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Location and Time-Dependent Control of Rejection by Regulatory T Cells Culminates in a Failure to Generate Memory T Cells

Manuela Carvalho-Gaspar, Nick D. Jones, Shiqiao Luo, Laurent Martin, Matthew O. Brook, and Kathryn J. Wood

Adaptive CD25+CD4+ regulatory T cells (Treg) can be induced following exposure to alloantigen and may function alongside naturally occurring Treg to suppress allograft rejection when present in sufficient numbers. However, the location of the Treg as they function in vivo and the mechanisms used to control donor-reactive T cells remains ill-defined. In this study, we used a CD8+ TCR transgenic model of skin allograft rejection to characterize in vivo activity of donor-reactive Treg cells during induction of transplantation tolerance. We demonstrate that, initially after skin transplantation, Treg attenuate the priming of donor-reactive naïve CD8+ T cells in the lymphoid tissue draining the graft site. However, with time, peripheral suppression is overcome despite the continued presence of Treg, resulting in the priming of donor-reactive CD8+ T cells and graft infiltration by the resultant effector T cells and induction of a “Tc1-like” intragraft gene expression profile. These intragraft effector CD8+ T cells are then prevented from eliciting rejection by Treg that simultaneously infiltrate the skin allografts, resulting in a failure to generate donor-reactive memory CD8+ T cells. Overall, these data demonstrate for the first time that donor-reactive Treg can suppress allograft rejection using distinct mechanisms at different sites in vivo with the overall outcome of preventing the generation of donor-reactive memory T cells. The Journal of Immunology, 2008, 180: 6640–6648.

Immunological tolerance to foreign organ grafts is one of the main goals in transplantation and indeed specific unresponsiveness or operational tolerance to allografts can be achieved through a variety of different strategies in animal models (1–3) and, at present, to a more limited extent in humans (4–6). Although the mechanisms by which specific unresponsiveness is achieved may vary with the tolerizing therapy, it is now clear that the generation/expansion of donor-reactive regulatory T cells (Treg) is required for both the induction and maintenance of tolerance in many models (7–9).

The existence of leukocytes able to suppress alloantigen-specific immune responses was first described by Billingham, Brent, and Medawar over 50 years ago (10). More recently, it has been shown that, in particular, naturally occurring, thymus-derived Treg play a critical role in the maintenance of peripheral tolerance to self-proteins (11–13). Alloantigen-reactive murine Treg appear to share many properties with naturally occurring Treg in that they reside primarily, but not exclusively, within the CD25+CD4+ T cell subset, constitutively express Foxp3, a gene that encodes a forkhead/winged helix transcription factor required for the development of Tregs (14–16), and are dependent on CTLA-4, IL-10, and TGF-β for their mode of action (7, 17–20). Although it is now clear that alloantigen-reactive Treg are required for the induction and maintenance of transplantation tolerance (8), the mechanisms involved and the location at which suppression is effected have yet to be fully characterized, particularly in the light of the fact that inflammation, a situation that inevitably occurs at the time of transplantation, has a dramatic effect on Treg generation and function (21, 22).

Blockade of the CD40–CD154 pathway at the time of transplantation facilitates the generation of alloreactive Treg, which are able to suppress skin allograft rejection mediated by CD8+ H2Kb-reactive BM3 TCR transgenic T cells (23). In this study, we sought to determine the impact of Treg generated by exposure to alloantigen in vivo on naive donor-reactive CD8+ T cells as they respond to alloantigen after transplantation. We demonstrate that donor alloantigen-reactive Treg initially markedly diminished the priming of naive BM3 T cells after transplantation of an H2Kb+ skin graft, but with time functional BM3 effector T cells were generated that infiltrated the skin graft. However, rejection did not occur as concomitantly, Treg also migrated to the graft preventing the rejection response locally and the generation of memory T cells in the periphery resulting in a deficit in memory T cells in animals exhibiting active regulation.

Materials and Methods

Mice

CBA.Ca RAG-1 knockout (CBA RAG−/−) and BM3 TCR-transgenic mice (H2b) were a gift from Dr. D. Kioussis (Mill Hill, London, U.K.) and Professor A.L. Mellor (Institute of Molecular Medicine and Genetics, Augusta, GA (24), respectively. BM3 mice were crossed to a CBA RAG−/−
background (BM3) for use in these studies. CBA.Ca (CBA; H2\(^b\)) and C57BL/10 (B10; H2\(^a\)) mice were originally purchased from Harlan Sprague Dawley. All mice were bred and housed in the BMS-JR, John Radcliffe Hospital, Oxford, U.K. in accordance with the Animals (Scientific Procedure) Act 1986 of the U.K. All experiments were performed with mice aged between 6–12 wk at the time of first procedure.

