Dominant transplantation tolerance impairs CD8+ T cell function but not expansion

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Alloreactive CD8+ T cells may persist in animals made tolerant of transplanted tissues; their function is controlled through continuous censorship by regulatory CD4+ T cells. We sought to establish the stage at which such censorship operates. We found that monospecific CD8+ T cells introduced into tolerant animals responded to the tolerated tissue antigen as if they had received CD4+ T cell "help": they proliferated and accumulated normally. However, they did show compromised graft rejection, interferon-γ production and cell-mediated cytotoxicity. These findings suggest that tolerance mediated by regulatory T cells acts by censoring immune effector functions rather than by limiting the induction of T cell responses.

It is now well documented that CD4+ regulatory T (T_R) cells can maintain transplantation tolerance[1–6] and prevent both autoimmune diseases and gut immunopathology[7–9]. T_R cells seem to be able to control the function of both CD4+ and CD8+ effector cells[10]. Under the constraints of regulation, alloreactive CD8+ T cells may still persist in tolerized animals while being continuously censored by CD4+ T_R cells[10,11]. Based on current *in vitro* data[12,13], one plausible explanation for censorship would be that T_R cells directly prevent CD8+ T cells from being activated or dividing. Another is that T_R cells might interfere with "help" provided by CD4+ T cells that would allow CD8+ T to expand and differentiate[14,15]. "Help" operates through antigen-presenting cells (APCs) and involves the "licensing" of these APCs through an interaction between CD40 and CD40 ligand (CD40L)[16,17] as well as the provision of paracrine cytokines operating between T cells brought into proximity by the APCs[15,18].

To study how CD8+ T cells develop in an environment in which tolerance is maintained by T_R cells, we introduced 5-and 6-carboxy-fluorescein succinimidyl ester (CFSE)-labeled, monospecific, T cell receptor (TCR)–marked alloreactive CD8+ T cells into mice tolerized to skin grafts mismatched for major histocompatibility complex (MHC) antigens, where the tolerant state depends on the action of T_R cells. When these marked CD8+ T cells were challenged with antigen, we observed that they proliferated at the same rate as the controls and were able to acquire help from CD4+ T cells, which was reflected as equivalent cell expansion. Despite any obvious impairment at this inductive end of the response, we observed that the progeny of the marked CD8+ T cells were disarmed in terms of the immune effector functions of cytotoxicity, interferon-γ (IFN-γ) production and graft rejection. These findings suggest that, *in vivo*, T_R cells may be able to censor CD8+ T cells without influencing their capacity to proliferate and survive. Based on these findings, it may be necessary to devise new *in vitro* assays for T_R cells.

Results

CD8+ T cells fail to reject grafts in tolerant hosts

When combined with anti-CD8, nondepleting antibodies to CD4 or CD154 can induce dominant tolerance to a variety of tissue grafts[19]. We found that a combination of all three nondepleting monoclonal antibodies (mAbs) can even tolerize H-2^k CBA/Ca (CBA) mice to fully MHC-mismatched H-2^b C57BL/10 (B10) skin grafts (**Web Fig. 1** online). This tolerance was antigen-specific because fresh donor B10 skins were accepted, whereas third party BALB/c grafts were rapidly rejected. Tolerance is maintained through dominant mechanisms[19], which are defined as follows. First, the inability to break tolerance by the transfusion of naïve spleen cells (this resistance is lost if host T cells are ablated); second, the adoptive transfer of suppression by CD4+ T cells from the tolerized host; and third, tolerized mice show linked suppression, so that tolerant CD4+ T cells are able to suppress rejection by CD4+ or CD8+ T cells directed to third party antigens when they are coexpressed, for example, on an F_1 graft. Thus, tolerant CD4+ T cells directed against B10 antigens presented by MHC class II can suppress the rejection of grafts mediated *via* the direct recognition of H-$2K^b$ by CD8+ T cells, and this was the system we used in the following experiments.

We used such tolerized CBA mice bearing two established B10 grafts to investigate the fate of naïve monospecific CD8+ T cells introduced into an environment in which host T_R cells actively maintain the tolerant state. As a source of naïve monospecific CD8+ T cells specific for tolerated transplantation antigen, we used CD8+ T cells derived from the KB5.C20 TCR–transgenic mouse[20] with TCR specificity for the MHC class I molecule H-$2K^b$. This TCR can be detected with the idiotypic antibody known as Desire (Des)[21]. CD4-depleted splenocytes (1×10^7 Des^+CD8^+ T cells) from these mice were labeled with CFSE and then adoptively transferred into mice

