Testicular Immune Privilege Promotes Transplantation Tolerance by Altering the Balance between Memory and Regulatory T Cells

Isam W. Nasr, Yinong Wang, Ge Gao, Songyan Deng, Lonnette Diggs, David M. Rothstein, George Tellides, Fadi G. Lakkis and Zhenhua Dai

J Immunol 2005; 174:6161-6168; doi: 10.4049/jimmunol.174.10.6161
http://www.jimmunol.org/content/174/10/6161

References
This article cites 52 articles, 29 of which you can access for free at:
http://www.jimmunol.org/content/174/10/6161.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Testicular Immune Privilege Promotes Transplantation Tolerance by Altering the Balance between Memory and Regulatory T Cells

Isam W. Nasr,* Yinong Wang,† Ge Gao,‡ Songyan Deng,* Lonnette Diggs,* David M. Rothstein,* George Tellides,† Fadi G. Lakkis,* and Zhenhua Dai*§

Immune responses are suppressed in immunologically privileged sites, which may provide a unique opportunity to prolong allograft survival. However, it is unknown whether testicular immune privilege promotes transplantation tolerance. Mechanisms underlying immune privilege are also not well understood. Here we found that islet transplantation in the testis, an immunologically privileged site, generates much less memory CD8^+ T cells but induces more Ag-specific CD4^+CD25^+ regulatory T cells than in a conventional site. These CD4^+CD25^+ cells exhibited the suppression of alloimmune responses in vivo and in vitro. Despite the immune regulation, intratesticular islet allografts all were rejected within 42 days after transplantation although they survived longer than renal subcapsular islet allografts. However, blocking CD40/CD40L costimulation induced the tolerance of intratesticular islet allografts. Tolerance to intratesticular islet allografts spread to skin allografts in the non-privileged sites. Either transfer of memory CD8^+ T cells or deletion of CD25^+ T cells in vivo broke islet allograft tolerance. Thus, transplantation tolerance requires both costimulatory blockade, which suppresses acute allograft rejection, and a favorable balance between memory and regulatory T cells that could favorably prevent late allograft failure. These findings reveal novel mechanisms of immune privilege and provide direct evidence that testicular immune privilege fosters the induction of transplantation tolerance to allografts in both immunologically privileged and non-privileged sites.


Immuneologists have long recognized that there are certain areas in the body where immune responses are “forbidden.” These sites are then referred to as “immunologically privileged” sites. The mechanisms underlying immune privilege are not well understood. It was originally thought that immune privilege results from “immunologic ignorance” as the immunologically privileged sites lack lymphatic drainage and possess physical blood-tissue barriers (1). In the 1970s, it became clear that foreign tissues in the privileged sites can evoke Ag-specific systemic immunity (2). Moreover, certain privileged sites were shown to possess an extensive effenter lymphatic pathway (3). Subsequent studies have shown that the mechanisms of immune privilege are much more complex. They showed that the constitutive expression of Fas ligand (FasL) on parenchymal cells in the eye contributes to its privilege by inducing the apoptosis of Fas-positive inflammatory cells (4) and that Sertoli cells of the testis express FasL that prevents graft rejection (5). However, more recent studies by others have generated contradictory data and indicated a paradoxical role of FasL in immune privilege (6, 7). They found that transgenic expression of Fasl on islet cells causes a more rapid rejection of islets accompanied by granulocyte or neutrophilic infiltration (8–10). The explanation for immune privilege has been expanded to include immune deviation and cytokines. Immune responses upon Ag re-encounter via the eye were deviated toward a Th2-like response (2, 11). Other studies have shown that the cytokine TGF-β is required for immune privilege (12, 13) and that infiltrating precursor CTLs fail to acquire direct cytotoxic function in the privileged sites (14). We have recently found the impaired CD8^+ memory recall in the testis (15). NK T cells have also been shown to participate in the ocular immune privilege (16, 17) and CD8^+ regulatory T cells have been generated following ocular Ag inoculation (16).

