

Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation

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After a brief period of antigenic stimulation, T cells become committed to a program of autonomous expansion and differentiation. We investigated the role of antigen-specific T cell precursor frequency as a possible cell-extrinsic factor impacting T cell programming in a model of allogeneic tissue transplantation. Using an adoptive transfer system to incrementally raise the precursor frequency of antigen-specific CD8⁺ T cells, we found that donor-reactive T cells primed at low frequency exhibited increased cellular division, decreased development of multifunctional effector activity, and an increased requirement for CD28- and CD154-mediated costimulation relative to those primed at high frequency. The results demonstrated that recipients with low CD4⁺ and CD8⁺ donor-reactive T cell frequencies exhibited long-term skin graft survival upon CD28/CD154 blockade, whereas simultaneously raising the frequency of CD4⁺ T cells to ~0.5% and CD8⁺ T cells to ~5% precipitated graft rejection despite CD28/CD154 blockade. Antigenic rechallenge of equal numbers of cells stimulated at high or low frequency revealed that cells retained an imprint of the frequency at which they were primed. These results demonstrate a critical role for initial precursor frequency in determining the CD8⁺ T cell requirement for CD28- and CD154-mediated costimulatory signals during graft rejection.

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Abbreviations used: DLN, draining LN; mOVA, membrane-bound OVA; MST, median survival time.

Organ and cell transplantation represents an important means of effectively treating a wide range of genetic and acquired diseases. A major challenge in transplantation is inhibiting the immune response mounted by the recipient against donor tissue. This immune attack, comprised of many cell types, is orchestrated by T cells that become activated after recognizing allo-antigens and receiving appropriate costimulatory signals (1, 2). One promising therapy designed to regulate the immune response to donor tissue and thereby prevent the rejection response involves the use of reagents that block the costimulatory signals required for T cell activation (3). The most well characterized of these are CD28, which is ligated by CD80 or CD86 (4) and can be blocked via a CTLA-4 Ig fusion protein (4), and the CD40 pathway, which can be blocked using mAbs reactive against CD40 or CD154 (CD40L) (5).

Although very effective in many experimental rejection models, it is widely recognized

and of considerable interest that combined blockade of the CD28 and CD40 pathways does not uniformly control rejection responses, indicating that not all allo-reactive T cell responses are dependent on these costimulatory signals during primary activation. For example, CD28/CD154 blockade results in graft survival of >120 d when BALB/c skin is grafted onto treated C3H recipients, whereas engraftment of BALB/c skin onto B6 recipients treated with the same regimen results in only moderate prolongation of graft survival (6–10). This breakthrough rejection could not be overcome simply by increasing the dose or duration of costimulation blockade treatment, further demonstrating that some T cell responses are fundamentally less dependent on CD28- and CD154-mediated costimulatory signals (7). The factors governing this observed relative independence of CD28/CD154-mediated signals are not well understood.

In recent years, the programmed differentiation model has emerged as a new paradigm to describe T cell responses based on evidence

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that after a brief period of antigenic stimulation, T cells become committed to a program of autonomous clonal expansion and differentiation into effector cells (11–14). T cell programs are flexible and can be altered by the initial priming conditions as well as by extrinsic factors during the execution of the program. Of particular relevance to transplantation immunology are two recent studies indicating that the initial T cell precursor frequency of the responding population is a powerful influence on the program, and that T cells that undergo activation at high frequency differ from those that become activated under conditions of low frequency in their ability to differentiate into central and effector memory T cells (15) and in their ability to survive as memory cells (16). In addition, evidence suggests that, in infection models, responding T cells can achieve the same clone burst size over a wide range of initial precursor frequencies (17, 18). In these instances, T cells that begin at low frequency must undergo many more rounds of division and hence may be more susceptible to antigen-induced cellular deletion (15, 19). Furthermore, studies in models of autoimmunity have revealed that high initial CD8⁺ frequencies can convert helper-dependent responses into helper-independent responses (20).

Comparatively little is known about the instructional developmental T cell programs that are executed during allograft rejection and whether they are influenced by initial precursor frequency. The initial frequencies of alloreactive T cells span a much broader range than those of pathogen-specific or autoreactive T cells. For responses across fully mismatched MHC disparities, up to 1 in 10–100 T cells may participate in the response (21), a number that is 2–3 logs greater than the estimated precursor frequency of pathogen-specific T cells. Although there is less quantitative information on the frequencies of donor-reactive T cells in the setting of MHC-identical transplants, the frequencies are likely to be closer to those observed in response to pathogens ($1:10^4$ – 10^6) (22, 23). Varying degrees of MHC class I and II matching would be expected to affect the initial frequency of responding CD8⁺ and CD4⁺ T cells, respectively. For this reason, the issue of how initial antigen-specific T cell precursor frequency influences the kinetics, magnitude, and functionality of the T cell program is particularly pertinent to the field of transplantation.

Work from several groups has provided correlative evidence that systems with low donor-reactive T cell frequency are more susceptible to the inhibitory effects of CD28 and CD154 blockade than systems with high precursor frequency (24–26). However, direct assessment of the effect of increased donor-reactive T cell precursor frequency has been limited by the availability of tools to incrementally increase both the donor-reactive CD4⁺ and CD8⁺ T cell compartments and to systematically analyze the magnitude and character of the responding T cell population under these conditions. In this study, we used a novel model in which membrane-bound OVA (mOVA) is constitutively expressed in all tissues under the control of the β -actin promoter (27). The mOVA protein contains the epitopes recognized by T cells that can be

isolated from the TCR transgenic mice OT-I and OT-II (OVA 257–264/K^b and OVA 323–339/I-A^b, respectively). Thus, the frequency of alloreactive T cells can be manipulated by titrating into naive recipients increasing numbers of OT-I and OT-II T cells. We therefore sought to address whether costimulation blockade-induced tolerance is influenced by the precursor frequency of naive donor-reactive T cells. The results indicate that a critical threshold exists above which antigen-specific T cells cannot be tolerized by CD28 and CD154 costimulatory pathway blockade, suggesting that variation in the initial precursor frequencies of donor-reactive CD4⁺ and/or CD8⁺ T cells is a critical determinant in the susceptibility or resistance to CD28/CD154 costimulation blockade-induced graft acceptance. We demonstrate that the initial frequency not only determines the fate of the graft, but also dictates both important quantitative and qualitative aspects of the developmental differentiation program executed by donor-specific T cell populations. These results therefore imply that antigen-specific T cell frequency during the priming phase of the immune response may influence the requirement for CD28- and CD154-mediated costimulation during T cell activation and create distinct opportunities for therapeutic interventions.