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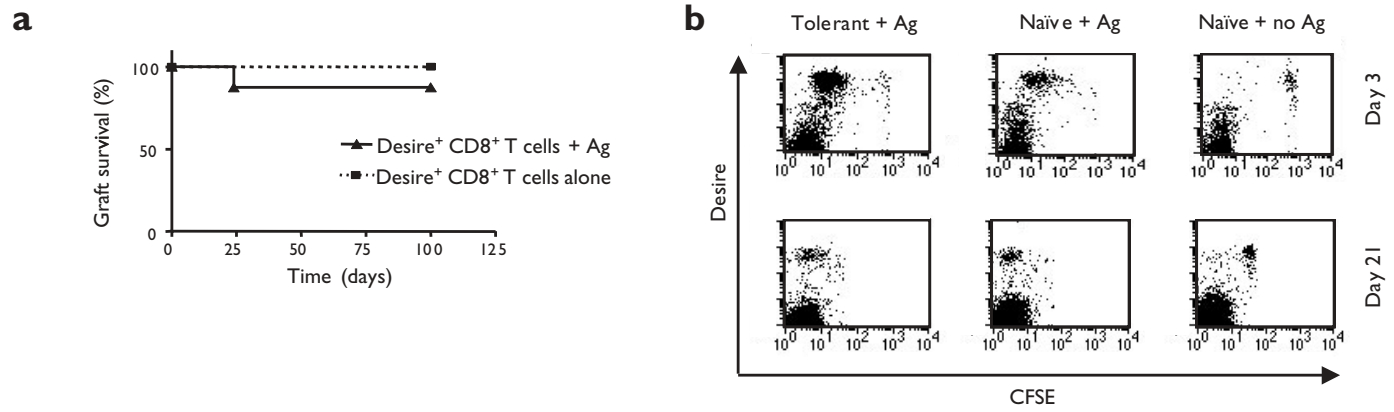

Figure 1. Graft survival despite CD8⁺ T cell proliferation in tolerized animals. CFSE-labeled Des⁺CD8⁺ T cells were infused into tolerized and naïve (control) CBA recipients. Some mice were then challenged intravenously with 1×10^6 (CBA × B10)F_1 APCs. Peripheral blood lymphocytes were stained and analyzed by flow cytometry 3 and 21 days later. (**a**) Skin graft survival after T cell infusion. No graft rejection was observed in tolerized mice that were not challenged with (CBA × B10)F_1 APCs (filled squares); MST >100 days; $n = 5$. Only one graft was rejected in mice challenged with (CBA × B10)F_1 APCs (filled triangles); MST >100 days; $n = 10$. There was no significant difference between these two groups ($P = 0.429$) (**b**) Proliferation profile of Des⁺CD8⁺ T cells in peripheral blood. Dot plots were gated on live (forward and side scatter) and CD8⁺ T cells. Ag, antigen.

that had been tolerized to B10 skin 160 days earlier. On the following day, 1×10^6 (CBA × B10)F_1 splenocytes were injected as a source of APCs. These APCs expressed H-2K^b (as well as other B10 antigens) and were not capable of mounting any response against the host. Only one animal rejected the established grafts after this antigen challenge and none were rejected without it (**Fig. 1a**). To assess the proliferation and survival of Des⁺CD8⁺ T cells in the peripheral blood, samples of blood were taken from the nonrejecting mice 3 and 21 days after injection of APCs. Because the CFSE intensity of all proliferating cells merged with background amounts on day 21, the percentage of dividing cells in the Des⁺CD8⁺ T cell population could be used as an indicator of proliferation status at day 3. We found there was no difference in terms of proliferation between cells transfused into tolerant or naïve mice after antigen challenge (**Fig. 1b**). The same results were seen for spleen cells sampled on day 3 (**Fig. 2**). It seems, therefore, that CD8⁺ T cell proliferation could be activated in tolerized mice, but CD8⁺ T cells could not reject established grafts in that environment.

The next series of experiments were designed to establish the cellular basis of this uncoupling of proliferation and rejection. First, we wondered whether the outcome could be explained by a lack of CD4⁺ T cell help in tolerant mice. We examined therefore the role played by CD4⁺ T cell help in the expansion and net accumulation of CD8⁺ T cells that responded to alloantigen and provided help for graft rejection.

Optimal CD8⁺T cell expansion requires CD4⁺T cell help

We adoptively transferred 1×10^7 CFSE-labeled Des⁺CD8⁺ T cells into naïve CBA mice or into mice that had been depleted of CD4⁺ T cells the previous day. The mice were challenged intravenously with 1×10^6 (CBA × B10)F_1 splenocytes (APCs) 1 day later. On day 4, mice were killed and spleen cells collected for flow cytometric analysis. We found that the extent of Des⁺CD8⁺T cell proliferation was only moderately impaired in CD4⁺ T cell–depleted (helpless) mice compared to CD4⁺ T cell–sufficient CBA mice challenged in the same way (**Fig. 2a,b**). However, the absolute number of