**Skin transplantation**

Individual full thickness skin grafts (~1 cm\(^2\)) were prepared to fit the graft bed on the left lateral thorax of anesthetized recipients 1 day after cell adoptive transfer. The grafts were inspected regularly until they were completely destroyed, at which time the grafts were considered rejected.

**Cell preparation and adoptive transfer**

**BM3 RAG\(^{−/−}\) CD8\(^+\) T cells (BM3 T cells)**

A single cell leukocyte suspension was made from spleens and mesenteric lymph nodes (MLN) harvested from BM3 and CD8\(^+\) T cells were purified as previously described (25). After purification, cells were CFSE labeled before adoptive transfer into CBA RAG\(^{−/−}\) mice 1 day before skin grafting.

**CD25\(^+\) CD4\(^+\) T cells**

CD4\(^+\) T cells were isolated from spleens and MLN from CBA mice that had accepted a donor-type B10 cardiac allograft for >100 days following administration of anti-CD154 mAb at the time of transplant by negative selection using an Ab mixture to deplete CD8\(^+\) cells, B cells, MHC class II\(^+\) cells, and monocytes and anti-rat-coated Dynabeads. Such cells were then stained with CD25-PE (PC61) and CD4-APC (RM4–5; both BD Biosciences) before sorting the CD25\(^+\) population using a FACSAria cell sorter (BD Biosciences). Typically CD25\(^+\) CD4\(^+\) T cells were isolated to >95% purity and were found to be >80% Foxp3\(^+\) (eBiosciences; data not shown).

**CFSE labeling**

Single cell suspensions were incubated for 10 min at 37°C with 10 μM CFSE (Molecular Probes), washed twice in ice-cold RPMI 1640 (Life Technologies), and resuspended in PBS (Oxoid) ready for i.v. injection.

**Flow cytometric analysis**

A single cell suspension was prepared from either spleen, MLN, or axillary lymph nodes and cell surface staining was conducted as described previously (25). All samples were then acquired immediately on a four-color FACSort (BD Biosciences) and analyzed using the CellQuest software package (BD Biosciences). Analysis of the forward scatter vs side scatter dot plot of a given sample enabled live cells (and beads, see below) to be distinguished clearly from dead cells and cell debris.

**Enumeration of cell numbers by flow cytometry**

We used a technique that allowed to determine the absolute number of BM3 T cells by flow cytometry using synthetic fluorescent beads (CaliBRITE beads, BD Biosciences) as described previously (25).

**Real-time PCR**

Skin grafts were harvested at different time points after transplantation, snap frozen, and DNase I-treated total RNA was later isolated using the Absolutely RNA MiniPrep kit (Stratagene) and reversed transcribed by the M-MLV reverse transcriptase (Invitrogen) (26). Real-time quantification was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) using either the fluorogenic probe (CD8\(^\alpha\) Taqman Gene Expression Assay from Applied Biosystems, hypoxanthine phosphoribosyltransferase (HPRT), CD3\(^\gamma\), IFN-γ, and perforin) or the SYBR Green technology (HPRT, CCL5, XCL1, CXCL9, and CXCL10) as described previously (26). Samples were standardized for HPRT and quantification of the gene of interest is given by 2\(^−ΔΔCt\), where ΔΔCt is obtained by calculating the difference between Ct of the gene of interest and HPRT (27).

**Histology**

Thin frozen sections (7–8 μm) were cut, air-dried overnight, and fixed in acetone (BDH, VWR International). Sections were then stained with either an anti-CD8-biotin (53–7.6, BD Biosciences) or anti-CD4-biotin mAb (RM4–5, BD Biosciences) for 1 h at room temperature and counterstained with Gill’s hematoxylin (BDH, VWR International) (25).

**Statistical analysis**

Data were analyzed using the statistical software Prism (version 3; GraphPad Software) and are reported as mean ± SD. Data for intragraft gene expression were analyzed using either two-tailed unpaired t test or one-way ANOVA with Bonferroni’s post test (multiple means) and p values <0.05 were considered statistically significant.

**Results**

**Alloreactive Treg prevent CD8\(^+\) T cell priming following allogeneic skin transplantation**

We have previously demonstrated that adoptive transfer of CD8\(^+\) H2K\(^b\)-reactive, TCR transgenic BM3 T cells into syngeneic RAG\(^{−/−}\) mice followed by transplantation of a B10 (H2K\(^b^\)) skin graft resulted in skin graft rejection (25). We have also shown that Treg purified from mice tolerized to B10 alloantigen were able to suppress skin allograft rejection mediated by BM3 CD8\(^+\) T cells in a donor-specific manner (23).

