

Mast cells are essential intermediaries in regulatory T-cell tolerance

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Contrary to the proinflammatory role of mast cells in allergic disorders, the results obtained in this study establish that mast cells are essential in CD4⁺CD25⁺Foxp3⁺ regulatory T (T_{Reg})-cell-dependent peripheral tolerance. Here we confirm that tolerant allografts, which are sustained owing to the immunosuppressive effects of T_{Reg} cells, acquire a unique genetic signature dominated by the expression of mast-cell-gene products. We also show that mast cells are crucial for allograft tolerance, through the inability to induce tolerance in mast-cell-deficient mice. High levels of interleukin (IL)-9—a mast cell growth and activation factor—are produced by activated T_{Reg} cells, and IL-9 production seems important in mast cell recruitment to, and activation in, tolerant tissue. Our data indicate that IL-9 represents the functional link through which activated T_{Reg} cells recruit and activate mast cells to mediate regional immune suppression, because neutralization of IL-9 greatly accelerates allograft rejection in tolerant mice. Finally, immunohistochemical analysis clearly demonstrates the existence of this novel T_{Reg}-IL-9-mast cell relationship within tolerant allografts.

The establishment of immunological tolerance requires both the induction of clonal deletion/anergy and active immune suppression. Immune suppression has been shown to be mediated by unique subsets of T cells called regulatory T cells (T_{Reg})¹. In general, there are two classes of regulatory T cells that impact on peripheral immunity. Naturally arising CD4⁺CD25⁺Foxp3⁺ T_{Reg} cells (nT_{Reg}) are derived from the thymus and have been extensively studied for their roles in autoimmunity and tolerance¹. In contrast, another population of T_{Reg} cells develops from the activation and differentiation of mature CD4⁺CD25⁻ T cells in the periphery; these T_{Reg} cells are called adaptive or induced T_{Reg} cells (iT_{Reg}). In multiple systems of allograft tolerance, the importance of these T_{Reg} cell populations to long-lived allograft survival has been shown by the fact that depletion of T_{Reg} cells before, and even after, the induction of transplantation tolerance results in rapid graft rejection^{2,3}. However, despite the increasing body of knowledge about T_{Reg} cells, the mechanisms by which these cells mediate immune suppression and prevent graft rejection are not well resolved.

Mast cells in tolerant allografts

Mast cells are derived from haematopoietic stem cells, which migrate into vascularized tissues and serosal cavities where they complete their maturation⁴. They are best known as primary responders in allergic reactions such as anaphylaxis and asthma. However, recent studies have shown that mast cells are heterogeneous, can produce an array of both pro- and anti-inflammatory mediators, can act as antigen-presenting cells and express a spectrum of co-stimulatory molecules. These findings indicate that mast cells are far more functionally diverse than previously imagined and can function as immunoregulatory cells that influence both innate and adaptive immunity^{4–6}. Previously, extensive serial analysis of gene expression (SAGE) in tolerant tissue showed that genes predominantly

expressed by mast cells were overexpressed in cultures of activated T_{Reg} cells and in tolerant allografts⁷. These unexpected findings linking mast cells to tolerance prompted our investigation of the potential functional role of mast cells in the establishment of T_{Reg}-cell-mediated allograft tolerance.

To confirm earlier results, the presence of mast cells and their gene products in tolerant allografts in a skin transplantation model was examined. In summary, mice were rendered tolerant to alloantigens by the intravenous infusion of allogeneic cells (a process known as donor-specific transfusion; DST) and concomitant administration of an antibody to CD154 (anti-CD154; ref. 2). This approach allowed for the long-term acceptance of allogeneic skin grafts compared with the non-tolerant control group, which rejected grafts approximately two weeks after grafting. To compare mast-cell-associated gene expression during graft rejection or tolerance, mice received a second graft (30 days after the first grafting), which was harvested seven days later⁷ (Fig. 1a). We then performed quantitative analysis of messenger RNAs in the infiltrating cells extracted from skin transplants. As expected, *Foxp3* and *Il10* expression was highly upregulated in the tolerant group. This indicated the presence of T_{Reg} cells that were producing immunosuppressive mediators in tolerant tissue (Fig. 1b)^{8,9}. In contrast, granzyme B and perforin expression in the rejecting group was much higher than in either the syngeneic or tolerant groups, as shown in other allograft models (Supplementary Fig. 1)¹⁰. Notably, in agreement with previous results⁷, all mast-cell-associated genes examined, including mast cell protease 1 (*Mcpt1*), mast cell protease 5 (*Mcpt5*), tryptophan hydroxylase (*Tph1*) and the high-affinity IgE receptor (*Fcer1a*), were upregulated in the tolerant group compared with the syngeneic and rejecting groups (Fig. 1c).