RESULTS

Initial precursor frequency of donor-specific T cells influences division rates and acquisition of effector function after transplantation

To explore the effects of precursor frequency on the primary immune response to transplanted tissues, we performed adoptive transfer experiments in which OVA-specific OT-I (CD8⁺) and OT-II T cells (CD4⁺) were titrated into B6 recipients of mOVA skin grafts, incrementally raising the effective donor-specific precursor frequencies. First, we varied only the initial CD8⁺ T cell frequency and supplied a constant (but elevated) frequency of CD4⁺ T cell help. 48 h before transplant, groups of B6 mice received either 10^6 or 10^7 OT-I T cells. Both groups also received a fixed dose of 10^6 OT-II CD4⁺ T cells. As expected, all groups rejected their mOVA skin grafts (not depicted). Median survival times (MSTs) were 20 d for mice that did not receive T cells, with slightly shorter survival times of 16 and 14 d for the groups receiving 10^6 and 10^7 OT-I T cells, respectively.

To assess the effect of antigen-specific precursor frequency on the functional differentiation of the responding T cell populations after allogeneic tissue transplantation, recipients were killed at various time points after transplantation for analysis of the donor antigen-specific T cell response. The transferred OT-I T cells were identified as CD8⁺ Thy1.1⁺, and CFSE division profiles were examined. Division of the transferred populations was detectable in the draining LN (DLN) by day 3, with increased cell division in the low frequency group (Fig. 1 A). Remarkably, by day 10 the population that began as ~ 10 -fold lower than the high frequency population attained nearly identical absolute numbers in the DLN at the peak of the response (Fig. 1 B). Similar results

were obtained after analysis of CD8⁺ Thy1.1⁺ OT-I T cells isolated from the spleens of these recipients (not depicted). The convergence in absolute cell numbers is likely due to the observed increase in cell division of the low precursor frequency population (Fig. 1 A), as annexin V and propidium iodide staining of donor-reactive CD8⁺ Thy1.1⁺ T cells did not suggest increased death rates in the cells stimulated at high frequency (not depicted). In fact, the kinetics of contraction of the cell populations after the peak of the response was more rapid in the cells stimulated at low frequency than in those stimulated at high frequency (Fig. 1 B). These results suggest that very significant differences exist in terms of the rates of T cell expansion and contraction for T cell populations responding from low versus high precursor frequency.

To assess the effect of priming at high or low precursor frequency on the acquisition of effector function by OT-I T cells after antigen encounter, DLN cells from mice that received 10⁷ (high) or 10⁶ (low) OT-I T cells were assayed for their ability to produce IFN- γ and TNF- α after peptide challenge in vitro. A higher percentage of CD8⁺ Thy1.1⁺

T cells stimulated under low frequency conditions produced IFN- γ at day 14 (55.2%) as compared with those stimulated at high frequency (14.5%) (Fig. 1 C). However, the total number of IFN- γ -producing cells per DLN was similar in both the high and low precursor frequency groups, suggesting that cells stimulated at lower frequency reached the same peak number of effector cells (Fig. 1 D). We also examined the number of OT-I cells that produced TNF- α after antigen rechallenge and found that although the DLNs of mice containing cells stimulated at high frequency had similar numbers of IFN- γ - and TNF- α -secreting cells, the DLNs of mice containing cells stimulated at low frequency had only about half the number of TNF- α -producing cells as IFN- γ -producing cells (Fig. 1 D), suggesting a difference in the quality of the response. Indeed, substantial evidence in the literature supports the idea that high quality effector cells are multifunctional in nature (28–30). Collectively, these results demonstrate that although cells stimulated at high and low frequency are eventually sufficient to reject a skin graft, cell populations stimulated at low frequency must undergo much more extensive

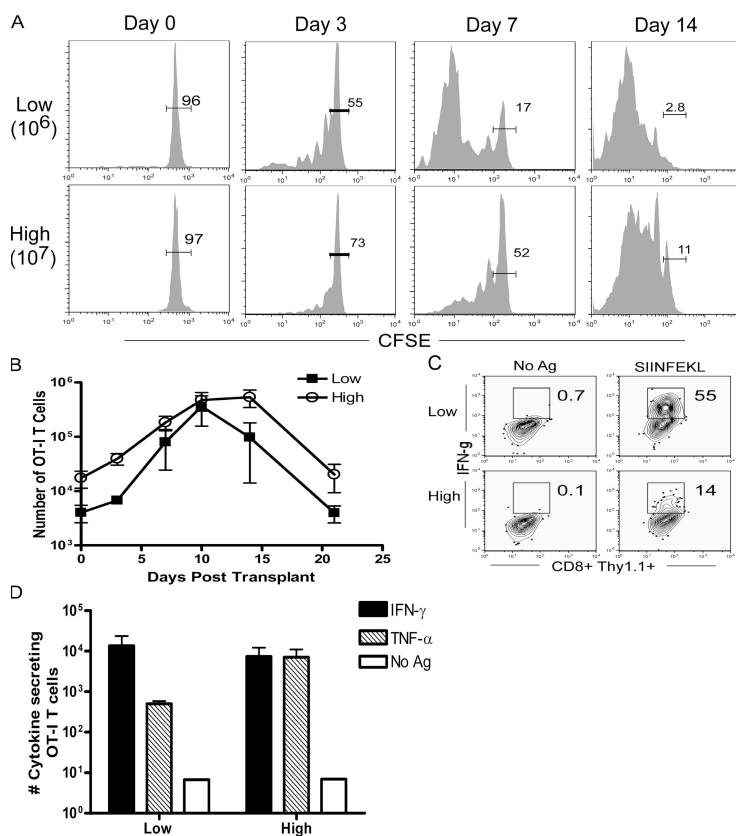


Figure 1. OT-I T cells primed at high precursor frequency undergo less division and take on characteristics of higher quality effector cells. 10⁶ or 10⁷ OT-I T cells (along with 10⁶ OT-II T cells) were adoptively transferred into naive B6 recipients, which then received an mOVA skin graft. (A) DLN OT-I T cells primed at low frequency underwent more rounds of division and with earlier kinetics at days 3, 7, and 14 after transplantation as measured by CFSE analysis. (B) Absolute numbers of OT-I T cells isolated from the DLN showed a convergence at the peak

of expansion in both the low and high frequency groups. (C) Cytokine-producing ability of cells primed at low versus high frequency was measured as the percentage of cells that stained positive for intracellular anti-IFN- γ after a 4-h in vitro peptide stimulation. (D) The absolute number of multifunctional effector cells that secreted both IFN- γ and TNF- α was calculated by multiplying the percentage of cytokine-positive cells by the total number of OT-I T cells as determined by TruCount analysis in B.

expansion to achieve this and possess fewer “high quality” multifunctional T cells.

