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Analysis of the Underlying Cellular Mechanisms of Anti-CD154-Induced Graft Tolerance: The Interplay of Clonal Anergy and Immune Regulation[1]

Sergio A. Quezada,* Kathy Bennett,* Bruce R. Blazar,† Alexander Y. Rudensky,‡ Shimon Sakaguchi,§ and Randolph J. Noelle[2]*

Although it has been shown that $CD4^+CD25^+$ regulatory T cells (T^{reg}) contribute to long-term graft acceptance, their impact on the effector compartment and the mechanism by which they exert suppression in vivo remain unresolved. Using a $CD4^+$ TCR transgenic model for graft tolerance, we have unveiled the independent contributions of anergy and active suppression to the fate of immune and tolerant alloreactive T cells in vivo. First, it is shown that anti-CD154-induced tolerance resulted in the abortive expansion of the alloreactive, effector T cell pool. Second, commensurate with reduced expansion, there was a loss of cytokine production, activation marker expression, and absence of memory T cell markers. All these parameters defined the tolerant alloreactive T cells and correlated with the inability to mediate graft rejection. Third, the tolerant alloreactive T cell phenotype that is induced by CD154 was reversed by the in vivo depletion of T^{reg}. Reversal of the tolerant phenotype was followed by rapid rejection of the allograft. Fourth, in addition to T^{reg} depletion, costimulation of the tolerant alloreactive T cells or activation of the APC compartment also reverted alloreactive T cell tolerance and restored an activated phenotype. Finally, it is shown that the suppression is long-lived, and in the absence of anti-CD154 and donor-specific transfusion, these T^{reg} can chronically suppress effector cell responses, allowing long-lived graft acceptance. ***The Journal of Immunology,*** **2005, 175: 771–779.**

Immunologic tolerance, as defined by Medawar (1), is an "indifference or non-reactivity" as measured by the acceptance of allogeneic skin grafts. Although initially conceived to be solely due to the lack of immune responsiveness, we now know that, in addition to clonal anergy, active suppression contributes extensively to graft persistence (2–9). To understand how anergy and suppression culminate in graft survival, we have studied the cellular and immunological fate of alloreactive $CD4^+$ TCR transgenic (Tg)[3] T cells under conditions of graft rejection and tolerance. The presented study demonstrates the profound, dominant impact of CD154 blockade on alloreactive T cell activation and differentiation and also illustrates the control that regulatory T cells (T^{reg}) exert in tolerized mice bearing allogeneic skin grafts.

Long-term graft acceptance has been achieved in a number of animal models through the blockade of costimulatory molecules (10–22). The i.v. infusion of allogeneic cells (donor-specific transfusion (DST)) with the concomitant administration of anti-CD154 has been shown to induce long-term acceptance of allogeneic skin (23–25), islets (24, 26, 27), and other tissues and organs in mice and nonhuman primates (26, 28, 29). Furthermore, this intervention causes the deletion of alloreactive $CD8^+$ T cells (30), anergizes $CD4^+$ T cells (31), and elicits regulatory T cell activities (7). Nonetheless, many important unresolved issues remain concerning the underlying cellular and molecular mechanisms of tolerance induced by anti-CD154. Two reports favor the proposition that costimulation blockade is not the mechanism of anti-CD154 action, and that direct cytotoxic deletion by anti-CD154 is critical for graft tolerance (32, 33). Others suggest that clonal anergy of the alloreactive T cell clones due to CD154 blockade is the operative effect of anti-CD154 in graft survival (34, 35). Although initially hypothesized that tolerance resulting from Ag delivery in absence of costimulation is exclusively due to T cell anergy, we and others have shown that active suppression by $CD4^+CD25^+$ T cells is critical for long-term graft acceptance. In fact, the deletion of T^{reg} previous to inducing transplantation tolerance results in graft rejection at a similar pace to that in nontolerized animals (4, 5, 7). These data suggest that anergy exerts only a modest impact on the rate with which immunogenic allografts are rejected. Although these studies provide valuable insights, questions about the fate and function of the alloreactive T cell compartment upon therapeutic intervention can only be answered in systems where alloreactive T cells can be directly followed.

To track T cell fate in the context of transplantation tolerance and to understand the cellular basis for graft tolerance induced by DST and anti-CD154, we used a TCR Tg $CD4^+$ T cell (TEa-Tg) with specificity to a major alloantigen (Eα in the context of IA^b) present on donor F_1 ($H\text{-}2^{bxd}$) APCs (DST) and F_1 skin grafts (36). Permanent graft survival has been previously achieved in this system by treatment of mice with DST and anti-CD154, showing that in response to immunogen (allogeneic skin), adoptively transferred TEa-Tg expand and differentiate to mediate graft rejection. In contrast, in response to DST (either F_1 ($H\text{-}2^{bxd}$) or BALB/c ($H\text{-}2^d$)),

*Department of Microbiology and Immunology, Dartmouth Medical School, and Norris Cotton Cancer Center, Lebanon, NH 03756; †Division of Bone Marrow Transplantation, University of Minnesota, Minneapolis, MN 55455; ‡Howard Hughes Institute, University of Washington, Seattle, WA 98195; and §Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto, Japan

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[2] Address correspondence and reprint requests to Dr. Randolph J. Noelle, Department of Microbiology and Immunology, Dartmouth Medical School, and Norris Cotton Cancer Center, Medical Center Drive, Lebanon, NH 03756. E-mail address: rjn@dartmouth.edu

[3] Abbreviations used in this paper: Tg, transgenic; DST, donor-specific transfusion; GITR, glucocorticoid-induced TNFR family-related protein; H-Ig, hamster Ig; LN, lymph node; T^{reg}, regulatory T cell.

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TEa-Tg expand via indirect presentation and become hyporesponsive (31). Combined treatment with anti-CD154 and DST (anti-CD154/DST) limits DST-induced expansion and enhances the unresponsiveness of the TEa-Tg, resulting in an enhancement in graft survival. After DST and anti-CD154, isolated TEa Tg T cells, when restimulated in vitro, were overwhelmingly (>95%) unresponsive to restimulation compared with those TEa Tg T cells that have seen graft. These studies were performed by the transfer of TEa-Tg T into $RAG^{-/-}$ recipients, and although graft longevity was enhanced with DST and constant administration of anti-CD154, long-term graft survival was not observed (31). We reasoned that the lack of T^{reg} in the $RAG^{-/-}$ model prevented the induction of long-term graft survival even in presence of constant costimulation blockade. Based on this hypothesis and by using TEa-Tg cells as tracers of the immune response, we tracked the fate of Tg alloreactive T cells in intact mice containing T^{reg} in response to graft rejection and tolerance. The studies presented in this report show that graft tolerance induced by blocking CD154 results in abortive T cell expansion and failed T cell differentiation. Surprisingly, this early response of the allospecific Tg T cells is governed by T^{reg}. Cytotoxic elimination of T^{reg} extinguishes graft tolerance and allows allospecific T cells to proliferate, differentiate, and develop effector function. In addition, tolerance induced by DST is sustained over time, because naive TEa Tg adoptively transferred into tolerized recipients partially expand in response to skin, but fail to accumulate and differentiate. Although the impact of DST and anti-CD154 is profound, it is fragile and reversible by overt costimulation. Taken together, the data present a comprehensive model for anti-CD154-induced graft tolerance that analytically represents the roles that clonal anergy and T^{reg} suppression play in allograft tolerance.

