

Mast Cell Interleukin-2 Production Contributes to Suppression of Chronic Allergic Dermatitis

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SUMMARY

The incidence of chronic allergic dermatitis is rapidly increasing. Regulatory control of this disease has not been adequately explored. Here we report that mast cell-derived interleukin-2 (IL-2) contributes to the suppression of chronic allergic dermatitis. Mice deficient in IL-2 production, or deficient in mast cells (*Kit^{W-sh/W-sh}*), showed exacerbated dermatitis upon repeated oxazolone challenge when compared to their wild-type counterparts. Adoptive transfer of wild-type, but not *Il2^{-/-}*, mast cells into *Kit^{W-sh/W-sh}* mice dampened the inflammatory response. During the course of disease, mast cell expansion occurred at the site of inflammation and also in the spleen, where production of IL-2 by mast cells was markedly enhanced. In the absence of mast cell IL-2 production, the ratio of activated to regulatory T cells at the site of inflammation was increased. Thus, MC-derived IL-2 contributes to the maintenance of suppression in chronic allergic skin inflammation.

INTRODUCTION

Eczematous dermatitis is a chronic inflammatory skin disease, associated with cutaneous hyperreactivity, which usually occurs in individuals with an allergic background (also referred to as atopic dermatitis [AD]). One of its most striking features is the rising incidence during the past two decades, currently approaching 15%–30% of children at some point during childhood (Bieber, 2008; Schultz Larsen et al., 1996). Both genetic and environmental factors contribute to the development of AD (Bieber, 2008; Leung et al., 2007), but the underlying mechanisms by which such factors contribute to the pathogenesis of dermatitis are poorly defined.

Animal models have been valuable in studying the mechanisms underlying the development and severity of dermatitis. Current models can be divided into three groups consisting of spontaneous dermatitis, genetically engineered mice, and hapten-induced inflammation (Jin et al., 2009). In this latter category, haptens such as oxazolone (Matsumoto et al., 2004) and dinitrofluorobenzene (DNFB) (Phanuphak et al., 1974) are used to

induce skin disease in a convenient and reproducible manner. Oxazolone induces a T cell-dependent allergic contact hypersensitivity with mast cell (MC) involvement (Evans et al., 1971; Pritchard and Micklem, 1972) but in the course of repeated challenges, it evolves into a chronic allergic inflammatory response similar to human AD (Matsumoto et al., 2004). In hairless mice (*hr/hr*), chronic treatment with oxazolone results in dermatitis that demonstrates barrier abnormalities, a T helper 2 cell (Th2 cell)-predominant inflammation, MC- and eosinophil-rich infiltrates, as well as elevated IL-4 and IgE (Man et al., 2008), all hallmarks of human AD. However, the mechanistic roles played by some of these features in the chronicity of disease are unexplored.

MCs can enhance inflammation not only in allergic diseases (Kalesnikoff and Galli, 2008) but also in autoimmune models such as experimental autoimmune encephalomyelitis (Secor et al., 2000). Alternatively, they can act as suppressors as demonstrated in their contribution to skin allograft tolerance (Lu et al., 2006). MCs can also exert stimulatory or inhibitory effects on both effector and regulatory T (Treg) cells (Galli et al., 2008; Gri et al., 2008; Hershko and Rivera, 2010; Mekori and Metcalfe, 1999). The potential mediators of MC-T cell communication are numerous and include secreted cytokines, chemokines, leukotrienes, and histamine as well as varied surface molecules (Bachellet and Levi-Schaffer, 2007; Hershko and Rivera, 2010; Nakae et al., 2006). The putative anatomical sites where the interaction between these two cell types takes place are also varied and may include such tissues as the skin (Lu et al., 2006), draining and remote lymph nodes (LN) (Gri et al., 2008), and spleen as well as other sites that harbor these cells.

