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Original Article

The immune response is a prerequisite for the development of CD4$^+$Foxp3$^+$ regulatory T cells in transplantation

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Abstract: CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) are critical in maintaining the peripheral tolerance and homeostasis of the immune system, yet their development and role in transplantation are poorly understood. Here we show that the levels of Tregs in neonatal transplant tolerant mice are similar to the levels in naive mice when they are kept in a state of homeostasis devoid of an immune response. An increased frequency of Tregs was observed only in recipients with allograft rejection, in naive mice that received alloantigens, or in tolerant mice adoptively transferred with alloreactive T cells. Even though an antigen-specific immune response is a prerequisite for the development of Tregs, both antigen-specific and nonspecific Tregs are generated in this process. We conclude that Tregs are induced and function in an inflammatory environment and in a negative feedback loop.

Keywords: CD4$^+$Foxp3$^+$ T cell, transplant tolerance, immune response, alloreactive T cell, allograft rejection

Introduction

Transplant rejection is still a major obstacle for the long-term survival of allografts [1]. Despite the fact that many immunosuppressants have been developed that efficiently prolong graft survival, the causes of chronic graft rejection remain an open question. It is well established that regulatory T cells, especially CD4$^+$ CD25$^+$Foxp3$^+$ T cells, are essential for the maintenance of peripheral tolerance. The transcription factor forkhead box P3 (Foxp3) is necessary for their development and function [2]. The CD4$^+$Foxp3$^+$ T cells (Tregs) have a potent suppressive activity that prevents allograft damage by regulating the development and proliferation of alloreactive T cells [3]. Because Tregs play essential roles in maintaining transplant tolerance, they are usually the target of rejection treatment [4-6]. Indeed, regulatory T-cell therapy is an emerging strategy for the prevention of allograft rejection by promoting antigen-specific tolerance [7]. Despite this, and the fact that there have been several studies on in vitro expanded Tregs, little is known about their induction in vivo in transplant recipients [8], limiting their clinical application.

In the present work, we explore the induction of Tregs in transplantation using naive and neonatal transplant tolerant mice in adoptive transfer experiments. Our results suggest that both antigen specific and nonspecific Tregs generated in the allograft immune response may generate feedback to control the strength of the immune response and maintain peripheral tolerance.

Materials and methods

Mice

C57BL/6, C3H, BALB/c and EGFP transgenic C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and housed in a specific pathogen free facility with free access to normal chow and tap water in the Department of Animal Research. Male C57BL/6 and female

BALB/c mice were bred to generate F1 mice in our animal laboratory. OVA (323–339) peptide-specific TCR-transgenic (OT-II) mice were obtained from Jackson laboratory, and the mice were crossed with EGFP transgenic C57BL/6 mice to obtain EGFP-OT-II mice. All mouse experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee, and the protocols were approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

Skin transplantation

Full-thickness trunk skin grafts from donor mice (Female F1 mice) were transplanted onto the dorsal flanks of the recipients. The skin grafts were secured with Vaseline-impregnated gauze and bandages for the initial 7 days. After the bandages were removed, the survival periods of the skin grafts were monitored and necrosis of the entire graft was defined as rejection.

Induction of neonatal transplant tolerance

Newborn C57BL/6 mice (< 24 h) were infused with 3×10^7 splenocytes (50 μL) from F1 mice via the orbital branch of the anterior facial vein. Six weeks later, the neonatal tolerant mice were implanted with skin grafts from F1 mice. The mice with the F1 skin grafts for > 60 days were regarded as having long-term tolerance.

