

Mast Cell Degranulation Breaks Peripheral Tolerance

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Mast cells (MC) have been shown to mediate regulatory T-cell (T^{reg})-dependent, peripheral allograft tolerance in both skin and cardiac transplants. Furthermore, T^{reg} have been implicated in mitigating IgE-mediated MC degranulation, establishing a dynamic, reciprocal relationship between MC and T^{reg} in controlling inflammation. In an allograft tolerance model, it is now shown that intragraft or systemic MC degranulation results in the transient loss of T^{reg} suppressor activities with the acute, T-cell dependent rejection of established, tolerant allografts. Upon degranulation, MC mediators can be found in the skin, T^{reg} rapidly leave the graft, MC accumulate in the regional lymph node and the T^{reg} are impaired in the expression of suppressor molecules. Such a dramatic reversal of T^{reg} function and tissue distribution by MC degranulation underscores how allergy may cause the transient breakdown of peripheral tolerance and episodes of acute T-cell inflammation.

Key words: Acute allograft rejection, allergy, allograft survival, anti-CD154 monoclonal antibody, CD4⁺CD25⁺ T cells, skin allograft

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Introduction

Mast cells (MC) are best known for their role in allergies and protecting our body from parasitic, bacterial and viral infection (1). IgE antibody against the allergen or infectious agent binds to the high-affinity IgE receptor on MC. Subsequent encounter with allergen leads to the release of the MC granular content with heightened inflammation. While the historical role of MC has been as regulators of inflammatory responses, MC have been recently been implicated as regulators of tolerance (2,3). The striking contrast in MC

function is exemplified by their pro-inflammatory roles in nematode infection and allergies (1) or their role in mediating suppression, as in UV-B damage (4), mosquito bites (5) and graft tolerance (6,7). Recently, the pivotal role of MC in the establishment of acquired tolerance to an allograft was shown (6,7). It was hypothesized that the secretion of immunosuppressive mediators by MC was critical for sustaining tolerance. Recently, the dynamic and reciprocal nature of MC-T^{reg} interactions was shown. It was reported that T^{reg} can suppress IgE-mediated degranulation through OX40-OX40L interactions (8,9) thereby showing a natural mechanism for MC stabilization. It is clear that the release of inflammatory mediators as a consequence of MC degranulation results in inflammation. How this impacts peripheral tolerance and T^{reg} function is not known. Therefore, studies were designed to determine if degranulation of MC present in tolerant allografts would impact on allograft survival. Data presented show that IgE-mediated degranulation, either within the graft or systemically, breaks established peripheral tolerance and leads to T-cell-mediated, acute rejection of the allograft. Degranulation causes the release of MC intermediaries, a rapid migration of both T^{reg} and MC from the graft as well as a transient demise in the expression of T^{reg} suppressive cytokines. Such a dramatic reversal of T^{reg} function and tissue distribution by MC degranulation underscores how allergy may cause the transient breakdown of peripheral tolerance and episodes of acute T-cell inflammation.

Material and Methods

Mice

C57Bl/6, CB6F₁ (C57Bl/6×BALB/c hybrid), C57Bl/6-Ly5.2⁺, and C57Bl/6-Rag^{-/-} mice were purchased from the Jackson Laboratory. FoxP3/GFP reporter mice were provided by Dr. A. Rudensky (University of Washington School of Medicine, Seattle, WA) (23).

Skin graft model

Skin grafting was performed as described previously (43). For dual grafting, the first graft was placed on the back near the base of the tail, whereas the second graft was placed on the back close to the neck 2 weeks later. Grafts were monitored for rejection for 30 days postdegranulation and were considered rejected when 80% of the original graft disappeared or became necrotic.

Degranulation

Chemical degranulation was done by application of 50 μ L Compound 40/80 (1 mg/mL, Sigma) directly under the graft. 'Active' immunization was achieved by 100 μ g of OVA/Alum (Pierce) i.p., 37 days prior to grafting or passive by transfer of IgE. For passive immunization, 2 or 5 μ g of either OVA-specific IgE (clone 2C6; Serotec) or TNP-specific IgE (clone A3B1;

cross-reactive with NP) was given intravenously 24 h prior to degranulation, respectively. Degranulation was induced either locally (50 μ L of 1 mg/mL OVA in PBS) or systemically (500 μ L of 1 mg/mL OVA in PBS intraperitoneal) for mice that received OVA-IgE or were active immunized. Degranulation in mice that received NP-IgE was done by injecting 20 ng of NP₁₇-OVA/NP₂₃-BSA locally. Blocking of degranulation was done by subcutaneous injection of 100 μ L of Cromolyn Sodium Salt (39 mM in PBS, Sigma-Aldrich) 30 min prior to degranulation.

Cytokine profile of the graft

Grafts were collected, cut to small pieces in HBSS (6 grafts/mL) 18 h post-degranulation and incubated for 1 h at 37°C. Cytokines in the supernatants were determined by multiplex analysis (Biorad) and verified by ELISAs (IL4, IL6, IL10 (Pharmingen), IL9 (PeproTech) and TNF α (eBioscience)).

Induction of inflammation

Mice were either treated with 200 μ L complete Freund's adjuvant (Sigma), 50 μ g TLR4 agonist (LPS, *E. coli* 055:B5, Sigma) or 50 μ g TLR9 agonist (CpG; ODN-1826, Eurogentech) by intraperitoneal injection. For induction of local inflammation, 8 μ L of 5 mg/mL FITC (Sigma) in 1:1 acetone (Fisher scientific)/dibutylphthalate (Sigma) was applied directly onto the preshaved graft. Positive controls included degranulation after passive immunization or intraperitoneal injection of 50 μ g of agonistic α CD40 (FGK; BioExpress).

Immunohistochemistry

Tissues were snap-frozen and 8 μ m sections were fixed with methanol prior to staining with α -CD117-A647. Hoechst was used for nuclear staining. Slides were analyzed by a confocal microscope (LSM510Meta, Zeiss) at a 100 $\times$ magnification. To analyze granulated MC, slides were stained with toluidine blue (Sigma). Toluidine blue-positive MC numbers in skin were determined by counting the number of stained MC in seven randomly chosen fields at 100 $\times$ magnification by two independent observers using an Olympus DP70 camera system operating on a BX60 transmitted microscope.

Cell isolation and analysis

Naïve T-cells were isolated from pooled spleen and LN. Prior to cell sorting, T-cells were pre-enriched by CD4 negative selection (biotin-selection kit, StemCell). Isolation of graft T-cells from FoxP3-GFP mice was performed by digestion with DNase, Liberase and Collagenase (10 mg/mL, Roche) for 45 min at 37°C after weighing the grafts. Analysis was performed after co-staining with CD4 (RM4-5, Pharmingen) by flow-cytometry. For determination of the total number of MC in the dLN, samples were stained with CD117 (clone: 2B8) and Fc ϵ RI (MAR-1, eBioscience) and analyzed by flow-cytometry.

T-cell studies in vivo

At day 27 and 35, mice were treated with 300 μ L intraperitoneal and 50 μ L locally at the site of the graft with mixture of α CD4 (GK1.5) and α CD8 (2.43) antibodies (kind gift from Dr. MJ Turk, Dartmouth, NH) to deplete T-cells. Depletion was confirmed at day 37. For the transfer of tolerance experiments, grafts dLN were collected at day 35 and T-cells purified by two rounds of CD4 negative depletion (StemCell). RAG^{-/-} received an F1 graft 2 weeks prior to adoptive transfer of 1×10^6 purified T-cells by i.v. injection.

