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Oral Exposure to Alloantigen Generates Intrgraft CD8+ Regulatory Cells

Juan Zhou,*† Ronald I. Carr,*† Robert S. Liwski,*† Andrew W. Stadnyk,*§ and Timothy D. G. Lee2*†

We have previously reported that oral administration of allogeneic rat spleen cells before kidney allotransplantation significantly prolongs graft survival. This prolongation was alloantigen specific and was associated with a decrease in graft-infiltrating cells (GIC) and an increase in transcription of IL-4 mRNA in the GIC. In this study increased splenic mixed lymphocyte responses from animals orally exposed to alloantigen before kidney transplantation suggested that the kidney allograft prolongation was not due to a masking of allore cognition, but to an immunomodulation of the immune response. We have assessed GIC T cell subsets on day 5 post-transplant and found decreased numbers of CD4+ T cells in fed animals compared with controls, but there was no change in CD8+ T cell numbers. The CD8+ GIC from fed animals transcribed substantial levels of perforin, granzyme, and Fas ligand mRNA, indicating the presence of active CTL. Direct CTL assays showed that the GIC from fed recipients exhibited higher allo-CTL activity than GIC from control unfed recipients. In addition, the CD8+ GIC exhibited high levels of IL-4 mRNA, suggesting Tc2-type regulatory cells. Prolonged graft survival in the face of active CTL and Tc2 cells suggests the presence of a CD8+ regulatory cell population in the allograft. To confirm this, cell transfer experiments were performed. Prolongation of graft survival was transferred from rats orally exposed to alloantigen to naive animals by transfer of CD8+ GIC. This is the first report that oral exposure to alloantigen prolongs kidney allograft survival by the generation of intrgraft CD8+ regulatory cells. The Journal of Immunology, 2001, 167: 107–113.

Oral administration of Ag to ameliorate autoimmune disease has been demonstrated in a number of experimental animal models (1–3). Recently, trials have been initiated to assess the feasibility of such treatments in the reduction of human disease (4). However, prolongation of allograft survival by oral administration of alloantigen has been less well established. Weiner and colleagues reported that oral administration of allogeneic spleenocytes ameliorated allosensitization by skin grafting, but only observed effects on second set (not primary) rejection of solid organ allografts with their feeding protocol (5, 6). We subsequently demonstrated, using a different protocol, that feeding allosplenocytes significantly prolonged kidney allograft survival in rats (7, 8). This prolongation was alloantigen specific (8). Recently, this oral tolerance to primary allografts has been confirmed by Ishido and colleagues in a rat cardiac model (9).

Oral tolerance has been suggested to be mediated by clonal deletion/anergy (1, 4) or active suppression (4, 10, 11). In active suppression, regulatory cells are thought to play a critical role by modulating the immune response through secretion of Th2 cytokines and/or TGF-β (4, 12, 13). Such regulatory cells have been shown to be capable of transferring tolerance to naive recipients. In an experimental allergic encephalomyelitis (EAE) model, the cells that transferred this tolerance following oral administration of Ag were CD8+ T cells (14). An important role of CD8+ regulatory T cells in the generation and maintenance of oral tolerance has also been suggested by experimentation in other models (15, 16). Recently, however, CD4+ T regulatory cells have been suggested to play an important role in the development of oral tolerance (17).

Although oral tolerance has been extensively studied in autoimmune disease models, it is only recently that oral tolerance has been demonstrated to prolong first-set transplant survival (7–9). As such, the mechanisms responsible for this graft prolongation by oral administration of alloantigen are still unclear. It has been reported that oral administration of alloantigen induces an Ag-specific reduction in delayed-type hypersensitivity responses (5, 9, 18), a decreased MLR (5, 9), and a reduction in allospecific CTL activity (18). Feeding alloantigen has also been suggested by some to induce a Th subset shift from Th1 responses to Th2 responses (5, 6, 9) that is associated with prolonged allograft survival (6, 9, 12). In this study we demonstrate the presence of active CD8+ regulatory cells infiltrating grafts in orally tolerated animals. Adoptive transfer of these CD8+ cells to naive rats transfers the graft tolerance seen in the original fed animals. The presence of high levels of mRNA for IL-4 in this CD8+ population suggests that Tc2 graft-infiltrating cells (GIC) result from oral transplantation tolerance and mediate the graft prolongation.

