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CD4⁺CD25⁺ regulatory T cells (T\textsubscript{Reg}) are critical for the acquisition of peripheral allograft tolerance. However, it is unclear whether T\textsubscript{Reg} are capable of mediating alloantigen-specific suppressive effects and, hence, contributing to the specificity of the tolerant state. In the current report we have used the ABM TCR transgenic (Tg) system, a C57BL/6-derived strain in which CD4⁺ T cells directly recognize the allogeneic MHC-II molecule I-A\textsuperscript{bm12}, to assess the capacity of T\textsubscript{Reg} to mediate allospecific effects. In these mice, 5–6% of Tg CD4⁺ T cells exhibit conventional markers of the T\textsubscript{Reg} phenotype. ABM T\textsubscript{Reg} are more effective than wild-type polyclonal T\textsubscript{Reg} at suppressing effector immune responses directed against I-A\textsuperscript{bm12} alloantigen both in vitro and in vivo. In contrast, they are incapable of suppressing responses directed against third-party alloantigens unless these are expressed in the same allograft as I-A\textsuperscript{bm12}. Taken together, our results indicate that in transplantation, T\textsubscript{Reg} function is dependent on TCR stimulation, providing definitive evidence for their specificity in the regulation of allograft immune responses. The Journal of Immunology, 2006, 176: 329–334.

T he emergence of T cell immunoregulation is considered the hallmark of peripheral allograft tolerance (1–5). Immunoregulatory networks active in tolerant recipients are characterized by donor specificity, capacity to mediate linked suppression, and dependence on the indirect pathway of allore cognition. Multiple reports have established that activation of CD4⁺CD25⁺ regulatory T cells (T\textsubscript{Reg})\textsuperscript{3} constitutes an essential element of the immunoregulatory pathways that create peripheral allograft tolerance (6–8). In the absence of this T cell subset, a variety of potent tolerizing therapies lose their ability to induce tolerance (9, 10). Indeed, some of these therapies appear to be acting, at least in part, by directly modulating the function of T\textsubscript{Reg} (10). Despite the pre-eminence of T\textsubscript{Reg} in transplant tolerance models, our understanding of how these cells account for the major features of the tolerant state is still incomplete.

Bulk T\textsubscript{Reg} populations are capable of suppressing a variety of interactions with both self and foreign MHC:peptide complexes (11–13). Given the heterogeneity of T\textsubscript{Reg} Ag recognition, monospecfic TCR transgenic (Tg) systems have been critical to understanding the specificity of T\textsubscript{Reg} function in response to nominal Ags. For instance, the demonstration that after specific TCR stimulation, T\textsubscript{Reg} suppression in vitro can be extended to bystander effector T cells (T\textsubscript{Eff}) bearing different specificities was facilitated by the use of influenza hemagglutinin-specific TCR Tg T\textsubscript{Reg} (14). Similarly, the Ag-specific nature of T\textsubscript{Reg} proliferation (15, 16) and suppressive function (17) in vivo was also demonstrated through the use of TCR Tg systems. In contrast to immune responses against nominal Ags, in the absence of a suitable TCR Tg alloreactive system, the elucidation of T\textsubscript{Reg} specificity in transplantation has been more difficult to achieve and is still controversial. A source for confusion has been the widespread use of lymphopenic adoptive transfer systems in which nonspecific suppression of homeostatic proliferation can mask the regulatory effects of polyclonal T\textsubscript{Reg} (18). In addition, in these models T\textsubscript{Reg} harvested from naive, alloantigen-inexperienced mice are capable of preventing T\textsubscript{Eff} from rejecting MHC-mismatched allografts when cell transfer is performed at high ratios of T\textsubscript{Reg} to T\textsubscript{Eff} (10, 19, 20). This finding, which most likely reflects the inherent alloantigen cross-reactivity of T\textsubscript{Reg} TCRs, can also be interpreted as indicating that alloantigen-specific T\textsubscript{Reg} are not required in transplantation tolerance. In contrast, we and others have shown that T\textsubscript{Reg} exhibit donor specificity, but only after alloantigen exposure in the presence of a tolerizing regimen (6, 8, 10), a phenomenon that is crucial for the induction of transplantation tolerance. It must be acknowledged, however, that these later experiments were not performed using a criss-cross design (21) and therefore cannot be considered unambiguous proof of specificity. Thus, elucidation of whether T\textsubscript{Reg} can mediate alloantigen-specific suppressive effects, which would be critical as a first step to understanding the mechanisms of donor specificity in transplantation tolerance, remains an unsolved question.