Mixed-lymphocyte reaction

$CD4^+CD25^-$ cells were purified from naive and neonatal tolerant mice using the $CD4^+CD25^+$ regulatory T cell isolation kit, according to the manufacturer's instructions (Miltenyi Biotec). A preparation of $CD4^+CD25^-$ cells with a purity of > 95% was used for the subsequent experiments. The purified $CD4^+CD25^-$ T cells (1.0×10^5) were co-cultured with irradiated (3000 rad) splenocytes from the F1 mice at a ratio of 1:1 in round-bottom 96-well plates in RPMI-1640, supplemented with 10% FCS, HEPES (25 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM) for three days. During the last 16 h, the cells in the individual wells were exposed to 1 μCi $[^3H]$ thymidine and the $[^3H]$ thymidine incorporation was quantified in a scintillation counter.

Flow cytometry

To monitor the chimerism, peripheral blood mononuclear cells (PBMCs) were isolated from neonatal tolerant C57BL/6 mice by Percoll density gradient centrifugation. PBMCs were stained with PE-anti-mouse $H\text{-}2^d$ (clone, SF1-1.1, BD Biosciences), and the percentages of $H\text{-}2^{d+}$ PBMCs were examined by flow cytometry to determine the degrees of chimerism in the recipients. For $CD4^+Foxp3^+$ T cell analysis, splenocytes were stained with PE-anti-mouse CD4 antibody (eBiosciences) for 30 min on ice, fixed and permeabilized, followed by intracellularly staining with eFl660-anti-mouse Foxp3 antibody. The cells were analyzed by flow cytometry on a FACSCanto II instrument (BD Biosciences).

Adoptive transfer experiments

Tolerant mice (skin graft survival for > 60 days) were randomized and injected intravenously with 5×10^7 splenocytes from naive, tolerant C57BL/6 mice and F1 mice, respectively, to detect changes in the $CD4^+Foxp3^+$ T cells in the immune response and tolerance. Spleen cells from naive EGFP-C57BL/6 mice were adoptively transferred into the naive, tolerant C57BL/6 mice and F1 mice, respectively, to determine whether non-alloreactive $CD4^+Foxp3^+$ T cells were also induced in the allograft immune response.

OVA-induced $CD4^+Foxp3^+$ T cell expansion

Splenocytes (8×10^7 cells per mouse) from EGFP-OT-II mice were adoptively transferred into C57BL/6 mice. Two days later, the mice were intraperitoneally (i.p.) injected with OVA peptide (200 μg OVA in 500 μL sterile phosphate-buffered saline). The Foxp3 expression within OT-II and host $CD4^+$ T cells was analyzed 7 days later after the cell transfer.

Statistical analysis

All data were expressed as the mean ± SD. The differences between the groups were evaluated using a one-way analysis of variance (ANOVA), followed by Student's t test. A *P*-value of < 0.05 was considered statistically significant.

Results

Alloreactive T cells and $CD4^+Foxp3^+$ T cells in naive and neonatal tolerant mice

Newborn C57BL/6 mice were infused with 3.0×10^7 splenocytes from F1 mice. At 6 weeks of age, the mice were grafted with a skin graft