We now sought to determine how such donor alloantigen-reactive Treg control and suppress the CD8\(^+\) T cell response that otherwise leads to skin graft rejection in this setting. To this end, 1 × 10\(^5\) CFSE-labeled BM3 T cells were adoptively transferred either alone or together with 5 × 10\(^5\) Treg into syngeneic RAG\(^{−/−}\) mice. Such mice received a donor-type B10 skin graft the day after adoptive transfer. We found that, in the presence of Treg, there was little evidence of proliferation of BM3 T cells in the first 10 days after transplantation, and few BM3 T cells with low or undetectable levels of CFSE (i.e., having undergone at least seven rounds of division) were detected in the draining lymph-node (dLN) (Fig. 1A). In clear contrast, in the absence of Treg, BM3 T cells had responded to the skin allograft, proliferating exclusively in the dLN during the first 10 days after transplantation, and had generated a substantial population of CFSE\(^−\) T cells (Fig. 1A). These results were confirmed by enumerating the absolute number of CFSE\(^−\) BM3 T cells in the dLN and contralateral axillary lymph nodes (cLN) in mice that had received BM3 T cells either alone or in combination with Treg. We found that very few CFSE\(^−\) BM3 T cells were found in the dLN compared with the cLN in mice that had also received Treg, whereas in mice that had received BM3 T cells only, priming in the dLN resulted in an 18-fold increase in the number of CFSE\(^−\) BM3 T cells in the dLN compared with the number of CFSE\(^−\) cells present in the cLN (Fig. 1B). Moreover, in mice that had also received Treg, the CFSE level in CFSE\(^−\) cells was the same in both dLN and cLN, clearly demonstrating that the markedly diminished generation of CFSE\(^−\) T cells during the first 10 days after grafting was a result of inhibition of BM3 T cell priming and not a result of a slower division rate (Fig. 1A). Very few CFSE\(^−\) BM3 T cells were found in other lymphoid tissues in all mice studied (Fig. 1B, data not shown) (25).

Homeostatic proliferation of T cells following adoptive transfer into lymphopenic mice may be slowed or prevented by the presence of Treg and could potentially mask the alloantigen-specific effects of the Treg (28, 29). Therefore, we determined whether the presence of Treg had any impact on the homeostatic proliferation of BM3 T cells by analyzing the CFSE levels of CFSE\(^−\) BM3 T cells in nondraining peripheral lymphoid tissues. We found that in mice that had received BM3 T cells but no skin graft (BM3 only; Fig. 1C), BM3 T cells underwent slow division as judged by the gradual loss of CFSE from day 5 to day 15 in the spleen, as well as in the MLN and cLN (data not shown). The loss of CFSE in these tissues was exactly the same as that observed in CFSE\(^−\) BM3 T cells in mice that received a B10 skin allograft (BM3 plus Skin Tx; Fig. 1C), except where priming was taking place in the dLN. More importantly, homeostatic proliferation of BM3 T cells was identical in mice that had also received Treg (BM3 plus Reg plus...
B10 Skin Tx), suggesting that, in this model, Treg do not impair homeostatic proliferation and that therefore the prevention of T cell priming was not simply due to suppression of homeostatic proliferation (Fig. 1C).

**Treg prevent CD8⁺ T cell infiltration of skin allografts**

The infiltration of skin allografts undergoing rejection by alloreactive T cells only occurs following priming and expansion of the T cells in the LN (25). Next, we sought to correlate the inhibition of BM3 T cell priming in mice that had also received Treg, assessing the degree of BM3 T cell infiltration of allografts 10 days after transplantation. B10 skin allografts were analyzed for the presence of BM3 T cells by immunohistochemistry and real-time RT-PCR for CD8 expression. Staining for CD8 revealed that by 10 days after transplantation, CD8⁺ T cells had infiltrated B10 skin grafts transplanted onto mice that had received BM3 T cells only (BM3 plus Skin Tx; Fig. 2A). In marked contrast, when Treg were co-transferred with the BM3 T cells (BM3 plus Reg plus Skin Tx), only the occasional infiltrating CD8⁺ T cell was found at day 10. Importantly, staining for CD8 was undetectable in skin allografts from recipients that had not received BM3 CD8⁺ T cells (data not shown), confirming that the CD8 staining observed was specific for infiltrating BM3 CD8⁺ T cells. In addition, no CD8 staining was found in skin grafts 5 days after transplantation in any of the groups. The absence of BM3 T cells in skin grafts from recipients that received both BM3 T cells and Treg 10 days after transplantation was confirmed by real-time PCR for CD8 expression (Fig. 2A).