As an immunologically privileged site, the testis may provide a unique environment for suppressing immune responses and enhancing graft acceptance. Researchers have attempted to transplant islet grafts in the testis of animals. They initially found that testicular islet graft survival is significantly extended without immunosuppression (18–20). However, it was not known whether islet transplantation in the testis achieved tolerance in those models. Despite those initial successes, studies by others have shown that intratesticular islet transplantation fails to prolong allograft survival (21) and that testicular islet allografts were rejected after cyclosporin A treatment was stopped (22). These findings indicated that intratesticular islet transplantation by itself is not as optimal as originally thought. Researchers thereafter have paid less attention to the role of testicular immune privilege in promoting allograft survival. We believe that the naturally occurring immunologically privileged sites, instead of excessive immunosuppression, should be exploited to facilitate allograft survival or tolerance. It is possible that long-term
allograft survival or tolerance can be induced under less stringent conditions in the testis than in a conventional site.

This study was set to investigate alloimmune responses in an immunologically privileged site, the testis, and the mechanisms by which testicular immune privilege promotes islet allograft survival or tolerance. We found that islet transplantation in the testis generates fewer CD8\(^+\) memory cells but induces more Ag-specific CD4\(^+\) CD25\(^+\) regulatory T cells than in a conventional site, the kidney capsule. Blocking costimulatory pathway CD40/CD40L induced tolerance of islet allografts transplanted in the testis but not under the kidney capsule. Tolerance to testicular islet allografts spread to skin allografts in non-privileged sites. These findings revealed novel mechanisms of testicular immune privilege and demonstrated that it is an advantage to induce transplantation tolerance in an immunologically privileged site over a conventional location.

**Materials and Methods**

**Mice**

2C TCR-transgenic C57BL/6 mice on a recombination activating gene knockout (Rag\(^1\) \^-) background (2C.Rag\(^1\)\^-) were generated by backcrossing 2C transgenic mice onto Rag\(^1\) \^- mice (The Jackson Laboratory). Wild-type (wt) BALB/c mice, wt C3H/HeJ, and C57BL/6 mice were purchased from The Jackson Laboratory. All mice were housed in a specific pathogen-free environment, and animal protocols were approved by Yale University Institutional Animal Care and Use Committee.

**Pancreatic islet transplantation**

Islet donors were 9- to 10-wk-old BALB/c mice. Donor mice were anesthetized, and a median laparotomy was performed. The common bile duct was exposed and 3 ml of collagenase V (Sigma-Aldrich) (1 mg/ml in PBS) was injected into the common bile duct to distend the pancreas. The pancreas was then removed, minced, and subjected to stationary digestion by incubation in a 37°C water bath for 12 min (23). Cold HBSS with 10% FCS were added to stop digestion, and the pancreatic tissue was shaken vigorously for 30 s. Islets were then washed three times in HBSS by centrifuging at 1000 rpm for 5 min, and the crude preparation was filtered through a 100-μm nylon cell strainer. After flushing with HBSS, the strainer was rinsed with HBSS to wash the islets into the dish (23). Islets were counted and picked up using a 23-gauge needle.

**Mouse priming with islet allografts and islet transplantation**

Islet allograft recipients were 6- to 8-wk-old C57BL/6 mice. Briefly, mice were anesthetized and a right flank incision was made. Islets (~400 per mouse) were injected into the subcapsular space of the right kidney using a 23-gauge needle, and the incision was closed with skin staples. For testicular islet transplantation, islets with same number were injected into the subcapsular space of the right kidney using a 23-gauge needle, and the incision was closed with skin staples. For testicular islet transplantation, islets with same number were injected into the subcapsular space of the right kidney using a 23-gauge needle, and the incision was closed with skin staples.

**Skin transplantation**

Skin donors were wt C3H/HeJ (third party) or BALB/c mice. Recipient mice were wt C57BL/6 mice that were treated with MR1 and transplanted with BALB/c islets in the testis 120 days ago. Full-thickness trunk skin was transplanted to the dorsal flank area (both sides) of recipient mice. The recipient mice received a skin allograft from both C3H/HeJ (third party) and BALB/c mice simultaneously. Skin rejection was defined as graft necrosis greater than 90%.