This is the first report to demonstrate that oral exposure to alloantigen generates intrgraft regulatory cells that are capable of transferring prolongation of allograft survival to naive animals.

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3 Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; GIC, graft-infiltrating cells; FasL, Fas ligand; MLN, mesenteric lymph nodes; BN, Brown Norway.
The intragraft regulatory cells are CD8+ T cells, which may regulate rejection responses by Tc2-type activities or by Fas/Fas ligand (FasL) interaction with alloreactive T cells.

Materials and Methods

Animal model

Male Brown Norway (BN; RT1a) and Lewis rats (RT1a) purchased from Harlan Sprague-Dawley (Indianapolis, IN) were used as donors and recipients, respectively. This strain combination is fully disparate at both major and minor histocompatibility complex loci. Animals were maintained in the Dalhousie University Faculty of Medicine animal care facility and were provided water and rodent chow ad libitum. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council on Animal Care.

Single-cell suspensions

Lymphocytes were isolated from the spleen or mesenteric lymph nodes (MLN) and single-cell suspensions were prepared following standard protocols (as we have described previously, Ref. 8). Cells were prepared, washed, and used for in vitro experiments in RPMI 1640 medium (Life Technologies, Burlington, Canada) supplemented with 20 mM HEPES (U.S. Biological Corp., Cleveland, OH), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (Life Technologies). Spleen cells were purged of RBC by lysis for 5 min with ACK erythrocyte lysing buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2EDTA, pH 7.4). After lysis of RBC, spleen cells were washed in RPMI 1640.

Oral tolerance induction

Lewis rats were fed, without anesthetic, by intragastric intubation using a 3 1/2 Fr premature human infant feeding tube (Sherwood Industries, St. Louis, MO). All rats were fed 1 × 10^8 BN splenocytes suspended in 300 μl PBS (0.15 M NaCl and 0.05 M Na2PO4, pH 7.4) on days −14, −13, −12, −11, −10, and −1, with the day of the kidney transplantation being day 0.

Kidney transplantation

Kidney transplantation was performed as described previously (8). Briefly, BN rats were anesthetized with sodium pentobarbital. After ligation of the abdominal aorta and vena cava proximal and distal to the renal artery and vein, the left kidney was perfused in situ with cold (4°C) heparinized 0.9% saline and removed with a cuff of the aorta and the renal vein as well as a long piece of the ureter. The donor kidney was kept in cold saline during preparation of the recipient. Following left nephrectomy of the recipient, the donor kidney was transplanted into an orthotopic position by end-to-end anastomosis of the renal artery to the abdominal aorta, and the renal vein was connected to the inferior vena cava. The ureter was attached by end-to-end anastomosis. Three days after the transplant the right native kidney was removed, leaving rat survival dependent on the transplanted kidney.

MLR reaction

Lewis rat spleen and MLN responder cells were recovered from kidney transplant recipients on day 5 post-transplant. BN spleen cells were used as stimulators. Responder cells (1 × 10^6 cells/well) were cultured with or without equal numbers of mitomycin C (25 μg/ml)-treated stimulator cells in RPMI 1640 medium. The plates were incubated at 37°C for 72 h, then pulsed with [3H]thymidine (1 μCi/well) for 18 h and harvested with an automated cell harvester (Skatron, Sterling, VA). Proliferation was assayed by [3H]thymidine incorporation.

Isolation of GIC

GIC were isolated as we have described previously (8). Briefly, at days 5 post-transplantation, rats were sacrificed, and kidney allografts were flushed in situ with heparinized (100 U/ml) 0.9% saline and removed from the rat. The kidney was minced and then digested in 25 ml collagenase solution (50 μg/ml collagenase (Life Technologies) and 20% FBS in RPMI 1640). To clear the debris, including dead tubular epithelial cells, the cell suspensions were rapidly passed down a loosely packed glass wool column (300 mg sterile glass wool in a 10-ml syringe), then mixed with isotonic Percoll (Pharmacia, Piscataway, NJ) to a concentration of 30% Percoll and centrifuged at 460 × g for 20 min at 10°C. The pellet was resuspended in RPMI 1640, then loaded on top of a cushion of 70% Percoll (in RPMI 1640) and centrifuged as described above. The cells overlying the cushion were removed for GIC examination.