Functionality of regulatory T-cells in vitro and in vivo

One day postdegranulation, FoxP3-GFP⁺ T-cells from the graft dLN were FACS sorted. For *in vitro* studies, Ly5.2⁺ naïve T-cells were purified by FACS sorting and labeled with carboxyfluorescein succinimidyl ester (5 μ M, CFSE). In total 5×10^4 were cultured with T-depleted irradiated Ly5.1⁺ splenocytes (3000 rad) at different ratios of T^{reg}:Tnaïve. After 3 days CFSE dilution, profiles were analyzed within the Ly5.2⁺ population. For *in vivo*

functionality, GFP⁺/Ly5.1⁺ T^{reg} were mixed at different ratios with naïve, polyclonal T-cells and adoptively transferred into F1 pregrafted RAG^{-/-}. Phenotypic analysis of T^{reg} was performed by staining T-cells of the dLN after CD4 enrichment. All samples were stained with FoxP3 in either FITC or PE depending on the co-staining. FoxP3⁺ cells were analyzed for the expression of Ki67 (Ki67 staining kit, BD), CTLA4(UC10-4F10-11, Pharmingen), ICOS (15F9, eBioscience), CD62L(MEL14, BioLegend), CD73(clone TY/11.8, eBioscience), CD103(2E7, eBioscience), GITR(YG1TR 765, BioLegend), and IL7R α (CD127; A7R34, eBioscience). For RT-PCR analysis, FoxP3⁺ T^{reg} from the grafted FoxP3-GFP mouse were flow sorted. The primers used for estimates of gene expression were: TGF- β 1 forward: 5'-TTGCTTCAGCTCCACAGAGA-3', TGF- β 1 reverse: 5'-TGGTGTAGAGGG-CAAGGAC-3', IL-10 forward: 5'-GGTTGCCAAGCCTTATCGGA-3', IL-10 reverse: 5'-ACCTGCTCCACTGCCTTGCT-3', Ebi3 forward: 5'-AGCAGCAGCTCCTAGCCT-3', Ebi3 reverse: 5'-GCGGAGTCGG-TACTTGAGAG-3', granzymeB forward: 5'-CTCCACGTGCTTTCACCAA-3', granzymeB reverse: 5'-AGGATCCATGTTGCTCTGTAGTAG-3'.

Statistics

All statistics have been calculated using the prism 4.03 software package (GraphPad Software, San Diego, CA). Survival data were analyzed using the Kaplan–Meier method, with the Wilcoxon rank test and the log-rank test. Luminex data were analyzed by the immune monitoring laboratory of the Norris Cotton Cancer Center/Dartmouth Hitchcock Medical Center and are shown as mean $\pm$ SD whereas other data are expressed as mean $\pm$ SEM. The latter were analyzed by one-tailed ANOVA and posttested by Tukey analysis. Statistical significance was calculated for a 95% confidence interval ($p < 0.05$). The exact p -values are denoted in the figures.

Results

MC degranulation results in the rejection of an established tolerant allograft

Previously we showed that in mice tolerized with α CD154 and donor-specific transfusion (DST) both T^{reg} and MC accumulate in the tolerant allograft and are functionally critical for allograft persistence (7). Herein, it was asked if IgE-mediated MC degranulation and the release of inflammatory mediators would alter T^{reg} function, distribution and impact on allograft persistent in the tolerant host.

Both tolerized and syngeneic control mice were treated as shown in Figure 1A, unless stated differently (10–12). Within 15 days, all tolerized mice bearing an allogeneic skin graft in which MC had been degranulated lost their grafts. Syngeneic mice maintained their grafts for the duration of the experiment regardless of treatment (Figure 1B). These results indicate that degranulation of MC facilitates the rejection of allogeneic skin grafts in tolerized hosts. Both active immunization with OVA-Alum 30 days prior to grafting, followed by challenge with OVA, as well as chemically induced (compound 40/80) (13,14) degranulation showed the same results as with passive immunization (Supporting Figure S1A and B, respectively). Cytokines known to be abundant after IgE-mediated MC degranulation were increased in groups where MC had been degranulated (Figure 1C), confirming that the MC released their granular content and established a pro-inflammatory