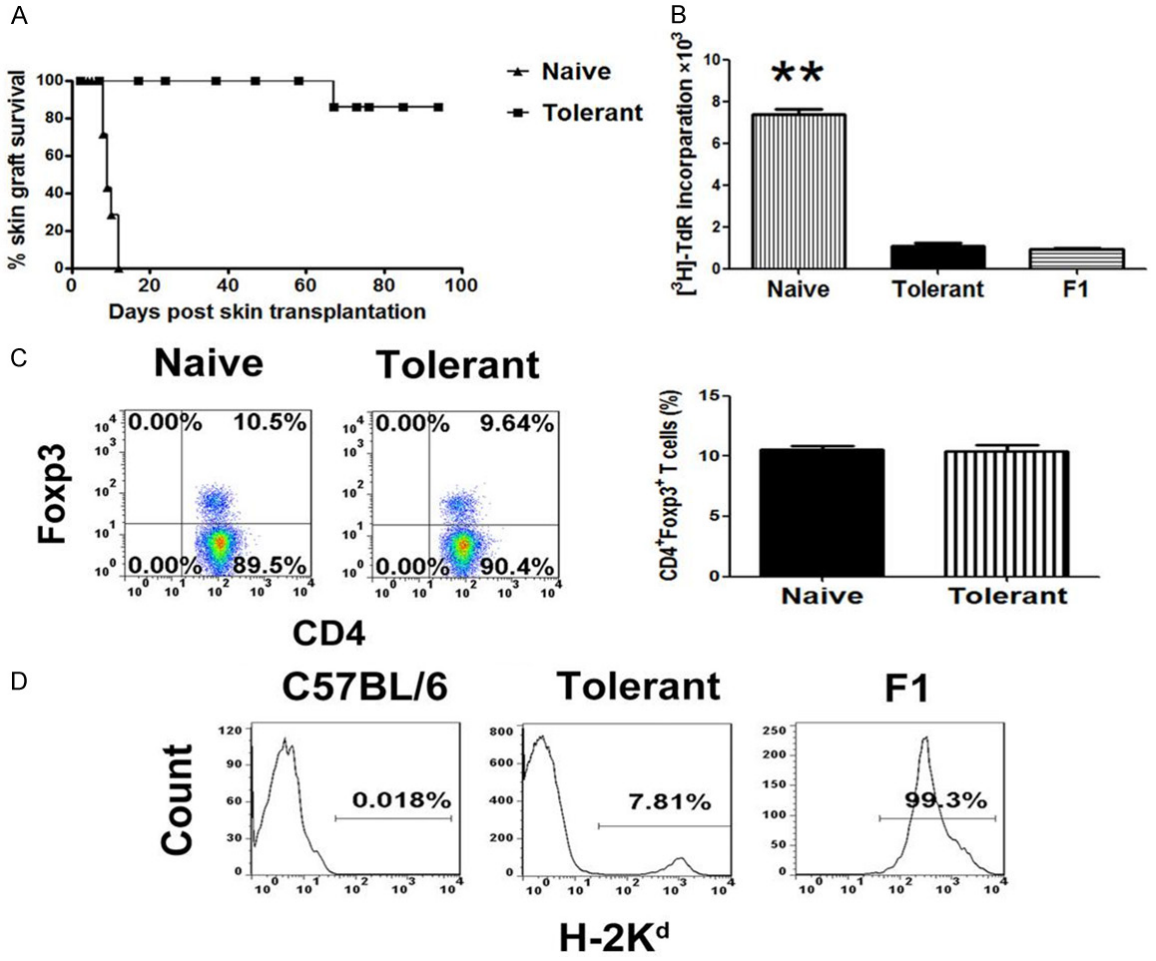

Figure 1. Alloreactive T cells and $CD4^+Foxp3^+$ T cells in neonatal tolerant and naive mice. A. Skin graft survival. Neonatal tolerant C57BL/6 mice at six weeks of age were transplanted with F1 skin grafts. Naive C57BL/6 mice served as the control (n = 6 per group). B. Alloreactive T cells in neonatal tolerant mice. Splenic $CD4^+CD25^-$ cells sorted from neonatal tolerant mice were co-cultured with irradiated F1 splenocytes at a ratio of 1:1. Cells from naive C57BL/6 (Naive) and F1 mice served as the positive and negative controls, respectively. Values are expressed as the mean ± SD from three individual experiments. **, $P < 0.01$ *vs*. the F1 group. C. The frequency of $CD4^+Foxp3^+$ T cells in tolerant and naive mice. D. Chimerism in neonatal tolerant mice.

from F1 mice. About 80% of the mice with the F1 skin grafts survived throughout the observation period (tolerant) (**Figure 1A**). Naive mice rejected the F1 skin grafts within 12 days with a MST of 8.4 ± 1.3 days. To determine the frequency of alloreactive T cells in tolerant mice, purified $CD4^+CD25^-$ T cells were co-cultured with irradiated F1 splenocytes. Cells sorted from naive and F1 mice were used as positive and negative controls, respectively. $CD4^+CD25^-$ T cells isolated from naive mice proliferated efficiently upon F1 antigen stimulation, while cells from tolerant mice did not show an increased response compared with cells from the F1 mice, indicating that the alloreactive T cells were almost completely depleted in the tolerant mice (**Figure 1B**).

