Mast cell degranulation breaks peripheral tolerance

Victor C. de Vries1, Anna Wasiuk1, Kathryn A. Bennett1, Micah J. Benson1, Raul Elgueta1, Thomas J. Waldschmidt2, and Randolph. J. Noelle1

1Department of Microbiology and Immunology, Dartmouth Medical School and Norris Cotton Cancer Center, Lebanon, NH 03756
2Department of Pathology, University of Iowa, Iowa City, IA 52242

Abstract

Mast cells (MC) have been shown to mediate regulatory T-cell (T\textsuperscript{reg}) dependent, peripheral allograft tolerance in both skin and cardiac transplants. Furthermore, T\textsuperscript{reg} have been implicated in mitigating IgE mediated MC degranulation, establishing a dynamic, reciprocal relationship between MC and T\textsuperscript{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\textsuperscript{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\textsuperscript{reg} rapidly leave the graft, MC accumulate in the regional lymph node and the T\textsuperscript{reg} are impaired in the expression of suppressor molecules. Such a dramatic reversal of T\textsuperscript{reg} function and tissue distribution by MC degranulation underscores how allergy may causes the transient breakdown of peripheral tolerance and episodes of acute T-cell inflammation.

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 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\textsuperscript{reg} interactions was shown. It was reported that T\textsuperscript{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 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, CB6F_1 (C57Bl/6xBALB/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 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 two weeks later. Grafts were monitored for rejection for 30 days post-degranulation and were considered rejected when 80% of the original graft disappeared or became necrotic.

Degranulation

Chemical degranulation was done by application of 50 \( \mu \)l Compound 40/80 (1mg/ml, Sigma) directly under the graft. “Active” immunization was achieved by 100 \( \mu \)g of OVA/Alum (Pierce) i.p., 37 days prior to grafting or passive by transfer of IgE. For passive immunization 2 or 5 \( \mu \)g of either OVA-specific IgE (clone 2C6; Serotec) or TNP-specific IgE (clone A3B1; cross-reactive with NP) was given intravenously 24h prior to degranulation, respectively. Degranulation was induced either locally (50 \( \mu \)g of 1mg/ml OVA in PBS) or systemically (500 \( \mu \)l of 1mg/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 20ng of NP\(_{17}\)-OVA/NP\(_{23}\)-BSA locally. Blocking of degranulation was done by subcutaneous injection of 100 \( \mu \)l of Cromolyn Sodium Salt (39mM in PBS, Sigma-Aldrich) 30 minutes prior to degranulation.

Cytokine profile of the graft

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

Induction of inflammation

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

Tissues were snap-frozen and 8 μm sections were fixed with methanol prior to staining with αCD117-A647. Hoechst was used for nuclear staining. Slides were analyzed by confocal microscope (LSM510Meta, Zeiss) at a 100× 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 100x 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 (10mg/ml, Roche) for 45 minutes 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 FcRI(MAR-1, eBioscience) and analyzed by flow-cytometry.

**T-cell studies in vivo**

At day 27 and 35 mice were treated with 300 μl intraperitoneal and 50 μl locally at the site of the graft with mixture of αCD4(GK1.5) and αCD8(2.43) antibodies (kind gift from Dr. MJ Turk, Dartmouth, NH, USA) 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 two weeks prior to adoptive transfer of 1x10⁶ purified T-cells by i.v. injection.

**Functionality of regulatory T-cells in vitro and in vivo**

One day post-degranulation 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 carboxyfluorescin succinimidyl ester (5 μM, CFSE). In total 5x10⁴ were cultured with T-depleted irradiated Ly5.1+ splenocytes (3000rad) at different ratios of Treg:Tnaive. After 3 days CFSE dilution, profiles were analyzed within the Ly5.2+ population. For in vivo functionality, GFP+Ly5.1+ Treg were mixed at different ratios naïve, polyclonal T-cells and adoptively transferred into F1 pregrafted RAG-/- mice. Phenotypic analysis of Treg 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(MEL-14, BioLegend), CD73(clone TY/11.8, eBioscience), CD103(2E7, eBioscience), GITR(YGITR 765, BioLegend), IL7R (CD127; A7R34, eBioscience). For RT-PCR analysis FoxP3+ Treg from the grafted FoxP3-GFP mouse were flow sorted. The primers used for estimation of gene expression were: TGF-1 forward: 5’-gtgctcaagctccagagca-3’ TGF-1 reverse: 5’-ggttgttagggcaggaac-3’ IL-10 forward: 5’-ggccagccttcgc3’ IL-10 reverse: 5’-cctgtctcaacctgcctgt-3’ Ebi3 forward: 5’-AGCAGCAGCTCCTAGCGTCT-3’ Ebi3 reverse: 5’-cgagctgctctctctgag-3’ granzymeB forward: 5’-ctcaggtgctccaa-3’ granzymeB reverse: 5’-gagcatgtgtggctttaggtag-3’