**FIGURE 1.** CD25⁺ CD4⁺ Treg prevent BM3 CD8⁺ T cell priming in the draining lymphoid tissue 10 days after B10 skin transplantation. One × 10⁹ CFSE labeled BM3 T cells were adoptively transferred into CBA RAG⁻/⁻ recipient mice, either alone (BM3 + Skin Tx) or together with 5 × 10⁶ CD25⁺ CD4⁺ Treg (BM3 + Reg + Skin Tx). The following day, mice were transplanted with a B10 skin allograft. Control CBA RAG⁻/⁻ recipient mice received 1 × 10⁶ CFSE-labeled BM3 T cells but no skin graft (BM3 only). Draining (dLN) and contralateral (cLN) axillary lymph nodes were harvested 5, 10, and 15 days after skin grafting. A, The CFSE fluorescence of BM3 T cells (shown in dot-plots of CD8 vs CFSE of gated BM3 T cells) present in both the cLN and dLN 10 days after skin grafting. Data shown are representative of three mice per group. B, The absolute number of CFSE⁻ BM3 T cells was determined in dLN and cLN either in the presence or absence of CD25⁺ CD4⁺ Treg 10 days after transplantation of a B10 skin allograft. Results are expressed as mean number of CFSE⁻ BM3 T cells per tissue ± SD (n = 3/group). *, p < 0.05. C, CD25⁺ CD4⁺ Treg fail to affect homeostatic proliferation of nonprimed BM3 T cells. The results represent the mean fluorescence intensity of CFSE in CFSE⁻ BM3 T cells in the Spleen 5, 10, and 15 days post transplantation. N.D., not determined; n = 3 per group per time point. Results were confirmed in a second independent study.

**FIGURE 2.** CD8⁺ T cells do not infiltrate skin allografts in the presence of CD25⁺ CD4⁺ Treg resulting in the abrogation of the intragraft inflammatory response. CBA RAG⁻/⁻ recipient mice were adoptively transferred with 1 × 10⁹ CFSE labeled BM3 T cells either alone (BM3 + Skin Tx) or together with 5 × 10⁶ CD25⁺ CD4⁺ Treg (BM3 + Reg + Skin Tx). The following day, mice were transplanted with a B10 skin allograft. B10 skin grafts were harvested 5 and 10 days after transplantation from both groups of mice (n = 3). A, Histological assessment of infiltrating CD8⁺ T cells was conducted by staining frozen sections for CD8. CD8⁺ T cells were enumerated by point counting at least five consecutive fields/graft (n = 3). B, Real-time PCR analysis was performed on skin allografts harvested from CBA RAG⁻/⁻ mice that had (BM3 + Reg + Skin Tx) or had not (BM3 + Skin Tx) received cotransfer of CD25⁺ CD4⁺ Treg together with BM3 T cells. Expression of CD8 (A) and IFN-γ (B), perforin, CCL5, XCL1, CXCL10, and CXCL9 in such grafts was compared with expression in naive untransplanted donor tail skin and/or to skin allografts harvested from CBA RAG⁻/⁻ mice that did not receive BM3 T cells (Skin Tx only). Results are expressed as mRNA (units/HPRT) ± SD. Two to three grafts were analyzed per time point per experiment. n.e., Not expressed. ***, p < 0.001; **, p < 0.01; *, p < 0.05.
We also investigated whether inhibition of BM3 T cell priming by Treg during the first 10 days after transplantation correlated with a reduced intragraft inflammatory response. Real-time RT-PCR analysis of skin allografts revealed that the markedly diminished priming and infiltration of the graft by BM3 T cells 10 days after transplantation in mice that had received Treg correlated with reduced intragraft expression of IFN-γ (84-fold), perforin (19-fold), CCL5 (11-fold), XCL1 (19-fold), CXCL10 (20-fold), and CXCL9 (37-fold) mRNA levels (genes known to be important in CTL activity and homing) when compared with the levels found in rejecting skin grafts analyzed at the same time-point after transplantation (BM3 plus Skin Tx; Fig. 2B). Overall, these data clearly show that one mechanism by which Treg control naïve T cells that have the potential to initiate skin graft rejection is by the suppression of priming of alloreactive T cells in the peripheral lymphoid organs up to 10 days after skin grafting.

With time, despite active regulation, effector CD8+ T cells are generated that infiltrate skin grafts resulting in the induction of proinflammatory genes in the graft

Next, we investigated whether the inhibition of alloreactive T cell priming by Treg persisted throughout the lifetime of the graft. To this end, we analyzed the peripheral response of BM3 T cells following skin grafting at a later time point after transplantation (Fig. 3). Surprisingly, we found that by 15 days after transplantation of a B10 skin graft, CFSE BM3 T cells were detectable in the dLN of mice that had also received Treg, although the numbers of such cells present were significantly reduced compared with those found in the dLN of mice that had received BM3 T cells alone (p = 0.0092; Fig. 3A). Analysis of the CFSE levels in the total BM3 T cell population in the dLN of mice revealed that a significant proportion of the BM3 T cells harbored intermediate levels of CFSE in the presence of Treg, suggesting that T cell priming had only recently been initiated, i.e., at a later time than when BM3 T cells were transferred alone where priming was evident by 10 days post-transplantation (Fig. 1A) and by day 15, the majority of BM3 T cells were CFSE− (Fig. 3B).