The expanding prevalence of AD and the considerable gaps in understanding its etiology and the regulation of its severity prompted us to investigate the strategic players that govern disease intensity. Here we report that MCs are protective in late stages of oxazolone-induced chronic allergic dermatitis. Protection occurs by a previously unrecognized regulatory mechanism in which MC-derived IL-2 is required to maintain the appropriate ratio of activated T cells to Treg cells at the site of inflammation during the chronic phase of disease.

RESULTS

IL-2 Controls the Severity of Chronic Allergic Dermatitis

Induction of oxazolone-induced chronic allergic dermatitis in mice recapitulates many of the characteristics of human AD

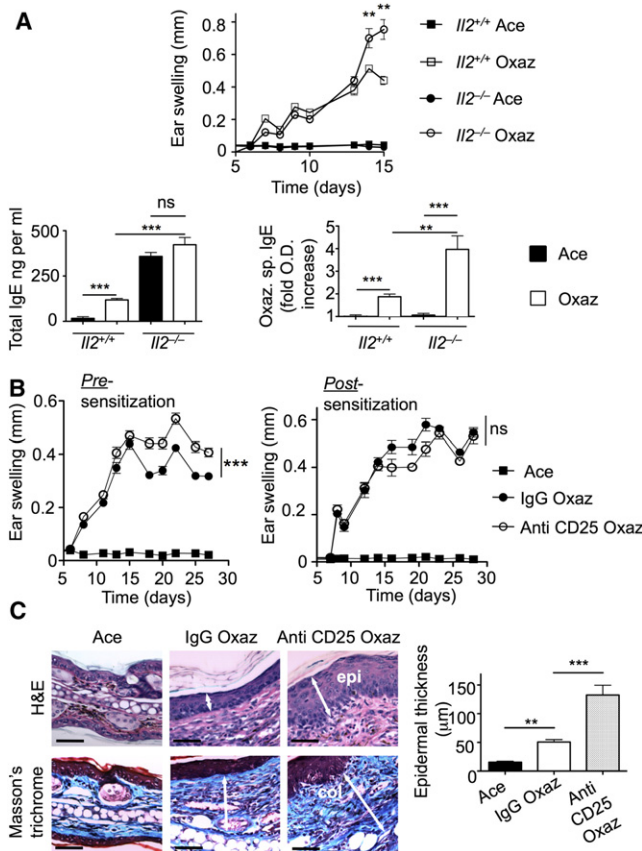


Figure 1. IL-2 Is Important in Suppression of Oxazolone-Induced Chronic Dermatitis

(A) Ear swelling during induction of disease in 8- to 10-week-old *IL2*^{-/-} and *IL2*^{+/+} mice (*n* = 4 mice/group) (top). Total IgE levels (bottom left) and oxazolone-specific (Oxaz sp) IgE (bottom right) as measured in the serum by ELISA.

(B) Disease course in WT C57BL/6 mice that were injected with anti-CD25 presensitization on day -1 (*n* = 5 mice/group) or postsensitization on day 7 (*n* = 4). Data are reported beginning at the day of challenge where detectable ear swelling might be expected. Each experiment was performed twice.

(C) Sections of ear tissue (left) stained as indicated and quantitation of epidermal thickening (right). Abbreviations: epi, epidermis; col, collagen. Scale bars represent 50 μm.

Statistical analysis was by two-way ANOVA (B) or by a two-tailed Student's *t* test (A and C [right]). Results are means ± SEM. ***p* < 0.01; ****p* < 0.001. See also Figure S1.

(Leung et al., 2004), which prompted us to use this model as a tool for investigating regulatory processes.