**Treg and memory T cell preparation, phenotyping**

To induce Ag-experienced CD4\(^+\) CD25\(^+\) Treg cells, B6 mice were transplanted with BALB/c islets in the testis or under kidney capsule. Two or four weeks later, spleen and lymph node cells were pooled and enriched for CD4\(^+\) T cells by positive selection on a magnetic cell separator (autoMACS) (Miltenyi Biotec). Cells were then stained with anti-CD4-PE and anti-CD25-FITC Abs, and CD4\(^+\) CD25\(^+\) Treg cells were sorted out using a FACSVantage (BD Biosciences). The purity of CD4\(^+\) CD25\(^+\) cells was >95%. Purified CD4\(^+\) CD25\(^+\) Treg cells together with naïve T cells were then adoptively transferred to Rag\(^1\) \^- mice to measure Treg-suppressive activity. For Treg cell phenotyping, cells from tissues, spleens, or lymph nodes were stained with anti-CD4-PE and anti-CD25-FITC Abs and analyzed by a FACScalibur (BD Biosciences).

To measure generation of CD8\(^+\) memory cells, naïve CD8\(^+\) T cells (CD8\(^+\) CD44\^-) from transgenic 2C Rag\(^1\) \^- mice were isolated by autoMACS and FACSVantage. 2C TCR-tg CD8\(^+\) cells specifically recognize L\(^a\) allantogen on BALB/c cells and can be tracked by a specific Ab (B2). Briefly, CD8\(^+\) T cells were first isolated by autoMACS using positive selection. Cells were then incubated with anti-CD8-PE and anti-CD44- FITC Abs (BD Pharmingen) and were sorted by FACSVantage after gating on CD8\(^+\) CD44\^- population. The purity of these cells was typically >98%. These transgenic naïve CD8\(^+\) T cells were then transferred to wt B6 mice for the generation of CD8\(^+\) memory. The CD8\(^+\) memory cells were finally detected by staining with anti-CD8-PE, B2, and rat anti-mouse IgG1-FITC and enumerated by a FACScalibur at 12 weeks after transplantation. CD8\(^+\) memory phenotype was further confirmed by staining with anti-CD62L-allophycocyanin or anti-CD44-allophycocyanin (BD Pharmingen) as described before (24, 25). To generate memory CD4\(^+\) and CD8\(^+\) T cells for their adoptive transfer to the tolerated recipients (Fig. 4A), wt B6 mice were immunized i.p. with irradiated BALB/c splenocytes. Two weeks later, memory CD4\(^+\) and CD8\(^+\) T cells (CD4\(^+\) CD44\^- and CD8\(^+\) CD44\^-) were isolated by autoMACS and FACSVantage as described above.

**Suppression of T cell proliferation in vitro**

To analyze Treg suppression of T cell proliferation in vitro, different numbers of CD4\(^+\) CD25\(^+\) Treg cells (up to 5 × 10\(^4\)/well), isolated from B6 mice after either renal or testicular islet inoculation, were cultured with CD4\(^+\) CD25\(^-\) cells (5 × 10\(^4\)/well) isolated from naïve B6 mice in 96-well plates (Corning Costar) in complete RPMI 1640 medium (10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Irradiated BALB/c splenocytes were added to the culture to set up a one-way MLR. Cells were cultured for 96 h and pulsed with [\(^3\)H]thymidine for the last 12 h. Cells were then harvested and analyzed by a scintillation counter (PerkinElmer Life Sciences).

**Real-time quantitative RT-PCR**

Total RNA was isolated from CD4\(^+\) CD25\(^-\) cells using RNeasy Mini kits (Qiagen), and its reverse transcription to cDNA was performed using SuperScript first strand synthesis system (Invitrogen Life Sciences) according to the manufacturer’s instructions. The message levels were quantified by an Opticon real-time PCR using the ABI 7700 sequence detection system (Applied Biosystems) with primers that span exon boundaries to minimize amplification of genomic DNA, and with hypoxanthine phosphoribosyltransferase (HPRT) primers for internal controls. PCR cycling conditions were 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run in triplicate. The mRNA levels were normalized to the HPRT level, where HPRT = 1. The PCR primer pairs are as follows: FoxP3, 5'-GGGCTTTTCCAGGACAGA-3', 5'-GCTGATCATGGCTG-3'; HPRT, 5'-AGCCTAAAGTGAGCCAGTAAG-3', 5'-TTACTAGGCGATGCGCA-3'.