The ABM TCR Tg mouse is a C57BL/6 (I-A\textsuperscript{b})-derived strain that expresses a V\textsubscript{α}2.1 and a V\textsubscript{β}8.1 TCR specific for the intact class II molecule I-A\textsuperscript{bm12} (expressed on a variant strain of
C57BL/6 called B6.C-H2bm12/KHeg, hereafter referred to as bm12) and does not recognize other alloantigens (22–24). This is, therefore, a CD4+ TCR Tg model of direct alloantigen presentation. I-A<sup>bm12</sup> and I-A<sup>A</sup> differ only at three amino acids in a span of five amino acids (25). Hence, bm12 and C57BL/6 mice have only a limited MHC class II mismatch, which is, nonetheless, sufficient to prompt rejection of bm12 skin allografts by C57BL/6 mice (26). In contrast, bm12 hearts are not acutely rejected by C57BL/6 recipients, although the grafts eventually develop severe arterial disease (chronic rejection) (24).

We have previously determined that ABM mice, in which 90–95% of peripheral CD4<sup>+</sup> T cells express the Vα2.1/Vβ8.1 TCR Tg (24), spontaneously accept bm12 heart allografts, provided recipients have not been previously sensitized by the placement of bm12 skin allografts (24). In addition, long-term surviving bm12 heart allografts from ABM recipients exhibit only minimal signs of chronic rejection. Thus, despite the very high frequency of alloreactive T cells, ABM recipients fail to acutely or chronically reject bm12 heart allografts. We report in this article that in ABM mice a small fraction of TCR Tg CD4<sup>+</sup> T cells constitutively express CD25 and are bona fide T<sub>Reg</sub>. These allospecific regulatory T cells are powerfully suppressive both in vitro and in vivo and are responsible for the capacity of ABM mice to spontaneously accept bm12 hearts. Using this system we show that during alloimmune responses, T<sub>Reg</sub> suppressive function is dependent on specific TCR stimulation. This suggests that one of the mechanisms contributing to the exquisite specificity of allograft tolerance could be the preferential activation of alloantigen-specific T<sub>Reg</sub>.

Materials and Methods

Mice

The ABM (anti-bm12) TCR Tg mice were generated by Dr. E. Palmer (University Hospital, Basel, Switzerland) (22). TEa CD4<sup>+</sup> TCR Tg mice were provided by Dr. R. J. Noelle (Dartmouth Medical School, Lebanon, NH). The TEa TCR recognizes the I-E-derived peptide ASFEAQGLA in the context of I-A<sup>A</sup>, which is expressed in all APCs from H-2<sup>b</sup>/1-E<sup>−</sup> strains (e.g., CB6F<sub>1</sub>, an F<sub>1</sub> hybrid of C57BL/6 and BALB/c) (27). Bm12, CB6F<sub>1</sub>, BALB/c, C57BL/6, and C57BL/6 nude mice were purchased from The Jackson Laboratory. F<sub>1</sub> (BALB/c × bm12) hybrids and ABM backcrossed into the Rag<sup>−/−</sup> background were generated in our laboratory. Only Rag<sup>−/−</sup> ABM mice were used to isolate TCR Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells. Mice were maintained under pathogen-free conditions at Beth Israel Deaconess Medical Center and were used at 6–8 wk of age. Animal experiments were approved by the Beth Israel Deaconess Medical Center institutional animal care committee.

Cell sorting

Single-cell suspensions, prepared from lymph nodes and spleens, were enriched for T cells using T cell enrichment columns (R&D Systems), and T cell subset sorting was achieved using a MoFlo cell sorter (DakoCytomation) after staining with fluorochrome-conjugated anti-CD25, anti-CD4, anti-Vα<sub>2</sub>.1, and anti-Vβ8.1 mAbs (all mAbs from BD Pharmingen). Purity was consistently >95% for Vα2.1 and Vβ8.1 double-positive cells. T cell subsets from C57BL/6 or TEa mice were sorted based on CD4 and CD25 markers only.