To determine the role of regulatory T cells in neonatal transplant tolerance, we characterized the frequencies of $CD4^+Foxp3^+$ T cells in naive and neonatal tolerant mice prior to F1 skin grafting. No significant difference was detected in the frequency of splenic $CD4^+Foxp3^+$ T cells between naive and tolerant mice (11.0 ± 1.1% and 10.6 ± 1.1%, respectively) (**Figure 1C**). Also, the tolerance could not be transferred to naive mice when they were adoptively transferred with lymphocytes from tolerant mice, indicating $CD4^+Foxp3^+$ T cells in the tolerant mice did not show an increased suppressive activity. Chimerism was confirmed in the tolerant mice whose PBMCs had 6.3 ± 2.7% of donor $H\text{-}2^{d+}$ cells (**Figure 1D**), consistent with previous reports [9, 10].

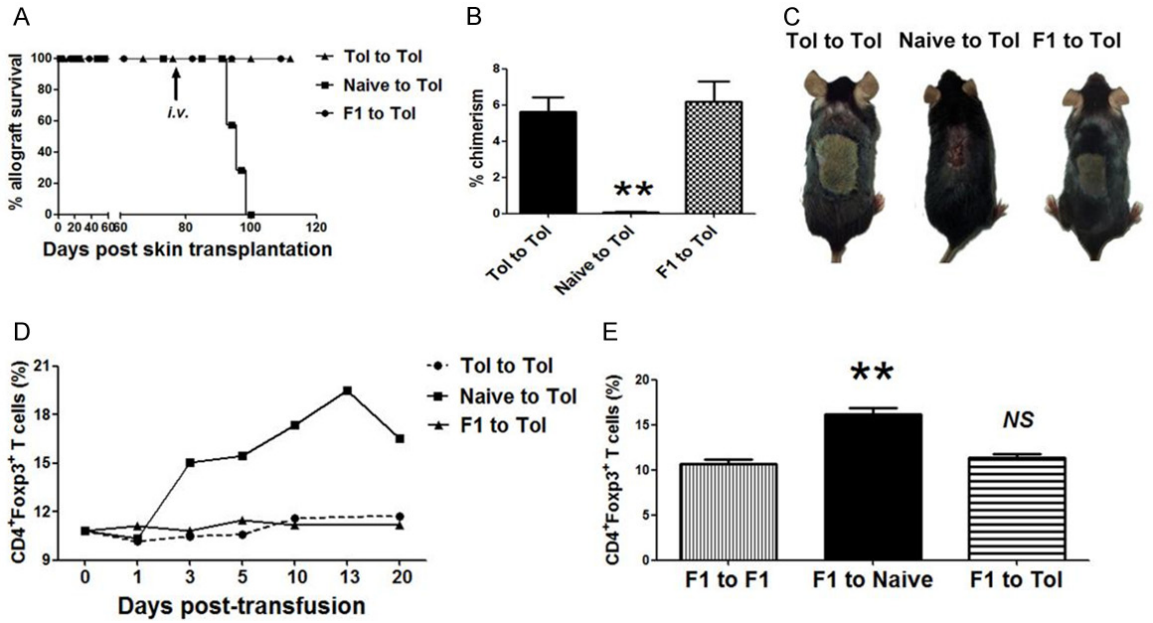

Figure 2. Induction of $CD4^+Foxp3^+$ T cells in allograft immune response. A. Skin graft survival in long-term tolerant mice after infusion with splenocytes from naive EGFP-C57BL/6, F1 or long-term tolerance mice (Tol). B. Chimerism loss induced by transferred alloreactive T cells. PBMCs were stained with PE-anti-H-$2K^d$ mAb to detect F1 cells (H-$2^{d/b}$) at day 20 post infusion. C. Images of skin graft rejection and tolerance (day 20). D. Variation of splenic $CD4^+Foxp3^+$ T cells in recipients after infusion with splenocytes (n = 5 per group). E. Naive and tolerant C57BL/6 mice were infused with 5×10^7 F1 splenocytes. F1 mice served as a negative control. Ten days later, the percentages of splenic $CD4^+Foxp3^+$ T cells were examined by flow cytometry. Data are expressed as the mean ± SD of each group (n = 4 - 5 per group) from three separate experiments. NS, no significance. **, $P < 0.01$ *vs*. the F1 mice.