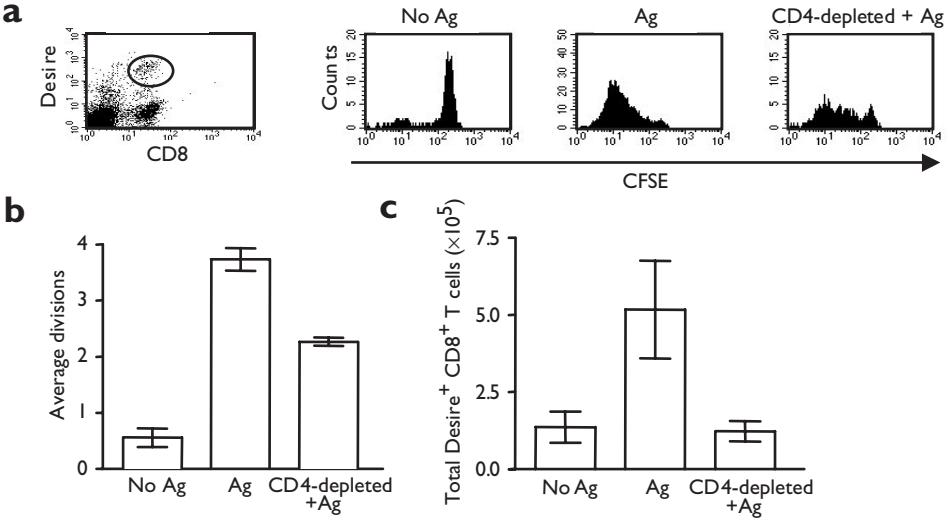

Figure 2. Limited expansion of monospecific CD8⁺T cells without CD4⁺T cell help. Euthymic or thymectomized and CD4-depleted CBA mice were injected intravenously with 1×10^7 CFSE-labeled Des⁺CD8⁺ T cells. The next day, mice were immunized intravenously with 1×10^6 (CBA × B10)F_1 APCs or no APCs as a control. Three days after T cell transfer, mice were killed and spleen cells were stained and analyzed by flow cytometry. (**a**) The intensity of CFSE gated on CD8⁺7AAD⁻Des⁺ lymphocytes. (**b**) The mean number of divisions was calculated as outlined in the Methods. (**c**) Total number of Des⁺CD8⁺ T cells in the spleens of host mice on day 3 after transfer; $n = 6$; data were pooled from three independent experiments. Ag, antigen.

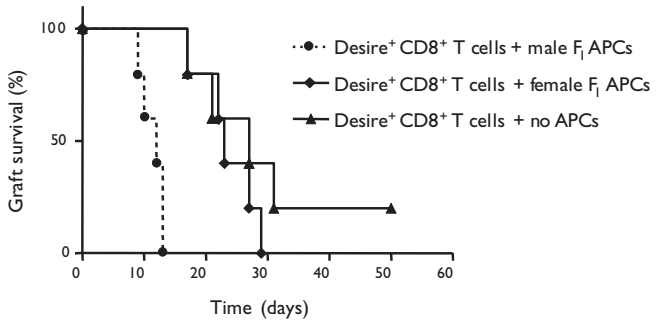

Figure 3. CD4⁺ T cell help enhances graft rejection by monospecific CD8⁺ T cells. Female A1(M) × RAG-1$^{-/-}$ mice were grafted with T cell–depleted B10 tail skins. One month later, mice received 1×10^7 Des⁺CD8⁺ T cells intravenously. The next day, mice were immunized intravenously with 1×10^6 mitomycin C–treated male (filled circles, $n = 5$, MST = 11.5 days), female (CBA × B10)F_1 APCs (filled diamonds, $n = 5$, MST = 23 days) or no (filled triangles, $n = 5$, MST = 27.5 days) APCs. Rates of graft rejection were then monitored. Rejection was seen in all groups, but was significantly delayed in the groups that lacked help, that is in those mice that were primed with female F_1 APCs or were given no APCs compared to the mice that were primed with male help-evoking F_1 APCs ($P = 0.015$).

Des⁺CD8⁺ T cells that accumulated in the spleens of CD4⁺ T cell–depleted mice was substantially lower compared to CD4⁺-sufficient mice challenged with H-2K^b, but was similar to CD4-sufficient mice that had not been exposed to antigen (**Fig. 2c**). Thus CD4⁺ T cell help is needed to ensure optimal net accumulation of CD8⁺ T cells responding to antigen.

Pre-activation of dendritic cells *via* CD40 bypasses the need for T cell help[16,17]. Consistent with this idea, the agonist CD40 antibody FGK.45 substituted for CD4⁺ T cell help and promoted similar expansion of Des⁺CD8⁺ T cells in CD4⁺ T cell–depleted mice (**Web Fig. 2** online) This suggested that activation of APCs *via* CD40 may still occur through the T helper pathway in antigen-challenged tolerized animals.

CD4⁺ help is not essential, yet enhances rejection

To determine whether the Des⁺CD8⁺ T cells could reject established grafts where T_R cells were not active, skin grafts from T cell–depleted B10 mice were grafted onto female recombination-activating gene 1–deficient TCR-transgenic mice bearing a receptor directed to a male minor transplantation antigen (H-Y) in the context of H-2E^k (A1(M) × RAG-1$^{-/-}$). Such mice have CD4⁺ T cells with the anti–H-Y receptor and no CD8⁺ T cells. Consequently, they cannot reject the female B10 graft that then becomes established. Because it was derived from T cell–depleted mice, the grafted skin did not provide T cells to colonize these mice. One month after skin grafting, 1×10^7 Des⁺CD8⁺ containing spleen cells were injected intravenously together with (CBA × B10)F_1 APCs to determine whether they were competent to reject the established grafts in the A1(M) × RAG-1$^{-/-}$ mice. One group of mice received no APCs, one received 1×10^6 male (help-evoking) splenocytes and the third group received a similar number of female (help-avoiding) splenocytes (APCs). Grafts were rejected with a median survival time (MST) of 27.5 days in the group without APC challenge and an MST of 23 days in the group challenged with female APCs (**Fig. 3**). In contrast, the group primed with male APCs rejected the grafts far more rapidly, with an MST of 11.5 days. This result suggested that 1×10^7 Des⁺CD8⁺ spleen cells can reject B10 grafts without CD4⁺ T cell help, but are much more efficient if given help from CD4⁺ T cells. One caveat to these experiments is that compared to the tolerant hosts, A1(M) × RAG-1$^{-/-}$ mice are still relatively lymphopenic and might, therefore, offer a more permissive environment for Des⁺CD8⁺ T cells to reject grafts. Thus, we concluded that the CD8⁺Des⁺ T cells were competent to reject established grafts, even without APC interaction. But why would these CD8⁺Des⁺ T cells not reject grafts in tolerized mice? One possibility was that dominant tolerance had censored CD4⁺ T cell help.