Materials and Methods

Mice

C57BL/6, C57BL/6 CD45.1 ($Ly5.2^+$) congenic, $CB6F_1$ mice (hybrid of C57BL/6 and BALB/c), and BALB/c mice were purchased from National Cancer Institute. TEa $CD4^+$ Tg cells (TEa-Tg) express a TCR that recognizes the peptide ASFEAQGALANIAVDKA in the context of $I\text{-}A^b$ (36). This peptide corresponds to positions 52–68 from the α-chain of I-E class II molecules and is presented in all APCs from $H\text{-}2^b/I\text{-}E^+$ strains ($CB6F_1$). C57BL/6 mice are $H\text{-}2^b$, but $I\text{-}E^-$, whereas BALB/c are $H\text{-}2^d$ and $I\text{-}E^+$, and therefore their F_1 hybrids are $H\text{-}2^b/I\text{-}E^+$ and able to directly present Ag. TEa Tg mice were bred to congenic C57BL/6 CD45.1 ($Ly5.2^+$) mice at the animal facility at Dartmouth Medical School. All mice were bred and housed in microisolator cages in a pathogen-free facility.

$F(ab')_2$ preparation

$F(ab')_2$ of the anti-CD154 Ab (MR1 clone) were prepared by pepsin digestion using the ImmunoPure $F(ab')_2$ kit (Pierce) and following the manufacturer's specifications. After digestion, samples where loaded onto a protein A column to eliminate undigested Ig, and the flow-through was further concentrated with a 50-kDa M_r cutoff membrane. Purified $F(ab')_2$ was run on an SDS gel to verify integrity and purity. No intact Ig was detected in any of our preparations. The ability of the $F(ab')_2$ to bind to murine CD154 was confirmed by testing the equimolar capacity of $F(ab')_2$ and intact MR1 to block staining of CHO-CD154 transfectants with intact MR1-FITC. For tolerance experiments, 300 μg or 1.0 mg of $F(ab')_2$/mouse/day i.p. were used as the low and high doses, respectively.

Skin grafting and immunization

Skin grafting was performed as a modification of the technique used by Markees et al. (25). Briefly, full thickness tail skin (~1 cm^2) from $CB6F_1$ (F_1) female donors was transplanted onto the dorsal area of age-matched C57BL/6 females. To induce T cell tolerance, recipients received 4×10^7 T-depleted spleen cells from F_1 donors on day −7 before skin graft (day 0) and 250 μg of anti-CD154 mAb (MR-1 clone) on days −7, −5, and −3. Control recipients received equivalent amounts of hamster Ig (H-Ig). Grafts were considered rejected when 80% of the original graft disappeared or became necrotic. This criterion for rejection is appropriate; it agrees with that used in many studies in transplantation tolerance (37) and continues to be used by many groups in the field (23, 25, 38–40). When necessary, 250 μg of antiglucocorticoid-induced TNFR family-related protein (DTA-1 clone) was administered i.p. three times every other day for 1 wk, either at the time of anti-CD154 injection (tolerance induction) or after tolerance had been established (maintenance experiments). Also, when used to reverse tolerance, three doses of 100 μg/mouse of agonistic anti-CD40 (FGK clone) were injected i.p. every other day.

In vivo depletion of $CD4^+CD25^+$ suppressor cells

To cytotoxically deplete $CD4^+CD25^+$ T^{reg}, C57BL/6 recipients were injected i.p. with 250 μg of anti-CD25 (PC61 clone) 4 days before the beginning of the experiments (DST administration). It has been previously shown that 4 days after administration of anti-CD25, >90% of the $CD4^+CD25^+$ cells are eliminated from peripheral blood and secondary lymphoid organs (7). This was confirmed in all experiments.

Adoptive transfer of TEa-Tg cells and assessment of in vivo cell division and expansion

One million TEa-Tg were i.v. injected into recipients either on day −7 together with DST (for tolerance induction) or on day 21 together with skin graft (maintenance experiments). When necessary, TEa-Tg were prelabeled with the intracellular dye CFSE (Molecular Probes). To determine TEa-Tg expansion and total number of TEa per mouse, lymph nodes (LNs) were harvested 5 days (induction experiments) or 7 days (maintenance experiments) after adoptive transfer into C57BL/6 recipients. Cell suspensions were analyzed by four-color flow cytometry by acquiring the same number of live cells (forward vs side scatter) in all samples. TEa-Tg were detected as $CD4^+CD45.1^+$ donor cells, and the total number of TEa-Tg was determined by multiplying the percentage of $CD4^+CD45.1^+$ T cells by the total number of cells.

Intracellular cytokine staining

CFSE-labeled cells harvested from LNs were incubated for 2 h in vitro at 37°C in the presence of 50 ng/ml PMA and 500 ng/ml ionomycin. Brefeldin A (5 μg/ml) was added for the last 1.5 h of incubation. Cells were then stained for CD4 and CD45.1, fixed and permeabilized (BD Pharmingen), and stained for IFN-γ or IL-2 following the manufacturer's instructions.

Statistical analysis

Survival data were analyzed using the Kaplan-Meier method, with the Wilcoxon rank test and the log-rank test used to verify the significance of the difference in survival between groups. A value of $p < 0.05$ was considered statistically significant. All experiments are representative of at least three repeats, and for kinetics of skin graft rejection, groups were eight or more mice were used.