Allograft immune response is prerequisite for $CD4^+Foxp3^+$ T cell expansion

The tolerant mice were adoptively transferred with splenocytes from naive EGFP-C57BL/6 mice, from tolerant or F1 mice. Infusion with cells from naive mice destroyed the chimerism and tolerized the skin grafts within 20 days (**Figure 2A-C**). Along with the allograft rejection, the proportions of $CD4^+Foxp3^+$ T cells increased significantly in the recipients (**Figure 2D**). Infusions with cells from tolerant C57BL/6 or F1 mice did not induce an immune response or an increase in $CD4^+Foxp3^+$ T cells in the tolerant recipients.

Further, F1 splenocytes were adoptively transferred to naive, tolerant C57BL/6 mice or F1 mice, respectively. The F1 cell infusion elicited an anti-donor immune response and triggered an increase in the frequency of splenic $CD4^+Foxp3^+$ T cells in the naive recipients (16.1 ± 1.5%, $P < 0.01$), but not in the tolerant recipients (11.4 ± 0.6%, $P = 0.68$), compared with 10.8 ± 1.0% in the F1 recipients (**Figure 2E**).

Antigen specific and nonspecific $CD4^+Foxp3^+$ T cells generated in the immune response

We determined Foxp3 expression within alloreactive and non-alloreactive $CD4^+$ T cells in the allograft immune response. Splenocytes from naive EGFP-C57BL/6 mice (H-2^b) were adoptively transferred into the neonatal tolerant mice. F1 (H-$2^{b/d}$) and naive C57BL/6 mice receiving the same numbers of splenocytes served as the positive and negative controls, respectively. Eight days later, increased frequencies of $CD4^+Foxp3^+$ T cells were detected in the transferred naive-($EGFP^+$, alloreactive) and host-derived ($EGFP^-$, non-alloreactive) $CD4^+$ T cells in the tolerant recipients compared with the negative control (**Figure 3**). An expansion of $CD4^+Foxp3^+$ T cells was also identified in both the transferred naive C57BL/6-derived alloreactive and the host-derived non-alloreactive $CD4^+$ T cells in the F1 recipients with graft-versus-host disease (GVHD) (**Figure 3**).

Further, the C57BL/6 mice were reconstituted with splenocytes from the EGFP-OT-II mice and