Dominant tolerance does not censor T cell help

Naïve and tolerant mice were transfused with CFSE-labeled Des⁺CD8⁺ T cells together with (CBA × B10)F_1 APCs. All mice were killed 3 days after APC challenge. CFSE division profiles of Des⁺CD8⁺ T cells in different compartments (spleen, draining lymph nodes and contralateral axillary lymph nodes) were compared between these two groups of mice. No difference in proliferation was observed between naïve and tolerant mice in the different cell compartments (**Fig. 4a**). Indeed, the accumulated numbers of Des⁺CD8⁺ T cells were similar in naïve and tolerant animals (**Fig. 4a**). By this criterion, and compared to the data in **Fig. 2**, the CD8⁺ T cells must have received help from host CD4⁺ T cells, yet they still failed to reject grafts. Thus, in tolerance maintained through active regulatory mechanisms, CD8⁺ T cells may still experience help in order to expand and accumulate, although this may not result in graft rejection.

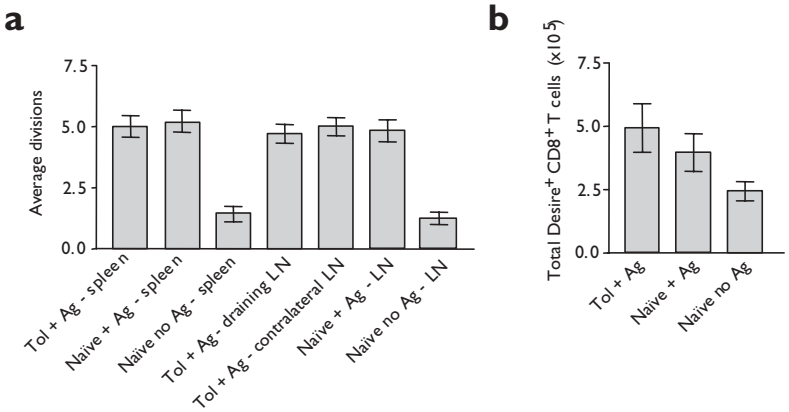

Figure 4. Monospecific CD8⁺ T cells can expand in tolerized mice CFSE-labeled Des⁺CD8⁺ T cells were transferred into tolerized and naïve (control) CBA recipients. Some mice were then challenged intravenously with 1×10^6 (CBA × B10)F_1 APCs. Three days later, peripheral blood lymphocytes were stained and analyzed by flow cytometry. (**a**) Proliferation responses of Des⁺CD8⁺ T cells in spleens and lymph nodes. Each group had eight mice and data were pooled from four independent experiments (**b**) Total number of Des⁺CD8⁺ T cells in spleen. Cells were enumerated as described in the Methods. Each group had eight mice and data were pooled from four independent experiments. LN, lymph node.