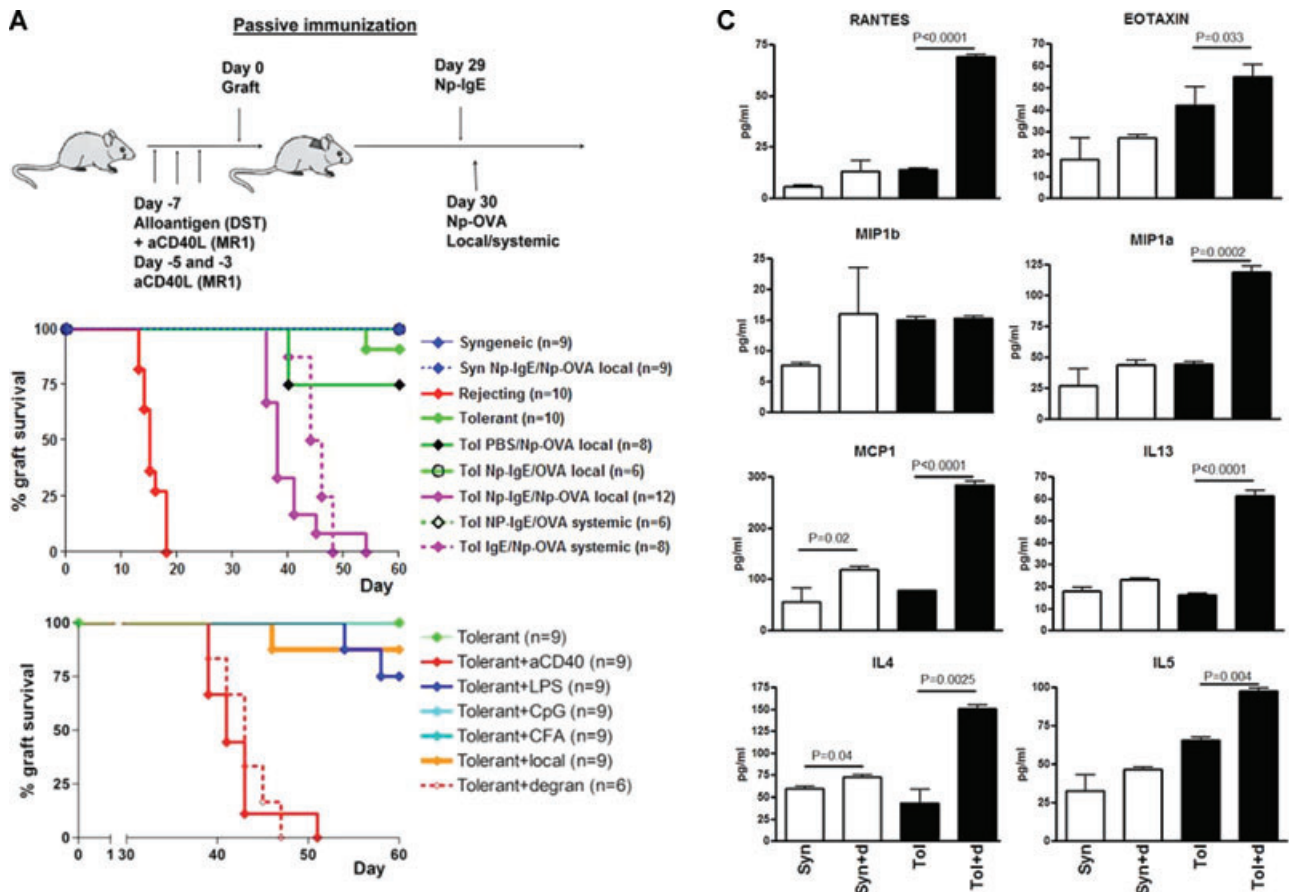


Figure 1: Degranulation of mast cell leads to acute rejection of established tolerant skin grafts. (A) To induce tolerance, C57BL/6 mice were administered a regimen of donor-specific transfusion (DST) of allogeneic splenocytes and anti-CD154 (200 μ g i.p.). Subsequently, mice received an allogeneic skin graft and survival was monitored. These mice maintain the graft for up to 100 days postgrafting, with day 0 designated as the day the mice received the graft. MC were degranulated in mice 30 days after engraftment receiving the tolerance-inducing regimen and an allograft, in all studies described. At day 0 mice received either a CB6F1 graft (designated tolerant/tol) or a syngeneic (syn) B6 graft. Syngeneic and rejecting mice did not receive pretreatment. At day 29, mice were sensitized with 5 μ g of NP-specific IgE (NP-IgE) and 24 h later challenged with NP₁₇-OVA or NP₂₃-BSA, systemically (20 ng/mouse intraperitoneal) or locally (2 ng/mouse intragraft). Graft rejection was monitored for another 30 days. (B) Survival of grafts for both systemic and local treatment. Controls included were either sensitized with PBS followed by local challenge with NP₁₇-OVA or sensitized with NP-IgE followed by local challenge with 20 ng of OVA. The total number of mice pooled from multiple independent experiments is shown in parentheses. (C) Tolerant and syngeneic with and without degranulation were collected 18 h after degranulation and incubated in HBSS for 1 h at 37°C. Supernatants were collected and tested for functionality in a proliferation assay (data not shown). Cytokine analysis was performed by multiplex, and results were confirmed by ELISA. Shown are chemokines and cytokines known to be abundantly present under allergic conditions (15). (D) In order to see whether degranulation induced rejection was solely the effect of inflammation, various strategies were used to induce overt inflammatory responses. Mice were treated with 50 μ g TLR4 or TLR9 agonist, 200 μ L CFA or local by chemically induced inflammation. As positive controls agonistic CD40 and degranulation were included. Data shown are combined from two independent experiments.

Th2-biased micro-environment similar to that observed in allergies (15).

Comparison of different inflammatory mediators in inducing graft rejection of tolerant allografts

A battery of inflammatory mediators was used to evaluate if any inflammatory insult under tolerizing conditions would inevitably lead to graft rejection. Mice were treated with inflammatory doses of the Toll-like receptor (TLR) 4 ago-

nist, LPS; the TLR9 agonist, CpG-ODN 1826; Complete Freund's Adjuvant (CFA) or an agonistic α CD40 antibody. Finally, local, chemical-induced Th2-biased inflammation was induced using acetone/dibutylphthalate/FITC applied directly onto the graft (16–18). No acute rejection was observed in any of the groups tested except for the tolerant mice administered α CD40 antibody, as previously reported (19). These data demonstrate that the acute rejection observed after degranulation was not due to the introduction

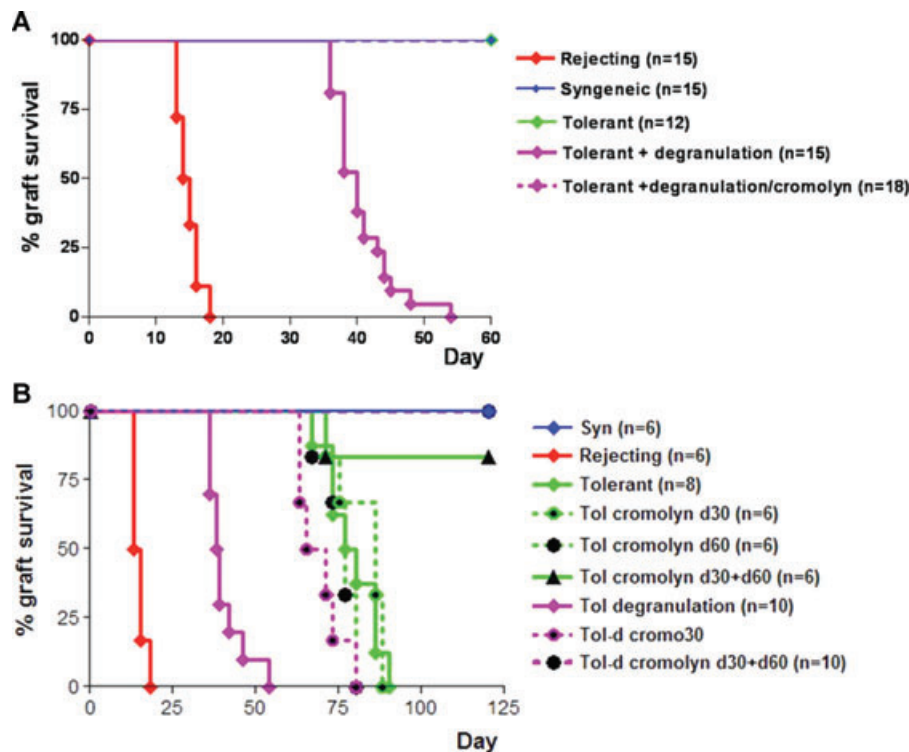


Figure 2: Mast cell stabilization protects against degranulation-induced rejection and prolongs graft survival in general. (A) Mice were treated according to Figure 1A. However, 30 min prior to local challenge with 20 ng of NP₁₇-OVA one group received an intragraft injection of 100 μ L of a 39 mM solution of cromolyn, a chemical known to stabilize MC thus preventing degranulation. The total number of mice pooled from three independent experiments is shown in parentheses. (B) Passively immunized mice were treated locally with Cromolyn at day 30 and/or day 60 postgrafting either with or without local degranulation. Grafts were monitored for a total of 120 days. The number of mice in each group, as shown in parentheses, is from multiple independent experiments. For both Figure A and B, the relevant syngeneic controls for this experiment did not show rejection for the entire duration of the experiments and are omitted from this figure for clarity.

of overt inflammation *per se* (Figure 1D) but due to the nature of the inflammatory microenvironment created by degranulation.

Mast cell stabilization leads to prolonged allograft survival

To seek further evidence that breakdown of allograft tolerance was the effect of MC degranulation, degranulation was blocked by local intragraft injection of sodium cromoglycate (cromolyn) (20). The observed acute rejection after MC degranulation was completely blocked with the administration of cromolyn (Figure 2A) thereby confirming that the acute allograft rejection was the result of degranulation.