We also determined whether the observed priming of BM3 T cells, despite the presence of Treg, resulted in the generation of immunocompetent effector T cells that were able to infiltrate the graft. Immunohistochemical staining for CD8 in grafts from these mice at day 15 after transplantation revealed that effector BM3 T cells had now infiltrated the skin grafts, albeit in lower numbers than in mice that had received BM3 CD8− T cells alone (p < 0.05) although the level of CD8 expression in such grafts was not significantly different (Fig. 4A). Infiltration of BM3 T cells in such mice was found to result in equivalent intragraft mRNA expression levels for all of the proinflammatory genes analyzed in skin grafts from mice that received either BM3 T cells alone or both BM3 and Treg (Fig. 4B). Furthermore, depletion of CD8 T cells from these grafts resulted in markedly diminished expression of a number of proinflammatory genes, including IFN-γ, CCL5, and CXCL9, confirming that BM3 T cells that infiltrate skin grafts despite Treg are fully functional effector cells (Fig. 4C).

Overall, these data indicate that while Treg initially severely curtailed the priming of alloreactive T cells in the dLN, inhibition of priming was transient and, even in the presence of Treg, BM3 T cell priming in the dLN was initiated by 15 days after transplantation, resulting in the generation BM3 T cells that infiltrated skin allografts and induced a proinflammatory gene expression profile no different to that generated during rejection.

**FIGURE 3.** BM3 T cell priming is initiated by 15 days after B10 skin transplantation despite the presence of CD25+CD4+ Treg. CBA RAG−/− recipient mice were adoptively transferred with 1 × 10⁶ CFSE labeled BM3 T cells either alone or together with 5 × 10⁵ CD25+ CD4+ Treg. The following day, mice were transplanted with a B10 skin allograft. Control CBA RAG−/− recipient mice received 1 × 10⁶ CFSE-labeled BM3 T cells but no skin graft (BM3 only). dLN and cLN were harvested 15 days after skin grafting. A, The absolute number of CFSE− BM3 T cells was determined in dLN and cLN. Results are expressed as mean number of CFSE− BM3 T cells per tissue ± SD (n = 3). *, p < 0.05. Both BM3 only and BM3 + Reg + Skin Tx groups harbored significantly more CFSE− BM3 T cells in the dLN compared with the cLN (p < 0.05). Results have been confirmed in a second independent experiment. B, The CFSE fluorescence of BM3 T cells (shown in dot-plots of CD8 vs CFSE of gated BM3 T cells) present in both the cLN and dLN of BM3 plus Skin Tx and BM3 plus Reg plus Skin Tx mice was determined 15 days after skin grafting. Data shown are from one representative recipient mouse out of three.

**Treg infiltrate skin allografts**

We first confirmed that Treg were present in the peripheral lymphoid tissue. Mice that had received Treg in addition to BM3 T cells were found to have significant numbers of Treg in the dLN (Fig. 5A; as well as in the MLN and spleen, data not shown). However, we failed to reveal any significant difference in the number of Treg present in the dLN compared with the cLN. It should be noted that the Treg we have used in these studies is a polyclonal population, i.e., from CBA mice with long term surviving B10 cardiac allografts, thus not all of the cells in this population will be donor-reactive. Therefore, it is possible that any differences in homing of donor alloantigen-reactive Treg would have been masked in this model.

As grafts were not rejected when Treg were present in vivo despite clear BM3 T cell priming from day 15 onwards, we postulated that following transient inhibition of T cell priming in the periphery, Treg migrated to and infiltrated the skin allografts, where they continued to maintain control of effector T cells that, in the absence of Treg, would have the potential to initiate graft rejection locally. To test this hypothesis, we used Foxp3 intragraft mRNA expression as a surrogate marker for Treg infiltration. Real-time RT-PCR analysis for intragraft Foxp3 mRNA expression clearly showed that Foxp3 mRNA was expressed only in grafts from mice that received Treg (Fig. 5B). Intragraft expression of Foxp3...
mRNA in such mice was significant 10 days post-transplantation and was found to increase with time until day 25 (66-fold compared with skin grafts from BM3-only mice). These findings were confirmed by immunohistochemical staining for CD4 (only Treg express CD4 in this model; Fig. 5C). CD4+ cells were detectable in skin grafts from mice that received Treg together with BM3 T cells by day 10 after transplantation. In contrast, no staining for CD4 was detectable in skin allografts from recipients that had received BM3 T cells alone, demonstrating that the CD4 staining observed was specific for graft infiltrating Treg (Fig. 5C). These data correlated with the expression of Foxp3 in skin grafts, thereby confirming that Treg had infiltrated the grafts from day 10 onwards and could therefore potentially control the infiltrating effector T cells locally in the graft.