Cell culture experiments

CD4<sup>+</sup>CD25<sup>+</sup> T cells (5 × 10<sup>5</sup>) were cultured with 3 × 10<sup>5</sup> irradiated allogeneic spleen cells or 10<sup>5</sup> allogeneic bone marrow-derived mature DCs, with or without 5 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells, and proliferation was measured by [3H]TdR incorporation. DCs were derived from bone marrow by culture for 6 days in RPMI 1640 plus 10% FCS, antibiotics, 50 μM 2-ME, and 10 ng/ml GM-CSF, with addition of LPS during the last 12 h, and were sorted based on high CD86 expression.

Real-time PCR

Real-time PCR was performed with the ABI 7700 sequence detector system using commercially designed primer/probe sets (Applied Biosystems). The expression of the target genes was normalized to that of the housekeeping gene GAPDH, and data were expressed as the relative fold difference between cDNA from the study samples and that from a calibrated sample.

Heterotropic cardiac transplantation

Cardiac transplants were performed in ABM recipients as previously described (28). In some cases thymectomized recipients were given 200 μg of rat anti-mouse CD25 mAb (PC61, 5.3; IgG1; ATCC TB222) i.p. 4 wk before transplantation. We have previously determined that at such doses anti-CD25 mAb eliminates >80% of CD4+CD25<sup>+</sup> T cells in secondary lymphoid tissues.

Adaptive cell transfer and skin transplantation

Lymphopenic C57BL/6 nude mice were injected with sorted CD4+CD25<sup>+</sup> and/or CD4<sup>+</sup>CD25<sup>+</sup> T cells transferred at different cell ratios 1 day before skin allograft transplantation. Full-thickness trunk skin grafts from donor mice were then grafted onto the dorsum of adoptively transferred recipient mice.

Results

TCR Tg T<sub>Reg</sub> can be identified in the ABM Tg model

In ABM mice, a small fraction (mean, 5%; n > 20) of CD4<sup>+</sup> T cells from secondary lymphoid tissues constitutively expressed CD25 as well as other markers typical of the T<sub>Reg</sub> phenotype (Fig. 1). The proportion of CD4<sup>+</sup>CD25<sup>+</sup> T cells was smaller than that observed in C57BL/6 controls (mean, 8.6%; n > 20; p < 0.005). Most of the CD4<sup>+</sup>CD25<sup>+</sup> T cells present in ABM mice bore the Vα2.1/Vβ8.1 TCR (mean, 71%; n > 20), indicating that they expressed the anti-bm12 TCR Tg, although this proportion was lower than that in ABM CD4<sup>+</sup>CD25<sup>+</sup> T cells (mean, 84%; n > 20; p < 0.004; Fig. 1A). As previously reported for other mice carrying Tg TCRs (14, 29, 30), in ABM mice, CD4<sup>+</sup>CD25<sup>+</sup> T cells were only found in conventional, not in Rag<sup>−/−</sup>, backgrounds (data not shown). This presumably reflects the need for endogenous TCR α-chain rearrangement for the thymic development of CD4<sup>+</sup>CD25<sup>+</sup> Tg cells (30). To characterize ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells, we quantified the expression of genes associated with T<sub>Reg</sub> function. Resting CD4<sup>+</sup>CD25<sup>+</sup>, but not CD4<sup>+</sup>CD25<sup>+</sup>, ABM Tg T cells expressed high levels of CTLA4, Forkhead/winged helix transcription factor gene (Foxp3), and CD103 (Fig. 1B). No significant differences were found in the expression of these genes between Tg and control C57BL/6 CD4<sup>+</sup>CD25<sup>+</sup> T cells (data not shown). Together, our results indicate that allospecific anti-bm12 Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells are present in ABM mice, and that these cells exhibit a similar phenotype to conventional T<sub>Reg</sub>.

ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells are allostrogenic specific and mediate powerful suppressive effects in vitro