The mast cell density in syngeneic, rejecting and tolerant grafts was quantified to determine if the increase in mast-cell-gene expression

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was due to increased mast cell infiltration in tolerant grafts. Immunohistochemical analysis of rejecting primary grafts at day 7 revealed an apparent increase in infiltrating CD4⁺ T cells relative to that seen in syngeneic or tolerant grafts. In addition, CD117⁺ mast cells that were present in syngeneic grafts were noticeably absent from rejecting grafts at day 7 (Fig. 2a; Table 1). Previously, it was shown that skin mast cells can migrate to the regional lymph node following antigen challenge to the skin¹¹. Therefore, the lack of mast cells in the rejecting allografts could be the result of mast cell migration. However, there was no significant increase in mast cell numbers in the draining lymph nodes in the rejecting group when compared to the syngeneic or tolerant groups (Supplementary Table 1). Hence, the loss of mast cells in rejecting grafts was unlikely to be due to the inflammation-induced migration of mast cells to the regional lymph node, but rather the direct cytotoxic elimination of mast cells by the host. In contrast to rejecting grafts, in established tolerant grafts (day 60) there was massive T_{Reg} (CD4⁺Foxp3⁺)-cell infiltration⁸ (Supplementary Table 2) as well as sustained, or increased, mast cell (CD117⁺) density (Fig. 2b; Table 1). The increased presence of mast

cells in tolerant versus rejecting grafts was consistent with the previous SAGE result, which was indicative of increased mast-cell-gene expression, and the reverse transcription–polymerase chain reaction (RT–PCR) data reported herein. Collectively, these data indicate that both mast cells and T_{Reg} cells increase in number in tolerant allografts and may be crucial in sustaining allograft survival.

No long-term allograft tolerance in mast-cell-deficient mice

Mast cells were functionally implicated in T_{Reg}-cell-mediated allograft survival through a series of studies in mast-cell-deficient mice (C57BL/6 *Kit*^{W^{sh}/sh}; *Kit*^{W^{sh}/sh} (W^{sh}) mice). W^{sh} mice have an inversion mutation in the transcriptional regulatory elements upstream of the *c-Kit* open reading frame, which influences *c-Kit* gene expression in a tissue- and age-specific manner^{4,12}. Mast cell numbers in W^{sh} mice decrease exponentially after birth owing to their developmental dependence on *c-Kit*^{13,14}, whereas the frequency of other haematopoietic and lymphoid cell populations remains relatively normal. To assess the role of mast cells in allograft survival, W^{sh} mice were administered anti-CD154 and DST to induce allospecific tolerance (Fig. 3a) and grafted with allogeneic skin. Co-administration of anti-CD154 and DST induced long-term acceptance of skin allografts in wild-type mice (median survival time (MST) >70 days), but not in the W^{sh} mice (MST = 17 days; *P* < 0.0001; log-rank test; Fig. 3b). In fact, anti-CD154/DST-treated W^{sh} mice rejected allografts at a rapid pace that was almost indistinguishable from the untreated control mice (MST = 13 days). Hence, mast cells were essential for anti-CD154/DST-induced tolerance. One of the control groups (syngeneic C57BL/6 skin transplant onto W^{sh} mice) demonstrated delayed rejection (MST = 45 days) with long-term survival of 40% of the grafts (Fig. 2b). This delayed rejection could be due to differences in minor histocompatibility molecules in addition to the impaired development of melanocytes in the W^{sh} mice¹³. W^{sh} mice express *c-Kit* in melanocytes at an early age, which should allow the central tolerance to become established; however, the lack of normal melanocyte development in the adult may allow for this minor, delayed rejection response.

To examine further the role of mast cells in transplantation tolerance, mast-cell-knockin mice were prepared⁴. Previously, it has been shown that mast cells can be reconstituted both systemically and/or regionally in mast-cell-deficient mice by adoptive transfer of bone-marrow-derived mast cells (BMMCs) generated *in vitro*^{4,14}. BMMCs were harvested five weeks after the initiation of culture (Supplementary Fig. 2a), and a total of 5 × 10⁶ cells (>99% c-Kit⁺FcεRIα⁺) were injected intradermally into the back skin of W^{sh} mice. Eight weeks after intradermal reconstitution, mice showed comparable mast cell frequency in back skin to that observed in wild-type C57BL/6 mice, as reported previously¹⁴ (Supplementary Fig. 2b, c). BMMC-reconstituted W^{sh} mice were then grafted with skin transplants after anti-CD154/DST co-administration and the acceptance of allografts was monitored over time (Fig. 3c). As shown in Fig. 3d, local reconstitution of mast cells in the back skin was able to extend graft survival on W^{sh} mice (MST = 44 days) that were initially unable to sustain allografts following anti-CD154 and DST treatment (MST = 20 days; *P* = 0.0052). Although the rapid