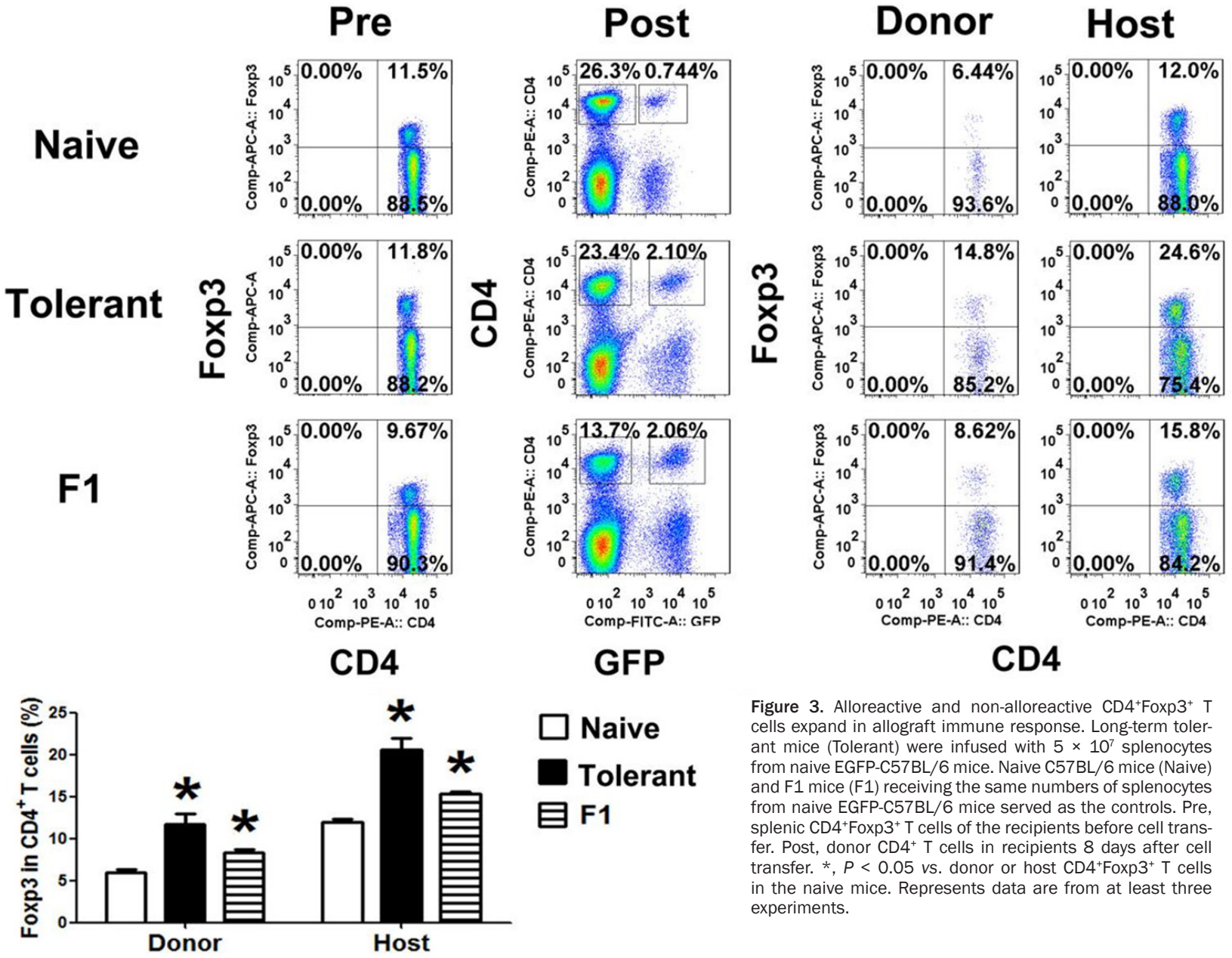

Figure 3. Alloreactive and non-alloreactive $CD4^+Foxp3^+$ T cells expand in allograft immune response. Long-term tolerant mice (Tolerant) were infused with 5×10^7 splenocytes from naive EGFP-C57BL/6 mice. Naive C57BL/6 mice (Naive) and F1 mice (F1) receiving the same numbers of splenocytes from naive EGFP-C57BL/6 mice served as the controls. Pre, splenic $CD4^+Foxp3^+$ T cells of the recipients before cell transfer. Post, donor $CD4^+$ T cells in recipients 8 days after cell transfer. *, $P < 0.05$ *vs*. donor or host $CD4^+Foxp3^+$ T cells in the naive mice. Represents data are from at least three experiments.