In this system of allograft tolerance, peripheral tolerance begins to decay at day 70 postgrafting in tolerant mice (21). To address if limited allograft survival in tolerized mice was due to 'low-grade' MC degranulation, cromolyn was applied to the allograft at day 30 and/or day 60 after engraftment. Single treatment did not impact graft survival. However, when performed on both days, allografts were

maintained for >120 days even when allergic exacerbations were mimicked right after cromolyn treatment (Figure 2B). These results show that allograft survival in tolerized mice can be extended by the administration of cromolyn and suggests that MC degranulation may contribute to the natural decay of tolerance in this system.

MC degranulation leads to an accumulation of MC in the draining lymph nodes

To define the cellular events occurring upon MC degranulation, tolerant and syngeneic skin grafts were collected at day 1 and 5 after degranulation. Degranulation leads to disappearance of MC from the allograft, as measured with by the number of c-Kit⁺ cells present within the graft (Figure 3A) and inversely correlated with the number of MC in the corresponding allograft-draining axillary and brachial LN as quantified by the number of c-Kit^{high}Fc ϵ R^{high} cells (Figure 3B). MC numbers were confirmed by toluidine blue staining of both graft and dLN (Supporting Figure S2). Taken together, these data suggest that upon degranulation, some MC degranulate and other MC egress from the skin and enter the draining lymph.

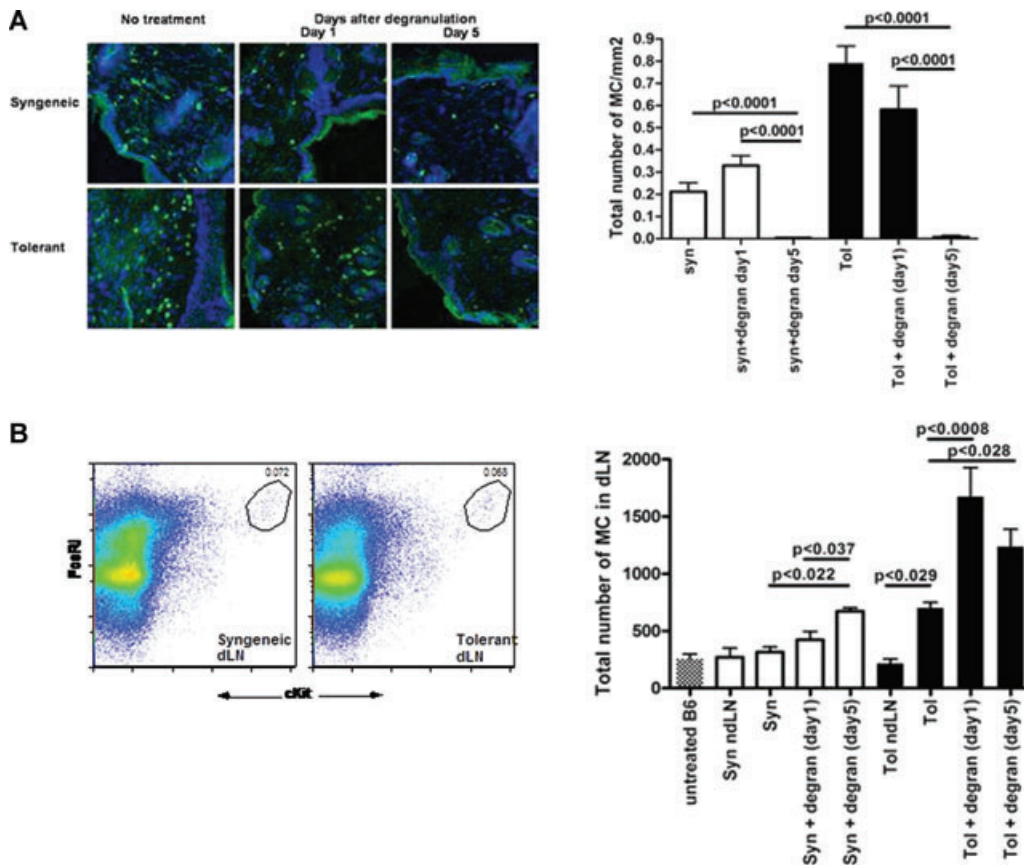


Figure 3: Local IgE-mediated degranulation leads to migration of MC from the graft to the draining LN. (A) Grafts were degranulated locally after passive immunization. Confocal images of representative tolerant and syngeneic grafts with MC (cKit⁺FcεRI⁺) are shown in green. Nuclear stains with Hoechst are shown in blue. Quantification of the number of MC in the skin was performed by counting seven randomly chosen fields in a total of six individual mice. (B) Flowcytometric analysis of the draining, pooled inguinal and axillary, lymph nodes. After digestion with DNase/liberase, single cell suspensions were counted and stained with cKit and FcεRI. A representative plot shows MC in the gate after pre-enrichment with FcεRI-PE on the left. Cell counts were performed before and after enrichment to calculate the absolute number of MC (cKit^{high}FcεRI^{high}) in the dLN as shown on the right. Data are combined from three independent experiments with a total of nine mice.

MC degranulation-mediated rejection is dependent on T-cells

In order to address whether the rejection observed after MC degranulation was dependent upon T-cells, CD4⁺ and CD8⁺ T-cells were eliminated with depleting antibodies. MC degranulation in the absence of T-cells did not lead to graft loss (Figure 4A). Similar results were obtained using compound 40/80 to degranulate the MC (Supporting Figure S3). This demonstrates that T-cells were mediating graft rejection after degranulation.

MC degranulation results in a breakdown of T-cell tolerance

It is known that in this system a balance of allogeneic effector T-cells (T^{eff}) and T^{reg} controls graft rejection and suggests that MC degranulation has an impact on this balance. To directly measure the intrinsic ability of T-cells from tolerant mice to mediate graft rejection or not, an adop-

tive transfer study was designed. Purified dLN T-cells from the different groups were transferred into RAG^{-/-} mice bearing an allograft. Adoptive transfer of CD4⁺ T-cells from tolerant mice after local MC degranulation rejected the allografts in the secondary recipients (Figure 4B). These data show that MC degranulation restores alloreactive T-cell responses in a tolerant mouse.

Regional degranulation results in the systemic decay of peripheral tolerance

Studies were designed to address whether local MC degranulation would facilitate a breakdown in allograft tolerance at the systemic level. To test this, tolerant mice received two skin allografts, one at day 0 and one at day 14. The first allograft (graft #1) was draining into the inguinal lymph nodes. The second graft (graft #2) drained to the brachial and axillary lymph nodes (day 14) (22). At day 30, graft #1 was challenged after passive immunization