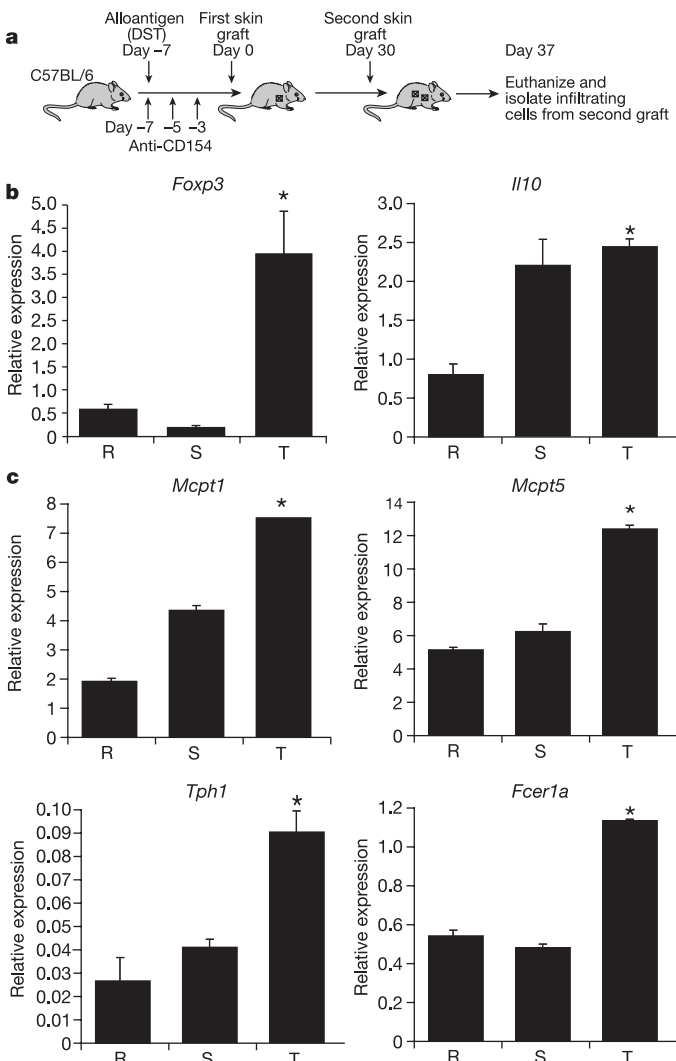


Figure 1 | Mast-cell-related gene expression in tolerant allografts.

a, Allograft tolerance was induced by anti-CD154 and DST. Thirty days after the first skin graft, mice received a second graft, which was harvested 7 days later. **b**, Infiltrating cells from rejecting (R), syngeneic (S) and tolerant (T) grafts were isolated and quantitative real-time RT–PCR (qRT–PCR) analysis was performed. **c**, qRT–PCR analysis of mast-cell-related gene mRNA levels in the indicated groups. Data in **b** and **c** are representative of two individual experiments; values represent the mean ± s.d., **P* < 0.05 (R versus T).

Table 1 | Numbers of mast cells in skin post-grafting

Day	Mast cells per mm ²		
	Rejecting	Syngeneic	Tolerant
7	19.3 ± 18.9	85.8 ± 16.4*	49.0 ± 24.4*†
60	NA	77.8 ± 16.8	90.9 ± 38.8‡§

Data are representative of two individual experiments (*n* = 3–6 mice per group); values represent the mean ± s.d. NA, not available.

**P* < 0.05 by analysis of variance versus values for R at day 7.

†*P* < 0.05 by analysis of variance versus values for S at day 7.

‡*P* = 0.5005 by analysis of variance versus values for S at day 60.

§*P* < 0.05 by analysis of variance versus values for T at day 7.