IL-2 is a key cytokine for Treg cell function (Malek and Bayer, 2004), which has been implicated in the control of allergic disease, particularly asthma (Boudousquie et al., 2009; Curotto de Lafaille et al., 2008; Wilson et al., 2008). *IL2*^{-/-} mice were used as an initial tool to investigate the regulatory features in control of chronic allergic dermatitis. On a C57BL/6 background, these animals exhibit a 40% reduction in Treg cells (Zheng et al., 2007) and have a profound impairment in Treg cell function (Malek and Bayer, 2004). In contrast to *Foxp3*^{sf} mice, the *IL2*^{-/-} mice have a longer life span, do not suffer from spontaneous dermatitis, and are thus a preferable model for hapten-induced

disease. When exposed to oxazolone, severe ear swelling was noted in *IL2*^{-/-} mice (Figure 1A, top). Hyperresponsiveness was accompanied by elevated total IgE titers and overproduction of oxazolone-specific IgE (Figure 1A, bottom). Given the known spontaneous manifestation of inflammation in these genetically altered mice (Sadlack et al., 1995), we confirmed the role of IL-2 through administration of a CD25 monoclonal antibody (Kohm et al., 2006). This antibody binds to the alpha chain of the IL-2 receptor (IL-2R), which is constitutively expressed on Treg cells, and causes transient inactivation (Kohm et al., 2006). Injection of anti-CD25 prior to sensitization with oxazolone resulted in enhanced inflammation at a late stage of the disease relative to the control injection of isotype identical IgG. This was manifested as a sustained increase in ear thickness (Figure 1B, left), at a range previously reported as physiologically significant (Grimbaldeston et al., 2007), as well as expansion of dermatitis to the adjacent skin (not shown). When antibodies were administered 1 week after sensitization, only a minimal effect on disease course was observed (Figure 1B, right). Examination of tissue sections disclosed that oxazolone-induced epidermal hyperplasia and collagen deposition are considerably enhanced after anti-CD25 administration (Figure 1C). Collectively, these experiments indicate that IL-2 is vital to the control of oxazolone-induced dermatitis.

MCs Suppress Oxazolone-Induced Dermatitis

We explored the role of MCs in the oxazolone-induced dermatitis by using MC-deficient *Kit*^{W-sh/W-sh} mice (Grimbaldeston et al., 2005). After five challenges with oxazolone, ear swelling was markedly increased in *Kit*^{W-sh/W-sh} mice, compared to their WT counterparts (Figure 2A, left). Adoptive transfer of WT bone marrow-derived MCs into the ear skin of *Kit*^{W-sh/W-sh} mice resulted in reduced ear swelling (Figure 2A, right), demonstrating that MCs dampened inflammation. Similarly, disease suppression was observed after the administration of IL-2-anti-IL-2 complexes, a manipulation known to increase the bioavailability of IL-2 and to promote Treg cell expansion (see Figure S1 available online; Kamimura et al., 2006; Wilson et al., 2008). Histological analysis of tissues from *Kit*^{W-sh/W-sh} mice revealed that, after intradermal (i.d.) reconstitution with MCs, engraftment occurs in the skin of the ear as well as in the cervical draining LN and spleen (Figures 2B and 2C). No MCs were found in LNs or spleens of unreconstituted *Kit*^{W-sh/W-sh} mice, but an occasional MC was found in ear skin, in mice with dermatitis (data not shown). After reconstitution, the distribution of MCs was similar to that of WT mice, although more MCs were found in the LNs of *Kit*^{W-sh/W-sh} relative to WT mice while the latter mice had more MCs in the ear (Figure S2). In both types of mice, induction of dermatitis caused an accumulation of MCs in the (ear) skin and an apparent redistribution of MCs from the subcapsular sinus to the LN cortex (Figure 2B). In reconstituted *Kit*^{W-sh/W-sh} mice, no MCs were found in tissues far from the site of inflammation, like a distal (brachial) LN or in the stomach (Figure 2C). However, MCs were observed in the spleens of unchallenged WT mice and i.d. reconstituted *Kit*^{W-sh/W-sh} mice, and for both, their numbers increased after oxazolone treatment. This accumulation was intriguing because of the fact that the spleen did not show dynamic changes in total cell numbers or even in CD4⁺ T cells, with the exception of a modest but distinct increase in Treg cells