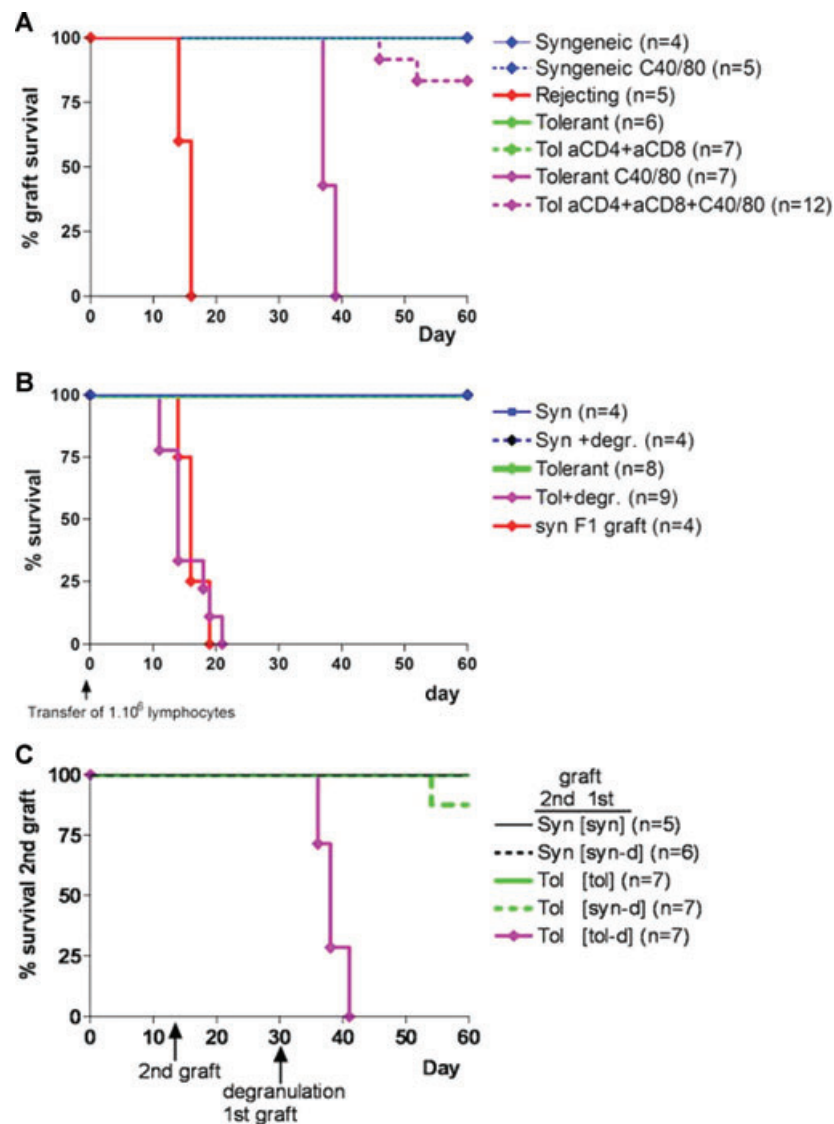


Figure 4: Break of peripheral tolerance by degranulation is T-cell dependent. (A) Graft survival after depletion of CD4 and CD8 T-cells with α CD4 and α CD8 antibodies (300 μ L intraperitoneal and 50 μ L local) 2 days prior to and 5 days after local degranulation at day 30. Grafts were monitored for another 30 days after degranulation, and the data shown are pooled from two independent experiments. (B) RAG^{-/-} were grafted 2 weeks prior to adoptive transfer of 1.10^6 lymphocytes from the inguinal and axillary draining LN of the indicated groups. After One day, degranulation lymph nodes were isolated and T-cells were purified by two rounds of negative depletion yielded over 92% purity. Graft survival measurement starts at the day of transfer of lymphocytes. Pooled data are shown from in total three independent experiments. (C) In order to see whether degranulation could lead to rejection of a graft in a distant location, mice received two graft 2 weeks apart. The first graft was placed close to the base of the tail and the second graft was in the neck to assure both grafts drained to separate lymph nodes. All mice receiving an allogeneic CB6F1 graft were pretreated with DST/ α CD154. At day 29, mice with two accepted grafts were included in the study. After passive immunization, the first graft was locally challenged. Graft survival of a secondary graft was monitored for another 30 days. In order to control for aspecific rejection due to antigen drainage to the second graft, a control was included where the first graft was syngeneic, and thus would not reject by degranulation, and the second graft was an allogeneic CB6F1 graft. Treatment and source of the first graft are indicated in between brackets. Data are combined from multiple experiments and the total number of mice used is shown in parentheses.

and rejected at the expected kinetics. Interestingly, graft #2 was also rejected after a delay of about 1–2 days (Figure 4C). As a control to ensure local degranulation of graft #1, a cohort of mice received a syngeneic graft

#1 and an allogeneic graft #2. Local degranulation of the syngeneic graft did not lead to rejection of the allogeneic graft confirming that allergen was not reaching the other graft. Together, these data demonstrate that local MC