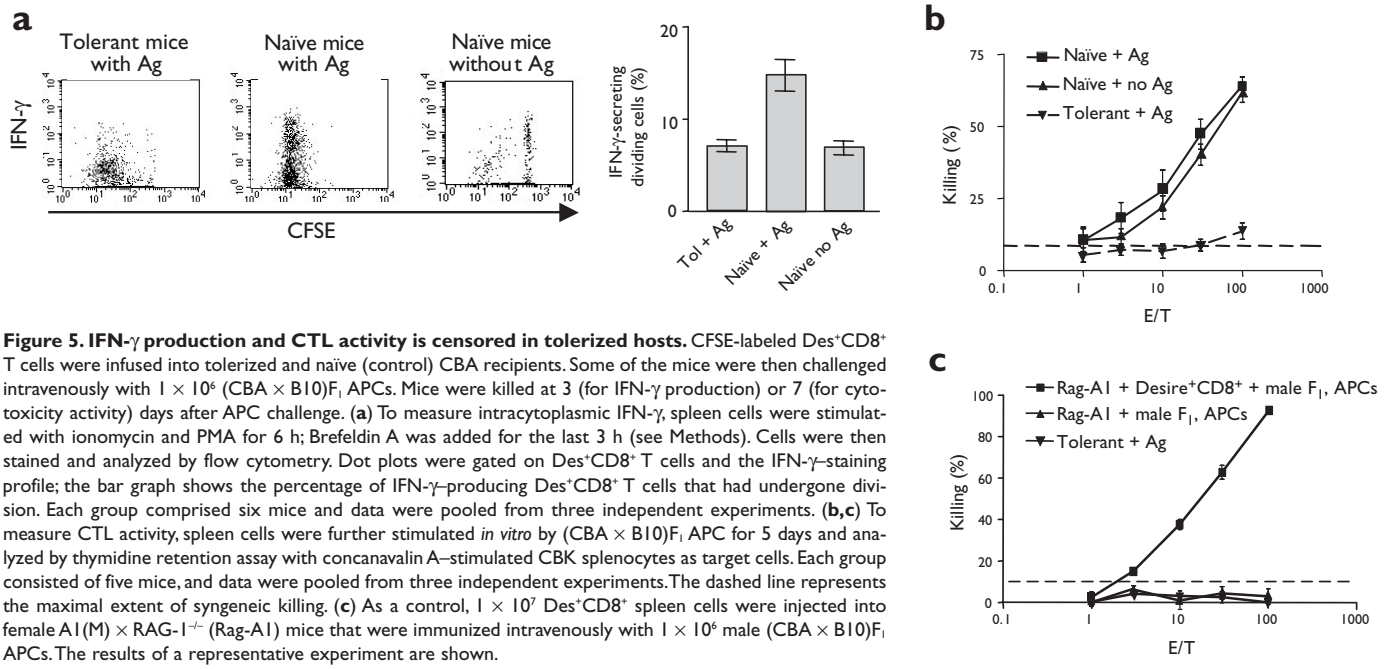

Figure 5. IFN-γ production and CTL activity is censored in tolerized hosts. CFSE-labeled Des$^+$CD8$^+$ T cells were infused into tolerized and naïve (control) CBA recipients. Some of the mice were then challenged intravenously with 1×10^6 (CBA × B10)F_1 APCs. Mice were killed at 3 (for IFN-γ production) or 7 (for cytotoxicity activity) days after APC challenge. (**a**) To measure intracytoplasmic IFN-γ, spleen cells were stimulated with ionomycin and PMA for 6 h; Brefeldin A was added for the last 3 h (see Methods). Cells were then stained and analyzed by flow cytometry. Dot plots were gated on Des$^+$CD8$^+$ T cells and the IFN-γ–staining profile; the bar graph shows the percentage of IFN-γ–producing Des$^+$CD8$^+$ T cells that had undergone division. Each group comprised six mice and data were pooled from three independent experiments. (**b,c**) To measure CTL activity, spleen cells were further stimulated *in vitro* by (CBA × B10)F_1 APC for 5 days and analyzed by thymidine retention assay with concanavalin A–stimulated CBK splenocytes as target cells. Each group consisted of five mice, and data were pooled from three independent experiments. The dashed line represents the maximal extent of syngeneic killing. (**c**) As a control, 1×10^7 Des$^+$CD8$^+$ spleen cells were injected into female A1(M) × RAG-1$^{-/-}$ (Rag-A1) mice that were immunized intravenously with 1×10^6 male (CBA × B10)F_1 APCs. The results of a representative experiment are shown.

Dominant tolerance disarms immune effector functions

To examine why the CD8$^+$ T cells could not reject grafts, despite seemingly normal cell division and accumulation, we measured two further effector functions of the Des$^+$CD8$^+$ T cells: the generation of IFN-γ and cytotoxic T cells (CTLs). The capacity of Des$^+$CD8$^+$ T cells to produce IFN-γ was measured in both naïve (control) and tolerant mice. CFSE-labeled Des$^+$CD8$^+$ T cells were transferred into the mice, which were then challenged with (CBA × B10)F_1 APCs. Splenocytes from these mice were collected 3 days later, and intracellular IFN-γ was measured after 6 h of polyclonal activation by ionomycin and phorbol 12-myristate 13-acetate (PMA). Although naïve mice clearly developed IFN-γ staining and dividing cells after stimulation the percentage of IFN-γ$^+$ cells in the dividing Des$^+$CD8$^+$ T cells from tolerant mice was no different from background (**Fig. 5a**). Neither interleukin 4 (IL-4) nor IL-10 were found in these cells, which ruled out the possibility of deviation from a T_H1 to T_H2 immune response.

We next assessed CTL activity after restimulation of splenocytes from the experimental groups with equal numbers of mitomycin C–treated (CBA × B10)F_1 APCs *in vitro* for 5 days. As a further control, female Des$^+$CD8$^+$ T cells were adoptively transferred into female A1(M) × RAG-1$^{-/-}$ mice and were challenged in the same way with male (CBA × B10)F_1 APCs. Des$^+$CD8$^+$ T cells failed to generate cytotoxic responses when transferred into tolerant hosts but not when transferred into naïve mice or female A1(M) × RAG-1$^{-/-}$ mice (**Fig. 5b,c**). These findings do not rule out the possibility that CTLs were blocked by host T_R cells in the *in vitro* phase of the experiment, but do show that residence in a tolerant host compromises CTL activity. Thus, although H-2K^b–specific CD8$^+$ T cells were able to expand and accumulate in antigen-primed, previously tolerized hosts, they were unable to develop at least three immune effector functions: graft rejection, IFN-γ expression and the capacity to generate CTLs.

Together, these findings suggest that, with this form of dominant tolerance, regulation does not act on the early inductive events of T cell expansion but rather on differentiation towards effector functions.

Discussion

Our study, which was done with a lymphocyte-replete immune system, examined whether dominant tolerance mediated by T_R cells blocked induction of the immune response or whether it prevented downstream effector function. We found that naïve monospecific CD8$^+$ T cells adoptively transferred into tolerant mice could be induced to proliferate to the same extent as in naïve hosts. In addition, they received help from CD4$^+$ T cells, as determined by their accumulation to the same absolute cell number as nontolerant controls. Instead, dominant tolerance resulted in the disarming of three crucial effector functions: cytotoxicity, IFN-γ production and graft rejection. These findings reflected what may happen to CD8$^+$ T cells during the maintenance phases of transplantation tolerance and were different to events that occur during the induction of tolerance where, despite ample cell proliferation, substantial loss of alloreactive CD8$^+$ T cells occurs[23,24].

Although we cannot rule out the possibility that effector cells might develop at some later time and then be deleted, this seems unlikely because the CD8$^+$ T cells were still viable after 21 days. In addition, the depletion of CD4$^+$ T_R cells from tolerant mice can lead to rapid rejection of grafts by previously primed CD8$^+$ T cells, which shows that alloreactive CD8$^+$ T cells may persist[10].