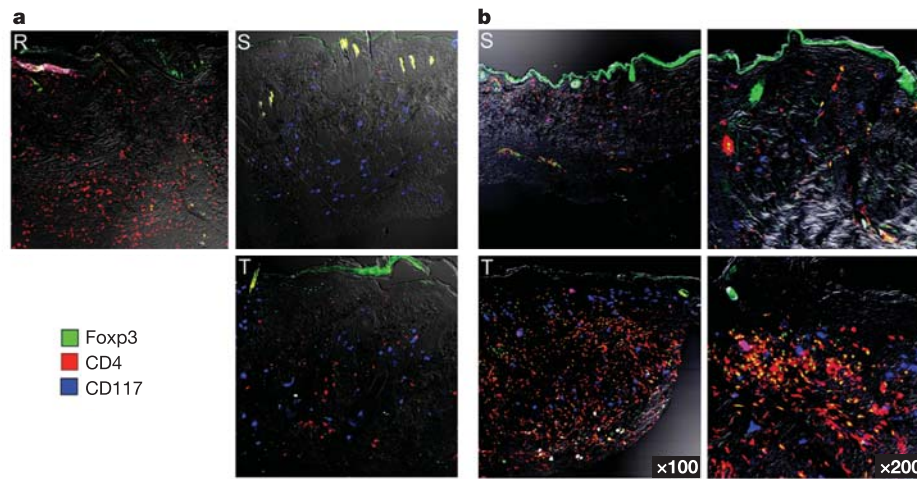


Figure 2 | Histological analysis of cell infiltration in tolerant allografts. **a**, Cryosections of skin transplants from the indicated groups (R, rejecting; S, syngeneic; T, tolerant) 7 days post-grafting were stained for infiltration of CD4⁺ T cells (red), CD4⁺Foxp3⁺ regulatory T cells (yellow) and CD117⁺

skin mast cells (blue). Magnification is × 100. **b**, For transplants at 60 days, a higher original magnification (× 200; right column) was used to show the colocalization of different cell populations.

allograft rejection in the anti-CD154/DST-treated W^{sh} mice indicates a role for mast cells in allograft survival, the data can merely implicate c-Kit⁺ cells. However, together with the mast-cell-reconstitution experiments, these results strongly suggest an indispensable role for mast cells in the establishment of skin transplant tolerance.

IL-9 links mast cells to T_{Reg}-cell-mediated allograft tolerance

The data presented thus far indicated that both T_{Reg} and mast cells were important in long-lived allograft tolerance. However, it remained unclear whether these two cell types functionally interacted to establish regional tolerance in the tolerant allograft. Analysis of gene array data from anti-CD3/anti-glucocorticoid-induced tumour necrosis factor related gene (GITR)-activated T_{Reg} cells indicated that *Il9* was highly upregulated on T_{Reg}-cell activation (Fig. 4a). IL-9 was initially cloned as a T-cell growth factor whose receptor shares the common γ-chain with IL-2 family members such as IL-2, -4, -7, -15

and -21 (refs 15, 16). Subsequently, IL-9 was shown to be a mast cell growth factor on the basis of its capacity to enhance the survival of primary mast cells and to induce their production of inflammatory cytokines, mast cell proteases and the high-affinity IgE receptor (FcεRIα)¹⁷. Furthermore, IL-9-deficient mice contained far fewer mast cells than their wild-type littermates¹⁸. As shown in Fig. 4a, following anti-CD3/anti-GITR stimulation, which has been shown to have an important role in controlling T_{Reg} cell activities^{19,20}, *Il9* was markedly upregulated in T_{Reg} cells, but not the CD4⁺CD25⁻ T-cell population. Upregulation of IL-9 protein expression in T_{Reg} cells was confirmed by an IL-9 enzyme-linked immunosorbent assay (ELISA; Fig. 4b). Moreover, CD28 co-signalling could induce levels of IL-9 production that greatly exceeded those observed with GITR co-signalling (Fig. 4b). IL-9 production seems to be a common feature of T_{Reg} cells because both nT_{Reg} cells (CD4⁺CD25⁺) as well as iT_{Reg} cells (CD4⁺CD25⁻ T cells cultured with anti-CD3 and transforming