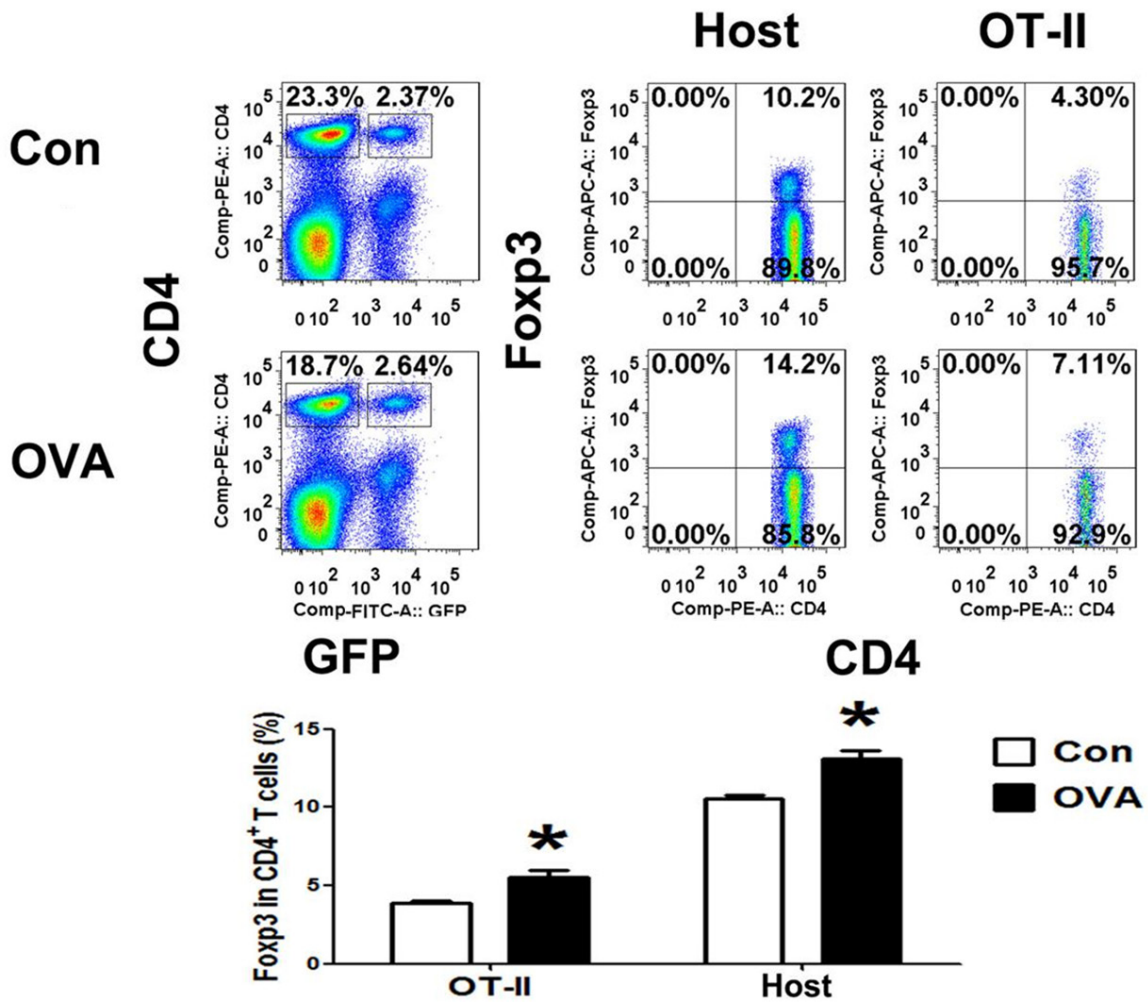

Figure 4. Expansion of OT-II and non-OT-II CD4$^+$Foxp3$^+$ T cells after OVA stimulation. C57BL/6 mice receiving splenocytes from EGFP-OT-II mice were challenged with OVA peptide by i.p. administration. Untreated mice served as the control (Con). Splenic CD4$^+$Foxp3$^+$ cells were examined 7 days after cell transfer (n = 5 per group). *, $P < 0.05$ *vs*. the Con group.

challenged with an OVA peptide two days after the cell transfer, with the unchallenged mice serving as a control. The OVA stimulation promoted an increase in the levels of Foxp3 expression in both the OT-II and host-derived CD4$^+$ T cells (**Figure 4**), indicating that both the antigen specific and nonspecific CD4$^+$Foxp3$^+$ T cells generated in an immune response.