We found that help from CD4$^+$ T cells enhances the survival, rather than proliferation, of CD8$^+$ T cells; this is supported by published observations on a transgenic animal model of diabetes[25]. We have shown here that CD4$^+$ T cell help is mandatory for the optimal accumulation of CD8$^+$ T cells, although not essential for graft rejection. This enabled us to examine whether there was any impairment of cellular proliferation and help in this model of dominant transplant tolerance.

The observation of relatively unaltered expansion and accumulation of CD8$^+$ T cells within the tolerant host was unexpected given the common use of the suppression of proliferation as an assay for T_R activity[12,26]. Our findings may indicate that the *in vitro* proliferation assay does not adequately reflect the physiological role of T_R cells *in vivo*, serving only as a surrogate indicator of their presence. Alternatively, it

may be that there is heterogeneity within T_R cells and that the different readout systems assess distinct populations[6,27–33]. As a consequence it would be useful to establish *in vitro* assays that adequately reflect the impact of T_R cell suppression on the later phase of an immune response.

The "disarming" or "censorship" we have described here is not unique to $CD8^+$ T cells in dominant tolerance. Similar disarmed $CD8^+$ T cells have been reported in other biological systems. In a murine model of lymphocytic cytomegalovirus (LCMV) infection in animals lacking $CD4^+$ T cells, $CD8^+$ T cells induced in response to viral challenge could be detected by specific tetramer binding, lacked effector functions (such as cytotoxicity and IFN-γ production), but could mediate viral clearance[34]. Similar disarmed $CD8^+$ T cells could also be found in patients with chronic HIV[35] and acute hepatitis C[36] infection. In addition, in patients with metastatic melanoma, a population of $CD8^+$ T cells detected by tetramer binding was unable to lyse the melanoma target cells or secrete cytokines after mitogen stimulation[37].

In a previous study of peripheral tolerance, $CD8^+$ T cells with antigenic specificity for hemagglutinin (HA) were adoptively transferred into mice expressing HA in pancreatic islet cells. Before eventually being deleted, these $CD8^+$ T cells became activated and proliferated, but lost the effector functions of cytotoxicity and IFN-γ production[38]. It has been postulated that these cells are arrested at some immature and functionally impaired stage during maturation into fully effective CTLs as a result of a lack of appropriate T cell help[35,39]. Therefore, it is possible that in an environment of dominant tolerance, the help available may enable optimal $CD8^+$ T cell accumulation, but is insufficient or inappropriate to enable complete maturation. It should be noted that censorship of the downstream effector functions of T cells appears not to be restricted just to $CD8^+$ T cells, but can also be demonstrated for $CD4^+$ T cells (unpublished data).

In conclusion, in the context of dominant transplantation tolerance, naïve and monospecific $CD8^+$ T cells respond to donor antigen by expansion and accumulation to an extent that is comparable to naïve nontolerant controls. However, these $CD8^+$ T cells are hindered from developing effector functions such as CTL activity and IFN-γ production and are unable to induce graft rejection.

Methods

Mice. CBA/Ca (H-2^k), C57BL/10 (H-2^b), CBK (CBA/Ca bearing a transgenic H-$2K^b$ molecule)[40], KB5.C20 (H-2^k) TCR–transgenic (CBA/Ca with Des^+CD8^+ T cells recognizing H-$2K^b$)[20] and A1(M) × RAG-$1^{-/-}$ TCR–transgenic mice (with only $CD4^+$ T cells, all recognizing H-Y presented by H-$2E^k$ and backcrossed more than ten generations to CBA/Ca)[41] were bred and maintained in the SPF facility of the Sir William Dunn School of Pathology (Oxford, UK). All procedures were done in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Thymectomy and skin grafting. Thymectomy was done as described[42]. Briefly, a longitudinal incision was made on the anterior surface of the neck, and the thymus was removed as two intact lobes by the application of negative pressure through a glass tip inserted in the anterior mediastinum. Skin grafting was done as described[43], with some modifications. Briefly, full-thickness tail skin (1 × 1 cm) was grafted on the lateral flank. Grafts were observed on alternate days after removal of the bandage at day 8 and considered rejected when no viable donor skin was present.

Cell depletion and tolerance induction. For depletion of $CD4^+$ T cells, intraperitoneal (i.p.) coinjection of 1 mg of each of the lytic CD4 antibodies YTA 3.1.2[44] and YTS 191.2[45] was done 5 and 3 days before the mice were used. Tolerance was induced in CBA/Ca recipients by giving three i.p. injections of 1 mg each of the non-depleting mAbs anti-CD4 (YTS 177.9.6)[44], anti-CD8 (YTS105.18.10)[44] and anti-CD40L (MR1)[46] over a period of 1 week, starting on the day of transplantation. All these mAbs, as well as the agonistic anti-CD40 mAb (FGK-45)[47] were produced by us by culture in hollow fiber bioreactors, purified from culture supernatants by 50% ammonium sulfate precipitation and dialyzed against PBS; the purity was checked by native and SDS gel electrophoresis (PhastGel, Pharmacia, St. Albans, UK).