ABM Tg T<sub>Reg</sub>, but not ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Reg</sub>, were anergic in vitro after direct stimulation with bm12 splenocytes (Fig. 2A). In addition, ABM Tg T<sub>Reg</sub>, but not wild-type C57BL/6 T<sub>Reg</sub>, powerfully suppressed the allospecific proliferation of ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Eff</sub> (Fig. 2B). To study the allospecificity of ABM Tg T<sub>Reg</sub> in vitro, we took advantage of the capacity of T<sub>Reg</sub> to proliferate if cultured with mature DCs (16). ABM Tg T<sub>Reg</sub> proliferated in response to mature bm12, but not third-party, bone marrow-derived mature DCs (Fig. 2C). Highly specific effects were also elicited when ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells were challenged with the two populations of mature DCs, albeit the effector T cell proliferation was significantly higher than that of T<sub>Reg</sub> (Fig. 2C). To determine whether ABM Tg T<sub>Reg</sub> could suppress the proliferation of T cells bearing different TCR specificities, we used TEa Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells, which mount strong proliferative responses when cultured with CB6F<sub>1</sub>, but not with bm12, irradiated splenocytes. ABM Tg T<sub>Reg</sub> did not suppress the proliferation
of TEa Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells in response to CB6F<sub>1</sub> stimulators (Fig. 2D, center column). In contrast, in the presence of mixed CB6F<sub>1</sub> and bm12 stimulators, ABM Tg T<sub>Reg</sub>s markedly inhibited TEa Tg CD4<sup>+</sup>CD25<sup>+</sup> T cell proliferation (Fig. 2D, right column). Taken together, our results indicate that ABM Tg T<sub>Reg</sub>s are absolutely dependent on their cognate alloantigen for activation and

FIGURE 2. ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells exhibit powerful, TCR-restricted, suppressive properties in vitro. A. ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells (5 < 10<sup>5</sup>) do not proliferate in response to 3 < 10<sup>5</sup> irradiated bm12 splenocytes and suppress the proliferation of 5 < 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells. B. The proliferation of 5 < 10<sup>5</sup> ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> T cells in response to 3 < 10<sup>5</sup> irradiated bm12 splenocytes is powerfully suppressed by increasing numbers of ABM Tg, but not polyclonal C57BL/6, T<sub>Reg</sub>s. C. Both 5 < 10<sup>4</sup> ABM Tg CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells proliferate in response to 10<sup>4</sup> bm12, but not CB6F<sub>1</sub>, bone marrow-derived mature dendritic cells. Cell proliferation was estimated in all cases by [<sup>3</sup>H]TdR incorporation. Data are expressed as the mean cpm of triplicate cultures ± SE. Data portrayed in all panels are representative of at least three independent experiments. D. ABM Tg T<sub>Reg</sub>s (5 < 10<sup>5</sup>) suppress the proliferation of 5 < 10<sup>5</sup> TEa CD4<sup>+</sup>CD25<sup>+</sup> T cells only when bm12 irradiated splenocytes are added to the stimulator CB6F<sub>1</sub> cell population.
proliferation. However, once activated, they can suppress the proliferation of T\textsubscript{Eff} specific for alloantigens expressed on different APCs (bystander suppression).

**ABM mice reject bm12 heart allografts in the absence of T\textsubscript{Regs}**

To determine the role of ABM T\textsubscript{Regs} in the prevention of both acute and chronic bm12 heart allograft rejection, we performed a set of heart transplants in ABM Tg recipients that had been thymectomized and depleted of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{Regs} by anti-CD25 mAb treatment. ABM mice universally rejected bm12 heart allografts in the absence of T\textsubscript{Regs} (Fig. 3A). Thus, allospecific T\textsubscript{Regs} powerfully suppress cytopathic alloreactive T cells in vivo and prevent both acute and chronic allograft rejection.

**ABM Tg T\textsubscript{Regs} exhibit more powerful suppressive properties than C57BL/6 T\textsubscript{Regs} after transfer into bm12 skin allograft recipients**