**Ab treatment of mice**

To block CD40/CD40L costimulation and suppress primary alloimmune responses, wt B6 mice were injected i.p. with MR1 (anti-CD40L) (BioExpress) at 0.25 mg on days 0, 2, 4, and 6 or isotype Ab after transplantation. To deplete CD4\(^+\) CD25\(^+\) Treg cells, mice were treated with depleting anti-CD25 Ab (PC61; BD Pharmingen) at 0.25 mg every other day for four doses (26). Over 80% of CD25\(^+\) T cells were deleted after this treatment (26).

**Isolation of graft-infiltrating cells**

Graft-infiltrating cells were isolated as described previously (15). Briefly, kidneys or testes that harbor islets were perfused in situ with heparinized 0.9% saline. They were then minced and digested at 37°C for 30 min in 20 ml of RPMI 1640 medium containing 5% FCS and 350 U/ml collagenase (Sigma-Aldrich). To clear the debris, cell suspensions were rapidly passed down a loosely packed glass wool column (300 mg of sterile glass wool in a 10-ml syringe), then mixed with Percoll solution (Sigma-Aldrich) to a
concentration of 30%, and centrifuged at 2000 rpm for 15 min at room temperature. The pellet was washed and resuspended before flow analysis. **In vivo analysis of T cell proliferation and apoptosis by BrdU labeling and TUNEL** Recipient mice were pulsed i.p. with 0.8 mg of BrdU (Sigma-Aldrich) 7 days after allogeneic islet transplantation. Twenty-four hours later, graft-infiltrating cells were isolated and stained using anti-CD8-PE or anti-CD4-PE (BD Pharmingen). Cells were then fixed in 70% ethanol followed by 1% paraformaldehyde and incubated with 50 U/ml of DNAse I (Sigma-Aldrich). Cells were finally stained with anti-BrdU-FITC (BD Biosciences) and analyzed by a FACSCalibur. To detect apoptosis, graft-infiltrating cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 solution, and labeled with fluorescein-tagged dUTP by the TUNEL method according to the manufacturer’s instructions (Roche) (24).

**Statistical analysis** The analysis of allograft survival data was performed using the Mann-Whitney U test. Comparison of means was conducted using a two-tailed t test and ANOVA.

**Results**

**Testicular immune privilege suppresses the generation of memory CD8⁺ T cells** Memory T cells constitute a threat to the long-term survival of transplanted organs by mediating allograft rejection despite ongoing immunosuppression (27) or costimulation blockade (28). It is unclear whether a comparable number of T cell memory can be generated upon encounter with Ag in the testis, an immunologically privileged site. We hypothesized that testicular immune privilege suppresses the generation of memory T cells since T cells undergo an accelerated apoptosis in the testis upon activation (4, 5). To observe CD8⁺ memory generation, we used 2C transgenic CD8⁺ T cells that recognize the Ld alloantigen and are tracked by a clonotypic Ab, 1B2, thereafter referred to as CD8⁺ 1B2⁺. Naive 2C cells were first transferred to mice that received islet allografts in the testis or under the kidney capsule 1 day earlier. Twelve weeks later, CD8⁺ memory cells (CD8⁺ 1B2⁺ CD44hi) were quantitated by FACS analysis. We found that the percentage of memory CD8⁺ 1B2⁺ T cells in both lymphoid and nonlymphoid organs after intratesticular islet inoculation was much lower than that after renal islet transplantation (Fig. 1A). CD8⁺ 1B2⁺ T cells were barely detectable after syngeneic islet inoculation (<0.1%, not shown in the figure). Accordingly, the absolute number of CD8⁺ memory cells in both lymphoid and nonlymphoid organs after intratesticular islet inoculation was dramatically decreased (~60–70% less, p < 0.05) compared with that after renal islet transplantation (Fig. 1B and C). These CD8⁺ 1B2⁺ T cells generated via both intratesticular and renal islet inoculation were...
confirmed to have memory phenotypes since ones from mesenteric lymph nodes were CD44\textsuperscript{high}CD62L\textsuperscript{high}, a central memory, whereas ones from livers or other nonlymphoid organs were CD44\textsuperscript{low}CD62L\textsuperscript{low}, an effector memory (29) (Fig. 1D). This finding suggests that testicular immune privilege hinders the generation of long-term immunity.