Preparation of CFSE-labeled monospecific Des^+CD8^+ T cells. Cells were from the spleens of $CD4^+$ T cell–depleted KB5.C20 mice. A single-cell suspension was obtained by passing the splenocytes through a 70-μm cell strainer (Becton Dickinson, San Jose, CA) and the erythrocytes were depleted by water lysis. Des^+CD8^+ T cells represented ~25% of total spleen cells. These cells were incubated with CFSE (Molecular Probes, Eugene, OR) in protein-free RPMI for 10 min at 37 °C. Cells were then washed three times with ice-cold RPMI with 10% fetal calf serum. Cells were counted, diluted in PBS and 1×10^7 cells per mouse were injected intravenously into the tail vein. On the following day, cell suspensions were obtained from the spleens of $(CBA \times B10)F_1$ mice. In one series of experiments, the cells were treated with mitomycin C (Fig. 3). Cells were then counted, diluted in PBS and 1×10^6 cells per mouse were injected intravenously as a source of APCs.

Flow cytometric analysis. Cells from spleen, lymph node or peripheral blood were stained with the following mAbs: phycoerythrin (PE)–CD8α (PharMingen, San Diego, CA), 7-Aminoactinomycin D (7-AAD, Sigma, Gillingham, UK) and biotin-Desire[47]. The biotinylated mAbs were detected by allophycocyanin-streptavidin (PharMingen). Four-color FACSCalibur analysis was done with CellQuest software (both from Becton Dickinson). Live cells were gated on lymphocytes by forward and side scatter profiles and identified as 7-AAD^-. Des^+CD8^+ T cells were identified as $CD8^+$ and $Desire^+$ cells.

To measure the cellular proliferation of Des^+ $CD8^+$ T cells, the intensity of CFSE fluorescence was analyzed. The average number of divisions was calculated by the equation: average number of divisions = $\log_2(A/B)$. Where A represents the mean intensity of the non-dividing group identified in the mice without antigen stimulation and B represents the mean intensity of the test T cell population.

Enumeration of Des^+CD8^+ T cells. A single-cell suspension of spleen cells was counted in Turks solution, subjected to water lysis to eliminate red blood cells and stained as described above. The percentage of Des^+CD8^+ T cells was estimated by flow cytometry. The total number of Des^+CD8^+T cells in spleen was calculated by measuring the total number of dividing spleen cells and correcting for the percentage of Des^+CD8^+ T cells.

Intracellular cytokine staining. Staining for intracellular cytokines was done as described[48,49]. Briefly, spleen cells were stimulated *in vitro* for 6 h with 50 ng/ml of PMA and 500 ng/ml of ionomycin at 37 °C; 10 μg/ml of Brefeldin A was added for the last 3 h (reagents were from Sigma). Thereafter, cells were washed three times and stained with cychrome-CD8α (PharMingen) and biotin-Desire, followed by allophycocyanin-streptavidin. Cells were permeabilized with 0.5% saponin (Sigma) and stained with PE–anti-IFN-γ, PE–anti-IL-4 or PE–anti-IL-10 (all from PharMingen). Four-color FACSCalibur analysis was done with CellQuest software.

Cytotoxicity assay. Single-cell suspensions from the spleen (1×10^7) were stimulated with equal number of mitomycin C–treated $(CBA \times B10)F_1$ splenocytes. Five days later, allospecific CTL activity was measured by the thymidine retention assay (JAM assay)[50] with concanavalin A–stimulated (2.5 μg/ml, Sigma) CBK splenocytes as target cells. Each experiment was done with concanavalin A–stimulated CBA splenocytes as syngeneic control target cells.

Note: Supplementary information is available on the Nature Immunology *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Qin, S. *et al.* "Infectious" transplantation tolerance. *Science* **259**, 974–977 (1993).
2. Onodera, K. *et al.* Induction of "infectious" tolerance to MHC-incompatible cardiac allografts in CD4 monoclonal antibody-treated sensitized rat recipients. *J. Immunol.* **157**, 1944–1950 (1996).
3. Waldmann, H. & Cobbold, S. How do monoclonal antibodies induce tolerance? A role for infectious tolerance? *Annu. Rev. Immunol.* **16**, 619–644 (1998).
4. Taylor, P.A., Noelle, R. J. & Blazar, B. R. $CD4^+CD25^+$ immune regulatory cells are required for induction of tolerance to alloantigen *via* costimulatory blockade. *J. Exp. Med.* **193**, 1311–1318 (2001).
5. Kingsley, C. I., Karim, M., Bushell, A. R. & Wood, K. J. $CD25^+CD4^+$ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J. Immunol.* **168**, 1080–1086 (2002).
6. Graca, L. *et al.* Both $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells mediate dominant transplantation tolerance. *J. Immunol.* **168**, 5558–5565 (2002).
7. Fowell, D. & Mason, D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the $CD4^+$ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* **177**, 627–636 (1993).
8. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **155**, 1151–1164 (1995).
9. Suri-Payer, E., Amar, A. Z., Thornton, A. M. & Shevach, E. M. $CD4^+CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* **160**, 1212–1218 (1998).