Results

Blockade of CD154 function and administration of DST induce the abortive expansion of effector T cells

To define the impact of DST and anti-CD154 on the $CD4^+$ effector T cell compartment, TEa-Tg were transferred into WT C57BL/6 mice 7 days before grafting. At the time of TEa-Tg administration, the mice also received anti-CD154 and DST (F_1 T-depleted spleen cells) following the protocol described in Fig. 1*A*. Long-term acceptance of skin allografts was observed only with anti-CD154 and DST coadministration (Fig. 1*B*). When TEa-Tg expansion was measured 5 days after administration of DST (Fig. 1*C*), it was evident that DST and control H-Ig induced expansion of TEa-Tg cells. In contrast, the DST and anti-CD154 group showed no increase in the size of the TEa-Tg population. To evaluate whether this was due to the direct cytotoxic activity of the anti-CD154, an $F(ab')_2$ of anti-CD154 was produced. Although an equivalent dose of $F(ab')_2$ anti-CD154 (300 μg/mouse) to intact anti-CD154 (250 μg/mouse) was not effective at diminishing alloreactive T cell expansion (probably due to its shorter in vivo half-life), a higher dose of $F(ab')_2$ (1 mg/mouse) was as effective as the low dose of intact anti-CD154 (Fig. 1*C*). In addition, agonistic anti-CD40 reversed the impact of anti-CD154 blockade (Fig. 1*C*). Therefore, the primary impact of anti-CD154 and DST was to reduce the alloreactive T cell compartment by costimulation blockade and not via the direct cytotoxic elimination of $CD154^+$ effector T cells.

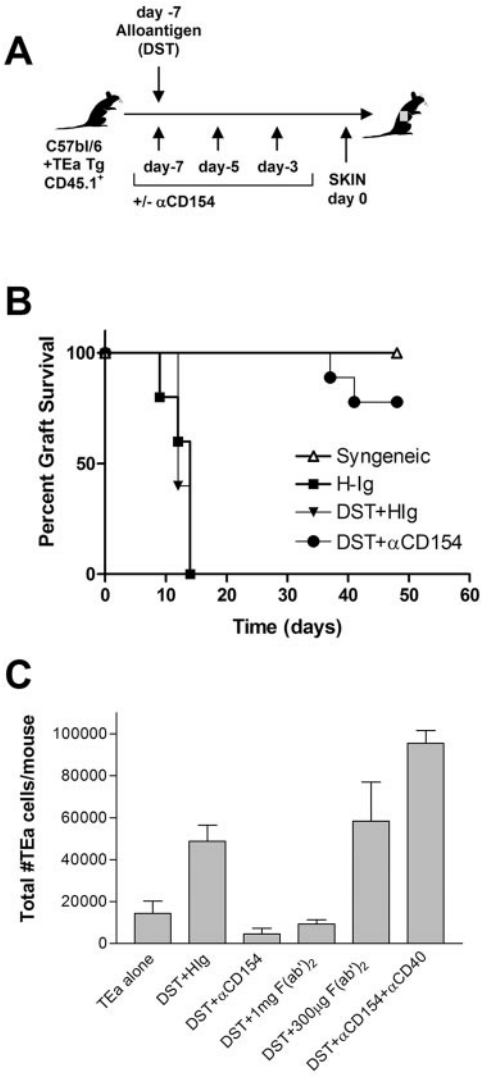

FIGURE 1. In vivo tolerance induction of TEa-Tg cells. TEa-Tg cells were adoptively transferred into C57BL/6 mice with or without DST and anti-CD154, then grafted with F_1 skin following the protocol described in *A*. Skin graft survival was followed over time (*B*) and compared with that in untreated mice or control mice grafted with syngeneic C57BL/6 skin. In parallel experiments, inguinal and axillaries LNs were harvested on day −2 (5 days after adoptive transfer), and the total number of TEa-Tg cells per mouse was determined to compare the effects of anti-CD154 and anti-CD154 $F(ab')_2$ and that of agonistic anti-CD40 on the expansion of alloreactive cells in response to DST (*C*).

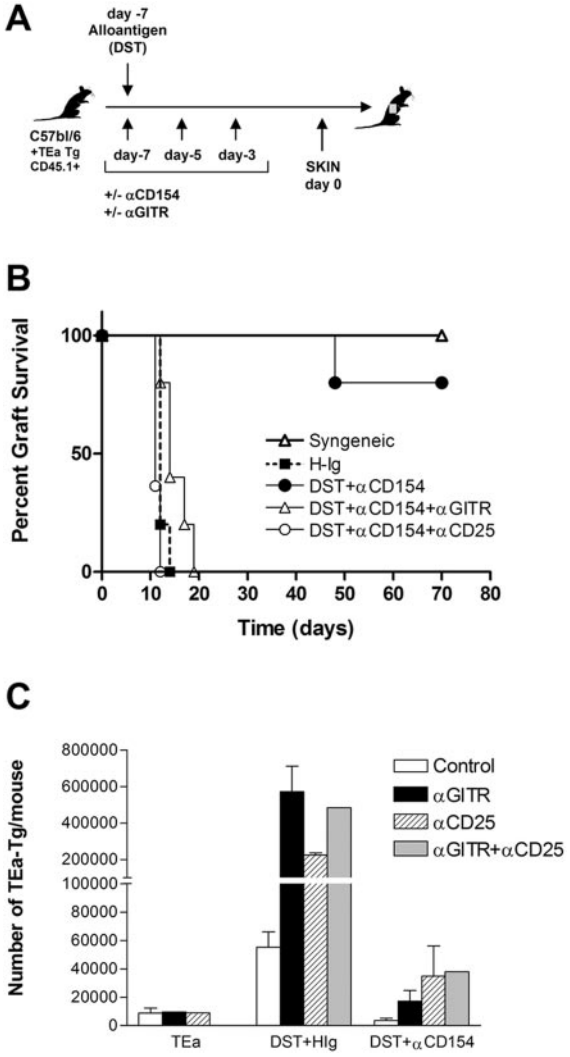

FIGURE 2. Ablation of T^{reg} cell activity during the induction phase prevents the establishment of tolerance. Mice where tolerized, or not, with DST and anti-CD154 7 days before skin grafting following the general protocol described in *A*. Four days before DST administration (day −11), a cohort of mice received anti-CD25 mAb to in vivo deplete the $CD4^+CD25^+$ T^{reg} population. Another group of mice received anti-GITR mAb together with anti-CD154, and skin graft survival was followed over time (*B*). Control groups grafted with C57BL/6 skin and receiving anti-CD25 or anti-GITR did not reject their grafts (not shown). In addition, LNs from a second group of mice were analyzed on day −2 (5 days after DST) for expansion of TEa-Tg in response to DST plus control H-Ig or DST plus anti-CD154 in the presence or the absence of anti-CD25 and/or anti-GITR (*C*).