Flow cytometry

The leukocytes isolated from the kidney graft were incubated with the following primary Abs: R73 (anti-αβ TCR; Pharmingen, San Diego, CA), OX-8 (anti-CD8; Serotec, Oxford, U.K.), and W3/25 (anti-CD4; Serotec) at a concentration of 10 μg/ml in PBS solution containing 0.5% (w/v) BSA (Roche, Indianapolis, IN). The secondary Ab was a polyconal FITC-la belled anti-mouse IgG (Organon Teknika, Durham, NC). Following incubation with the FITC-labeled Ab at a 1:500 dilution, the cells were fixed with 1% paraformaldehyde in PBS and examined on a FACScan (BD Biosciences, Mountain View, CA).

Purification of CD8+ or CD4+ T cells from GIC

Graft-infiltrating T cells were enriched by incubating GIC in warm nylion wool (Polysciences, Warrington, PA) columns (1 g nylon wool in a 10-ml syringe) with RPMI 1640 at 37°C for 1 h to remove the majority of the B cells, macrophages, and other accessory cells. The enriched population was usually 85–90% T cells as analyzed by flow cytometry. Enriched T cells were incubated with W3/25 or OX-8 (anti-CD4 or anti-CD8 Ab, supplied by Biotex, Edmonton, Canada) for 30 min before passage through immunoenrichment columns (Biotex), which enrich for CD8+ or CD4+ cells by negative selection. The purity of CD8+ or CD4+ cells obtained by this method was normally >97% (assessed by flow cytometry).

Direct CTL assay

Five days after transplantation, allograft kidneys were removed. Single-cell suspensions of CD8+ cells from the kidney GIC were prepared as effector cells. 51Cr (100 μCi for 1 h at 37°C)-labeled BN spleen Con A (2 μg/ml for 72 h) blasts were used as target cells. The effector and target cells were plated in V-bottom 96-well microtiter plates (1 × 10^5 target cells/well) at E/T cell ratios ranging from 100:1 to 12.5:1 in RPMI 1640 medium. After incubation at 37°C for 12 h, the plates were centrifuged, and lysis was measured by 51Cr release.

RT-PCR

Relative (rather than quantitative) RT-PCR was performed as we have described previously (8). Briefly, total RNA was obtained from the CD8+ GIC (1–2 × 10^6 cells) using TRizol (Life Technologies) as suggested by the manufacturer. RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies) and random hexamer primers (Life Technologies) according to the manufacturer’s instructions. PCR amplification of the product of the RT was achieved with a mixture containing 1× PCR buffer, 0.2 mM dNTP, 0.4 μM each of sense and antisense primers, and 2.5 U Taq polymerase (BRL, Rockville, MD) for 40 cycles. Ten-microliter aliquots of PCR products were analyzed in ethidium bromide-stained agarose gels. Primers were developed from the published sequences for the respective rat cytokines as we have previously described (8). Experimental amplicons were compared with the housekeeping β-actin amplicon, and relative amplification within samples was assessed.

Cell transfer

To generate GIC for cell transfer, Lewis rats were fed 1 × 10^8 BN splenocytes on days −14, −13, −12, −11, −10, and −1 and given kidney transplants on day 0 as described above. For control GIC, unfed Lewis rats were used as recipients. On day 5 post-transplantation, whole GIC, CD8−, or CD4+ cell populations were isolated from kidneys of fed and unfed animals. GIC were transferred i.v. into naive rats that had received a kidney transplant 1 day before the GIC injection. The GIC population isolated from one kidney was transferred into a single paired naive Lewis rat. Three days after kidney transplantation, the recipient native right kidney was removed, leaving rat survival dependent only on the transplanted BN kidney.

Results

Effects of oral exposure to alloantigen on allore cognition

Five days after kidney transplantation, the spleen and MLN were removed from the rats orally exposed or not exposed to alloantigen. Cell proliferation was assessed by MLR using mitomycin C-treated donor spleen cells as stimulators. In this experiment sensitization to alloantigen occurs in vivo in response to a kidney transplant. The MLR was used to assess whether prior oral exposure to alloantigen would decrease this sensitization induced by the graft. Fig. 1 shows the results of the MLR using spleen cells (Fig.