10. Marshall, S. E. *et al.* Tolerance and suppression in a primed immune system. *Transplantation* **62**, 1614–1621 (1996).
11. Tran, H. M. *et al.* Distinct mechanisms for the induction and maintenance of allograft tolerance with CTLA4-Fc treatment. *J. Immunol.* **159**, 2232–2239 (1997).
12. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25$^+$CD4$^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* **10**, 1969–1980 (1998).
13. Piccirillo, C. A. & Shevach, E. M. Cutting edge: control of CD8$^+$ T cell activation by CD4$^+$CD25$^+$ Immunoregulatory cells. *J. Immunol.* **167**, 1137–1140 (2001).
14. Cantor, H. & Boyse, E. A. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly$^+$ cells in the generation of killer activity. *J. Exp. Med.* **141**, 1390–1399 (1975).
15. Bach, F. H., Bach, M. L. & Sondel, P. M. Differential function of major histocompatibility complex antigens in T-lymphocyte activation. *Nature* **259**, 273–281 (1976).
16. Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R. & Melief, C. J. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480–483 (1998).
17. Ridge, J. P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4$^+$ T-helper and a T-killer cell. *Nature* **393**, 474–478 (1998).
18. Keene, J. A. & Forman, J. Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. *J. Exp. Med.* **155**, 768–782 (1982).
19. Waldmann, H. & Cobbold, S. Regulating the immune response to transplants. a role for CD4$^+$ regulatory cells? *Immunity* **14**, 399–406 (2001).
20. Schonrich, G. *et al.* Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* **65**, 293–304 (1991)
21. Hua, C., Boyer, C., Buferne, M. & Schmitt-Verhulst, A. M. Monoclonal antibodies against an H-2K^b-specific cytotoxic T cell clone detect several clone-specific molecules. *J. Immunol.* **136**, 1937–1944 (1986).
22. Zelenika, D. *et al.* The role of CD4$^+$ T cell subsets in determining transplantation rejection or tolerance. *Immunol. Rev.* **182**, 164–179 (2001).
23. Li, Y. *et al.* Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nature Med* **5**, 1298–1302 (1999).
24. Wells, A. D. *et al.* Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nature Med* **5**, 1303–1307 (1999).
25. Kurts, C. *et al.* CD4$^+$ T cell help impairs CD8$^+$ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. *J. Exp. Med.* **186**, 2057–2062 (1997).
26. Thornton, A. M. & Shevach, E. M. CD4$^+$CD25$^+$ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296 (1998).
27. Polanski, M., Melican, N. S., Zhang, J. & Weiner, H. L. Oral administration of the immunodominant B-chain of insulin reduces diabetes in a co-transfer model of diabetes in the NOD mouse and is associated with a switch from Th1 to Th2 cytokines. *J. Autoimmunity* **10**, 339–346 (1997).
28. Groux, H. *et al.* A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737–742 (1997).
29. Hall, B. M. *et al.* Anti-CD4 monoclonal antibody-induced tolerance to MHC-incompatible cardiac allografts maintained by CD4$^+$ suppressor T cells that are not dependent upon IL-4. *J. Immunol.* **161**, 5147–5156 (1998).
30. Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* **190**, 995–1004 (1999).
31. Seddon, B. & Mason, D. Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor β and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4$^+$CD45RC$^-$ cells and CD4$^+$CD8$^-$ thymocytes. *J. Exp. Med.* **189**, 279–288 (1999).
32. Bushell, A., Niimi, M., Morris, P. J. & Wood, K. J. Evidence for immune regulation in the induction of transplantation tolerance: a conditional but limited role for IL-4. *J. Immunol.* **162**, 1359–1366 (1999).
33. Hara, M. *et al.* IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo*. *J. Immunol.* **166**, 3789–3796 (2001).
34. Zajac, A. J. *et al.* Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med.* **188**, 2205–2213 (1998).
35. Appay, V. *et al.* HIV-specific CD8$^+$ T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* **192**, 63–75 (2000).
36. Thimme, R. *et al.* Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J. Exp. Med.* **194**, 1395–1406 (2001).
37. Lee, P. P. *et al.* Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nature Med.* **5**, 677–685 (1999).
38. Hernandez, J., Aung, S., Redmond, W. L. & Sherman, L. A. Phenotypic and functional analysis of CD8$^+$ T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J. Exp. Med.* **194**, 707–718 (2001).
39. Kalams, S. A. & Walker, B. D. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J. Exp. Med.* **188**, 2199–2204 (1998).
40. Tarazona, R. *et al.* Effects of different antigenic microenvironments on the course of CD8$^+$ T cell responses *in vivo*. *Int. Immunol.* **8**, 351–358 (1996).
41. Zelenika, D. *et al.* Regulatory T cells overexpress a subset of Th2 gene transcripts. *J. Immunol.* **168**, 1069–1079 (2002).
42. Monaco, A. P., Wood, M. L., Gray J. G. & Russel P. S. Studies on heterologous anti-lymphocyte serum in mice. II. Effect on the immune response. *J. Immunol.* **96**, 229–232 (1966).
43. Billingham, R. E., Brent, L. & Medawar, P. B. Actively acquired tolerance to foreign cells. *Nature* **172**, 603–606 (1953).
44. Qin, S. X. *et al.* Induction of tolerance in peripheral T cells with monoclonal antibodies. *Eur. J. Immunol.* **20**, 2737–2745 (1990).
45. Cobbold, S. P., Jayasuriya, A., Nash, A., Prospero, T. D. & Waldmann, H. Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*. *Nature* **312**, 548–551 (1984).
46. Noelle, R. J. *et al.* A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA* **89**, 6550–6554 (1992).
47. Rolink, A., Melchers, F. & Andersson, J. The SCID but not the RAG-2 gene product is required for Sμ-Sε heavy chain class switching. *Immunity* **5**, 319–330 (1996).
48. Jung, T., Schauer, U., Heusser, C., Neumann, C. & Rieger, C. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Meth.* **159**, 197–207 (1993).
49. Zelenika, D. *et al.* Rejection of H-Y disparate skin grafts by monospecific CD4$^+$ Th1 and Th2 cells: no requirement for CD8$^+$ T cells or B cells. *J. Immunol.* **161**, 1868–1874 (1998).
50. Matzinger, P. The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Meth.* **145**, 185–192 (1991).