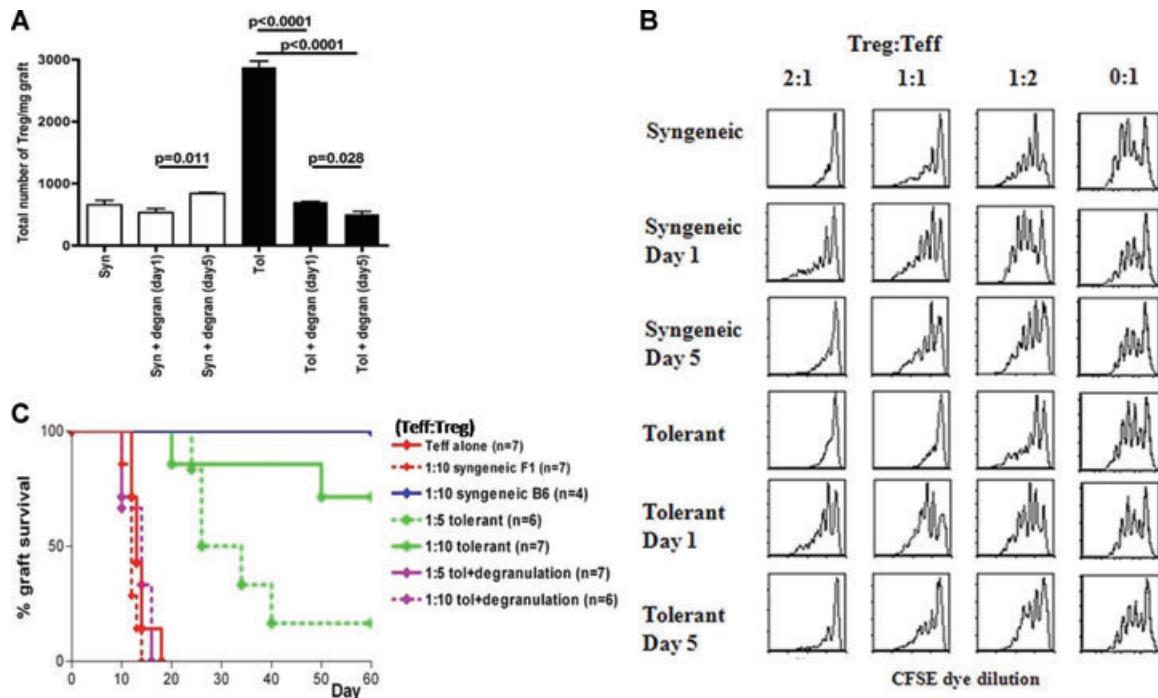


Figure 5: Mast cell degranulation leads to efflux of T^{reg} from the graft and a transient block in regulatory T-cell function. (A) Quantification of CD4⁺FoxP3⁺ T-cells (Treg) in the grafts of tolerized and syngeneic mice with and without local degranulation after passive immunization. Grafts were collected and individually weighted before digestion with DNase/Dispase/Collagenase. Single cell suspension were counted and stained with CD4 before analysis by flowcytometry. Total number of Treg/mg graft tissue is shown for a total of six mice from two independent experiments. (B) Following degranulation of tolerized, allografted mice, Ly5.2⁺ T^{reg} were recovered from the dLNs of Foxp3-GFP mice and FACS-purified based on GFP expression at day 1 or day 5 after local degranulation to be used in a standard suppressor assay. Briefly, naïve CD4 T-cells from wildtype, C57Bl/6 mice (T^{eff} cells), expressing Ly5.1 were purified and labeled with CFSE. FACS-purified Ly5.2 T^{reg} based from either degranulated or non-degranulated hosts were mixed at different ratios with T^{eff} cells, and T^{reg}-dependent suppression was measured by CFSE dye dilution. Grafting of mice was staggered in order to be able to isolate the Treg on the same day. Cells were flow sorted and mixed with congenically (Ly5.2⁺) marked CFSE-labeled naïve polyclonal T-cells at the indicated ratios. After 4 days of culture, the Ly5.2⁺ cells were analyzed for CFSE dye dilution. Shown are representative histograms of multiple independent experiments. (C) T^{reg} were purified from tolerized, allografted FoxP3⁺-GFP mice which were untreated or degranulated with allergen. As in Figure 5B, 24 h after degranulation, T^{reg} from tolerized controls or degranulated, tolerized groups were FACS purified based on GFP expression. T^{reg} from each of these groups were mixed at ratios with WT, T^{eff} (T^{eff}: T^{reg}) to be used in an *in vivo* suppressor assay. RAG^{-/-} mice were grafted 2 weeks prior to adoptive transfer of in total 1.10⁶ lymphocytes. Grafts were monitored for rejection for 60 after transfer of the T-cells. Data are pooled from three independent experiments with the total number of mice shown in parentheses.

degranulation leads to a systemic breakdown of peripheral tolerance.

Degranulation facilitates T^{reg} and MC loss from the graft and impairs T^{reg} function

MC and T^{reg} are present in tolerant allografts and are required to maintain allograft tolerance (7). It was hypothesized that degranulation may change T^{reg} composition and functionality, thereby altering graft longevity. Following degranulation, T^{reg} were enumerated in the grafts by using Foxp3-GFP mice (23) as hosts. At day 1 following MC degranulation, a dramatic reduction of CD4⁺Foxp3⁺ was observed in allografts (Figure 5A). At this time point, MC were still present in the allograft (Figure 3A), demonstrating that the efflux of T^{reg} precedes the efflux of MC from the skin allograft following MC degranulation.

In addition to the altered distribution of T^{reg}, the *in vitro* and *in vivo* functions of T^{reg} were also evaluated following MC degranulation. T^{reg} function was measured *in vitro* by a suppressor assay. T^{reg} from both tolerized, allografted mice and from those bearing syngeneic grafts suppressed WT T^{eff} proliferation, as measured by CFSE dye dilution. However, T^{reg} from mice whose MC were degranulated 1 day prior were less effective at suppressing T^{eff} proliferation (Figure 5B). The impairment in T^{reg} suppression was transient, because T^{reg} recovered at day 5 postdegranulation, regained their ability to suppress T^{eff} indistinguishably from the non-degranulated controls. These results indicate that MC degranulation leads to a transient reduction in T^{reg} function. Furthermore, T^{reg} function was also impaired after MC were degranulated in mice bearing syngeneic grafts, suggesting that this is a general mechanism of immune regulation mediated by MC. Of note, T^{eff} in the same

groups as tested in Figure 5B could still be suppressed by WT- T^{reg} (data not shown).

To examine whether T^{reg} after MC degranulation are impaired *in vivo*, an *in vivo* T^{reg} suppressor assay was performed. T^{reg} were mixed with WT- T^{eff} and adoptively transferred into pregrafted RAG $^{-/-}$ mice. The majority of mice, which received T^{reg} from non-degranulated mice at a 1:10 $T^{\text{eff}}:T^{\text{reg}}$ ratio, retained their allogeneic grafts for the duration of experiment. However, grafts were rejected when T^{reg} were isolated after MC degranulation at the same ratio of $T^{\text{eff}}:T^{\text{reg}}$ (Figure 5C). In sum, these data indicate that after MC degranulation, T^{reg} transiently lose their ability to mediate graft survival.

MC degranulation leads to a reduced expression of key T^{reg} immunomodulatory molecules

To define the underlying molecular mechanisms for the loss in T^{reg} function after degranulation, the expression of key surface molecules and cytokines was analyzed. Comparative FACS analysis revealed no changes in the expression of markers involved in maturation/migration (CD62L, CD103), peripheral homeostasis (IL7 α R), proliferation (Ki67) and contact-dependent suppression (CTLA4, ICOS, CD73, GITR). Additionally, the level of FoxP3 expression was not changed as shown by histogram overlay and bar diagram of the MFI for the individual conditions (Figure 6A). Together, these results indicate that the prominent T^{reg} markers noted are not affected by MC degranulation.

The expression of suppressive mediators molecules (TGF β , IL10, Granzyme B(GzB) and EBI3 (Ebstein Bar virus Induced gene 3)) secreted by FACS-purified FoxP3-GFP T^{reg} were determined by RT-PCR. TGF β mRNA expression was reduced over 50% in T^{reg} recovered 24 h after MC degranulation but was restored at day 5 postdegranulation when compared to T^{reg} from non-degranulated controls. The mRNA levels of IL10 and GzB were nearly absent at 24 h postdegranulation, with a modest recovery of expression for IL10 at 5 days postdegranulation. EBI3, however, was not impacted by MC degranulation at 24 h postchallenge yet declined at day 5 (Figure 6B). Taken together, these data suggest that MC degranulation leads to the loss of multiple suppressive mediators utilized by T^{reg} which may account for the loss in peripheral tolerance upon degranulation.

Discussion

The studies presented significantly advance our knowledge of MC- T^{reg} interactions and how degranulation impacts on the behavior and function of T^{reg} . The data show (1) that antibody or chemically induced MC degranulation, either regionally or systemically, leads to a T-cell-dependent loss of tolerant skin allografts; (2) the 'natural' loss of tolerant allografts can be reversed by the blockade of MC