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or MLN cells (Fig. 1B) derived from fed (orally exposed to alloantigen before kidney transplantation) or unfed rats. As shown in Fig. 1, prior oral exposure to alloantigen led to increased allo-sensitization, as seen by an increased response to allochallenge. Data are shown for a representative experiment and also shown is the mean percent increase for five experiments. Previous alloexposure by the oral route does not lead to reduced allorecognition and activation, which would be indicated by a proliferative response lower than that seen in the control MLR, where sensitization is generated by the kidney transplant. In fact, in all experiments there was a significant increase over control MLR with effector cells from the animals previously exposed to alloantigen by the oral route (271.3 ± 77.4% for spleen and 302.7 ± 56.2% for MLN, taking the control mean as 100%). In contrast, effector cells from animals orally exposed to syngeneic spleen cells or to third-party (PVG) spleen cells did not show increased MLR after kidney transplantation compared with control animals without prior alloexposure before kidney transplantation (syngeneic, 137.2 ± 41.4% of control; third-party exposure, 113.3 ± 28.6% of control). These results show that the feeding of allogeneic cells to induce Ag-specific immune regulation does not prolong allograft survival by decreasing allorecognition. On the contrary, the treatment regimen appears to increase alloreactivity.

**Phenotype of GIC**

The data reported above show that increased survival of kidney allografts by prior alloexposure through the oral route was not due to decreased recognition of the allochallenge. Therefore, we hypothesized that the increased survival would be due to a modulation in the generation or effector activity of CD8+ CTL in the graft.

To examine T cell responses in the graft, we isolated GIC on day 5 post-transplant. Using flow cytometry, we found that the GIC contained predominately T cells (~65%; labeled with anti-TCR mAb; data not shown) and macrophages (~35%; labeled with anti-Mac-1 mAb; data not shown). We have previously observed that on day 5 post-transplant, the total number of graft-infiltrating T cells was decreased by 30% compared with that in controls (8). The data in Fig. 2 show that this decreased number of T cells is due to a decrease in the number of CD4+ cells. In this figure data from a representative experiment (Fig. 2A) and the mean of five separate experiments (Fig. 2B) are shown. In fed animals CD4+ GIC are present at only 60% of control levels, whereas there is no significant difference in the CD8+ GIC levels between fed and control animals. Thus, there appears to be no defect in the ability of CD8+ T cells to transit to the kidney allograft. This suggests either that this CD8+ T cell population does not contain CTL or that the CTL activity is somehow modulated.

**CTL activity of GIC**

To examine whether the CD8+ T cell population in the GIC of the animals orally exposed to alloantigen contained alloresponsive CTL, we assessed the expression of cytotoxic mediators in the

![FIGURE 1. Cell proliferation of lymphocytes harvested from Lewis rat spleen (A) and MLN (B) in response to mitomycin C-treated allogeneic BN splenocytes. Spleen and MLN were harvested 5 days post-transplantation. The proliferation of splenocytes and MLN cells from fed animals significantly increased compared with that in unfed controls. As and Ba, The data are presented as mean ± SD of triplicate wells from a representative experiment. Ab and Bb, The mean of all five experiments, depicted as a percentage of proliferation compared with that of cells isolated from the unfed control (p < 0.02, by one-way ANOVA).](http://www.jimmunol.org/)

![FIGURE 2. Phenotype of GIC from day 5 post-transplanted kidneys. GIC were isolated, labeled with w3/25 (anti-CD4) or OX-8 (anti-CD8) mAb, and analyzed by flow cytometry. A, Representative experiment of five separate experiments shows the total number of infiltrating T cells obtained from kidney transplanted into fed and control animals. B, Mean (of five separate experiments) percentage of cells of the particular phenotype compared with GIC isolated from unfed controls (CD4 vs control, p < 0.01; CD8 vs control, p > 0.05; by one-way ANOVA).](http://www.jimmunol.org/)
CD8+ T cell population of the GIC, and we also conducted a direct CTL assay. The mediators we assessed (by RT-PCR) were those well known to be associated with CTL activity, granzyme B and perforin. In addition, we assessed the presence of mRNA for FasL, which mediates apoptosis in target cells by ligation of Fas. The results, shown in Fig. 3, clearly demonstrate the presence of abundant mRNA for the CTL mediators granzyme B and perforin in the CD8+ GIC T cell population from both control and fed animals. There is no obvious diminution in the expression of the message for these important cytotoxic mediators, suggesting that CTL are present in the CD8+ T cell compartment of the GIC. Furthermore, FasL expression appears to be increased, rather than decreased, in the CD8+ T cell compartment of the GIC from the transplanted kidneys of animals previously exposed to alloantigen. These data indicate that CTL are present in the CD8+ T cell compartment of these GIC.