Analysis of the impact of cytotoxic elimination of T^{reg} on graft tolerance and effector T cell expansion

A number of studies have documented that T^{reg} can prevent graft rejection, yet how they impair effector T cell function is not fully resolved. To determine whether T^{reg} are critical for the induction of graft tolerance and exert a dominant suppressive effect on Tg T cell growth and differentiation, T^{reg} were cytotoxically eliminated in vivo. PC61, a rat anti-CD25 mAb, was used to deplete T^{reg} in vivo. Previous studies have demonstrated that treatment of mice with PC61 reduced the percentage of $CD4^+CD25^+$ T cells in >90% of the animals after 4 days (7). With regard to graft tolerance, the treatment of mice with anti-CD25 (Fig. 2*A*) prevented tolerance induction, allowing skin rejection at a tempo equivalent to that of nontolerized mice (Fig. 2*B*). The impact of T^{reg} depletion on the in vivo expansion of the TEa-Tg in response to DST/anti-CD154 was studied on day 5 after DST injection. As shown previously (Fig. 1*C*), administration of DST/anti-CD154 resulted in a profound reduction in the number of TEa-Tg compared with DST/H-Ig (Fig. 2*C*). Elimination of T^{reg} activity with anti-CD25 significantly increased the expansion of TEa-Tg in mice treated with DST/H-Ig and partially restored accumulation of T cells in the DST/anti-CD154 group (Fig. 2*C*).

Analysis of the impact of DST and anti-CD154 on effector T cell proliferation, cytokine production, and differentiation

To gain additional insight into the impact of this tolerance-inducing regimen on T cell growth and differentiation, an expanded analysis of TEa-Tg T cell behavior was performed. CFSE-labeled TEa-Tg were transferred into mice together with DST/H-Ig or DST/anti-CD154

(Fig. 3*A*). Five days later, division, phenotype, and function were studied. The data show that compared with priming with a strong immunogen (skin), DST is capable of inducing TEa-Tg division, but with markedly reduced differentiation. Concomitant analysis of division and activation markers showed only partial T cell activation by DST compared with the high levels of expression of CD44 and CD69 and the clear down-regulation of CD62L observed in response to skin (Fig. 3*B*). In the presence of anti-CD154, DST still induced the TEa-Tg to divide, but with diminished accumulation and completely impaired expression of activation markers (no CD44 or CD69 up-regulation or CD62L down-regulation; Fig. 3*B*). Impaired T cell activation by DST/anti-CD154 was also observed when the cytoplasmic expression of IL-2 and IFN-γ by the TEa-Tg was studied. That is, allogeneic skin induced high cytoplasmic levels of IL-2 and IFN-γ, DST alone only induced partial IL-2 production and no IFN-γ, whereas DST/anti-CD154 completely ablated cytokine production (Fig. 3*C*). IL-4 and IL-10 were not detected under any experimental condition, thus ruling out a possible Th2 skewing of the alloreactive compartment (data not shown). In summary, the data show that upon encounter with DST and anti-CD154, TEa-Tg abortively expand and become functionally anergic.

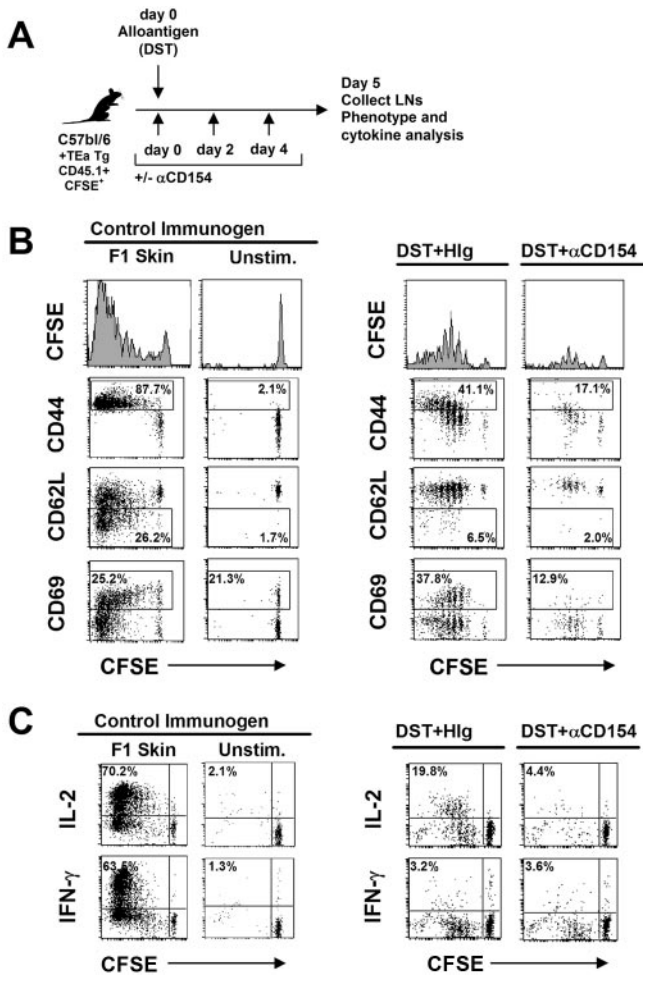

FIGURE 3. TEa-Tg divide in vivo and develop a unique tolerant phenotype in response to DST and anti-CD154. CFSE-labeled CD45.1$^+$ TEa-Tg were transferred into CD45.2$^+$ C57BL/6 mice on day 0 with DST/H-Ig or DST/anti-CD154 following the protocol shown in *A*. On day 5 after DST administration, mice were killed, and LNs (inguinal and axillaries) were harvested. LN cells were stained for the donor markers CD45.1$^+$ and CD4$^+$, and double-positive cells were analyzed for CFSE dilution and expression of the activation markers, CD44, CD62L, and CD69 (*B*). Another fraction of the cells was analyzed for the expression of intracellular cytokines (IFN-γ and IL-2) after a 2-h in vitro restimulation with PMA and ionomycin (*C*). As positive controls, mice received allogeneic F_1 skin instead of DST and syngeneic C57BL/6 skin as a negative control of expansion and differentiation. *B*, Numbers on the dot blot represent the percentage of TEa-Tg cells acquiring an activation phenotype; *C*, numbers represent the percentage of TEa-Tg cells that express IL-2 and IFN-γ upon division.

Analysis of the impact of GITR engagement on graft tolerance, effector T cell expansion, and differentiation

GITR is a recently described receptor of the tumor necrosis receptor family (41, 42) that is expressed constitutively on CD4$^+$CD25$^+$ T^{reg} and also by activated effector CD4$^+$CD25$^-$ T cells (43, 44). Although initial studies have shown that anti-GITR triggering of T^{reg} silences T^{reg} in vivo (43, 44), new emerging data indicate that it acts as a positive costimulatory signal for CD4$^+$CD25$^-$ effector T cells (45–47) and, in fact, may render effector CD4$^+$CD25$^-$ T cells resistant to T^{reg} suppression (48). Therefore, although the mechanism of action in vivo is not fully resolved, anti-GITR treatment can be considered a means to instill a state of resistance to T^{reg} action, either through its effects on T^{reg} or effector T cells. Similar to the cytotoxic elimination of T^{reg}, anti-GITR treatment (Fig. 2*A*) also restored skin graft rejection (Fig. 2*B*) and TEa-Tg expansion within the DST/H-Ig and DST/anti-CD154 groups (Fig. 2*C*). In the absence of anti-CD154, the magnitude of TEa-Tg expansion was greater in mice treated with anti-GITR than in those treated with anti-CD25. Similar to anti-GITR treatment, coadministration of anti-CD25 and anti-GITR increased expansion in both groups (Fig. 2*C*). Thus, loss of T^{reg} activity or costimulation through GITR significantly increased TEa-Tg expansion in response to alloantigen and partially restored expansion in response to DST/anti-CD154. Remarkably, a small increase in the TEa-Tg compartment (Fig. 2*C*; in the DST/anti-CD154 group plus anti-CD25 and/or anti-GITR) directly correlated with rapid rejection of the donor skin graft (Fig. 2*B*).