CTLA-4 Ig and anti-CD154–induced tolerance can be overcome by increasing CD4⁺ and CD8⁺ T cell precursor frequencies

Based on these studies demonstrating that T cell stimulation at high precursor frequency resulted in less rigorous proliferation and more multifunctional effector T cells, we hypothesized that high initial precursor frequency of donor-specific T cells might also reduce the cells’ requirement for CD28- and CD154-mediated costimulation during primary activation. To directly test this, TCR transgenic OT-I and OT-II T cells were adoptively transferred into naive B6 recipients, incrementally raising the precursor frequency of antigen-specific cells before engraftment with mOVA skin and treatment with CTLA-4 Ig/anti-CD154 mAbs (costimulation blockade) (Fig. 2 A). To quantify the “artificial” precursor frequency resulting from the adoptive transfer of increasing numbers of antigen-specific T cells, splenocytes and LN cells from recipients of 10⁵, 10⁶, or 10⁷ OT-I and OT-II T cells were analyzed for the presence of Thy1.1⁺ OT-I or OT-II T cells 48 h after transfer. Results indicated that the percentage of Thy1.1⁺ OT-I and OT-II T cells both increased in a step-wise fashion, with recipients of 10⁵ cells having a precursor frequency of ~0.04%, recipients of 10⁶ cells having a frequency of ~0.4%, and recipients of 10⁷ cells having a frequency of ~4% (Fig. 2 A). The artificial precursor frequencies of these adoptive transfer recipients therefore span the range that is thought to represent the frequencies normally observed for allogeneic T cell responses (21).

To assess the effect of this incremental increase in precursor frequency on skin graft rejection, recipients of 10⁵, 10⁶, or 10⁷ OT-I/OT-II cells were transplanted with mOVA skin and treated on days 0, 2, 4, and 6 with CTLA-4 Ig/anti-CD154. Although control mice that received no T cell transfer or costimulation blockade rejected their grafts with an MST of 21 d, mice that received costimulation blockade and no supplemental T cells maintained their grafts for >150 d. Concomitantly raising the frequency of CD4⁺ and CD8⁺ T cells to 0.05 or 0.5% did not affect the ability of costimulation blockade to induce allograft tolerance; however, at antigen-specific T cell frequencies of 5% for both CD4⁺ and CD8⁺ compartments, mice rapidly and consistently rejected mOVA skin grafts despite treatment with CTLA-4 Ig/anti-CD154 (MST 13 d; *P* < 0.001 as compared with all other treated groups) (Fig. 2 B). These results demonstrate that high antigen-specific T cell precursor frequency is sufficient to convert costimulation blockade-induced allograft tolerance into costimulation blockade-resistant rejection due to a reduced requirement for CD28- and CD40L-mediated costimulatory signals in cells stimulated at high precursor frequency.

CD8⁺ OT-I T cells become “costimulation independent” at a lower precursor frequency than do CD4⁺ OT-II T cells

Next, we sought to identify the contribution of a high precursor frequency of each T cell compartment to the

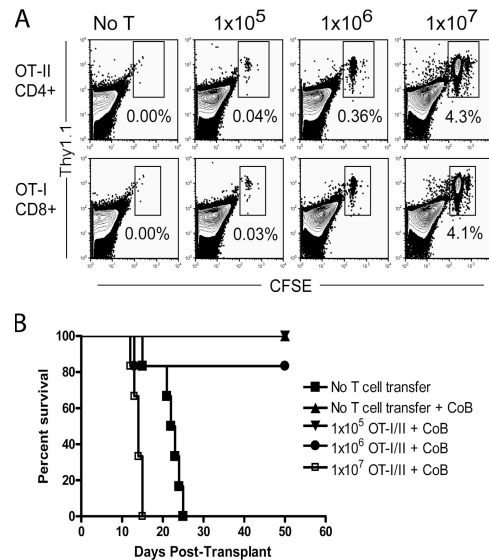


Figure 2. High frequency graft-specific CD4⁺ and CD8⁺ T cells precipitate costimulation blockade-resistant rejection. Increasing numbers (10⁵, 10⁶, or 10⁷) of mOVA-specific OT-I and OT-II T cells were adoptively transferred into naive B6 recipients. (A) Mice were analyzed 48 h after transfer to determine the precursor frequency of graft-specific T cells in the axillary LNs resulting from the transfer of each number of cells. Data are representative of three mice per group. (B) Recipients of the indicated numbers of graft-specific T cells received mOVA skin grafts and CTLA-4 Ig and anti-CD154 where indicated (*n* = 6 mice group, two independent experiments). Results demonstrate that raising the precursor frequency of graft-specific T cells to the level observed in fully allogeneic situations resulted in costimulation blockade-resistant rejection (*P* < 0.001 for mice receiving 10⁷ OT-I/OT-II T cells as compared with all other groups treated with CTLA-4 Ig and anti-CD154).

development of costimulation blockade-resistant rejection by independently varying the frequencies of either compartment before mOVA skin transplantation and CTLA-4 Ig/anti-CD154. Results demonstrate that high frequencies of either subset alone were insufficient to overcome the tolerizing effects of costimulation blockade and induce rapid and consistent costimulation blockade-resistant rejection (Table I). Furthermore, although CD4⁺ T cell frequency of 0.05% was not sufficient to precipitate maximum rejection even at high frequencies of OT-I cells, CD4⁺ cell frequencies of 0.5 and 5% both provided adequate help for 10⁷ OT-I T cells to induce maximum costimulation blockade-resistant rejection (Table I). Importantly, even 10⁷ OT-II T cells were insufficient to induce costimulation blockade-resistant rejection at lower frequencies of OT-I T cells (0.05 and 0.5%, or 10⁵ and 10⁶ transferred cells), implicating a high precursor frequency of antigen-specific CD8⁺ T cells as a major barrier to costimulation blockade-induced allograft tolerance.