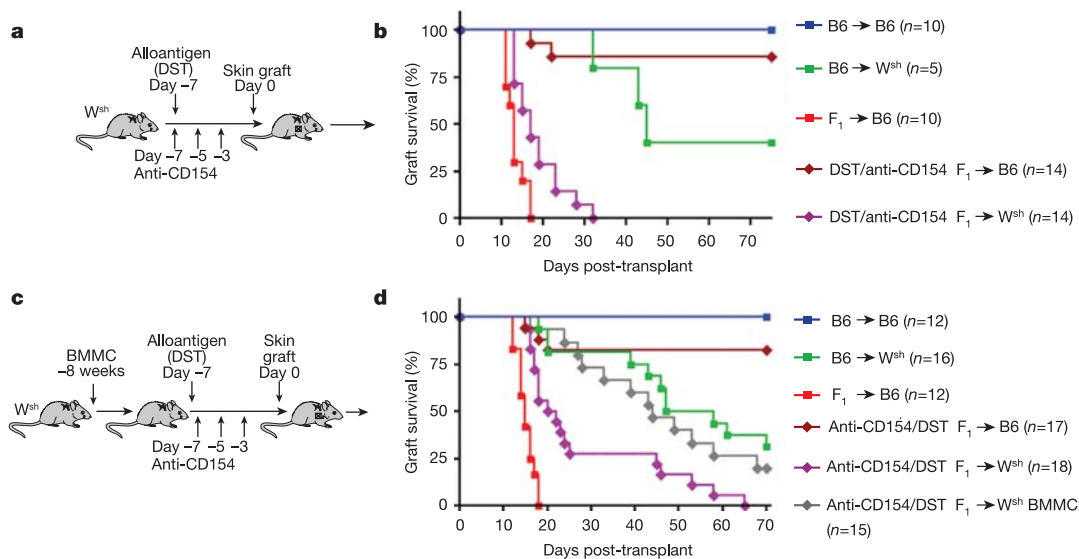


Figure 3 | Mast-cell-deficient mice are not capable of establishing long-term allograft tolerance. **a**, Anti-CD154 and DST were co-administered to mast-cell-deficient mice (W^{sh}) as described in the text. **b**, Allogeneic and syngeneic graft survival in treated W^{sh} and C57BL/6 (B6) mice was followed over time. F₁ is a hybrid of C57BL/6 and BALB/c. **c**, A group of W^{sh} mice

received BMMCs subcutaneously, 8 weeks before anti-CD154/DST co-administration and skin grafting. **d**, Graft survival was followed over time and compared with the control group without BMMC reconstitution. All skin graft rejection assays described above have been confirmed by at least three individual experiments.

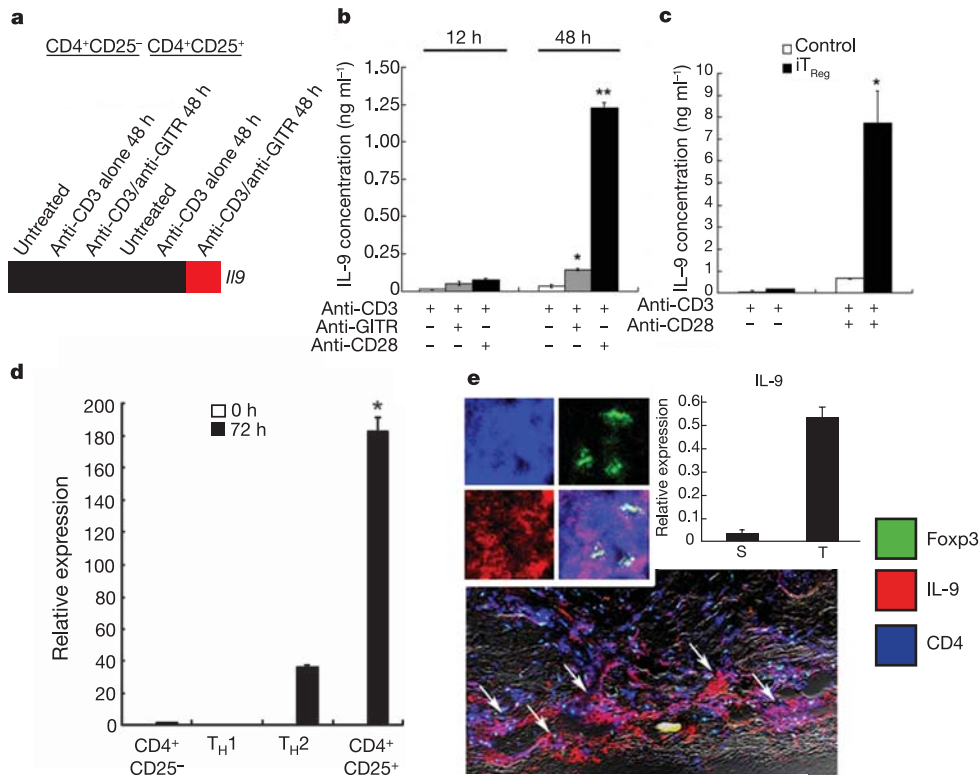


Figure 4 | **T_{Reg} cells produce high levels of IL-9 on activation both *in vitro* and *in vivo*.** **a**, CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were cultured *in vitro* with anti-CD3 and IL-2 with or without anti-GITR for 48 h. RNA was hybridized to the Affymetrix A430 array. **b**, IL-9 concentration in T_{Reg} cell culture supernatant with different treatments for 12 h and 48 h. Values represent mean \pm s.d., * $P < 0.001$ and ** $P < 10^{-7}$ versus anti-CD3 alone. **c**, IL-9 production in iT_{Reg} cell culture on activation at 48 h. Values represent mean \pm s.d., * $P < 0.05$ versus anti-CD3 alone. **d**, qRT-PCR of IL-9 expression in different T-cell subsets. T-cell subsets were stimulated with anti-