GITR costimulation and cytotoxic elimination of T^{reg} restore alloreactive T cell function and phenotype in vivo

In addition to the effects on cell accumulation and rejection, the impact of T^{reg} and anti-GITR on TEa-Tg division, function, and differentiation was determined. Mice were pretreated, or not, with anti-CD25 and then received CFSE-labeled TEa-Tg with or without DST/anti-CD154 and anti-GITR (Fig. 4*A*). Five days after DST administration, mice were killed, and cell cycle progression, T cell function, and phenotype were studied. As shown in Fig. 2*C*, T^{reg} depletion with anti-CD25 or costimulation via anti-GITR treatment resulted in an increase in the number of TEa-Tg in response to DST. This correlated with a greater number of cell divisions and a marked increase in differentiation, as evidenced by the expression of the activation markers CD44, CD69, and CD62L (Fig. 4*B*) and by an increase in cytoplasmic IL-2 and IFN-γ production (Fig. 4*C*). anti-CD25 or anti-GITR had only a moderate effect on T cell accumulation (Fig. 2*C*), Fig. 4 shows how both these treatments recovered the TEa-Tg from their anergic state, allowing them to progress further through the cell cycle, up-regulate activation markers (Fig. 4*B*), and recover the capacity to produce IL-2 (Fig. 4*C*). Finally, these data correlate with the breakdown of tolerance and skin rejection in the groups receiving DST and anti-CD154 (Fig. 2*B*).

Taken together, the data suggest that in concert with costimulation blockade, T^{reg} activities contribute to the deletion of alloreactive T cells and the control of their maturation (phenotype) and function (cytokine production). Finally, the restoration of these

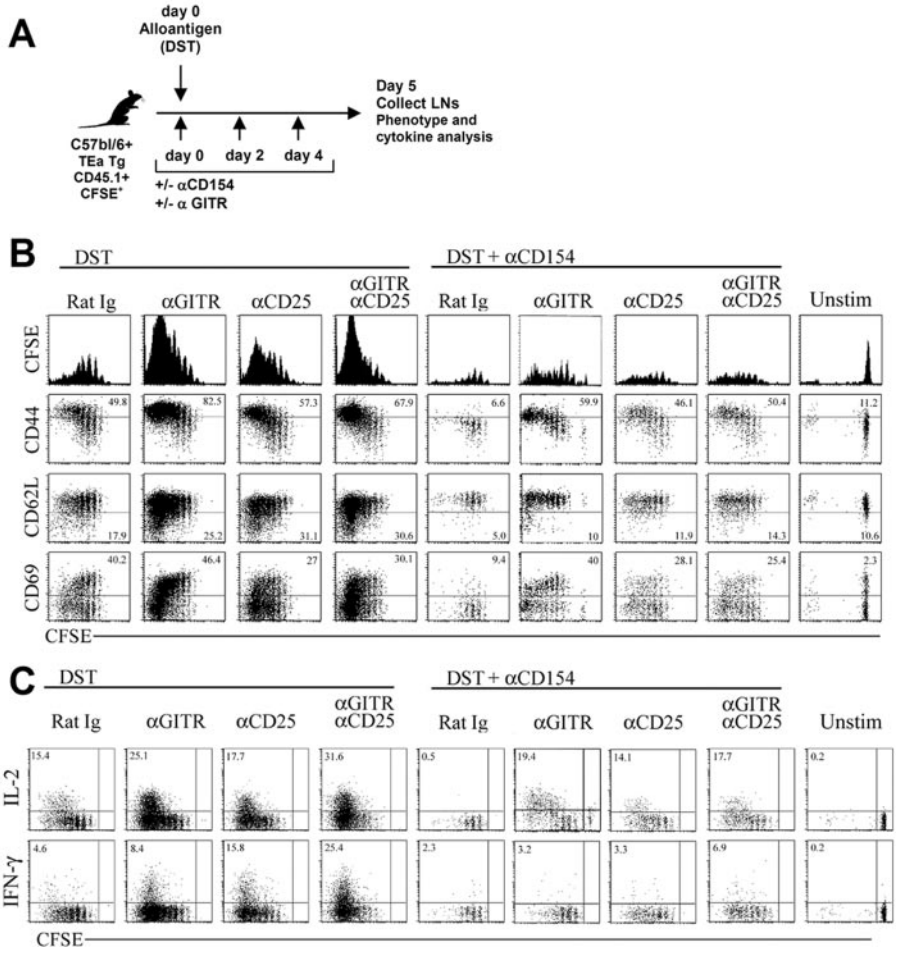

FIGURE 4. T^{reg} activities control the in vivo expansion and differentiation of TEa-Tg cells in response to DST/anti-CD154. CFSE-labeled $CD45.1^+$ TEa-Tg were transferred into $CD45.2^+$ C57BL/6 mice on day 0 with DST/H-Ig or DST/anti-CD154 and in the presence or the absence of anti-CD25 and/or anti-GITR following the protocol shown in *A*. On day 5 mice were killed, and LNs (inguinal and axillaries) were harvested. LN cells were stained for $CD45.1^+$ and $CD4^+$, and double-positive cells were analyzed for CFSE dilution and expression of the activation markers, CD44, CD62L, and CD69 (*B*). Another fraction of the cells was analyzed for the expression of intracellular cytokines (IFN-γ and IL-2) after a 2-h in vitro restimulation with PMA and ionomycin (*C*). *B*, Numbers on the dot blot represent the percentage of TEa-Tg cells acquiring an activation phenotype; *C*, numbers represent the percentage of TEa-Tg cells that express IL-2 and IFN-γ upon division. Mice that received anti-CD25 mAb were injected i.p. 4 days before DST administration (day −4).

effector cell activities by T^{reg} ablation and GITR ligation correlated with vigorous graft rejection.