**Treg prevent the generation of alloreactive memory CD8+ T cells**

We have previously shown that the majority of memory BM3 T cells are generated between 15 and 25 days post transplantation, suggesting that memory cells were generated from effector rather than naive T cells (data not shown). As shown in Fig. 2, effector T cells were found to traffic to the skin grafts beginning around 10 days after grafting. We therefore tested the hypothesis that the bulk of memory, CD4+ T cells generated in this model developed from intragraft effector cells that had migrated to the periphery during rejection. To this end, RAG−/− mice received BM3 T cells...
and a B10 skin allograft and were left for 10 days to allow effector T cells to infiltrate skin allografts (as shown in Fig. 2). Ten days post-transplantation, skin allografts were removed and retransplanted to secondary naive RAG\(^{−/−}\) mice. Such mice were analyzed 90 days after retransplantation (100 days after the initial transplant). B, The absolute cell number and phenotype of CD44\(^{+}\) BM3 T cells in pooled lymphoid tissues (spleen, MLN, cLN, and dLN) from regrafted mice 90 days post-retransplantation is shown compared with grafts retransplanted from primary CBA RAG\(^{−/−}\) mice that did not receive BM3 T cells (n = 3; mean ± SD).

As BM3 T cells were clearly primed, albeit with delayed kinetics, and infiltrated skin grafts in the presence of Treg, we assessed whether memory T cell generation was affected in these mice. To this end, we analyzed the number of CD44\(^{+}\) BM3 T cells present in the periphery 25 days after skin transplantation in the presence and absence of Treg (Fig. 7A). As expected, mice that had rejected their skin allografts as a consequence of the adoptive transfer of BM3 T cells alone had significant numbers of CD44\(^{+}\) Ag-experienced BM3 T cells in all lymphoid tissues (Fig. 6B), compared with control mice where no BM3 T cells were transferred to primary recipients. Not surprisingly, such BM3 T cells were found to have an Ag-experienced phenotype with all cells showing up-regulated expression of CD44 (Fig. 6B). These data demonstrate directly that the memory BM3 T cell pool is generated from intragraft effector BM3 T cells.

As BM3 T cells were clearly primed, albeit with delayed kinetics, and infiltrated skin grafts in the presence of Treg, we assessed whether memory T cell generation was affected in these mice. To this end, we analyzed the number of CD44\(^{+}\) BM3 T cells present in the periphery 25 days after skin transplantation in the presence and absence of Treg (Fig. 7A). As expected, mice that had rejected their skin allografts as a consequence of the adoptive transfer of BM3 T cells alone had significant numbers of CD44\(^{+}\) Ag-experienced BM3 T cells in all lymphoid tissues (Fig. 7A) (25). In contrast, mice that received both BM3 and Treg were found to harbor few circulating CD44\(^{+}\) BM3 T cells in non-draining lymphoid tissue such as the spleen and cLN (Fig. 7A). Indeed, no more CD44\(^{+}\) BM3 T cells were present in these tissues than were detectable in mice that had been transplanted with syngeneic skin grafts (Fig. 7A). However, Ag-experienced CD44\(^{+}\) BM3 T cells were detected in the dLN of these mice at this time.

The distribution of CD44\(^{+}\) BM3 T cells 100 days after transplantation was also determined (Fig. 7B). We found that the results were similar to those seen 25 days postgrafting with the exception that with time Ag-experienced BM3 T cells also showed a marked reduction in the dLN as well as in other lymphoid tissues in mice that received both BM3 and Treg cells.

To exclude the possibility that the absence of CD44\(^{+}\) putative BM3 T cells was a result of the inhibition of homeostatic proliferation of memory BM3 T cells by the Treg, we allowed BM3 T cell priming and the development of memory T cells following skin transplantation to proceed for 15 days (a time when putative memory BM3 T cells begin to seed the peripheral lymphoid tissues [data not shown]) before adoptively transferring Treg. Analysis of these mice 100 days after skin grafting (85 days after Treg adoptive transfer) revealed that the number of CD44\(^{+}\) memory BM3 T cells in these recipients was restored to levels similar to that seen in mice where BM3 T cells were allowed to reject skin grafts in the absence of Treg (Fig. 7C). Moreover, the deferred adoptive transfer of Treg at day 15 delayed but was unable to prevent rejection (data not shown). These data clearly demonstrate that the absence of CD44\(^{+}\) putative memory BM3 T cells in this model is part of the regulatory mechanism that inhibits skin graft rejection and is
not simply an artifact of using lymphopenic recipients. Overall, these data suggest that skin graft tolerance is maintained via the deletion of intragraft effector T cells mediated by Treg, which results in a severe deficit in memory CD44^+ T cell generation in mice undergoing active regulation.