**Inoculation with islet allografts in the testis induces allospecific CD4\textsuperscript+CD25\textsuperscript+ Treg cells**

CD4\textsuperscript+CD25\textsuperscript+ Treg cells play a key role in the maintenance of immunologic tolerance to both self and foreign Ags by suppressing aggressive T cell responses (30). They prevent autoimmunity and allograft rejection in many animal models (31–37). However, it is unclear whether Treg cells are involved in the formation of testicular immune privilege. To answer this question, B6 mice were transplanted with BALB/c islets either in the testis or under kidney capsule. Two weeks later, CD4\textsuperscript+CD25\textsuperscript+ Treg cells from lymph nodes, spleens, and kidney/testis were stained and enumerated by FACS analysis. As shown in Fig. 2, A and B, the number of CD4\textsuperscript+CD25\textsuperscript+ Treg cells per lymph node or spleen after intratesticular islet inoculation was comparable to that after renal islet transplantation and similar to that from PBS control group (p > 0.05). The number of local Treg cells in the testis after testicular islet inoculation was also similar to that in the kidney after renal subcapsular islet inoculation (Fig. 2C). However, there were very few Treg cells in the kidney when islets were inoculated via testis, and vice versa, suggesting that Treg cells barely migrate to a solid organ in the absence of local inflammation. Similar results were observed at 4 wk after islet transplantation (data not shown). These findings suggest that CD4\textsuperscript+CD25\textsuperscript+ Treg cell pool in secondary lymphoid organs remains steady all the time.

To examine the suppressive function of Treg cells in vivo, CD4\textsuperscript+CD25\textsuperscript+ Treg cells from spleens and lymph nodes of mice inoculated with islet allografts, together with naive T cells, were transferred to the Rag\textsuperscript{1-} mice that received a skin allograft 1 day earlier. As shown in Fig. 2D, Treg cells generated via intratesticular islet (H-2\textsuperscript{d}; BALB/c) inoculation were much more efficient in suppressing allograft (H-2\textsuperscript{d}) rejection mediated by naive T cells (median survival time (MST) = 100 days) than Treg cells generated via renal islet (H-2\textsuperscript{d}) inoculation (MST = 41 days) or third-party Treg cells generated via intratesticular islet (H-2\textsuperscript{d}, C3H/HeJ) inoculation (MST = 35 days) (p < 0.05). These findings suggest that more Ag-specific Treg cells are generated via intratesticular than renal islet inoculation, although overall number of CD4\textsuperscript+CD25\textsuperscript+ cells in vivo remains the same.

To confirm CD4\textsuperscript+CD25\textsuperscript+ cells generated in our system are all or mostly Treg cells, a one-way MLR was set up to measure the suppression of alloreactive CD4\textsuperscript+CD25\textsuperscript+ T cell proliferation in vitro by these CD4\textsuperscript+CD25\textsuperscript+ cells. As shown in Fig. 3A, Treg cells derived from intratesticular islet recipients, at low doses (<4 \times 10\textsuperscript{4} cells/well), were much more efficient in the suppression of
CD4+CD25− T cell proliferation than Treg cells derived from renal islet recipients \((p < 0.05)\), although both Treg cells at high dose \((5 \times 10^6, \text{ratio of } 1:1 \text{ for suppressor/responder})\) suppressed T cell proliferation at a similar magnitude. This finding suggests that CD4+CD25− cells are mostly Treg cells, although they could still contain some effecter T cells. To further investigate whether increased suppressive function of CD4+CD25− cells after testicular islet transplantation is due to increased generation of Treg component or more potent suppression after Treg activation, FoxP3 mRNA expression by CD4+CD25− cells was determined by a real-time quantitative PCR. As shown in Fig. 3B, CD4+CD25− cells derived from testicular islet recipients expressed higher FoxP3 mRNA than ones derived from renal islet recipients \((p < 0.05)\), suggesting that these CD4+CD25− cells contain more regulatory cells after testicular than renal islet inoculation. Hence the reason why overall CD4+CD25− cell number in the periphery after testicular islet inoculation is not increased compared with one after renal islet inoculation (Fig. 2) could be that the increased CD4+CD25− regulatory cell number in testicular islet recipients is offset by the decreased CD4+CD25− effecter T cells that resulted from the accelerated effecter T cell apoptosis in the testis, an immune privileged site.