To test the capacity of ABM Tg T\textsubscript{Regs} to mediate alloantigen-specific effects in vivo, we conducted adoptive transfer experiments using lymphopenic skin allograft recipients. In this model, the transfer of as few as 1 × 10\textsuperscript{5} CD4\textsuperscript{+}CD25\textsuperscript{−} or CD25\textsuperscript{−} wild-type naive T cells into skin allograft recipient results in rapid graft rejection, whereas transferred T\textsubscript{Regs} cells do not induce rejection and prevent CD4\textsuperscript{+}CD25\textsuperscript{−} T\textsubscript{Eff} populations from destroying the grafts (10). The median survival time of bm12 skin grafts challenged with 10\textsuperscript{5} ABM Tg CD4\textsuperscript{+}CD25\textsuperscript{−} T cells was 10 days (Fig. 3B). C57BL/6 T\textsubscript{Regs} (10\textsuperscript{5}) did not prevent 10\textsuperscript{5} ABM Tg CD4\textsuperscript{+}CD25\textsuperscript{−} T cells from rapidly rejecting bm12 allografts. In contrast, ABM Tg T\textsubscript{Regs} significantly delayed the occurrence of graft rejection (median survival time, 12 vs 40 days; p < 0.01; Fig. 3B). C57BL/6 T\textsubscript{Regs} only prevented skin allograft rejection when transferred at a high ratio (3:1) of T\textsubscript{Reg} to BM Tg CD4\textsuperscript{+}CD25\textsuperscript{−} T cell, albeit this protective effect was less marked than after administering an equivalent number of ABM Tg T\textsubscript{Regs} (data not shown). The need to transfer very high T\textsubscript{Reg} to T\textsubscript{Eff} ratios to ensure effective suppression when using naive polyclonal T\textsubscript{Regs} has been previously reported (10). ABM mice have a 30-fold higher frequency of I-A\textsuperscript{bm12}-reactive CD4\textsuperscript{+} T cells than polyclonal C57BL/6 mice (24). Hence, our data indicate that the net suppressive effects exerted by bulk T\textsubscript{Reg} populations in transplantation critically depend on the frequency of alloreactive T\textsubscript{Regs} among them.

**ABM Tg T\textsubscript{Regs} do not prevent the rejection of third-party skin allografts**

To elucidate the fine specificity of T\textsubscript{Reg} function in vivo, we performed additional experiments transferring C57BL/6 CD4\textsuperscript{+}CD25\textsuperscript{−} T cells together with ABM Tg T\textsubscript{Regs} into recipients of bm12 or third-party (BALB/c) skin allografts. Polyclonal CD4\textsuperscript{+}CD25\textsuperscript{−} T cells are capable of rejecting any MHC-mismatched allogeneic skin allograft (10). ABM Tg T\textsubscript{Regs}, in contrast, do not mediate immunosuppressive effects in vitro unless direct recognition of intact bm12 alloantigens takes place (Fig. 2). Furthermore, ABM Tg CD4\textsuperscript{+}CD25\textsuperscript{−} T cells fail to reject third-party BALB/c skin allografts (our unpublished observations). Hence, we hypothesized that ABM Tg T\textsubscript{Regs} would prevent wild-type CD4\textsuperscript{+}CD25\textsuperscript{−} T cells from rejecting bm12, but not third-party strain, allografts. As predicted, the cotransfer of ABM Tg T\textsubscript{Regs} had no effect on the capacity of C57BL/6 CD4\textsuperscript{+}CD25\textsuperscript{−} T cells to reject BALB/c skin allografts (Fig. 4A), whereas a protective effect was exerted upon bm12 allografts (Fig. 4B). The failure of transferred ABM Tg T\textsubscript{Regs} to delay BALB/c skin allograft rejection persisted even after markedly increasing the ratio of T\textsubscript{Reg} to T\textsubscript{Eff} at variance with the effect of transferring polyclonal C57BL/6 T\textsubscript{Regs} (Fig. 5C). These findings indicate that adoptively transferred T\textsubscript{Regs} suppress cytopathic alloimmune responses only when T\textsubscript{Regs} are stimulated by allografts expressing their cognate Ags.

**ABM Tg T\textsubscript{Regs} can mediate linked suppression**

Linked suppression, a phenomenon in which T\textsubscript{Regs} can suppress the rejection of third-party alloantigens provided they are expressed on the same APC as the tolerated Ags (4), is considered one of the hallmarks of peripheral allograft tolerance. To test the capacity of T\textsubscript{Regs} to mediate this process, we used F\textsubscript{1} (BALB/c × bm12) allografts as a source of APCs expressing both bm12 and third-party (BALB/c) alloantigens. The cotransfer of ABM Tg T\textsubscript{Regs} and C57BL/6 CD4\textsuperscript{+}CD25\textsuperscript{−} T cells at a 1:1 ratio into hosts grafted with F\textsubscript{1} skin did not delay the occurrence of allograft rejection (Fig. 5A). Nonetheless, the administration of a higher T\textsubscript{Reg} to CD4\textsuperscript{+}CD25\textsuperscript{−} T cell ratio resulted in significant prolongation of F\textsubscript{1} allograft survival (Fig. 5B). These results are at variance with those of experiments performed using BALB/c allografts (Fig. 5C). Taken together, our findings indicate that T\textsubscript{Reg} suppressive effects can extend to T\textsubscript{Eff} responding to third-party alloantigens present on the same graft that stimulates the T\textsubscript{Regs} (linked suppression).
Discussion