To directly assess CTL presence in the CD8+ T cell GIC population we performed a direct CTL assay (19) using BN allogeneic cells as targets. In this assay CD8+ T cells from GIC were immediately incubated with allogeneic target cells without an in vitro expansion of CTL precursors to assess the presence of mature fully active CTL in the fresh GIC population. The results, shown in Fig. 4, clearly demonstrate that the CD8+ T cell population in the GIC of both fed and unfed animals contains mature alloresponsive CTL. Indeed, the CTL activity in the animals that had been orally exposed to alloantigen before kidney transplant is consistently higher than the CTL activity in the animals without prior exposure to alloantigen by the oral route (p < 0.04).

These data confirm that the prolongation of allograft survival mediated by allogeneic exposure by the oral route is not due to a deficiency in the generation of competent alloresponsive CTL or their presence to the graft. This indicates that this population must be exposed to intragraft modulatory effects.

Cytokine transcription of CD8+ GIC

It has been suggested that oral tolerance can generate CD8+ regulatory cells (14, 20) and/or switch immune responses toward type 2 responses (7, 12, 17). Because in our experiments the number of CD8+ GIC T cells remains the same in fed and unfed animals, but grafts survived much longer in the fed animals, we postulated that the phenotype of the CD8+ GIC T cells may have shifted to a type 2 CD8+ T cell phenotype (Tc2). Therefore, we investigated cytokine mRNA from these cells. RT-PCR was used to reveal the relative amount of cytokine mRNA in these cells. As shown in Fig. 5, substantial amounts of IFN-γ and TGF-β mRNA were detected in CD8+ GIC T cells from both fed and unfed animals. However, compared with these cytokines, and to β-actin, the amount of IL-4 mRNA detected was markedly greater in the fed animals than in the controls. Indeed, in multiple repeats, IL-4 amplicons were not detected after RT-PCR of mRNA from CD8+ GIC from control animals, but were always detectable in fed animals. In contrast, IFN-γ was always detectable in both groups in comparable amounts relative to the β-actin control and other cytokines. In fact, IL-4 mRNA was only detectable in the CD8+ GIC T cells from fed animals, not in the CD8+ GIC T cells from unfed controls. These data suggest that in fed animals at least some of the CD8+ GIC are Tc2 cells and potentially regulatory cells.

Transfer of CD8+ intragraft regulatory cells

The data presented above suggest that the CD8+ GIC may be involved in intragraft modulation as a regulatory cell in animals that have been exposed to alloantigen by the oral route. To investigate the existence of regulatory T cells in the kidney, we transferred allograft GIC from fed or unfed animals into animals that had received a kidney allograft 1 day previously. GIC for transfer were taken on day 5 post-transplant, because our previous experimentation had shown significant damage to kidney grafts at this time point in control animals, but preservation of kidney architecture in animals orally exposed to alloantigen (thus indicating the presence of active regulatory events at this time point).

FIGURE 3. Expression of perforin, granzyme, and FasL mRNA (by RT-PCR) in CD8+ graft-infiltrating T cells. T cells were harvested from the transplanted kidneys on day 5 post-transplant from control (left) or fed (right) animals. β-Actin amplification was used as a control. These data are representative of five separate experiments.

FIGURE 4. Direct CTL activity of CD8+ GIC isolated from day 5 post-transplanted kidney allografts from fed and control animals. 51Cr-labeled allogeneic spleen Con A blasts were used as targets. These data are representative of three separate experiments (p < 0.04, by Mann-Whitney test).