Impact of CD40 and GITR costimulation on TEa-Tg cells and graft acceptance during the maintenance phase of tolerance

Both elimination of T^{reg} and GITR signaling could prevent the development of tolerance. The next set of studies was designed to test whether GITR triggering could break established tolerance in mice that have stably accepted an allogeneic skin graft. To this end, mice were first tolerized with DST/anti-CD154 and grafted with allogeneic skin. Forty-five days after skin grafting, mice with stable and healed allografts were treated with three doses of anti-GITR or control rat IgG. Grafts were rapidly rejected after anti-GITR treatment (Fig. 5*A*). As a control, syngeneic C57BL/6 skin was not rejected when recipient mice were treated with anti-GITR. The data reveal that the instilled state of tolerance induced by costimulation blockade and T^{reg} activities is long-lived, but reversible upon further costimulation.

Infectious nature of graft tolerance on naive T cell responses in vivo

Early studies by Waldmann et al. (2, 49) showed that regulatory activities are long-lived and can be adoptively transferred to mediate the suppression of graft rejection (infectious tolerance). The next set of studies was designed to assess whether the tolerance instilled in a mouse could suppress the responses of adoptively transferred naive TCR Tg T cells. To this end, C57BL/6 mice were treated with DST/anti-CD154 (tolerized mice), DST/H-Ig, or H-Ig (nontolerized mice) on day 0. Twenty-one days later (at a time when no Ag (DST) was detectable and more than one half-life of the Ab had occurred (50)), naive TEa-Tg were adoptively transferred into those recipients together with a skin graft as the immunogenic stimuli (Fig. 5*B*, *top panel*). The data show that naive TEa-Tg transferred into tolerized mice had a reduced capacity to reject F_1 skin (Fig. 5*B*). In contrast, when nontolerized mice received naive TEa-Tg, all of them rejected with rapid kinetics. Hence, tolerance induced by costimulation blockade and T^{reg} activities can suppress naive TEa-Tg function in the absence of overt tolerogen, and this hyporesponsive state can be ablated by late treatment with anti-GITR or agonistic anti-CD40 (Fig. 5*B*).

We then examined the impact of long-lived tolerance on the phenotype and function of naive TEa-Tg cells transferred into tolerant mice (Fig. 6*A*). Briefly and as described in Fig. 6*A*, 1×10^6 CFSE-labeled TEa-Tg cells were transferred into mice receiving, 21 days previously, DST and anti-CD154 (tolerized mice) or H-Ig (nontolerized). One day after TEa-Tg transfer, mice were grafted with allogeneic F_1 skin or control syngeneic B6 skin; 7 days later, mice were killed, and TEa-Tg were analyzed for division and differentiation. The data obtained from these experiments showed a clear difference in TEa-Tg cell cycle progression and differentiation. When transferred into nontolerized mice (H-Ig), TEa-Tg cells, in response to skin, progressed through the cell cycle at the same time that they up-regulated CD44 and CD69, and decreased the expression of CD62L on their cell surface (Fig. 6*B*). In contrast, when transferred into a tolerized host (DST plus anti-CD154), TEa Tg cells failed to accumulate and only partially divided in response to skin. The striking difference seems to be at the level of their differentiation into effector/memory cells, because

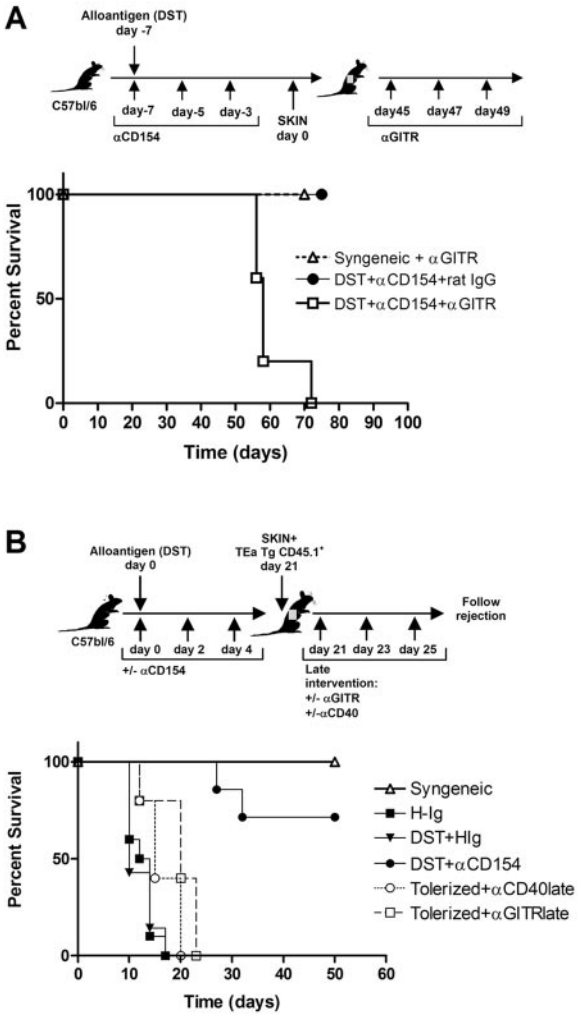

FIGURE 5. GITR triggering ablates the maintenance phase of tolerance. C57BL/6 mice were tolerized with DST and anti-CD154 before skin grafting. Forty-five days after skin grafting, mice received three injections of anti-GITR, and the kinetics of rejection were followed (*A*). Control groups included mice tolerized with DST/anti-CD154 and control rat IgG, and nontolerized mice grafted with C57BL/6 skin and treated with anti-GITR (*B*). C57BL/6 mice received DST in the presence or the absence of anti-CD154. Twenty-one days after treatment initiation, mice received CD45.1$^+$ naive TEa-Tg, were grafted with F_1 skin, and were treated, or not, with anti-CD40 or anti-GITR (*upper panel*). Skin graft rejection was followed over time. As a positive control for rejection, mice were treated with H-Ig and grafted with F_1 skin; as a negative control, mice were grafted with C57BL/6 skin (syngeneic).

they completely failed to up-regulate CD44 and down-regulate CD62L (Fig. 6*B*). In addition, IFN-γ and IL-2 production by TEa-Tg was ablated in tolerant mice compared with TEa-Tg transferred into nontolerized mice (H-Ig group; Fig. 6*C*). When anti-GITR was administered together with the skin graft and TEa-Tg (Fig. 6*A*), a partial reversion of the effect of DST/anti-CD154 was observed with respect to the acquisition of an activation/memory phenotype (Fig. 6*B*) and cytokine production (Fig. 6*C*), which correlates with graft rejection (Fig. 5*B*). Triggering with an agonistic anti-CD40 has been reported to ablate T^{reg} function and release T^{reg} control over the APC compartment (51); in this particular case, it overcame the suppressive impact of DST/anti-CD154 to restore TEa-Tg expansion and differentiation (Fig. 6*B*), cytokine production (Fig. 6*C*), and graft rejection (Fig. 5*B*).