**Testicular immune privilege promotes induction of allograft tolerance that is dependent on diminished CD8+ memory but enhanced CD25+ Treg regulation**

It is unclear whether allograft tolerance is induced in the testis under less stringent conditions than in a conventional site. To test this possibility, we transplanted BALB/c islets into the testis or kidney capsule of wt B6 mice that were treated with or without MR1 (anti-CD40L). We found that, despite the impaired generation of CD8+ memory and increased suppressive activity of Ag-specific CD4+CD25− Treg cells, intratesticular islet transplantation by itself only slightly prolongs allograft survival compared with islet transplantation under the kidney capsule with all allografts rejected within 42 days (Fig. 4A). As controls, syngeneic islet grafts transplanted either in the testis or under kidney capsule were not rejected \((>120 \text{ days, } n = 6, \text{ not shown in the figure})\).

As shown in Fig. 4A, although blocking CD40/CD40L interaction with MR1 significantly prolonged survival of allografts transplanted under the kidney capsule \((\text{MST} = 69 \text{ days})\), it failed to induce allograft tolerance at this location. In contrast, MR1 induced the tolerance of islet allografts transplanted in the testis in six of seven recipients \((>120 \text{ days})\). These recipients displayed donor-specific tolerance because they did not reject a skin allograft from a BALB/c \((H-2^b)\) mouse \((>100 \text{ days})\) but did reject a skin allograft from a third-party donor C3H/HeJ \((H-2^k)\) mouse within 22 days (Fig. 4B). These findings suggest that tolerance to intratesticular islet allografts also spreads to skin allografts in non-privileged sites. Treatment with isotype control Ab did not significantly alter allograft survival \((\text{data not shown})\). Importantly, islet allografts transplanted in the testis of mice that were treated with MR1 but received exogenous memory CD8+ cells were all rejected \((\text{MST} = 76 \text{ days})\), indicating that allospecific CD8+ memory cells were capable of breaking the tolerance induced by testicular immune privilege. Interestingly, the adoptively transferred CD4+ memory cells were much less efficient in breaking the immune privilege-mediated tolerance than CD8+ memory cells since only two of six intratesticular islet recipients that received MR1 and CD4+ memory rejected allografts. Finally, deletion of CD25+ Treg cells in vivo by a depleting anti-CD25 mAb hindered tolerance induction as most of intratesticular islet recipients that received both MR1 and anti-CD25 mAb rejected allografts within 120 days \((\text{MST} = 52 \text{ days})\). Histopathology confirmed that there is no infiltration with intact islet allografts in the testis in MR1-treated alone group but “massive” infiltration in the testis in control, MR1 plus CD8+ memory or plus depleting anti-CD25 Ab groups (Fig. 4C).
FIGURE 5. Blocking CD40/CD40L costimulation suppresses T cell proliferation but preserves T cell apoptosis and Treg cell number.