Discussion

Studies have indicated that Tregs play an important role in maintaining transplant tolerance and may be exploited for the therapeutic induction of operational tolerance [11, 12]. However, because we lack sufficient information about the role and induction of Tregs in transplantation, their clinical application is limited. In the present study, neonatal infusion with a high dose of donor cells induced a stable chimerism and the deletion of most alloreactive T cells. Tregs were maintained in the recipients at the same level as in naive mice, when they were under immune homeostasis without an overall immune response. The allograft immune response is a prerequisite for the development of Tregs, and more importantly, both antigen specific and nonspecific Tregs are generated in the process. Tregs may be induced and function in the allograft immune response in a negative feedback loop.

It was previously reported that Tregs developed during a spontaneous allograft acceptance,

after an acute graft rejection or antigen stimulation [13]. However, the conditions required for the development of Tregs are not well understood. The present study clearly shows Tregs can only be induced in the immune response to alloantigens or other stimuli. Alloreactive T cells in neonatally tolerant mice, just like autoreactive T cells in naive mice, have been clonally deleted in the thymus; therefore, the transplant tolerance is maintained without any increased suppression by Tregs. It has been reported that prolonged allograft acceptance is accompanied by increased numbers of $CD4^+Foxp3^+$ T cells, and a depletion of the regulatory T cells often abrogates the state of tolerance [14]. To explain this phenomenon, we propose that lower numbers of alloreactive T cells still remain in the recipients and will therefore mount an immune response to allografts, and Tregs are generated in the process, which feedback control the strength of the immune response, making the strength too low to efficiently eliminate the grafts. Other evidence supporting the thesis that regulatory T cells are triggered by an immune response includes that fact that higher levels of regulatory T cells were only observed in inflammation sites, such as in the central nervous system during experimental autoimmune encephalomyelitis [15, 16], despite their failing to control autoimmune inflammation, probably due to the increased effector ratio: regulatory T cells in situ. Also, the fact that inflammatory cytokines produced by active immune cells, such as IL-2, TGF-β and IFN-γ, are required for the development of Tregs [7, 17], provides further evidence for the finding that Tregs are generated in immune responses in a feedback loop.

Tregs are a critical subset of T cells that act to suppress the activation of autoreactive T cells that have escaped the thymus and thereby maintain a peripheral tolerance [18]. Tregs developed in graft rejections may function by suppressing the expansion and activation of alloreactive T cells in a negative feedback control system, and their tolerance is maintained when the number of alloreactive T cells in the periphery is low enough, a mechanism consistent with Tregs-mediated self-tolerance. This view is supported by the evidence that long-term graft acceptance was observed in some conditioned recipients, in which a minor population of alloreactive T cells still remained and depletion of Tregs often destroyed the tolerance [9, 19].

Despite the fact that both antigen-specific and nonspecific Tregs were generated in immune responses, the increase of antigen-specific Tregs may be specifically evident for antigen specific clonal expansion. In light of this, it is easy to understand the fact that the longevity of graft tolerance is dependent on constant immune suppression by Tregs triggered by a constant supply of graft antigens [4-6], and alloantigen specific Tregs displayed a significantly higher efficacy than polyclonal Tregs in preventing allograft rejection and graft versus the host disease [20], for Tregs only inhibit T cells bearing TCRs for the same antigen [21]. Besides antigen specific Tregs, antigen-nonspecific Tregs were also generated in immune responses, which makes it possible that Tregs-mediated tolerance can pass on from donor antigen to third party antigen. This view is supported by the evidence that third-party regulatory T cells prevent murine acute graft-versus-host disease [22].

In summary, our data provide evidence that an immune response is a prerequisite for the expansion of Tregs in transplantation, and both antigen specific and nonspecific Tregs are generated in the process.

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Disclosure of conflict of interest

None.

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