Discussion

The contributions of CD4$^+$CD25$^+$ T^{reg} to graft tolerance are profound, yet the manner in which they control immune effector mechanisms remains enigmatic. Although Medawar (1) may have envisioned that graft tolerance was due to an indifference or a central failure, the studies presented clearly demonstrate that central failure (anergy within the effector T cell compartment) does not prohibit graft rejection. Graft survival requires the parallel induction of anergy within the effector cell population (central failure) and the development of T^{reg} activities. These T^{reg} activities can control the clonal expansion and differentiation of the minimized, surviving effector T cells. It is only under these conditions that graft tolerance is achieved. The important findings presented in this study are the following. 1) The early response of alloreactive T cells to DST and anti-CD154 is robust and abortive, resulting in a reduction in the alloreactive T cell compartment, loss of the ability to produce cytokines, and loss of the acquisition of a memory T cell phenotype. 2) The abortive response of the alloreactive T cell pool induced by anti-CD154 is due not to the direct cytotoxic activity of the Ab for CD154$^+$ T cells, but to its blocking of CD154 function. 3) Silencing of T^{reg} activity reverses the induction of alloreactive T cell tolerance by restoring T cell expansion, the expression of memory T cell markers, cytokine production, and graft rejection. 4) The impact of costimulation blockade and T^{reg} activity is long-lived and, even in the absence of overt tolerogen, can control the expansion, differentiation, and function of allospecific, effector T cells, resulting in graft persistence.

The findings presented in this report together with the results of earlier studies (7, 31) show that immunologic tolerance to an allograft cannot be attained simply through the deletion or silencing of alloreactive T cells. Using recipients devoid of T^{reg}, our previous studies in TEa-reconstituted RAG$^{-/-}$ recipients showed that graft rejection occurs even after extensive clonal anergy of the TEa-Tg (<5% of the nontolerized controls) induced by indirect Ag presentation and even in the continued presence of anti-CD154 (31). It is worth clarifying that in that previous model, homeostatic expansion was ruled out as a possible mechanism for rejection, because CFSE-labeled TEa-Tg cells did not divide in the absence of Ag. In yet another model, we show that anti-CD154/DST treatment will not prevent rejection in RAG$^{-/-}$ mice reconstituted with purified polyclonal CD4$^+$CD25$^-$ effector T cells (7). Taken together, these data show that purified effector T cells are resistant to tolerance induction, as measured by graft rejection. Together with the present studies, it is evident that allograft tolerance requires the concomitant reduction in the size of the functional, alloreactive T cell pool (52–57) and the development of active suppression to control residual effector cell activities. It is surprising that T^{reg} play a critical role in the early abortive expansion, contraction, and anergy of the alloreactive, effector T cells.

Direct analysis of the fate and function of alloreactive T cell effectors and T^{reg} in mice treated with DST/anti-CD154 has allowed a far greater understanding of the multicomponent nature of this intervention. Together with our previous studies (31), it appears that DST is indirectly presented by host APCs as a tolerogen, causing rapid abortive expansion of the alloreactive TEa-Tg. Although most cells fail to survive or differentiate to an effector/memory phenotype, a small number do express CD44^{high} and are CD62L^{low} (Fig. 3*B*). Despite the nonimmunogenic profile elicited by DST, it fails to induce tolerance to a skin graft, due to a residual small subset of T cells that carries an activation and memory phenotype, CD44^{high}/CD62L^{low} (Fig. 3*B*), and the capacity to secrete IL-2 (Fig. 3*C*). In contrast to DST alone, DST/anti-CD154 extinguishes the small residual pool of effector TEa-Tg. This effect of