FIGURE 5. Expression of IL-4, IFN-γ, and TGF-β mRNA (by RT-PCR) in CD8+ graft-infiltrating T cells. T cells were harvested from kidney on day 5 post-transplant from control (left) and fed (right) animals. β-Actin amplification was used as a control. These data are representative of five separate experiments.
The results, shown in Fig. 6, confirm the presence of a regulatory cell in the kidney allograft after oral exposure to alloantigen. Survival of the kidney grafts in the animals receiving GIC from allogeneic kidney grafts of animals orally exposed to alloantigen was significantly increased compared with the survival of grafts in animals receiving GIC from control grafts. The mean survival in the control group was 9 days, with the longest survival being 12 days. The mean survival of the kidneys in animals receiving GIC transferred from orally treated animals was 22 days, with the longest survival being 46 days (p < 0.03). These data suggest that regulatory cells are present in the GIC of the allograft kidney of orally treated animals.

To confirm that the regulatory GIC are CD8⁺ T cells, we transferred purified CD8⁺ or CD4⁺ GIC populations from allogeneic kidney grafts of animals orally exposed to alloantigen. As shown in Fig. 7, transfer of CD8⁺ allograft GIC from orally pretreated rats into naive rats significantly prolonged kidney allograft survival in those rats (mean survival, 89 days; longest survival, 215 days; p < 0.01). CD4⁺ GIC, in contrast, did not confer increased survival (mean survival, 10 days; longest survival, 13 days; p > 0.05). These data indicate that a population of CD8⁺, but not CD4⁺, intragraft regulatory T cells is present in the kidney allografts of animals orally exposed to alloantigen. Note that purified CD8⁺ regulatory cells showed much more modulatory activity than whole GIC (compare Figs. 6 and 7).

Discussion

Oral tolerance has been extensively studied in a variety of experimental models (4, 10, 11). Depending on the dose and timing of the antigen challenge, oral tolerance has been reported to result in clonal anergy or active suppression (4, 10, 11). Recently, positive results ameliorating EAE by inducing oral tolerance to myelin basic protein have led to clinical trials of oral tolerance as a potential therapy for multiple sclerosis (4, 13). There is considerable evidence (15, 16) that CD8⁺ regulatory T cells play an important role in the generation and maintenance of oral tolerance. In the EAE model, oral tolerance-induced protection from disease could be adoptively transferred to naive hosts with CD8⁺ T cells (14). This is not to say that oral tolerance is absolutely dependent on the ability to generate CD8⁺ regulatory cells. Weiner and colleagues (20), for example, have provided evidence that both CD4⁺ and CD8⁺ T cells play a role in the induction of oral tolerance in EAE and have also postulated the existence of a Th3, TGF-β-secreting T cell as a mediator in this complex interaction (17). Others (11, 21, 22) have demonstrated that oral tolerance can be induced in CD8 knockout mice and in mice depleted of CD8⁺ T cells by anti-CD8 Ab treatment. Thus, it appears that the generation of CD8⁺ regulatory cells may be sufficient for, but not essential to, the development of oral tolerance.

In the experiments described here we have examined whether the generation of such regulatory cells plays a role in the significant prolongation of primary kidney allograft survival that we have observed following oral exposure to alloantigen (7, 8). We first assessed allorecognition by MLR in fed vs control animals. The MLR showed an increase in reactivity in the cells from the animals that had been previously exposed to alloantigen by the oral route before transplantation. From this we conclude that there is no defect in allorecognition produced by induction of immune modulation by pre-exposure to alloantigen by the oral route. These data confirm that prolongation of kidney allograft survival is not due to masking of allorecognition, but to an immunomodulatory effect on the immune response, presumably the development of an immunomodulatory cell.

Past research in oral tolerance has primarily concentrated on events in the draining node (MLN) or spleen. However, the response to allogeneic transplants is defined at the level of the organ transplant. We were the first to show that oral tolerance could prolong primary solid organ transplants (7, 8), and we chose to use the kidney as our model so that cells infiltrating the graft would be readily available. On the premise that rejection is defined at the level of the organ, we postulated that critical immunomodulatory events would also be defined at the level of the transplanted organ, and examining the graft-infiltrating lymphocytes would be of prime importance for developing an understanding of oral transplantation tolerance. We isolated GIC on day 5 post-transplant. This is the latest time point at which we can obtain GIC from control animals. We have reported previously that the total number of allograft-infiltrating T cells in orally treated animals was decreased at this time by ~30% compared with that in normal untreated control allografts (8). In the current study we have found that this decreased number of T cells is due to a decrease in the number of CD4⁺ cells. In contrast, we found that the CD8⁺ cells, a subset of which is presumably allospecific CTL, remain intact. CD8⁺ T cells are the major source of CTL and have been suggested to destroy graft tissue predominantly through direct cytotoxic effects against foreign class I MHC-expressing cells (23).
Many treatments that prolong allograft survival, including anti-
CD4 mAb (24), portal venous inoculation with alloantigen (25),
and nematode infection (26), are associated with decreased
numbers of CD8\(^+\) T cells or decreased CTL activity. The undiminished
numbers of intragraft CD8\(^+\) T cells in our study suggests that
the animals that have been orally pre-exposed to alloantigen do not
have a defect in the generation or transit of active CTL to the
allograft. However, it was possible that the CD8\(^+\) T cells in the
graft did not contain active CTL and that the modulation lay in a
defect in CD8\(^+\) CTL differentiation.