Precursor frequency affects susceptibility of the donor-specific CD8⁺ T cell populations to CD28 and CD154 blockade

Given the finding that a high precursor frequency of CD8⁺ T cells was critically important in the generation of costimulation

Table I. Impact of increased antigen-specific CD4⁺ or CD8⁺ precursor frequencies on the requirement for CD28/CD154-mediated costimulation during skin graft rejection^a

		Frequency ^b (no. ^c) of CD8 ⁺ cells				
Frequency (no.)		1 in 10 ⁶ (None)	0.05% (10 ⁵)	0.5% (10 ⁶)	5% (10 ⁷)	
Frequency (no.) of CD4 ⁺ cells	1 in 10 ⁶ (None)	>150 ^d	>150	>150	>150	
	0.05% (10 ⁵)	>150	>150	>150	>150	
	0.5% (10 ⁶)	>150	>150	>130.7 ± 68	17.2 ± 4.7	
	5% (10 ⁷)	>150	>150	>150	13.8 ± 1.2	

^aB6 mice received the indicated number of OT-I or OT-II T cells and an mOVA skin graft 48 h later. All mice were treated with CTLA-4 Ig and anti-CD154 mAbs as described in Materials and methods.

^bResulting frequency after adoptive transfer of exogenous T cells.

^cAbsolute number of adoptively transferred OT-I or OT-II T cells.

^dData is expressed as the mean survival time (in days) ± SEM of 10 mice per group.

blockade-resistant rejection, we sought to define the effects of altered CD8⁺ T cell precursor frequency on the programmed expansion and differentiation of donor-specific T cells in the presence of costimulation blockade. Two groups of B6 mice with varying precursor frequencies of donor-specific CD8⁺ T cells were prepared by transferring 10⁶ or 10⁷ OT-I T cells. Both groups also received a fixed dose of 10⁶ OT-II CD4⁺ T cells, an mOVA skin graft, and CTLA-4 Ig/anti-CD154. Again, we observed that the initial CD8 precursor frequency had a profound impact on graft survival after treatment with costimulation blockade. Mice that received 10⁶ OT-II cells and 10⁶ OT-I T cells accepted an mOVA skin graft for >150 d after treatment with costimulation blockade, whereas mice that received 10⁶ OT-II cells and 10⁷ OT-I T cells showed rapid and consistent costimulation blockade-resistant rejection resulting in graft rejection with an MST of 17 d ($P < 0.001$) (Fig. 3 A). Clinical rejection was confirmed by immunohistochemical analysis of the donor skin tissue, which revealed Thy1.1⁺ infiltration at day 14 after transplant in CTLA-4 Ig/anti-CD154-treated mice possessing a high but not low frequency of OT-I/OT-II T cells (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20062319/DC1>).

To assess the effect of initial precursor frequency on the programming of the T cell response, mice were killed at various time points after transplantation for analysis of the donor-reactive T cell response (Fig. 3 B). Frequencies of Thy1.1⁺ CD8⁺ T cells in the DLN at 10 d after transplant revealed a marked decrease in cells primed at low but not high frequencies in the presence of CTLA-4 Ig/anti-CD154. Furthermore, the absolute number of untreated CD8⁺ Thy1.1⁺ cells primed at low frequency increased substantially such that by day 10 it equaled the number of untreated CD8⁺ Thy1.1⁺ cells primed at high frequency (Fig. 3 C). Strikingly, cells primed at low frequency in the presence of costimulation blockade exhibited blunted expansion in total cell numbers. Conversely, cells stimulated at high frequency in the presence of costimulation blockade exhibited essentially overlapping magnitude and kinetics of expansion as cells from untreated recipients (Fig. 3 C), suggesting that blockade of CD28- and CD154-mediated signals has little effect on the magnitude

and kinetics of expansion of graft-specific T cells primed at high frequency.

We also performed CFSE analysis to compare the amount of cell division occurring under each set of conditions. We observed that the more rapid division of the low frequency groups is dramatically stunted by costimulation blockade on days 3 and 7 after transplant (Fig. 4 A, top two rows). Strikingly, division was less notably inhibited by costimulation blockade at high T cell frequency (Fig. 4 A, bottom two rows). Comparisons of absolute numbers of OT-I T cells that had undergone each number of rounds of division (0, 1, 2, etc.) revealed that more of the cells stimulated at low frequency had divided more than five times, whereas the majority of the cells stimulated at high frequency had divided fewer than five times (Fig. 4 B). Notably, cells stimulated at low frequency in the presence of CTLA-4 Ig/anti-CD154 were present at lower numbers in all stages of division and failed to accumulate in the later stages of division (more than seven rounds). In contrast, the division profile of cells stimulated at high frequency in the presence of costimulation blockade exhibited minimal cell loss relative to untreated controls (Fig. 4 B). These experiments suggest that costimulation blockade quantitatively impacts the expansion of donor-reactive T cell populations when primed under low frequency conditions, but has relatively little impact on cells primed under high antigen-specific precursor frequency conditions.

Precursor frequency qualitatively affects the phenotype and differentiation of donor-specific T cell populations in response to allogeneic tissue transplantation

Based on our observations that OT-I T cells primed at low frequency in vivo were quantitatively susceptible to the effects of costimulation blockade, whereas OT-I T cells primed at high frequency were able to overcome the induction of tolerance, we hypothesized that T cells primed at high versus low frequency in the absence of CD28- and CD154-mediated costimulatory signals might also be qualitatively different on a per cell basis in terms of their abilities to produce the effector cytokines IFN- γ and TNF- α . Although cells stimulated at high frequency in the presence or absence of costimulation blockade exhibited similar frequencies of