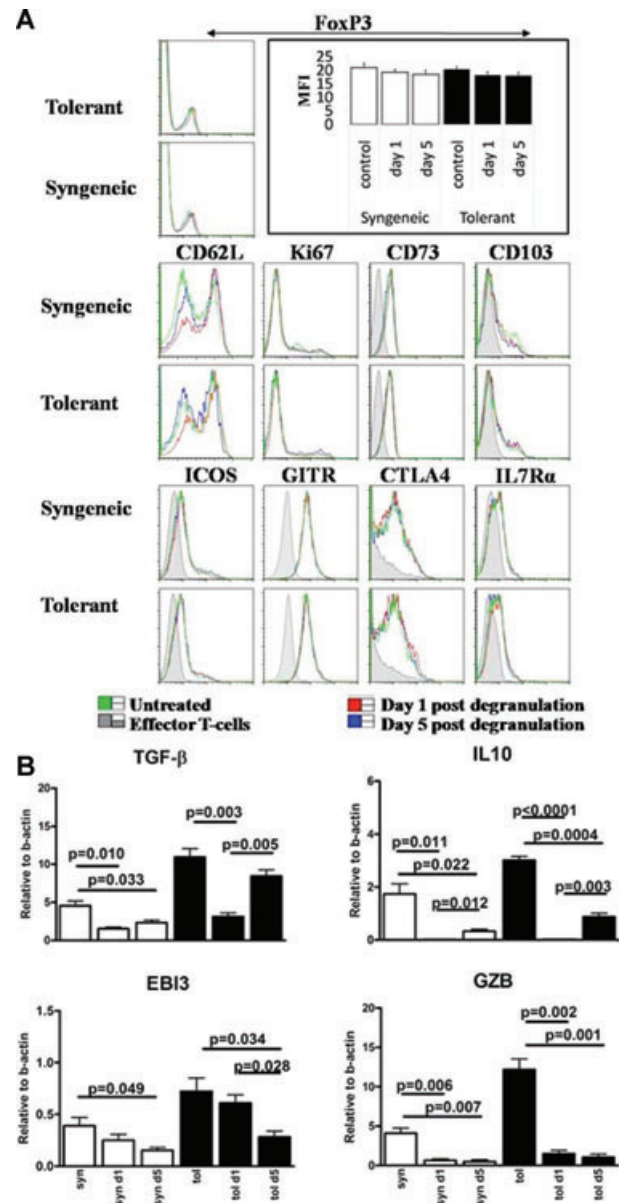


Figure 6: Degranulation blocks transcription of multiple important mediators used by T^{reg} for suppression. (A) Phenotypic analysis of T^{reg} isolated from the draining lymph nodes of treated and control mice by flowcytometry. T-cells were isolated from the draining, brachial and axillary, lymph nodes of the graft at day 1 and day 5 after degranulation, and grafting was staggered in order to analyze all samples on the same day. Single cell suspensions were stained with CD4 and FoxP3 in combination with the shown markers. Histograms shown are from CD4 $^{+}$ /FoxP3 $^{+}$ lymphocytes except for the FoxP3 histogram where all CD4 $^{+}$ cells within the lymphocyte gate are shown with the mean fluorescence intensities (MFI) of FoxP3 in the bar diagram. (B) RT-PCR for TGF β , IL10, EBI3 and GzB on flow sorted T^{reg} of the dLN of FoxP3-GFP mice 1 and 5 days after degranulation. In every experiment, mice were pooled in order to get sufficient numbers of T^{reg} . Mean and SEM are calculated from triplicate wells.

degranulation *in vivo*; (3) degranulation of MC causes a rapid change in the cellular composition of the tolerant microenvironment, with a loss in both MC and T^{reg} ; (4) regional degranulation ultimately results in the systemic breakdown of T-cell tolerance and (5) MC degranulation causes a transient loss in T^{reg} function.

Prior studies have documented the persistence of MC in tolerant allografts and their functional involvement in allograft survival. Histological examination indicated that these intragraft MC were not degranulated (6,7). Based on these observations, it was suggested that the secretion of immunosuppressive mediators by MC was critical for maintaining the allograft (6,7). In light of these findings, the impact of MC degranulation on the delicate balance of T^{reg} and T^{eff} under tolerant conditions was investigated. The various methods used for degranulation all led to acute T-cell-dependent allograft rejection.

MC-mediated graft loss could be blocked by using the MC stabilizing agent cromolyn providing additional proof that degranulation imparts an immunostimulatory impact leading to a breakdown in tolerance. Cromolyn has been effectively and safely used for over 30 years as a prophylactic treatment in allergies (24). A recent retrospective study demonstrated that blocking MC degranulation by a single application of cromolyn is effective in preventing allergies later in life. Most likely a permanent change in the composition of T-cell compartment was induced by the brief stabilization of the MC (25). Also preservation of transplant organs is prolonged by cromolyn by protecting the tissues from *ex vivo*, MC-mediated, ischemia (26,27). In the study presented, while a single application of cromolyn did not impact graft survival, a sequential treatment on day 30 and 60 postengraftment prolonged allograft tolerance over 120 days. We suspect that in the absence of MC stabilization, erosion of allograft tolerance over time as seen in this model could be partially explained by low levels of MC degranulation occurring even in the absence of allergens.

The regional or systemic delivery of allergen results in the tempered degranulation of MC with a significant proportion of MC retaining their granule content. MC degranulation resulted in the sequential egress of T^{reg} and MC from the allograft. Unresolved is the role of MC graft egress and putative LN accumulation to the process of graft rejection. However, the lack of complete degranulation of the MC upon administration of allergen may be the result of the immunosuppressive impact that T^{reg} exert on MC degranulation. Recent evidence has shown that T^{reg} can suppress MC degranulation by contact-dependent OX40-OX40L interactions as well as by a contact-independent manner through IL10 secretion *in vitro* (8,28,29). However, even in the absence of T^{reg} , MC degranulation is likely never complete since only a 50% reduction of granulated MC has been reported after C40/80 treatment (30).

It has been previously reported that the adoptive transfer of T^{reg} from untreated animals can suppress ongoing allergy (32,33). This observation suggests a functional defect in the T^{reg} compartment during allergy. No phenotypic differences were observed, and the level of FoxP3 expression was similar at all analyzed time points. This is in contrast to a recent report that during rejection, levels of FoxP3 of T^{reg} are downregulated (34). Either this could be induced by using a different transplant model or degranulation leads to a different type of rejection as normal allograft rejection based on minor histocompatibility differences. Both *in vitro* and *in vivo* analyses revealed a temporary impaired ability of the T^{reg} to suppress T^{eff} responses after degranulation. This suggests a regulatory role for MC in T^{reg} functionality. The underlying cause for the loss of T^{reg} suppression may be due to the loss of the expression of GZB as well as TGF β , and/or IL10. Many studies have examined the function of T^{reg} in atopic patients, but the findings from these studies are inconclusive (34–39). Some of the discrepancies from these studies can be explained, however, by the observation herein that there is only a brief window of time where the T^{reg} lose their suppressive function after degranulation.

The findings over the past 5 years underscore the enormous plasticity of the MC compartment and their impact on acquired immunity and acquired immune privilege (40). While MC have been extensively described in type I hypersensitivity diseases as being pro-inflammatory, it is evident that MC control the development and persistence of peripheral tolerance and immunity (6,7). Unlike T-cells, which can be decisively segregated into multiple effector and regulatory subsets, there are no defined subsets of functionally diverse MC (2). Emerging studies implicate MC as accessory cells for both T^{eff} and T^{reg} alike to mediate inflammation and suppression, respectively. The findings of this study are the first to show the reciprocal impact of MC degranulation on T^{reg} function. The implications in allergy and in peripheral tolerance are significant. The clinical relevance for the presented data in regard to allograft transplant recipients becomes evident when the data from a retrospective study in patients who received a kidney transplant are taken into consideration. Atopic individuals, in this case allergic rhinitis, with a kidney transplant show more severe and acute episodes of rejection than individuals without any history of allergy (41). These data are concordant with our observation that MC degranulation breaks peripheral tolerance systemically.