To study mechanisms by which blocking CD40/CD40L costimulation induces allograft tolerance mediated by testicular immune privilege, graft-infiltrating cells were isolated and analyzed for T cell proliferation by BrdU uptake and apoptosis by a TUNEL method at 1 wk after transplantation. As shown in Fig. 5A, MR1 suppressed in vivo proliferation of CD8+ T cells derived from both the kidney and testis. CD8+ T cells from the testis underwent an accelerated apoptosis compared with ones from the kidney (14 vs 5%) whereas blocking CD40L with MR1 preserved CD8+ T cell apoptosis in the testis (14 vs 16%), suggesting that accelerated T cell apoptosis could contribute to testicular islet allograft tolerance. Similar results were also found for CD4+ T cell components (data not shown). The total number of graft-infiltrating T cells was decreased in the kidney with MR1 treatment but was even lower in the testis after MR1 treatment (Fig. 5B). Hence it is possible that CD40/CD40L costimulatory blockade induces testicular islet tolerance by suppressing T cell proliferation/activation or acute rejection whereas the impaired generation of CD8+ memory and enhanced Treg regulation by testicular immune privilege may help stop late allograft rejection. Moreover, the total number of CD4+CD25+ Treg cells that infiltrated the kidney or testis was similar to that after MR1 treatment (Fig. 5C, p > 0.05), suggesting that blocking CD40/CD40L interaction does not hinder the generation of CD4+CD25+ Treg cells although B7/CD28 costimulation is required for the homeostasis and development of Treg cells (38, 39). Finally, an intact proliferation but increased apoptosis of T cells in the testis supports the notion that the impaired generation of memory T cells is due to the increased T cell death but not decreased proliferation in the testis.

Discussion

Using a testicular islet transplant model, we found that islet transplantation in the testis, an immunologically privileged site, generates fewer CD8+ memory cells but induces more Ag-specific CD4+CD25+ Treg cells than in a conventional site. Despite the features of testicular immune privilege, intratesticular islet transplantation by itself did not induce allograft tolerance or long-term survival due to vigorous acute rejection. However, blocking CD40/CD40L costimulation induced tolerance of islet allografts transplanted in the testis but not under the kidney capsule. These studies revealed novel mechanisms of immune privilege and provided the first evidence that testicular immune privilege fosters transplantation tolerance induction.

This study demonstrates that the phenomenon of immune privilege is not “all or none” but inducible and that immunologically privileged sites may have a lower or easier threshold for attaining tolerance than conventional sites. One may argue that it is very limited to use immunologically privileged sites for attaining tolerance due to their location/size. Hence, they have not widely been viewed as clinically useful. However, our findings show that the tolerance to islet allografts in the privileged sites “spreads” to skin allografts in nonprivileged sites. This will dramatically increase the therapeutic potential and feasibility of transplantation in immunologically privileged sites. Thus, they may be used as a stepping-stone toward peripheral tolerance by simply implanting alloantigen rather than being just the sites of organ/tissue grafting. This study therefore provides insight into designing strategies to induce transplantation tolerance.

The mechanisms underlying immune privilege are not well understood. A major mechanism proposed by earlier studies is Fas-mediated apoptosis of T cells or inflammatory cells because FasL is highly expressed by resident cells in the eye and testis (4, 5). However, recent studies have shown that transgenic expression of FasL on islet cells causes a rapid rejection of islet grafts, instead of graft acceptance (8–10). It is possible that overexpression of FasL causes acute inflammatory responses that are irrelevant to the rate of T cell death and cannot be offset by an accelerated T cell death.
by Fas signaling. Indeed, we found that intratesticular islet inoculation generates fewer memory CD8\(^+\) T cells than renal islet transplantation. The shrinking CD8\(^+\) memory pool was important for the maintenance of testicular immune privilege since transplantation tolerance was broken when CD8\(^+\) memory cells were transferred back to those recipient mice. This finding identified CD8\(^+\) memory cells as a target to induce transplantation tolerance via immune privilege. Previous studies have also shown that memory T cells constitute a threat to long-term graft survival because they are resistant to immunosuppression (27) or costimulation blockade (28, 40, 41). Therefore, testicular immune privilege helps overcome a major hurdle to achieving allograft acceptance or tolerance by shutting down CD8\(^+\) memory. We studied CD8\(^+\) but not CD4\(^+\) memory T cells because 1) allospecific CD8\(^+\) T cells from transgenic mice are available for tracking in vivo; 2) immune regulation results in a long-lasting CD8\(^+\) memory but a declining CD4\(^+\) memory pool (42, 43); and 3) CD8\(^+\) memory cells are much more efficient in breaking tolerance induced by testicular immune privilege than CD4\(^+\) memory cells (Fig. 4A).