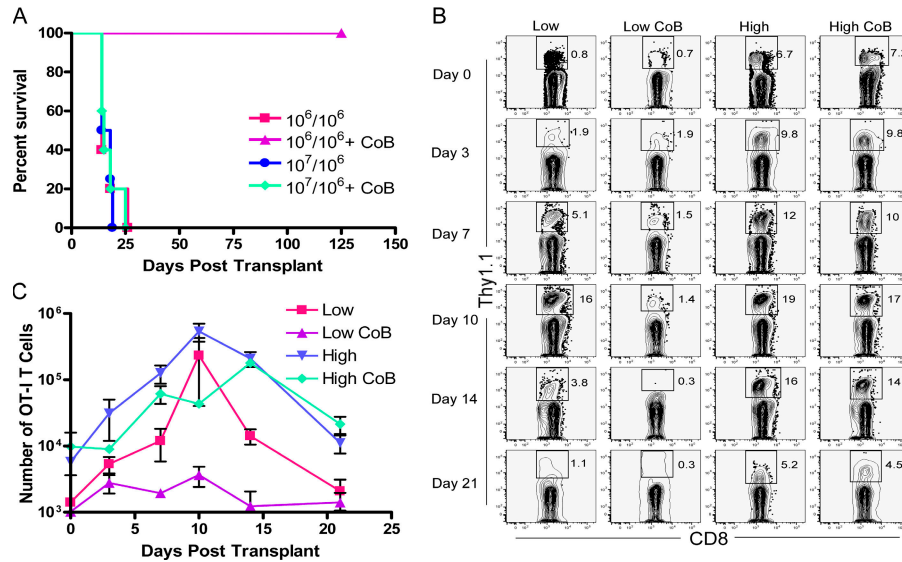


Figure 3. Decreased OT-I T cell expansion in the presence of CTLA-4 Ig/anti-CD154 after priming at low, but not high, antigen-specific precursor frequency. 10^6 or 10^7 OT-I T cells (along with 10^6 OT-II cells) were adoptively transferred into naive B6 recipients that then received an mOVA skin graft. (A) Results showed that mice receiving a low frequency (10^6) of OT-I T cells were protected from rejection by CTLA-4 Ig/anti-CD154, whereas mice receiving a high frequency (10^7) rapidly rejected their grafts ($P < 0.001$). (B) Analysis of the percentage of Thy1.1⁺ CD8⁺ T cells in the DLN revealed that costimulation blockade inhibited the expansion/accumulation of OT-I T cells in mice responding

from low frequency, whereas the expansion/accumulation of OT-I cells responding from high frequency was not as potently affected. (C) Absolute numbers of OT-I T cells isolated from the DLN showed a convergence at the peak of expansion at day 10 in both the low and high frequency untreated groups as well as the CTLA-4 Ig/anti-CD154-treated high frequency group. In contrast, the low frequency, CTLA-4 Ig/anti-CD154-treated group exhibited minimal expansion of OT-I T cell numbers. These data are representative examples of four independent experiments, with two to three mice per group, per time point, per experiment.

IFN- γ - and TNF- α -producing cells 4 h after in vitro peptide rechallenge, cells primed at low precursor frequency in the presence of costimulation blockade exhibited a dramatically diminished ability to make IFN- γ and TNF- α as compared with their untreated counterparts (Fig. 5 A and not depicted). The same result was observed when the total number of IFN- γ - or TNF- α -secreting cells per spleen was analyzed (Fig. 5 B). This striking result closely parallels the observed clinical outcome of graft rejection, as mice that received a low precursor frequency of OT-I T cells in the presence of costimulation blockade were the only group in which long-term tolerance was induced (Fig. 3 A).

Evidence of programming: CD8⁺ T cells primed at high or low frequency retain the imprint of these conditions after secondary challenge with antigen

It is becoming increasingly apparent that T cells retain an imprint of their initial priming conditions, which may then influence characteristics of the response upon subsequent encounters with antigen (11–13, 31–33). To test the hypothesis that precursor frequency during the priming phase of T cell activation is an important factor influencing the T cell program, we established a system where OT-I T cells were stimulated at high or low frequency with OVA peptide in vitro in the presence of CTLA-4 Ig/anti-CD154, and later rechallenged with an mOVA skin graft in vivo. B6 splenocytes were added to the low frequency in vitro cultures to keep the

total number of cells per well constant. Consistent with our in vivo studies, OT-I T cells primed at low frequency in vitro underwent more extensive division than cells primed at high frequency (Fig. 6 A). 3 d later, equal numbers (5×10^6) of live T cells primed at an initial high or low antigen-specific T cell frequency were adoptively transferred into P14xRAG^{-/-} recipients. These mice have a T cell compartment restricted to the recognition of LCMV gp33-41 and therefore cannot reject an mOVA skin graft without the provision of exogenous antigen-specific T cells (unpublished data). In contrast to polyclonal CD4⁺ and CD8⁺ T cell populations (34 and unpublished data), OT-I T cells do not undergo significant homeostatic expansion in these hosts (unpublished data), thus allowing analysis of the fate and function of the adoptively transferred cells in this system, relatively free from the effects of homeostatic proliferation.

48 h after T cell transfer, mice were challenged with an mOVA skin graft without any further CTLA-4 Ig/anti-CD154 treatment. Naive OT-I T cells, adoptively transferred to an additional group as a positive control, were able to reject an mOVA skin graft with an MST of 16 d (Fig. 6 B). Mice that received OT-I T cells primed at high or low frequency in the absence of CTLA-4 Ig/anti-CD154 both rapidly rejected their grafts with MSTs of 14 and 15 d, respectively. In sharp contrast, mice receiving OT-I T cells stimulated in vitro at low precursor frequency in the presence of costimulation blockade experienced prolonged survival of

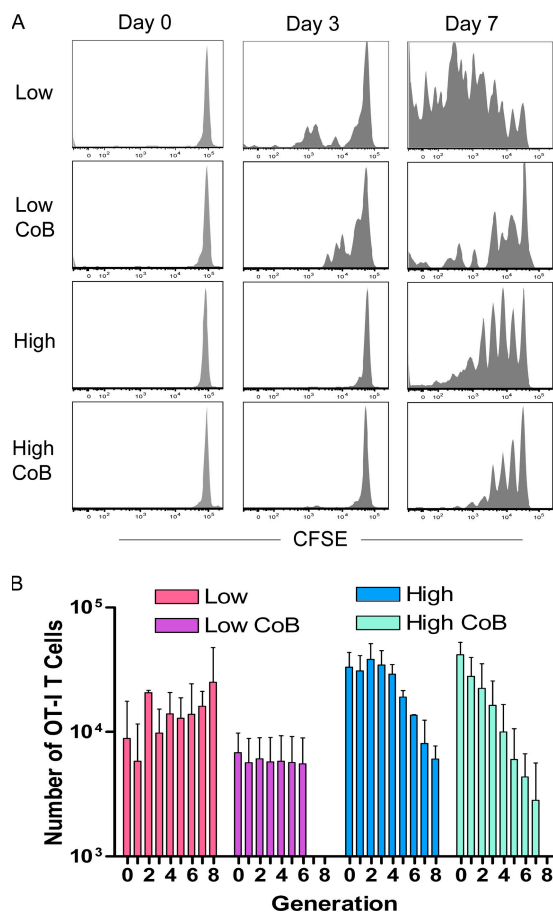


Figure 4. OT-I T cells primed at low frequency in the presence of CTLA-4 Ig and anti-CD154 fail to accumulate in later rounds of cell division. 10^6 or 10^7 OT-I T cells (along with 10^6 OT-II cells) were CFSE labeled and adoptively transferred into naive B6 recipients, which then received an mOVA skin graft. (A) Analysis of Thy1.1⁺ CD8⁺ DLN cells demonstrated reduced rounds of cell division in OT-I T cells responding from low frequency in the presence of CTLA-4 Ig/anti-CD154 relative to those left untreated (top two rows). In contrast, the cell division profile of OT-I cells responding from high frequency was not as potently affected by costimulation blockade (bottom two rows). (B) Absolute numbers of DLN OT-I T cells in each cell generation (0, no cell division) were calculated from the percentage of each population in peaks 0–7 or >7 (generation 8+). Data show that CTLA-4 Ig/anti-CD154-treated graft-specific cells responding from high frequency exhibited a division profile similar to those in the untreated controls, whereas cells responding from low frequency divided but failed to accumulate in the presence of CTLA-4 Ig/anti-CD154. These data are representative examples of four independent experiments, with two to three mice per group per experiment.