In the current study we have focused on the elucidation of the capacity of natural T Regs to mediate both alloantigen-specific and linked suppressive effects in transplantation. To do so we have used a unique TCR Tg system in which CD4\(^+\) H11001 T cells directly recognize the allogeneic MHC-II molecule, I-A\(^b\) bm12. We report in this paper for the first time that in ABM TCR Tg mice, a fraction of anti-I-A\(^b\) bm12-specific CD4\(^+\) H11001 T cells are bona fide T Regs capable of mediating alloantigen-specific suppressive effects both in vitro and in vivo. In addition, the regulatory properties of ABM TCR Tg T Regs are not restricted to bm12 allografts, but can also extend to F_1 (BALB/c \times bm12) allografts (linked suppression), although in this case effective prevention of graft rejection requires transfer of a high T Reg to T Eff cell ratio. We have previously determined that bm12 alloantigens placed into C57BL/6 hosts are not effectively processed by host APCs (28). Hence, bm12 allografts are only capable of stimulating polyclonal C57BL/6 T cells through direct alloantigen presentation. In contrast, the use of grafts with multiple mismatches at both major and minor histocompatibility Ags (e.g., BALB/c or F_1 grafts into B6 recipients) results in direct and indirect allore cognition events. These data indicate that the requirement for a high ABM Tg T Regs to wild-type CD4\(^+\) CD25\(^-\) T cell ratio to ensure effective protection of F_1, but not bm12, grafts may be due to 1) stimulation of a higher frequency of effector T cells by F_1 than by bm12 grafts, and/or 2) inefficient suppression by T Regs of CD4\(^+\) CD25\(^-\) T cells activated through the indirect allore cognition pathway. The latter would imply that effective suppression of alloimmune responses requires T Regs and T Eff sharing a common APC.

Our data also show that the spontaneous acceptance of bm12 heart allografts by ABM TCR Tg recipients is absolutely dependent on the presence of anti-I-A\(^b\) bm12 TCR Tg T Regs. This is remarkable given that in ABM mice, the overall anti-I-A\(^b\) bm12 T Eff responder frequency is \(~70\%\) (24), whereas only 5% of TCR Tg CD4\(^+\) T cells express T Reg markers. These findings indicate that in nonlymphopenic situations, natural T Regs with a defined specificity exhibit a very strong capacity to prevent allo graft rejection when their cognate Ag is expressed on the graft. This is in keeping with
a recent report using mice expressing a Tg TCR directed against the minor histocompatibility Ag, HY (31). Taken together, these studies, using nonlymphopenic hosts, suggest that both in vitro assays and in vivo adoptive transfer systems, in which unphysiological ratios of T<sup>reg</sup> to T<sup>eff</sup> are commonly required to ensure effective suppression, most likely underestimate the regulatory properties of natural T<sup>reg</sup>. These are clinically relevant observations, suggesting that administration of a limited number of allospecific T<sup>reg</sup> to nonlymphopenic transplant recipients might be an effective strategy to induce graft acceptance.

The use in our experiments of natural, alloantigen-inexperienced, ABM TCR Tg T<sup>reg</sup> (i.e., T<sup>reg</sup> obtained from naive unmanipulated ABM mice) precludes us from directly addressing the current controversy of whether tolerizing regimens result in the generation of allospecific T<sup>reg</sup> (5, 6, 10, 21). Our observation that regulation of transplant rejection by T<sup>reg</sup> critically depends on specific TCR stimulation raises the possibility that tolerance-inducing strategies might be active, at least in part, by preferentially expanding alloantigen-specific T<sup>reg</sup>. However, the use of polyclonal T<sup>reg</sup> harvested from tolerized recipients to assess T<sup>reg</sup> specificity has resulted in much less clear-cut results (5, 6, 10, 21, 32). In a polyclonal population of T cells, the expression of two TCR heterodimers by a single cell or Ag cross-reactivity by a given TCR may create a situation in which a T<sup>reg</sup> with alloantigen-specificity may be activated by another Ag. Moreover, the use of adoptive transfer systems involving lymphopenic hosts may exacerbate these effects. Alternatively, other regulatory T cell subsets might also be participating in ensuring transplantation tolerance allospecificity. Additional studies are required to completely elucidate these hypotheses.

Disclosures
The authors have no financial conflict of interest.

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