CD4\(^+\)CD25\(^+\) Treg cells play a key role in the maintenance of immunologic tolerance to both self and foreign Ags by suppressing aggressive T cell responses (30, 44, 45). They suppress autoreactivity and prevent graft rejection in many animal models (26, 31–35, 46, 47). However, it is unknown whether Treg cells participate in testicular immune privilege. Our data indicated that Ag-specific CD4\(^+\)CD25\(^+\) Treg cells are induced by intratesticular islet inoculation. These Treg cells were present in both lymphoid organs and inflammatory nonlymphoid organs such as the kidney and testis. They were essential for maintaining transplantation tolerance as depletion of CD25\(^+\) Treg cells in vivo broke the tolerance (Fig. 4A). A recent study by others has also shown that ovarian carcinoma fosters immune privilege by recruiting Treg cells to tumor sites (48). However, it was unclear whether more tumor-specific Treg cells were generated in the ovary than in a conventional site in their model. Interestingly, although Ag-specific Treg cells were induced by intratesticular islet inoculation, the overall number of CD4\(^+\)CD25\(^+\) cells in both lymphoid and nonlymphoid organs was not significantly altered. Therefore, T cell homeostatic mechanisms are capable of maintaining a stable CD4\(^+\)CD25\(^+\) cell pool after an Ag-specific immune response in vivo. It is likely that CD4\(^+\)CD25\(^+\) Treg cells specific for certain Ags are induced after encounter with these Ags at the cost of nonspecific Treg cells or other Ag-specific Treg cells, which are subsequently decreased in number because of the limited spaces or niches available. The mechanisms responsible for the increased induction of Ag-specific Treg cells by testicular immune privilege remain to be defined. We speculate that an accelerated effector T cell death in the testis would create more niches or spaces for CD4\(^+\)CD25\(^+\) Treg cells to grow since Treg cells do proliferate in vivo (49, 50).

Previous studies have generated contradictory data regarding the role of testicular immune privilege in prolongation of islet allograft survival (18–22). It remains unanswered whether testicular immune privilege promotes transplantation tolerance. We found for the first time that intratesticular islet transplantation, although by itself does not induce long-term allograft survival, does promote allograft tolerance induced by CD40/CD40L costimulatory blockade. Studies by Qian et al. (51, 52) have shown that blocking CD40/CD154 costimulation prolongs survival of conal allografts in the eye, another immunologically privileged site, but that termination of anti-CD154 treatment results in considerable loss of corneal allografts. It is unclear why blocking CD40/CD154 costimulation is capable of inducing tolerance to intratesticular islet allografts in our model but not ocular corneal transplants in their model. It is possible that testicular immune privilege forms via different mechanisms from ocular immune privilege. Interestingly, intratesticular islet transplantation by itself did not induce long-term allograft survival despite the severely impaired generation of allospecific memory CD8\(^+\) cells and increased induction of Ag-specific CD4\(^+\)CD25\(^+\) Treg cells. It is possible that an accelerated T cell apoptosis and induction of Treg cells are still insufficient to prevent acute allograft rejection in the testsis. However, the reduced CD8\(^+\) memory and increased Ag-specific Treg cells do bode well for long-term allograft survival or tolerance induction. Requirement for both CD40/CD40L costimulatory blockade and intratesticular islet transplantation to induce tolerance indicates that transplantation tolerance induction could include the following: 1) prevention of acute rejection by costimulatory blockade or other approaches; 2) “exhaustion” of long-term immunity mediated by Ag-specific T cell memory; and 3) immune regulation by Treg cells. This study may provide insight into the mechanisms underlying immune privilege and has clinical implications for allograft tolerance induction.

**Disclosures**

The authors have no financial conflict of interest.

**References**