their mOVA skin grafts (MST >40 d; $P < 0.001$ when compared with all other groups), whereas mice receiving an equal number of T cells that had been primed under high frequency conditions in the presence of costimulation blockade rapidly and consistently rejected their grafts with an MST of 16 d (Fig. 6 B). Analysis of the peripheral blood of these mice 40 d after transfer indicated that this outcome was not due to a failure of the cells primed at low frequency to survive after

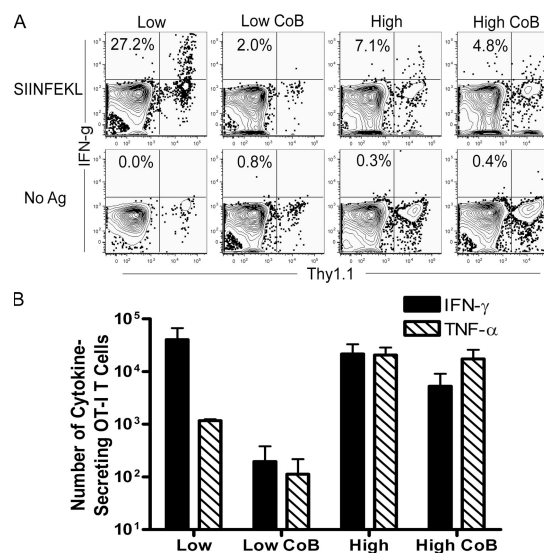


Figure 5. Reduced numbers of functional effector cells in CTLA-4 Ig⁻ and anti-CD154-treated mice with low precursor frequency of graft-specific cells. 10^6 or 10^7 OT-I T cells (along with 10^6 OT-II cells) were adoptively transferred into naive B6 recipients before transplantation with an mOVA skin graft. (A) IFN- γ production by cells primed at low versus high frequency in the presence or absence of CTLA-4 Ig/anti-CD154 was measured as the percentage of Thy1.1⁺ CD8⁺ T cells that stained positive for intracellular anti-IFN- γ after a 4-h in vitro peptide stimulation. (B) The absolute number of cytokine-producing effector cells that secreted IFN- γ or TNF- α was calculated by multiplying the percentage of cytokine-positive cells by the total number of OT-I T cells as determined by TruCount analysis. Results indicate that both the percentage (A and not depicted) and absolute numbers (B) of IFN- γ - and TNF- α -secreting cells were diminished in mice primed at low but not high frequency in the presence of CTLA-4 Ig/anti-CD154. These data are representative examples of four independent experiments, with two to three mice per group per experiment.

transfer, as comparable numbers of CD8⁺ Thy1.1⁺ cells were present in the two groups (Fig. 6 C). Because both cell types were present at equal frequencies during secondary antigenic challenge, these results suggest that the low initial precursor frequency and absence of costimulation during the priming phase of the immune response programmed the formation of functionally inactive effector cells that were incapable of rejecting a skin graft.

DISCUSSION

The use of tools to track the fate and function of antigen-specific T cells in experimental models of pathogen infection has supplied critical quantitative information that defined the working paradigm that, after initial encounter with antigen, T cells execute a programmed response that is imprinted during primary activation and includes expansion, death, and maintenance phases (13, 31, 35). Recent studies in other models have revealed how the initial precursor frequency can dictate the phenotypic and functional outcome of the T cell response to a pathogen (15, 23), and can influence cell survival and memory cell formation after immunization (16).

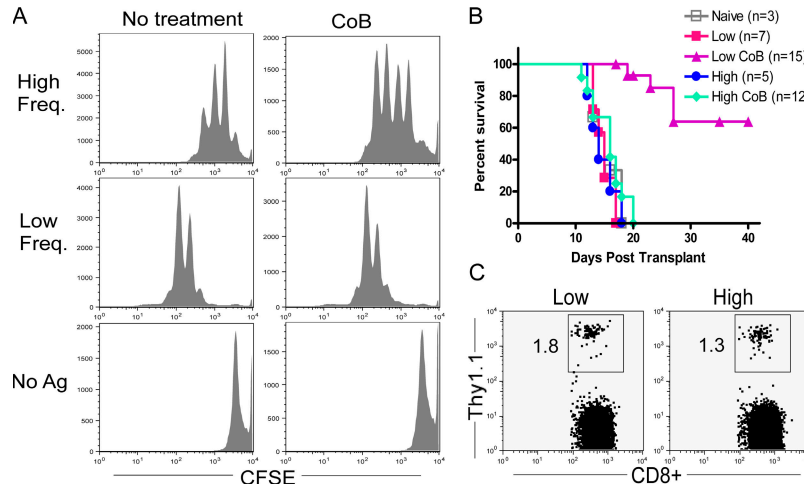


Figure 6. CD8⁺ T cells stimulated at initial high or low frequency retain the imprint of the conditions under which they were primed after secondary antigenic challenge. OT-1 T cells were CFSE labeled and stimulated with OVA peptide at high (3×10^6 cells/well) or low frequency (3×10^5 cells/well) in the presence of 100 $\mu\text{g/ml}$ CTLA-4 Ig/anti-CD154. B6 splenocytes were added to the low frequency cultures to keep the total number of cells per well consistent. (A) 3 d later, CFSE dilution of live CD8⁺ Thy1.1⁺ cells was measured by flow cytometry. Cells stimulated at low frequency underwent more rounds of division than those primed at high frequency, and CTLA-4 Ig/anti-CD154 did not inhibit cell division in

the 72-h culture period (data shown are representative examples from three independent experiments). (B) 5×10^6 T cells from cultures primed at an initial high or low antigen-specific T cell frequency were adoptively transferred into P14xRAG^{-/-} recipients. Mice received an mOVA skin graft 48 h later and were monitored for signs of rejection (data shown are cumulative results from three independent experiments, with the total number of mice indicated in the legend). (C) Analysis of peripheral blood of recipients of cells stimulated in vitro at low or high frequency at day 40 after transplantation revealed similar frequencies of CD8⁺ Thy1.1⁺ cells.