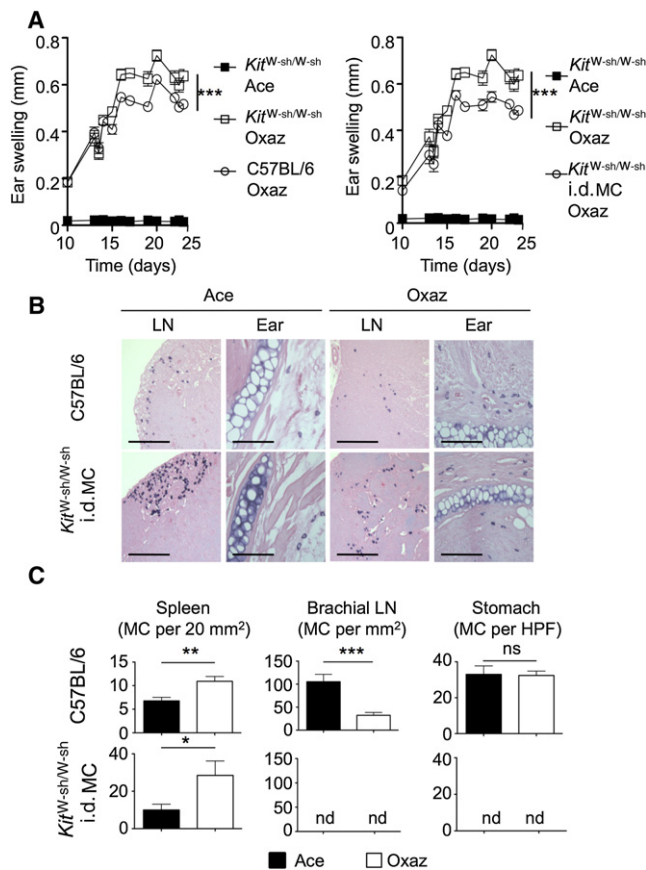


Figure 2. MCs Suppress Dermatitis and Expand in the Spleen
(A) Comparison of ear swelling between *Kit^{W-sh/W-sh}* ($n = 7$ mice/group) and C57BL/6 (WT) mice ($n = 6$) (left) or *Kit^{W-sh/W-sh}* mice i.d. injected with C57BL/6-derived MCs ($n = 5$) (right). Graphs are from the same experiments but presented individually in comparison to WT for clarity. Two independent experiments were performed. Statistical analysis was by two-way ANOVA.
(B) Toluidine blue staining for MC in the ear skin and draining cervical lymph node (LN). Tissues were harvested from WT and *Kit^{W-sh/W-sh}* mice i.d. injected with WT MCs after full treatment with oxazolone. Scale bars represent 200 μ m.
(C) Quantitation of MC numbers in tissues distal to the inflammatory site. All tissues were collected after completion of chronic dermatitis induction. HPF, high power field, $\times 200$.
Statistical significance was determined by a two-tailed Student's t test. Results (A and C) are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See also Figure S2.

(Figure S2). Because MCs were introduced only into the ears of *Kit^{W-sh/W-sh}* mice, this finding argues that MCs migrate from the skin to the draining LN and then to the spleen but not to the brachial LN or the stomach. This view was further supported by i.d. injection of small numbers of GFP-expressing BMDCs in the ear skin of *Kit^{W-sh/W-sh}* mice, because these MCs were found to migrate to the draining LNs and the spleen with concomitant disease suppression (Figure S3).

Dermatitis Is Enhanced by the Spleen and Dampened by Splenic MCs

The observation of increased MC recruitment to the spleen (Figure 2C) upon oxazolone challenge of WT or i.d. reconstituted

Kit^{W-sh/W-sh} mice led us to explore the relevance of the spleen and of MCs within it to disease regulation. Mice skewed toward Th1 (C57BL/6) or Th2 (BALB/c) cell responses were assessed for accumulation of MCs in the spleen upon oxazolone challenge. As shown in Figure S4A, C57BL/6 or BALB/c mice developed a similar response to oxazolone challenge, although BALB/c mice had a more rapid onset. Both strains of mice showed marked changes in MC numbers in the ear skin and the draining LN after oxazolone challenge (Figures S4B and S4C). Although the absolute numbers of spleen-resident MCs differed considerably in the two strains (Figure S4D), oxazolone challenge increased their numbers by approximately 3- to 5-fold in both strains (Figure S4E). This shows that oxazolone-induced dermatitis followed a similar pattern in both C57BL/6 and BALB/c mice and caused an increase of MCs in the spleen of both strains.