To address this we examined whether the known charac-
teristics of CTL, that is the expression of perforin, granzyme,
and FasL (27), were present in a highly purified CD8\(^+\) T cell popula-
tion from the GIC derived from allografts in orally treated animals.
We found no substantial difference in the level of mRNA for the
cytotoxic mediators perforin and granzyme in the GIC from orally
reated animals vs controls. Furthermore, FasL expression ap-
peared to be increased, rather than decreased, in these highly en-
riched CD8\(^+\) GIC T cell populations. This supports the hypothesis
that similar numbers of potentially active mature CTL are present
in the grafts of orally treated animals even in the face of prolonged
graft survival. This hypothesis was confirmed by our experimen-
tation showing increased, rather than decreased, cytotoxicity to
allogeneic targets in a direct CTL assay, thus amply demonstrating
the presence of mature CTL in the GIC population. This increase
in cytotoxicity could be due to the increased levels of FasL on
these cells.

Although many studies have demonstrated that CD8\(^+\) T cells
are not absolutely essential for allograft rejection, including heart,
kidney, and skin allografts (28, 29), CTL have been suggested to
play an important role in normal MHC-mismatched allograft re-
jection (30). The potential mechanism of CTL involvement in
acute renal allograft rejection has been suggested to be mediated
by cytotoxic granule-based killing, but not FasL-induced killing.
This is because granule proteins are expressed on biopsies of kid-
ney undergoing acute rejection, whereas FasL is expressed mostly
in the absence of acute rejection episodes (31, 32). In addition,
there is a paucity of FasL-induced apoptosis seen in renal allograft
rejection, which may be due to low levels of Fas expression on
graft cells (31) or resistance of kidney tubule cells to FasL-mediated
events (33). In our experiments the presence of these mature
alloreactive CTL in the orally treated animals exhibiting prolonged
graft survival was perplexing. Interestingly, similar observations
of increased CTL activity exhibited by GIC from kidney allografts
have also been reported in studies of blood transfusion transplan-
tation tolerance, although further characterization of the cells was
not performed (34). One potential explanation could be that the
mature alloreactive CTL fall under the control of regulatory ele-
ments in situ, and that they are somehow freed from those regu-
ulatory elements in the artificial conditions of the in vitro assay.
Another possibility is that the increased CTL activity observed in
our in vitro experiments is mediated by increased FasL expression,
which may be less significant in vivo, as described above. How-
ever, increased FasL expression by CD8\(^+\) intragraft regulatory
cells may, in fact, represent another level of regulation. Some im-
une-privileged sites, such as the testes and the anterior chamber
of the eye, are thought to express their privileged phenotype be-
cause of the high levels of FasL expression in these sites (35–38).
Indeed, Swenson and coworkers (39) have provided evidence that
kidney allografts transsected with FasL cDNA were protected from
rejection, presumably through interference with alloreactive T
cells by a Fas/FasL interaction. In addition, high levels of FasL
expression in renal biopsies from kidneys that are not undergoing
acute rejection support the idea that some degree of FasL-depen-
dent immune privilege occurs in renal graft protection (32). There-
fore, it is possible that the observed CD8\(^+\) T cells may be involved
in graft protection, rather than destruction, due to their level of
FasL expression, which may interact with alloreactive Fas-bearing
T cells.

This study focused on CD8\(^+\) GIC T cells isolated from allo-
grafs on day 5 post-transplant, a time point when robust graft
destruction occurs in control animals and the latest time point that
we could obtain GIC from control transplants. We currently have
no information on whether these CD8\(^+\) T cells persist throughout
the entire period of graft prolongation.