**Discussion**

Despite the current consensus that Treg play a key role in both the induction and maintenance of transplantation tolerance (8) and the prevention of autoimmune disease (30), the mechanisms involved and the location where they impose their suppressive activity in vivo are still not well defined and appear to differ depending on the model studied (reviewed in Refs. 8, 11–13). In this study, we show for the first time in a kinetic study that Treg prevent rejection mediated by CD8^+ T cells via multiple mechanisms and act at different locations in vivo depending on the time after transplantation.

One of the mechanisms identified by which alloantigen-reactive Treg initially suppress rejection in this model was through the attenuation of the generation of heavily divided (CFSE^−) BM3 effector T cells in the draining lymph tissue (Figs. 1 and 2). Although one possibility to explain these data is that Treg kill activated BM3 T cells, we favor the hypothesis that Treg suppress the activation/priming of BM3 T cells. Certainly this would be consistent with many other studies that have reported that Treg prevent T cell priming by acting on the APC either by direct killing (31) or by the modification of their activity by down-regulation of costimulatory molecules such as CD80 and CD86 (32, 33). Real-time imaging in a nonobese diabetic mouse model revealed that Treg are able to stably interact and modify Ag-bearing DC before Ag-specific T cell activation can occur (34). Treg were shown to home to the T cell area in the dLN where they accumulated in the T-B cell zone, in a similar way to that previously described for “effector” T cells, but that in this situation, the Treg interacted with Ag-bearing DCs before they had the chance to interact with Ag-specific naive T cells, thereby preventing T cell priming (34). A similar suppressive effect of Treg has also been described in a transgenic mouse model of experimental autoimmune encephalomyelitis (35).

Interestingly, the inhibition of BM3 T cell priming that we observed in this transplant model was found to be transient, and despite the continued presence of alloantigen reactive Treg in the dLN, by 15 days after transplantation BM3 T cells had begun to proliferate (Fig. 3), gained the ability to migrate to the graft, and induced an intragraft Tc1 type inflammatory response (Fig. 4). However, despite this evidence for activation of T cells with the ability to initiate rejection, the skin grafts were not rejected. Therefore, our data show that in addition to the suppression of BM3 T cell priming in the dLN other regulatory mechanisms exist, evident at later time points in this model, through which Treg prevent allograft rejection locally in the graft. It is also important to note that it appears that both inhibition of T cell priming in the lymphoid tissue as well as the suppression of rejection locally in the graft are required for long-term skin graft acceptance in this model as the transfer of Treg 10 or 15 days post transplantation was unable to prevent BM3 T cell mediated rejection (data not shown).

Certainly, one possibility is that Treg control infiltrating BM3 effector function. There are a number of reports that have suggested that Treg may control CD8^+ effector cell function following allo (36) or tumor Ag challenge by via the production of TGF-β (37). Even though we have shown a similar proinflammatory gene expression profile induced in the graft by BM3 T cells (primed in the presence or absence of Treg; Fig. 4), we cannot fully exclude that in part Treg prevent graft rejection via the inhibition of effector function. Although we were unable to detect any differences at the level of gene expression in this study, it is possible that small but significant decreases would not be revealed due to the extent of up-regulation of proinflammatory gene expression upon infiltration of grafts by activated BM3 T cells (typically between 20- and 200-fold increase; Figs. 2 and 4). However, where CD8^+ T cells were depleted from grafts in mice that had received both BM3 T cells and Treg, we found that many of the proinflammatory genes were significantly reduced, suggesting that infiltrating BM3 T cells were fully functional effector cells (Fig. 4C).

In Fig. 6, we showed that a cohort of graft infiltrating CD8^+ effector BM3 T cells traffic back out of the graft before rejection and form the peripheral memory T cell pool, resulting in a significant number of CD44^+ BM3 T cells being present in all lymphoid organs by 25 days after transplantation (25). In this study, we present evidence that while Treg failed to inhibit the priming and infiltration of effector BM3 T cells into skin grafts, at later time points after transplantation, the presence of Treg prevented the generation of a peripheral memory BM3 T cell pool (Fig. 7). This suggests that intragraft deletion of BM3 effector T cells mediated by intragraft Treg played a role in the maintenance of graft survival in this model, a conclusion consistent with other reports, suggesting that both mouse and human Treg are able to kill target cells (either CD4 and CD8 T cells, monocytes, DCs, and B cells) through a perforin/granzyme-dependent pathway (38–41). Although, the lack of memory BM3 T cells during active regulation provides only circumstantial evidence for intragraft deletion of BM3 T cells, it is unlikely that we would be able to demonstrate apoptosis of intragraft BM3 T cells directly. This is due in part to the number of macrophages present in tolerated grafts (data not shown) which are likely to clear apoptotic cells rapidly and, as discussed below, as intragraft effector cells are deleted, continuous generation of “new” effector cells means that grafts are likely to harbor significant numbers of BM3 T cells that have yet to be deleted. One trivial explanation for the diminished pool of memory T cells in tolerant mice is that memory T cells that have been generated are prevented from homeostatically proliferating due to the presence of Treg. However, this proved not to be case (Fig. 7C) demonstrating that the failure to generate memory T cells in mice that had received both BM3 and Treg relates to active regulation and is not an artifact relating to the use of lymphopenic recipients.