CD3 and anti-CD28 for 72 h as described. Values represent mean \pm s.d., * $P < 0.001$ versus T_H2 cell culture. **e**, Skin transplants from tolerant group 60 days post-grafting were stained for infiltration of CD4⁺ T cells (blue), CD4⁺Foxp3⁺ regulatory T cells (light blue) and IL-9 (red). IL-9-secreting CD4⁺Foxp3⁺ regulatory T cells are shown in purple (white arrows). Magnification is $\times 100$. Higher magnification (800 \times) of IL-9-secreting CD4⁺Foxp3⁺ regulatory T cells are shown with and without merging in the upper left panels. The right panel shows the qRT-PCR for IL-9 mRNA levels of the grafts in the indicated groups (mean \pm s.d.).

growth factor (TGF)- β *in vitro*²¹) produced high levels of IL-9 on activation (Fig. 4c). CD4⁺CD25⁻ T cells cultured in the absence of TGF- β produced $<10\%$ of the IL-9 compared with iT_{Reg} cells (Fig. 4c). As reported and shown in Fig. 4d, T-helper-2 (T_H2) cells secreted IL-9 on activation; however, T_{Reg} cells were superior in terms of IL-9 production on a per-cell basis. Notably, although high levels of IL-9 were produced, IL-9 did not seem to have a role in autocrine T_{Reg} cell growth or suppressive activity *in vitro* (Supplementary Fig. 3a, b).

Given the high levels of IL-9 produced by T_{Reg} cells and the role of IL-9 in mast cell homeostasis, a functional link between IL-9, mast cells and allograft tolerance was examined. First, immunohistochemical and quantitative real-time PCR analysis of skin transplants indicated that IL-9 was detected in tolerant grafts but not in syngeneic grafts (Fig. 4e and Supplementary Fig. 4). Second, our functional studies *in vivo* indicated a role for IL-9 in T_{Reg}-cell-mediated suppression. To investigate this, we first applied neutralizing anti-IL-9 to the anti-CD154/DST skin allograft model previously described. However, we could see only a partial effect with regard to alterations in graft rejection kinetics (data not shown). Owing to the complexities of the anti-CD154/DST system, which may obscure the potential involvement of IL-9 in T_{Reg}-cell-mediated tolerance, a Rag^{-/-} reconstitution system was employed that allowed the use of defined, enriched populations of T_{Reg} cells and effector T cells to study allograft survival²². Purified CD8⁺ T cells were transferred with or without purified CD4⁺CD25⁺ T cells into grafted Rag^{-/-} mice (Fig. 5a). As reported, the co-transfer of T_{Reg} cells delayed the onset of graft rejection (MST = 42 days) mediated by CD8⁺ T cells in this model (MST = 19.5 days; $P = 0.0243$). The T_{Reg}-cell-mediated delay

in graft rejection could be completely reversed with anti-IL-9 treatment (MST = 23 days; $P = 0.0161$; Fig. 5b). As CD8⁺ T cells did not produce IL-9, and there were no other CD4⁺ T cells in this system, this approach allowed us to speculate that IL-9 production by T_{Reg} cells delayed allograft rejection. To ascertain whether anti-IL-9 administration resulted in a reduction of mast cell accumulation in the tolerant grafts, mast cell numbers in various tissues were quantified. A reduced number of mast cells in the skin of mice treated with anti-IL-9 compared with those treated with control-mouse immunoglobulin was observed at day 10 after the transplantation of the allograft into Rag^{-/-} mice (Supplementary Fig. 5a, b). Hence, the regional production of IL-9 within the tolerant allograft facilitated mast cell accumulation. Although at this time we cannot definitively say IL-9 production by T_{Reg} cells mediates mast cell recruitment and function, IL-9 has clearly been shown to be instrumental in peripheral suppression of alloreactive CD8⁺ T cells.

Discussion

There are a number of novel, unanticipated findings that emerge from the studies presented here. First, even though recent studies have underscored the plasticity of mast cells in regulating acquired immune responses^{4-6,23}, the fact that mast cells may be instrumental in orchestrating T_{Reg}-cell-mediated peripheral tolerance is unprecedented. It is known that host-derived TGF- β is crucial for the peripheral immunosuppression mediated by T_{Reg} cells, and it is tempting to speculate that T_{Reg}-cell-activated mast cells are responsible for TGF- β production, or the liberation and activation of TGF- β via other known or unknown factors that mast cells secrete²⁴. In