**Statistics**

All statistics have been calculated using the prism 4.03 software package (GraphPad Software, San Diego California USA). 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 +/- SD whereas other data are expressed as mean +/- SEM. The latter were analyzed by one-tailed ANOVA and post-tested 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 αCD154 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 MCs had been degranulated lost their grafts. Syngeneic mice maintained their grafts for the duration of the experiment regardless of treatment(Fig.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(Supplementary fig.1A and 1B, respectively). Cytokines known to be abundant after IgE mediated MC degranulation were increased in groups where MCs had been degranulated(Fig.1C), confirming that the MC released their granular content and established a pro-inflammatory 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 agonist, LPS; the TLR9 agonist, CpG-ODN 1826; Complete Freund's Adjuvant (CFA) or an agonistic αCD40 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 αCD40 antibody, as previously reported(19). These data demonstrate that the acute rejection observed after degranulation was not due to the introduction of overt inflammation per se(Fig.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(Fig.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 post-grafting 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 (Fig. 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 (Fig. 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-KithighFcεRIhigh cells (Fig. 3B). MC numbers were confirmed by toluidine blue staining of both graft and dLN (Supplementary fig. 2). Taken together, these data suggest that upon degranulation, some MC degranulate and other MCs egress from the skin and enter the draining lymph.

**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 (Fig. 4A). Similar results were obtained using compound 40/80 to degranulate the MC (Supplementary fig. 3). 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 (Teff) and Treg 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 adoptive 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 tolerized mice after local MC degranulation rejected the allografts in the secondary recipients (Fig. 4B). These data show that MC degranulation restores alloreactive T-cell responses in a tolerized 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, tolerized 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 and rejected at the expected kinetics. Interestingly, graft #2 was also rejected after a delay of about 1-2 days (Fig. 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 allergen was not reaching the other graft. Together, these data demonstrate that local MC degranulation leads to a systemic breakdown of peripheral tolerance.

**Degranulation facilitates Treg and MC loss from the graft and impairs Treg function**

MCs and Treg are present in tolerant allografts and are required to maintain allograft tolerance (7). It was hypothesized that degranulation may change Treg composition and functionality, thereby altering graft longevity. Following degranulation, Treg 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(Fig.5A). At
this time point, MCs were still present in the allograft(Fig.3A), demonstrating that the efflux
of Treg precedes the efflux of MC from the skin allograft following MC degranulation.

In addition to the altered distribution of Treg, the in vitro and in vivo functions of Treg were
also evaluated following MC degranulation. Treg function was measured in vitro by a
suppressor assay. Treg from both tolerized, allografted mice and from those bearing
syngeneic grafts suppressed WT Teff proliferation, as measured by CFSE dye dilution.
However, Treg from mice whose MC were degranulated 1 day prior, were less effective at
suppressing Teff proliferation(Fig.5B). The impairment in Treg suppression was transient,
because Treg harvested at day 5 post-degranulation, regained their ability to suppress Teff
indistinguishably from the non-degranulated controls. These results indicate that MC
degranulation leads to a transient reduction in Treg function. Furthermore, Treg function was
also impaired after MCs were degranulated in mice bearing syngeneic grafts, suggesting this
is a general mechanism of immune regulation mediated by MC. Of note, Teff in the same
groups as tested in Fig.5B could still be suppressed by WT-Treg(data not shown).

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

**MC degranulation leads to a reduced expression of key Treg immunomodulatory molecules**

To define the underlying molecular mechanisms for the loss in Treg 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 (IL7R), 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(Fig.6A). Together, these results indicate that the
prominent Treg markers noted are not affected by MC degranulation.

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

**Discussion**

The studies presented significantly advance our knowledge of MC-Treg interactions and how
degranulation impacts on the behavior and function of Treg. 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 degranulation \textit{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 systemic breakdown in T-cell tolerance, 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\cite{6, 7}. Based on these observations, it was suggested that the secretion of immunosuppressive mediators by MC was critical for maintaining the allograft\cite{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\cite{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\cite{25}. Also preservation of transplant organs is prolonged by cromolyn by protecting the tissues from \textit{ex vivo} MC mediated ischemia\cite{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 post-engraftment 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 \textit{in vitro}\cite{28, 29, 8}. 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\cite{30}.