In closing, with the increasing prevalence of allergies in Western countries (42), the durability of allografts in atopic patients may suffer increasing jeopardy. Long-term MC stabilization in transplant recipients could be beneficial in maintaining intact MC and functional T^{reg} at the site of the graft and preventing temporal impairment of suppression due to degranulation. Since the regulation of T^{reg} function seems to be a one component of MC function, defining MC products leading to downregulation of suppressive

pathways in T^{reg} will be a great asset for developing new treatment strategies in a variety of diseases where either loss of suppression needs to be restored or ablated. Altogether, MC seem to be able to regulate the immune response in general and T^{reg} function in particular, either by supporting tolerance via secretion or by inducing inflammation via degranulation.

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Conflict of Interest Statement

The authors have no conflicting financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

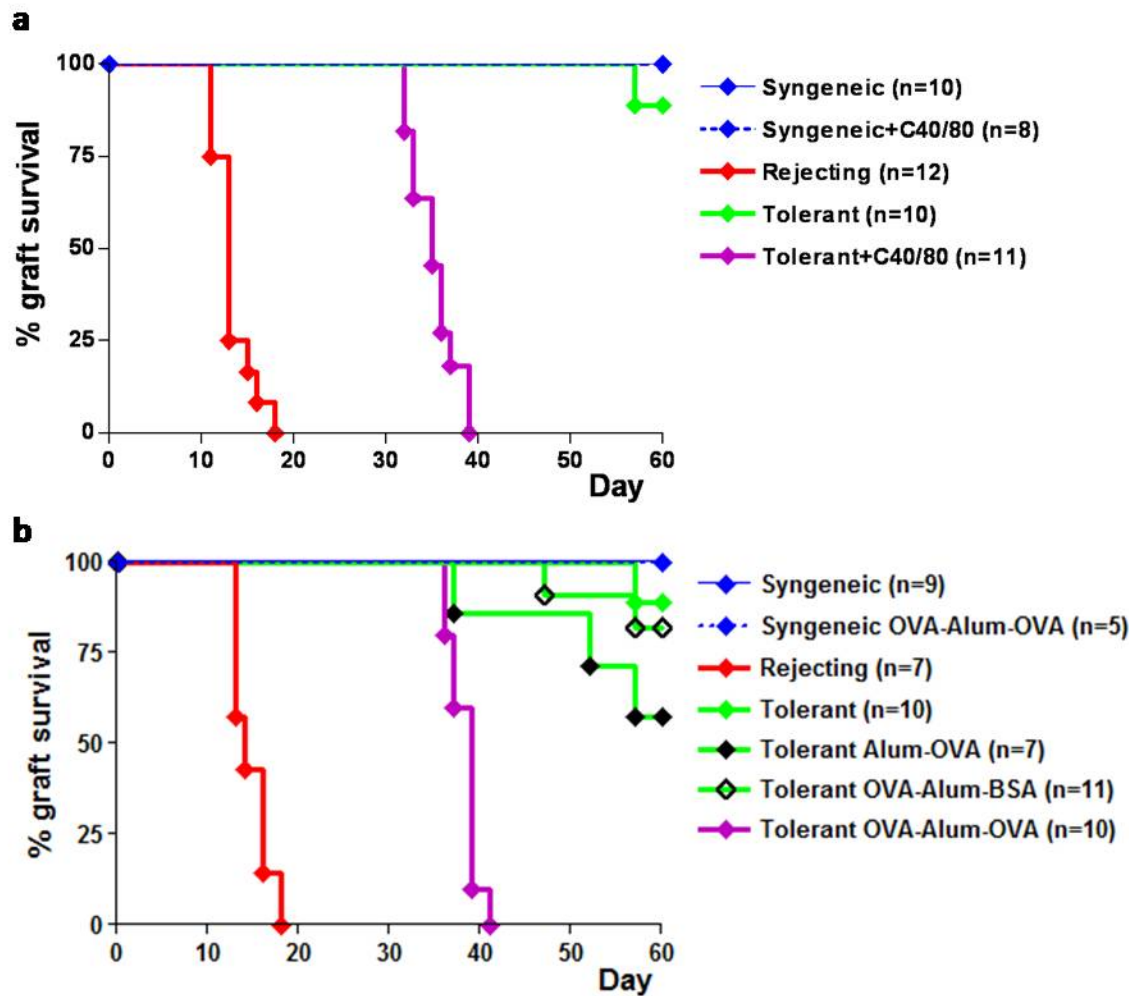
Figure S1: Degranulation by either compound 40/80 or by active immunization leads to acute rejection of established tolerant grafts.

Figure S2: Degranulation under tolerant conditions is not complete.

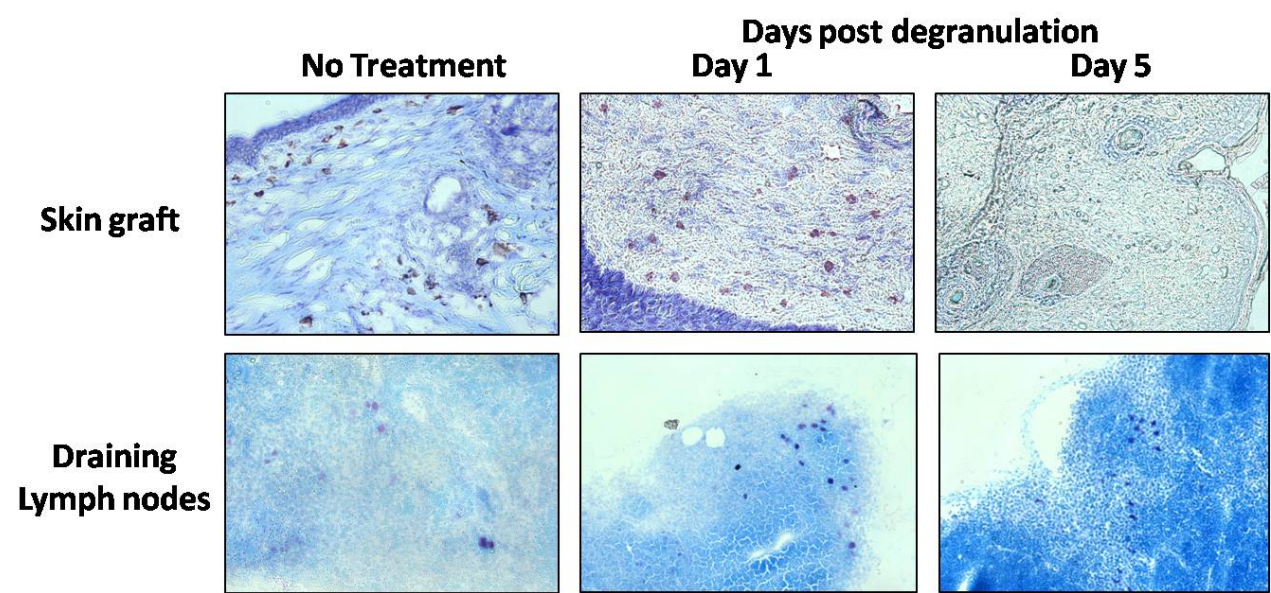
Figure S3: Chemically induced degranulation leads to a T-cell dependent break of tolerance.

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Supplementary figure 1



Supplementary figure 2



Supplementary figure 3