To explore the role of the spleen in the course of disease, wild-type (C57BL/6) mice were splenectomized and challenged with oxazolone. These mice developed a mild allergic dermatitis relative to their WT controls (Figure 3A) and were resistant to pre-sensitization injection of anti-CD25 (Figure 3A). These findings demonstrated that the spleen enhances the inflammatory response and that the increased inflammation that results from blocking the IL-2 receptor also requires the spleen. Conversely, splenectomy did not affect the response of draining LNs (Figure 3B) and oxazolone-specific IgE production (Figure S5A) upon oxazolone challenge and administration of anti-CD25. Likewise, CD4⁺ T cell and Treg cell proliferation in the LN were not affected by the absence of a spleen (Figure S5B). To directly address whether the suppression mediated by MCs was independent of their localization to the skin, we adoptively transferred MCs into *Kit^{W-sh/W-sh}* mice by intravenous (i.v.) injection. As previously described (Grimbaldeston et al., 2005), the i.v. introduction of MCs failed to reconstitute the ear pinna and these cells were not recruited to the ear after oxazolone challenge (Figure 3C), whereas the spleen of i.v. injected mice showed large numbers of MCs relative to their WT counterparts (Figure 3C). When challenged, *Kit^{W-sh/W-sh}* mice i.v. reconstituted with MCs showed a marked reduction in dermatitis relative to MC-deficient *Kit^{W-sh/W-sh}* mice (Figure 3D), as well as milder epidermal hyperplasia (Figure S6). Furthermore, splenectomized *Kit^{W-sh/W-sh}* mice mounted an attenuated response to oxazolone, with no additional alleviation after MC reconstitution (Figure 3E). In this experiment, however, we introduced MCs i.d. to ensure engraftment, because i.v. injected cells showed a strong predilection for the spleen and its removal altered reproducible engraftment. Regardless, the findings demonstrate that the spleen contributes to the inflammation induced by oxazolone challenge and that the presence of MCs in the spleen is associated with suppression of chronic allergic dermatitis.

IL-2R Blockade Enhances IgE-Dependent Accumulation of Splenic MCs

Having found that pre-sensitization treatment with anti-CD25 inhibits regulation of disease (Figure 1B), we examined the impact of this manipulation on the expansion of MCs in the spleen. Consistent with our previous result (Figure 2C), MC numbers in the spleen increased with oxazolone treatment and were further elevated when IL-2R was inactivated by anti-CD25 treatment (Figure 4A). Oxazolone-specific IgE also increased in a similar