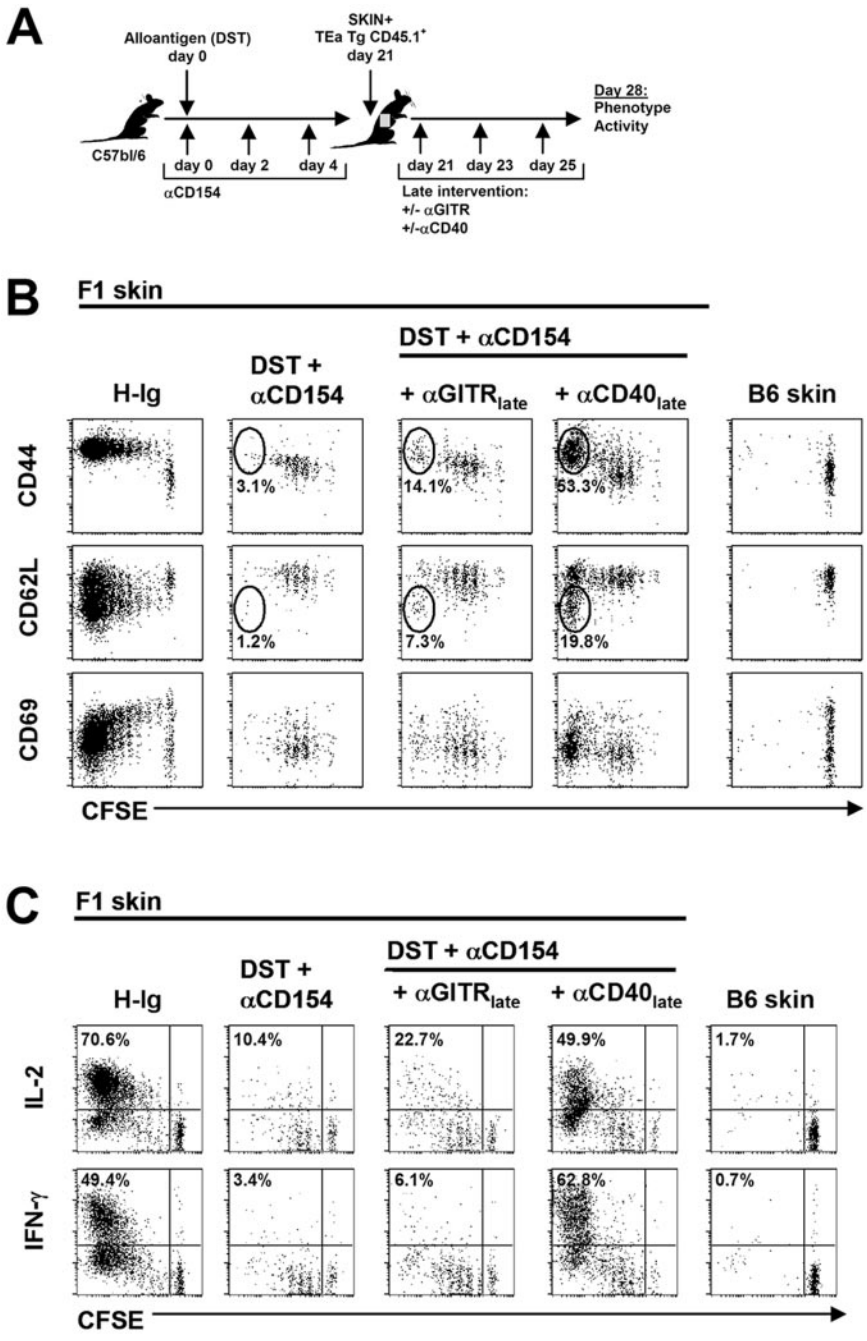

FIGURE 6. During the maintenance phase of tolerance, naive TEa-Tg were rendered hyporesponsive to highly immunogenic skin grafts. Tolerance was induced in C57BL/6 recipients with DST and anti-CD154; 21 days later, mice received CD45.1$^+$ naive CFSE$^+$ TEa-Tg cells and were skin grafted with F_1 skin or control syngeneic C57BL/6 skin. *A*, Seven days after skin grafting, mice were killed, and draining LNs were harvested. During the last 7 days, two of the DST/anti-CD154-tolerized groups were treated with anti-GITR or anti-CD40 agonistic mAb. *B*, Harvested cells were analyzed by flow cytometry to determine the expression of cell surface CD44, CD62L, and CD69 on CD45.1$^+$ CD4$^+$ TEa-Tg. *C*, CD45.1$^+$CD4$^+$ TEa-Tg were also analyzed for intracellular cytokine production (IFN-γ and IL-2) after short-term in vitro restimulation with PMA and ionomycin.

anti-CD154 treatment is due to blockade of CD154 and an indirect effect via the presence of T^{reg} activities. This conclusion is based on the fact that depletion of T^{reg} with anti-CD25 restores graft rejection in DST/anti-CD154-treated mice and allows the TEa-Tg to attain a partial effector/memory phenotype. In addition, the reduction of the alloreactive T cell pool and the lack of differentiation into effector cells (especially with regard to CD62L expression) strongly correlate with previous findings by our group that DST and anti-CD154 treatment has a significant negative impact on graft infiltration (31).

Although one might argue that the TEa-Tg is not representative of the endogenous alloreactive compartment, a similar trend of expansion and phenotype was observed in the endogenous CD4$^+$CD45.2$^+$ population (not show). Granting that the behavior of the TEa-Tg represents that of the endogenous alloreactive pool, it is remarkable how modest a change in cell number has to occur (compare DST/anti-CD154 with DST/anti-CD154/anti-GITR) to allow rapid unimpeded graft rejection.

In addition to arresting T cell function during the early phases of T cell activation, T^{reg} are critical for continued suppression of effector T cell activities and graft persistence, and the tolerogenic state is ablated by GITR signaling as well as by CD40 stimulation. In the second model presented, mice were tolerized with DST/anti-CD154, and then 21 days later were administered TEa-Tg and grafted. First, it is clear that T^{reg} activity persists for at least 3 wk after the administration of DST/anti-CD154. Second, this infectious phase of tolerance, like the early phase, can effectively arrest the expansion and differentiation of alloreactive effector T cells. This time period was specifically chosen because at 3 wk after anti-CD154 treatment, the direct biologic activity of the Ab is minimal (50). That is, the reduced responsiveness of the TEa-Tg is not due to blocking of CD154. This is demonstrated by the fact that

pretreatment of mice with anti-CD154 alone has only a partial impact on TEa responses 3 wk later (data not shown). Although this infectious system differs from that originally described by Waldmann et al. (2, 49), in this work we wanted to take a novel and distinctive approach, free of the manipulations and complications of adoptive transfer into leukopenic recipients (such approaches pose some significant questions with regard to the manipulation of the transferred cells and, most importantly, the homeostatic proliferation of effectors and suppressors within the immunodepleted recipient). In the studies presented in this report, we simply transferred naive TEa Tg T cells into a mouse that was tolerized so we could assess the full impact of the tolerized host over naive TEa to better understand the maintenance phase of tolerance.

At the time that these studies were initiated, a number of published reports indicated that anti-GITR selectively ablated T^{reg} function in vivo and in vitro (43, 44). However, more recently it has been shown that anti-GITR can act as a costimulator of activated $CD4^+CD25^-$ T cells (45–47); furthermore, most recent data suggest that anti-GITR mAb renders $CD4^+CD25^-$ effector T cells resistant to suppression by $CD4^+CD25^+$ T^{reg} (48). In light of these new data, we believe that GITR signaling is clearly responsible for breaking tolerance and the re-establishment of immunity against the graft, most likely by acting at the level of the effector T cell.

The fact that the cytotoxic elimination of T^{reg} using anti-CD25 mAb treatment relieves graft tolerance provides compelling evidence that the elimination of T^{reg} or their activities is sufficient for graft rejection. The enhanced expansion and accumulation of TEa-Tg T cells with anti-GITR treatment over that observed with anti-CD25 treatment (Fig. 2*C*) may represent the ability of anti-GITR to shut down T^{reg} activities in addition to its costimulatory potential, but that remains to be proven. With regard to treatment with anti-CD25 mAb, it has been suggested that it might have an impact on the effector compartment. In our system, anti-CD25 mAb is injected 4 days before donor transfusion and 11 days before skin grafting, thus having a minimal impact on the effector compartment. We strongly believe that if the anti-CD25 Ab was having a significant impact on the effector compartment, then we would not see the same kinetics of skin rejection when anti-CD25 was used (Fig. 2*B*). In addition, we have ruled out the possibility that $CD25^+$ depletion might induce homeostatic expansion of $CD4^+CD25^-$ effector cells. This conclusion is based on the fact that CFSE-labeled TEa Tg T cells do not divide in anti-CD25-treated hosts unless their cognate Ag is present.

We believe that together these results are the first to show the in vivo joint impact of costimulation blockade and T^{reg} activities on the short- and long-term functions of the effector compartment and have important implications for understanding physiologic mechanisms of transplantation tolerance in vivo. Future clinical approaches may not only target costimulatory pairs, but also enhance the function of the T^{reg} compartment to prolong graft survival. Newer approaches that have been developed in our laboratory and others include the generation and in vitro expansion of Ag-specific T^{reg} that together with costimulation blockade may induce sustained states of tolerance, including permanent graft acceptance (58) and control of autoimmune disorders (59, 60).

The fate and function of tolerant and immune alloreactive T cells have been addressed. We have shown that clonal anergy and suppression elicit a population of alloreactive T cells that expresses a unique tolerant phenotype that correlates with cellular unresponsiveness and graft acceptance. The underlying molecular mechanisms that mediate the suppression of allogeneic T cell expansion and differentiation by T^{reg} are the major focus of future studies.

Disclosures

The authors have no financial conflict of interest.

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