In contrast to the wealth of information on protective responses against pathogens, because of a lack of tools, relatively little is known about the programming of donor-specific T cells in response to an allograft. Although some pioneering work has been done with transgenic mice in transplant models (27, 36–38), to date there have not been systematic studies tracking the programmed response of both donor-specific CD4⁺ and CD8⁺ populations during rejection responses starting across the range of precursor frequencies that approximate the spectrum of frequencies that have been measured for allo-immune responses. Here, we demonstrate very profound differences in the T cell response to allografted tissue depending on the initial T cell frequencies. Specifically, we found that cells that were stimulated at low frequency exhibited higher and more rapid rates of replication (Fig. 1 A) and poor multi-functional cytokine production (Fig. 1 D). At the other end of the spectrum, the transfer of higher numbers of CD8⁺ (10^7) donor-specific T cells resulted in similar peak absolute numbers of CD8⁺ T cells after transplantation, but the cells had undergone fewer divisions on a per cell basis and showed a much higher frequency of CD8⁺ T cells with multi-cytokine production capacity (Fig. 1). From these findings, we conclude that initial antigen-specific T cell precursor frequency critically impacts the character of the primary immune response to allogeneic tissue transplantation. A recent seminal study by Marzo et al. (15) demonstrated that T cells primed at high frequency are more likely to differentiate into central memory T cells than those primed at low frequency, showing that precursor frequency can influence the character

of CD8⁺ T cell memory. Hataye et al. (16) found that clonal frequency influenced T cell survival and degree of memory cell formation. However, initial antigen-specific T cell frequency is just beginning to be appreciated as an important cell-extrinsic factor governing the antigen-specific T cell program. Here, we demonstrate for the first time that initial T cell precursor frequency critically impacts the functional character of the primary immune response as well.

We also addressed the effect of initial T cell frequency on the requirement for CD28/CD154-mediated costimulatory signals during immune activation. The results of our studies reveal a significant negative effect of CTLA-4 Ig and anti-CD154 on the proliferative burst size and effector function of cells stimulated at low frequency (Fig. 3). Analysis of absolute numbers of donor-reactive T cells that had undergone 0, 1, 2, etc. rounds of divisions revealed that CD28/CD154 blockade did not inhibit cells from entering cycle; rather, it resulted in abortive proliferation, failure of cells that had divided more than five times to accumulate as early as 7 d after transplantation (Fig. 4 B), and a dramatic decrease in the number of cytokine-producing effector cells (Fig. 5). The persistence of antigen-specific but functionally ineffective T cells is a phenotype reminiscent of the state of anergy originally described by Schwartz (39), but with some important differences. Although anergy is defined as diminished proliferation and IL-2 production after antigenic rechallenge with the relative sparing of effector cytokine secretion (IFN- γ /IL-4) (39), we observed abortive proliferation and a dramatic inhibition of IFN- γ and TNF- α secretion after in vitro rechallenge

of cells stimulated at low frequency in the presence of costimulation blockade. This phenotype may be more closely related to that observed in exhausted T cells in models of chronic viral infection (35). In contrast, our data reveal that at initial high frequency, costimulation blockade had a very limited impact on the magnitude of the response, CFSE division profile, and acquisition of effector function of graft-specific CD8⁺ T cells (Figs. 3 and 4). Based on these results, we conclude that high initial precursor frequency of antigen-specific CD8⁺ T cells can obviate the need for CD28/CD154-mediated costimulatory molecules and is a powerful determinant of the susceptibility or resistance to costimulation blockade-induced graft acceptance. Although it is commonplace for high frequencies of cells to be used in *in vivo* adoptive transfer experiments investigating the effects of costimulation on primary T cell responses to facilitate their identification and analysis (15, 40, 41), our findings suggest that experimental design should take into account the precursor frequency appropriate for the model being studied.

Although our study demonstrated that one of the critical factors impacting CD8⁺ T cell requirement for CD28- and CD40L-mediated costimulatory signals during primary immune activation is initial precursor frequency, the precise mechanism of this phenomenon remains to be determined. Our results demonstrate that a hallmark characteristic of costimulation-independent cells primed at high frequency is that they undergo fewer cell divisions after antigen encounter than those primed at lower cell frequency. We speculate that this increase is due to reduced competition for antigen or access to APCs among the antigen-specific T cells, as has been demonstrated for CD4⁺ T cells (42), and that reduced competition manifests as a stronger antigenic stimulus. A lack of costimulatory signals during T cell priming in cells that receive this stronger stimulus and thus are programmed to divide many times may be a catastrophic event. In this way, cells may calibrate the amount of costimulation they require against the amount they receive, and in the absence of sufficient costimulation, cells may fail to accumulate at later rounds of division and to differentiate into competent effector cells.

An alternate, but not mutually exclusive, possibility that may explain the relative costimulation independence of T cells stimulated at high frequency is that the number of T cells on a given APC may affect the outcome of the T cell response. It is possible that the low amounts of soluble factors such as IL-2 that are secreted even in the presence of CTLA-4 Ig/anti-CD154 are sufficient to provide external growth signals when produced by many cells in the same microenvironment. Alternatively, the presence of many T cells on a single APC may lead to increased or differential activation of the APC, such that it is now better able to stimulate the responding T cells. Such changes could include increased expression of costimulatory molecules other than CD80/CD86 and CD40, such that costimulation is provided by molecules other than the ones blocked in our study. Increased frequency of antigen-specific T cells within the LN environment could

also increase the likelihood of these cells interacting with nontraditional APCs that express costimulatory molecules other than CD80, CD86, and CD40.

The fact that the number of competent effector cells reaches a similar apex in mice that reject their grafts, regardless of initial precursor frequency, indicates that some critical threshold exists for the successful rejection of an allogeneic skin graft. Studies by Jones et al. (36) have provided additional evidence that T cell-mediated allograft rejection requires the generation of a critical threshold of differentiated effector T cells. Interestingly, this rejection threshold is specific to the allograft type, with larger numbers of effectors being required for the rejection of heart as compared with skin or islet allografts (36); therefore, differences in the initial precursor frequency of donor-specific CD8⁺ and/or CD4⁺ T cells may result in distinct effects on graft survival and the character and magnitude of the T cell program elicited by different allograft types. Certainly, the signals and feedback loops used by the immune system to determine the critical number of cells needed to reject a particular graft are unknown and represent new areas for study that may result in novel targets for therapeutic intervention.