It has been previously reported that the adoptive transfer of T_{reg} from untreated animals can suppress ongoing allergy\cite{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\cite{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 \textit{in vitro} and \textit{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 expression of GZB as well as TGF-$\beta$ and/or IL10. Many studies have examined the function of T_{reg} in atopic patients, but the findings from these studies are inconclusive\cite{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<sup>reg</sup> 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<sup>eff</sup> and T<sup>reg</sup> 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<sup>reg</sup> function. The implications in allergy and in peripheral tolerance are significant. The clinical relevance for the presented data in regards to allograft transplant recipients becomes evident when the data from a retrospective study in patients who received a kidney transplant is taken into consideration. Atopic individuals, in this case allergic rhinitis, with a kidney transplant show more severe and acute episodes of rejection then 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<sup>reg</sup> at the site of the graft and preventing temporal impairment of suppression due to degranulation. Since the regulation of T<sup>reg</sup> function seems to be a one component of MC function, defining MC products leading to down regulation of suppressive pathways in T<sup>reg</sup> 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<sup>reg</sup> function in particular, either by supporting tolerance via secretion or by inducing inflammation via degranulation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


22. the description I had was exactly pinpointing the location as used in medicine


To induce tolerance, C57BL/6 mice were administered a regimen of donor specific transfusion (DST) of allogeneic splenocytes and anti-CD154 (200 μg i.p.). Subsequently, mice received an allogeneic skin graft and survival was monitored. These mice maintain the graft for up to 100 days post-grafting, with day 0 designated as the day the mice received the graft. MCs were degranulated in mice 30 days after engraftment receiving the tolerance-inducing regiment and an allograft, in all studies described. At day 0 mice received either an 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 μg of NP specific IgE (NP-IgE) and 24 hours later challenged with NP\textsubscript{17}-OVA or NP\textsubscript{23}-BSA, systemically (20ng/mouse intraperitoneal) or locally (2ng/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 NP\textsubscript{17}-OVA or sensitized with NP-IgE followed by local challenge with 20ng of OVA. The total number of mice pooled from multiple independent experiments is shown in brackets. C. Tolerant and syngeneic with and without degranulation were collected 18 hours after degranulation and incubated in HBSS for 1h 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 μg TLR4 or TLR9 agonist, 200 μl CFA or local by chemically induced inflammation. As positive controls agonistic CD40 and degranulation were included. Data shown is combined from two independent experiments.
Figure 2. Mast cell stabilization protects against degranulation induced rejection and prolongs graft survival in general
A. Mice were treated according figure 1A. However, 30 minutes prior to local challenge with 20ng of NP17-OVA one group received an intragraft injection of 100 μl of a 39mM 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 brackets. B. Passively immunized mice were treated locally with Cromolyn at day 30 and/or day 60 post-grafting 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 brackets 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.
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^+) shown in green. Nuclear stain with Hoegrst are shown in blue. Quantification of the number of MC in the skin was performed by counting 7 randomly chosen fields in a total of 6 individual mice. B. Flowcytometric analysis of the draining, pooled inguinal and axillary, lymph nodes. After digestion with DNAse/iberase single cell suspensions were counted and stained with cKit and FcεRI. A representative plot is showing MC in the gate after pre-enrichment with FcεRIIPE 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 is combined from 3 independent experiments with a total of 9 mice.
Figure 4. Break of peripheral tolerance by degranulation is T-cell dependent

A. Graft survival after depletion of CD4 and CD8 T-cells with \( \alpha \)CD4 and \( \alpha \)CD8 antibodies (300 \( \mu \)l intraperitoneal and 50 \( \mu \)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 is pooled from two independent experiments. 

B. RAG\(^{-/-} \) were grafted 2 weeks prior to adoptive transfer of \( 1.10^6 \) lymphocytes from the inguinal and axilliary draining LN of the indicated groups. One day after 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 is shown from in total 3 independent
In order to see whether degranulation could lead to rejection of a graft in a distant location, mice received two grafts two 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/αCD154. 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 is indicated in between squared brackets. Data is combined from multiple experiments and the total number of mice used is shown in round brackets.
Figure 5. Mast cell degranulation leads to efflux of Treg 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 6 mice from 2 independent experiments. 

B. Following degranulation of tolerized, allografted mice, Ly-5.2+ Treg were harvested 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 (Teff cells), expressing Ly5.1 were purified and labeled with CFSE. FACS-purified Ly5.2 Treg based from either degranulated or non-degranulated hosts were mixed at different ratios with Teff cells, and Treg 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. Treg were purified from tolerized, allografted FoxP3+FACS-purified based on GFP expression. Treg from tolerized controls or degranulated, tolerized groups were FACS purified based on GFP expression. Treg from each of these groups were mixed at ratios with WT, Teff (Teff, Treg) to be used in an in vivo suppressor assay. RAG-/- mice were grafted 2 weeks prior to adoptive transfer of in total 1.10^6 lymphocytes. Grafts were monitored for rejection for 60 after transfer of the T-cells. Data is pooled from 3 independent experiments with the total number of mice shown in brackets.
Figure 6. Degranulation blocks transcription of multiple important mediators used by Treg for suppression

A. Phenotypic analysis of Treg 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 sample 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 TGFβ, IL10, EBI3 and GzB on flow sorted Treg
of the dLN of FoxP3-GFP mice one and five days after degranulation. In every experiment mice were pooled in order to get sufficient numbers of Treg. Mean and SEM are calculated from triplicate wells.