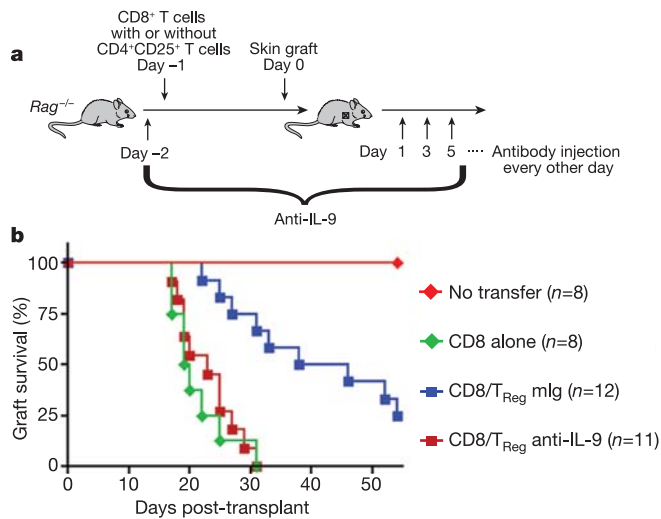


Figure 5 | IL-9 secreted by T_{Reg} cell functionally links mast cells to T_{Reg} -cell-mediated allograft tolerance. **a**, $Rag^{-/-}$ mice were reconstituted with purified $CD8^{+}$ T cells with or without T_{Reg} cells at a 5:1 ratio, 1 day before grafting. In the IL-9-treated group, neutralizing anti-IL-9 monoclonal antibody was administered intraperitoneally every other day starting 2 days before grafting as shown in the figure. **b**, Allograft survival was followed over time and compared with the control group treated with mouse immunoglobulin (mlg). All assays assessing the kinetics of skin graft rejection described above have been confirmed by at least three individual experiments.

addition, TPH1, like indoleamine-pyrrole 2,3-dioxygenase (Indo), is an enzyme that can metabolize tryptophan and create a tryptophan-deficient environment²⁵. As such, this may be a mechanism used by mast cells to limit T-cell activation. Second, recruitment of mast cells into sites of peripheral tolerance may be a common mechanism to control long-lived immune unresponsiveness at that site. In addition to allograft tolerance, a number of tumour models have documented the accumulation of mast cells, as well as T_{Reg} cells, to the tumour sites. Also, it has been shown that mast cells may contribute to tumour growth and metastasis^{23,26}. Thus, like in the allograft model presented, the T_{Reg} cell–mast cell partnership may also have an immunosuppressive role in dampening the immune response to tumours. Third, both n T_{Reg} cells and iT T_{Reg} cells seem to be producers of IL-9, and through the production of IL-9—and other effector molecules—may mediate the activities of mast cells *in vivo*. Hence, IL-9, mast cells and their gene products may become attractive therapeutic targets to ameliorate the impact of T_{Reg} cells *in vivo*.

Active suppression/regulation by T_{Reg} cells is essential to establish and sustain self-tolerance. We present a new paradigm through which T_{Reg} cells operate, and describe a novel set of cells and mediators that control peripheral tolerance. The finding that mast cells are critical in T_{Reg} -cell-dependent allograft tolerance further expands the knowledge of the interplay of different cellular components in controlling immune responses. Future studies will continue to unravel this pathway and will allow us to understand other mediators and cells that ultimately control peripheral immune suppression.

METHODS

Mice. C57BL/6, CB6F₁ (hybrid of C57BL/6 and BALB/c), C57BL/6 *Kit*^{W^{sh}; *Kit*^{W^{sh} (*W^{sh}*) and C57BL/6 *Rag*^{-/-} mice were purchased from the Jackson Laboratory. In all skin transplantation experiments, *W^{sh}* mice were at least 8 weeks old before grafting, to ensure mast cell deficiency¹³. All animals were maintained in a pathogen-free facility at Dartmouth Medical School.}}

Skin grafting and immunization. Skin grafting was performed following the procedure described previously². In brief, full-thickness tail skins from CB6F₁ (F₁) donors were transplanted onto the dorsal area of age-matched C57BL/6 recipients. Seven days before skin grafting, 4×10^7 T-cell-depleted splenocytes

from an F₁ donor were transferred into recipients through intravenous injection along with three injections of 250 μ g anti-CD154 monoclonal antibody (clone MR-1) on days -7, -5 and -3 to induce allograft tolerance. For T_{Reg} cell depletion, 250 μ g of anti-CD25 antibody (clone PC61) was administered through intraperitoneal injection 4 days before skin grafting. For blocking IL-9 activities *in vivo*, 200 μ g of neutralizing anti-IL-9 antibody (clone MM9C1)²⁷ was administered through intraperitoneal injection every other day throughout the duration of the experiments. Control recipients received identical amounts of mouse immunoglobulin.

Skin-infiltrating-cell isolation. Skin infiltrating cells were isolated following the modified protocol described previously⁷. Briefly, secondary-challenge skin transplants from different groups were removed 7 days after grafting. Skin grafts were then cut into small pieces, followed by trypsin digestion at 37 °C for 1 h. The remaining pieces were washed with RPMI 1640 medium over nylon mesh. Cell debris was removed by filtration through a 100- μ m nylon cell strainer and a 40- μ m nylon cell strainer, sequentially. The resulting cell suspension was then washed twice in cold HBSS media and used for further analysis.

