation and abolished the infectious tolerance pathway (4).

In addition to soluble mediators, cell-cell contact may be critical in allotransplantation tolerance (23). As shown by several recent studies, anergic T cells can act as suppressor cells both in vitro and in vivo, possibly via a mechanism of competition for the APC surface and locally produced IL-2 (13, 24, 25). In mouse autoimmune disease models, anergic CD4+ regulatory T cells express IL-2R α-chain and fail to proliferate upon TCR stimulation, yet they effectively suppress the proliferation of naive T cells (11). Exogenous IL-2 or anti-CD28 mAb restore the proliferation of CD25+ CD4+ T cells by anti-CD3 stimulation and abolish suppression in a CD25− and CD25+ T cell mixture (11, 26).

In summary, we have established an in vitro experimental system to analyze the function of regulatory T cells from an in vivo model of infectious transplantation tolerance in rats. Our results are the first to provide direct evidence for nondeletional, anergy-like active regulatory mechanisms that operate in vitro via the CD45RC+ subset in the spleen, but not LN, compartment of tolerant hosts. Future in vitro studies should identify distinctive CD4+ T cell subsets (CD25, CD62 ligand) that may give rise to the alloimmune regulatory T cells and determine whether close cell-cell contact and APCs, on the one hand, and type 2 cytokines, on the other, are indeed required for the acquisition of infectious tolerance in transplant recipients.

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References