The studies described here have important potential clinical implications. As new costimulation blockade-based modalities enter clinical trials in transplantation, it will be important to understand the circumstances that predict a greater likelihood of success or portend breakthrough rejection, such as high initial CD8⁺ T cell frequency. If initial precursor frequency also proves to have a powerful effect in human allo-specific T cell responses, this knowledge could be used to prompt consideration of alternative approaches to organ allocation, for example, reemphasizing class I matching as a means to effectively lower the graft-specific CD8⁺ T cell frequency. More likely, knowledge of low CD8⁺ donor-reactive precursor frequency in a particular patient could be used to direct the reduction of immunosuppression or prompt attempts at tolerance induction, whereas detection of high frequencies could direct the intensification of immunosuppression for the control of breakthrough CD8⁺ T cell responses. In addition, our studies predict that costimulatory blockade may synergize with agents designed to reduce the initial frequency of all T cells, such as anti-CD52 (43, 44) or anti-CD3 mAbs (45), in that these agents would effectively lower the initial frequency of graft-specific T cells and thereby increase the efficacy of treatment with costimulatory blockade.

MATERIALS AND METHODS

Mice. 6–8-wk-old adult male C57BL/6 mice were purchased from The Jackson Laboratory. TCR transgenic OT-I and OT-II mice were purchased from Taconic, Inc. and bred onto RAG^{-/-} and Thy1.1⁺ backgrounds. Act-mOVA mice were provided by M. Jenkins (University of Minnesota, Minneapolis, MN). Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

Skin grafting and costimulation blockade. Full thickness dorsal ear and tail skin grafts (~1 cm²) were transplanted onto the dorsal thorax of recipient

mice and secured with a plastic adhesive bandage for 5 d. Graft survival was monitored by daily visual inspection. Rejection was defined as the complete loss of viable epidermal tissue. Where indicated, recipients of skin grafts received treatment with 500 μg each of hamster anti-mouse CD154 mAb (MR-1; Bioexpress) and human CTLA-4 Ig (Bristol-Meyers Squibb) administered i.p. on the day of transplantation (day 0) as well as after transplant on days 2, 4, and 6.

T cell adoptive transfers. OT-I and OT-II TCR transgenic T cells were harvested from the spleens of OT-IxThy1.1⁺xRAG^{-/-} and OT-IIxThy1.1⁺xRAG^{-/-} mice, respectively. Single cell suspensions were prepared and counted, and the frequency of OT-I or OT-II T cells within the splenocyte preparation was determined before adoptive transfer by staining with anti-V α 2 (used by both TCRs) and anti-CD8 or anti-CD4, respectively (BD Biosciences). Cells were labeled with 5 μM CFSE before adoptive transfer. Mice received a single i.v. injection of the indicated number of OT-I or OT-II T cells along with syngeneic B6 carrier splenocytes.

Flow cytometric analyses for frequency and absolute number. At the indicated time points, recipients of OT-I and/or OT-II T cell transfers were killed, spleens and draining axillary LNs were harvested, and single cell suspensions were prepared. Cells were stained with Thy1.1-PerCP, CD8-PacOrange, and CD4-PacBlue (all BD Biosciences) for flow cytometric analysis on a BD LSRII multicolor flow cytometer. In some cases, the absolute number of antigen-specific T cells was determined by TruCount Bead Analysis (BD Biosciences) according to the manufacturer's instructions. Flow cytometric data were analyzed using FlowJo Software (Treestar).

Intracellular cytokine staining. For measurement of IFN- γ and TNF- α -secreting cells, single cell suspensions of splenocytes or draining axillary LN cells from adoptive transfer/skin graft recipients (10^6 per well) were incubated in a 96-well plate with 10 μM OVA257–264 (SIINFEKL; Emory University Microchemical Core Facility) and 10 $\mu\text{g}/\text{ml}$ brefeldin A (BD Biosciences). After 6 h in culture, cells were processed using an intracellular staining kit (BD Biosciences) according to the manufacturer's instructions and stained with anti-TNF- α -PE, anti-IFN- γ -APC, anti-Thy1.1-PerCP, anti-CD8-Pacific Orange, and anti-CD4-Pacific Blue (all from BD Biosciences).

In vitro T cell stimulation, separation, and adoptive transfer. OT-I T cells were stimulated with OVA peptide at high (3×10^6 cells/well) or low frequency (3×10^5 cells/well) in a 24-well plate in the presence of 100 $\mu\text{g}/\text{ml}$ CTLA-4 Ig and anti-CD154. B6 splenocytes were added to the low frequency cultures to keep the total number of cells per well consistent. Culture media consisted of RPMI 1640 supplemented with 10% FBS (Mediatech), 2 mM L-glutamine, 0.01 M Hepes buffer, 100 $\mu\text{g}/\text{ml}$ gentamycin (Mediatech), and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich). 3 d later, live cells were isolated by Ficol-Hypaque centrifugation (Mediatech) and 5×10^6 T cells from cultures primed at an initial high or low antigen-specific T cell frequency were adoptively transferred into P14xRAG^{-/-} recipients as described above.

Statistical analyses. Skin graft rejection is represented by Kaplan-Meier survival curves. Statistical comparisons of mean survival times were performed using nonparametric one-way ANOVA (Kruskal-Wallis test), followed by Dunn's multiple comparisons posttest comparing the relevant datasets (GraphPad Prism Software).

Online supplemental material. Fig. S1 shows donor skin graft tissue that was harvested on day 14 after transplant, and 100% acetone-fixed tissue was frozen in OCT compound (Tissue-Tek) to test for the presence of Thy1.1⁺ OT-I/OT-II T cell infiltrates. Frozen tissue sections were counterstained with hematoxylin, labeled with biotinylated anti-Thy1.1, and visualized using the Dako LSAB + labeled streptavidin-HRP kit. Tissue sections from three mice per group (representative samples are shown) revealed

the presence of Thy1.1⁺ cells in both groups receiving no treatment (both left panels) and the mice receiving a high frequency of OT-I T cells with CTLA-4 Ig/anti-CD154 (bottom right). No Thy1.1⁺ cells were detectable in the tissue of mice receiving a low frequency of OT-I T cells and CTLA-4 Ig/anti-CD154 (top right). Fig. S1 is available at <http://www.jem.org/cgi/content/full/jem.20062319/DC1>.

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