Cell preparation, BMDC generation and cell reconstitution. Single-cell spleen suspensions were prepared from 8–10-week-old mice. $CD8^{+}$, $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+}$ T cells were purified by magnetic separation with MACS (Miltenyi Biotec) according to the manufacturer's instructions. Enriched cell populations and purified cells were phenotypically analysed by fluorescence-activated cell sorting (FACS). The purity of each population was around 90–95%. To generate different T-cell subsets, purified $CD4^{+}CD25^{-}$ T cells were cultured with plate-bound anti-CD3 monoclonal antibody (clone 145-2C11) at 10 μ g ml⁻¹ and soluble anti-CD28 monoclonal antibody (clone PV-1) at 1 μ g ml⁻¹. For T_{H1} cell preparation, recombinant mouse IL-12 (5.0 ng ml⁻¹; PeproTech) with neutralizing anti-IL-4 monoclonal antibody (10 μ g ml⁻¹; clone 11B11; BD Pharmingen) were added; for T_{H2} cell preparation, recombinant mouse IL-4 (5.0 ng ml⁻¹; PeproTech) with neutralizing anti-interferon (IFN)- γ monoclonal antibody (10 μ g ml⁻¹; clone 37895.11; R&D Systems) were added. Cells were harvested after 5 days of culture and their purities were verified by real-time PCR analysis of lineage-specific gene expression (*Tbx21* for T_{H1} and *Gata3* for T_{H2} ; data not shown). For iT T_{Reg} cell preparation, recombinant human TGF- β (1 ng ml⁻¹; PeproTech) and human IL-2 (100 U ml⁻¹; PeproTech) was added. After 5 days of culture, cells were harvested and their purities were verified by FACS analysis of *Foxp3* expression (data not shown). For mast cell reconstitution, BMDCs were generated by culturing bone marrow cells with IL-3 (20 ng ml⁻¹; PeproTech) for 5 weeks as shown previously^{28,29}. The purity was assessed by anti-CD117 (c-Kit) and anti-Fc ϵ R1 α staining. A total of 5×10^6 BMDCs were then injected intradermally into the *W^{sh}* recipients 8 weeks before grafting. For $Rag^{-/-}$ mice reconstitution, 1×10^6 $CD8^{+}$ T cells were adoptively transferred through intravenous injection with or without 2×10^5 $CD4^{+}CD25^{+}$ T cells 1 day before grafting.

Real-time PCR and gene array analysis. Total RNA from isolated skin-infiltrating cells or different T-cell subsets was purified using the RNeasy system (Qiagen). Complementary DNA was then prepared and applied to real-time PCR analysis (SYBR green; BioRad). Relative expression of various gene targets normalized to β -actin was calculated as:

$$(2 - (\text{experimental CT} - \beta\text{-actin CT})) \times 1,000$$

where CT is the cycle threshold of signal detection. For gene array analysis, as shown in the previous literature³⁰, RNAs purified from different cell populations with various treatments were analysed using Affymetrix mouse genome A430 oligonucleotide arrays.

Cytokine secretion assay and immunohistology. Secretion of IL-9 was assayed by ELISA. Different T-cell populations (1×10^6) were cultured in 24-well plates precoated with 1 μ g ml⁻¹ anti-CD3 (clone 2C11) with or without 10 μ g ml⁻¹ anti-GITR (also known as Tnfrsf18; clone DTA-1) or anti-CD28 (clone PV-1). Supernatants were collected at the time indicated. IL-9 was quantified according to the manufacturer's instructions (Peprotech). For immunohistology, previously grafted skins were snap frozen, cryocut, and acetone-fixed. Slides were blocked with normal mouse serum. Tissue sections were stained for CD4 (clone GK1.5), CD117 (clone 2B8) and *Foxp3* (clone FJK.16s). For IL-9 staining, biotinylated rabbit polyclonal anti-mouse IL-9 antibodies (Peprotech) and PE-conjugated streptavidin (eBioscience) were used. The specificity of IL-9 staining has been confirmed by the absence of staining in skin tissue from *Il9*^{-/-} mice (our unpublished data).

Received 16 May; accepted 20 June 2006.

Published online 20 August 2006.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. Galli for critical review of the manuscript during preparation. This work was supported by NIH grants; J.V.S.'s research was funded in part by the Belgian Federal Service for Scientific, Technical and Cultural Affairs and by the Actions de Recherche Concertées, Communauté française de Belgique, Direction de la recherche scientifique.

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