Despite the absence of CD44^− memory BM3 T cells in nondraining lymphoid tissue, CD44^+ BM3 T cells were detectable in the dLN 25 days after transplantation (Fig. 7A). We believe that these cells are recently activated BM3 and not circulating memory cells. It should be noted that in this model not all BM3 T cells are activated at the same time, indeed naive BM3 T cells are still detectable in lymphoid tissue for >25 days after grafting (data not shown). Suppression of T cell priming by Treg is transient and by 15 days after transplantation, priming of naive BM3 T cells has been initiated, thus theoretically, priming would continue until all peripheral naive BM3 T cells were activated. This appears to be the most likely explanation for the presence of CD44^+ BM3 T cells exclusively in the dLN 25 days after transplantation as when we analyzed mice that had been reconstituted with Treg and BM3 T cells there was a marked deficit in CD44^− BM3 T cells in the dLN 100 days after skin grafting, as well as all other lymphoid tissues examined, as the naive BM3 T cell pool had been exhausted (Fig. 7B). Therefore, although BM3 memory T cells were absent from mice that harbored Treg, the priming of naive BM3 T cells continued. This also provides an explanation as to why there was no diminution in intragraft proinflammatory gene expression in the face of intragraft deletion (Fig. 4) as it is likely that as effector BM3 T cells are deleted, they are replaced by recently activated
BM3 T cells that continue to infiltrate grafts until such a time as all peripheral naive T cells have been primed.

The location of Treg in vivo is likely to be exceptionally important for the effective inhibition and control of aggressive Ag-reactive cells. Treg have been found not only in the lymphoid tissue of transplanted mice (Fig. 5) (7, 23, 42) but also at later time points in the graft site itself, suggesting that T cell suppression of graft rejection operates beyond secondary lymphoid tissue (Fig. 5) (20, 36, 43, 44). What remains unclear is why the location of regulation changes with time following transplantation resulting in a switch from control of T cell priming to control of effector-mediated skin graft rejection within the graft itself. One possibility is that in the lymphoid tissue, draining the graft site the effector T cell response becomes more potent with time and simply overrides the suppression imposed by peripheral Treg. Alternatively, it may be that T cells initially recognize alloantigen on migrating donor APCs, but that with time they are primed as a result of alloantigen recognition on vascular endothelium (45). As mouse vascular endothelial cells are not thought to express MHC class II (46) Treg may be no longer able to suppress T cell priming resulting in T cell infiltration of the skin graft. Clearly, if this were the case then the site and mechanisms of regulation may be different between control of alloreactive CD8+ T cells and the migration of Treg to the graft whereupon the control of BM3 T cell responses switches to within the graft. We are currently performing experiments to attempt to delineate between these possibilities.

Taken together, these data expand current knowledge on the mechanisms used by alloreactive Treg during suppression of T cell immune responses that otherwise would lead to graft rejection. In this study, we show that Treg prevent graft rejection using multiple mechanisms in both the peripheral lymphoid tissue as well as in the graft itself. Initially, Treg prevent the priming and proliferation of alloreactive CD8+ T cells to skin allografts in the dLN. However, with time, Treg traffic to the transplanted organ and inhibit skin graft rejection mediated by effector CD8+ T cells by inducing the deletion of effector cells such that memory T cells fail to be generated. In conclusion, we believe that these data revealing multiple mechanisms of regulation elicited by Treg within the same model serve to provide a plausible explanation for the different mechanisms of regulation identified in a number of other studies and suggest that many of the mechanisms, both in lymphoid tissue as well as at the site of inflammation, may coexist in all models of regulation. Which mechanisms are implicated in the control of pathogenic T cells by Treg is likely to depend on the time studied after Ag challenge, the location and longevity of Ag recognition, and the relative strength of the potential T cell response. Furthermore, extrapolation of these data to clinical transplantation suggests that the identification of patients that have become “operationally” tolerant to their foreign organ grafts will require a multifaceted approach involving both peripheral and perhaps more importantly analyses that focus on the transplanted organ itself (47).

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References