It has been suggested that type 1 immunity, mediated mainly by
CD4\(^+\) Th1 cells, is the major effector mechanism of allograft re-
jection, whereas type 2 immunity favors allograft survival (40, 41).
A shift from type 1 T cell to type 2 T cell responsiveness has been
reported to prolong allograft survival in several experimental mod-
elos (6, 12, 26), and this shift has been associated with an increased
presence of IL-4 (6, 9, 42). Because IL-4 is the only type 2 cyto-
kine that can be reliably correlated with type 2 T cell activity,
the presence of increased levels of IL-4 mRNA in the CD8\(^+\) GIC from
allografts of fed animals indicates an increased intragraft type 2
response. IL-4 mRNA has often been found in whole graft extracts
or the whole GIC population of control rejecting grafts and in
biopsies of human grafts, but has not been previously demon-
strated in a graft-infiltrating CD8\(^+\) T cell population. This finding,
in the context of the tolerizing protocol, suggests that the regula-
tory cell is a CD8\(^+\) Th2 cell (43, 44).

The presence of regulatory cells can be demonstrated by in vivo
transfer (45, 46). Others have shown that oral tolerance can
induce regulatory cells in the spleen and draining lymph nodes,
using autoimmune disease models (14, 17). We also found that
regulatory cells are present in the spleen (the reactive node for
kidney transplants) and MLN (the draining node of the gastroin-
testinal tract) following oral exposure to alloantigens. We were
able to transfer graft protection using cells from these sites from
fed animals, but not from unfed control animals (mean survival of
rats that received splenocytes or MLN cells from fed animals, 38
and 24 days, respectively; mean survival of rats that received
splenocytes or MLN cells from unfed control rats, 9 day). The
presence of regulatory cells in the reactive node may indicate an
effect of oral tolerance and the inductive phase of the responses.
Importantly, however, we demonstrated that regulatory cells were
also present in the kidney allograft of fed animals. Transfer of
kidney GIC from orally tolerized animals into naive animals pro-
longed graft survival in these animals. This confirms that oral
transplantation tolerance mediates a change at the effector site (i.e.,
within the transplant). We chose to harvest cells from the kidney
allografts of orally pre-exposed animals on day 5 post-transplant
because 1) our preliminary histologic evidence has shown that
immune modulation is ongoing at this point; 2) this is the point at
which we know there are mature CTL in the GIC population of the
graft; and 3) this is the latest time point that we could reliably
obtain GIC from control transplants. We compared the effect of
transfer of these cells harvested from orally treated animals with
that in cells obtained from control non-pretreated animals on al-
lograft survival in naive recipients. Our results confirmed that oral
exposure to alloantigen generates intragraft regulatory cells that are
present in the kidney allograft.

Once we had demonstrated that regulatory T cells exist in the
kidney allograft of animals orally exposed to alloantigen, it was
important to differentiate between CD4\(^+\) and CD8\(^+\) effects.
Because others have previously shown the existence of a CD8\(^+\)
regulatory cell in the spleens and lymph nodes of orally tolerized
animals in other models (14), we suspected that regulation would
occur in this compartment. The data obtained from our subsequent transfer experiments confirmed that CD8+, but not CD4+, T cells in the GIC transfer tolerance. Thus, animals that have been orally exposed to alloantigen develop CD8+ regulatory cells that are present in the kidney on day 5 post-transplant. This cell population will transfer graft prolongation to a naive animal receiving a kidney transplant. Interestingly, the transferred CD8+ GIC were much better at transferring graft prolongation than transferred whole GIC, even though the same number of CD8+ T cells was transferred in each instance (compare Figs. 6 and 7). This suggests that the whole GIC population contains both effector and regulatory cells.

These data do not rule out a contribution of CD4+ T cells to oral transplantation tolerance in ways other than active regulation. However, these data confirm that generation of CD8+ regulatory cells, a response to oral pre-exposure to alloantigen, is sufficient to induce graft prolongation. These intragraft regulatory cells are capable of transferring prolongation of graft survival to naïve animals and may mediate their effects by IL-4 secretion and/or Fas/FasL interaction with alloreactive T cells.

References


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