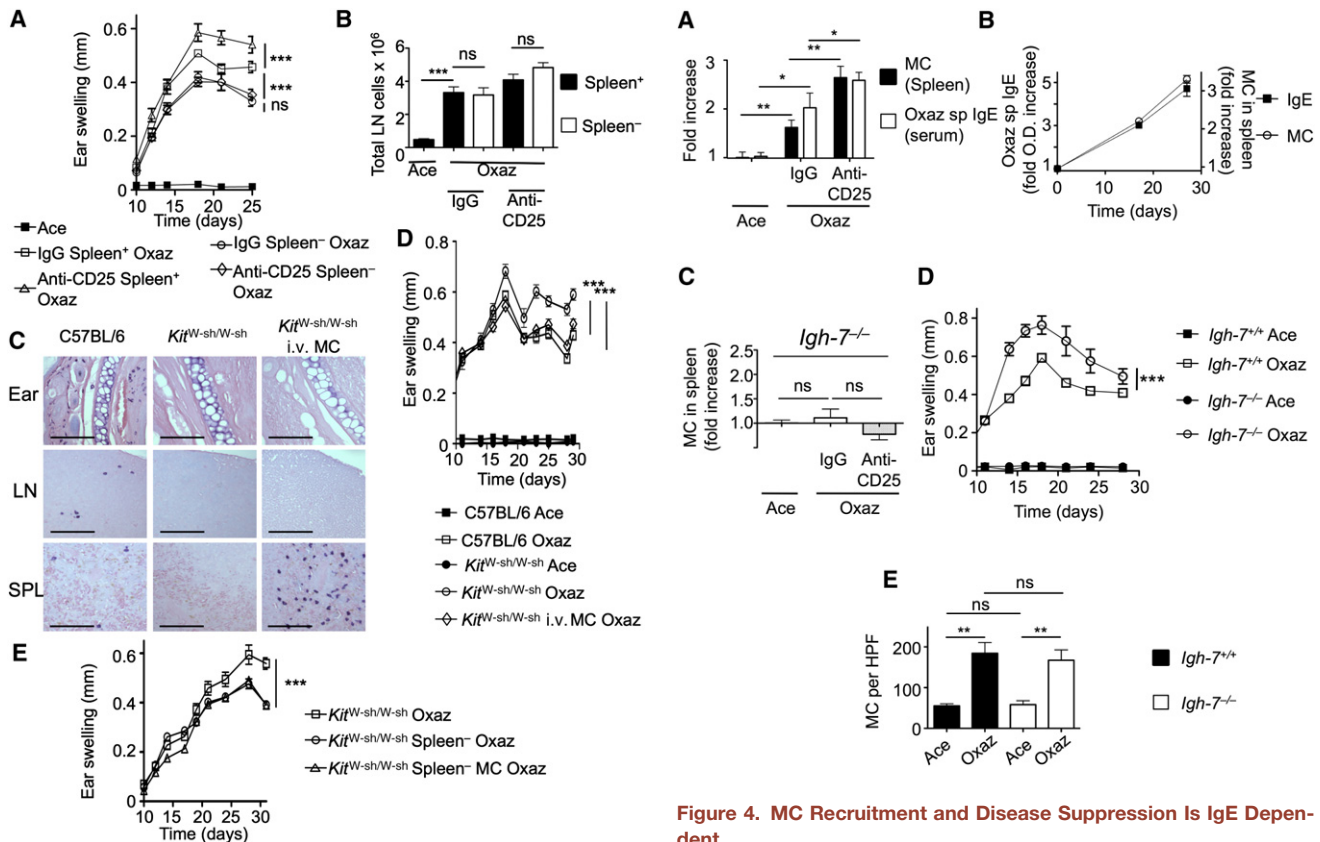


Figure 3. The Spleen Exacerbates Dermatitis and Facilitates Anti-CD25 Enhancement or MC Suppression of Disease

(A) Splenectomized (Spleen⁻) C57BL/6 (WT) mice (n = 5) were treated with either control IgG or anti-CD25 and with oxazolone to induce dermatitis. Ear swelling was compared to nonsplenectomized (Spleen⁺) littermates. (B) Draining cervical LNs were harvested for evaluation of total cell numbers. The LNs were extracted from normal and splenectomized mice shown in (A) after full treatment with acetone or oxazolone. (C) MCs were introduced through the tail vein prior to induction of oxazolone dermatitis, and tissues from the inflamed ear, draining lymph nodes (LN), and spleens (SPL) were stained with toluidine blue. Scale bars represent 100 μ m. (D) Disease course in WT, *Kit^{W-sh/W-sh}*, or *Kit^{W-sh/W-sh}* mice after i.v. injection of MCs (n = 5). Two independent experiments were conducted. (E) Comparison of ear swelling between *Kit^{W-sh/W-sh}* mice and *Kit^{W-sh/W-sh}* mice after splenectomy or splenectomy and i.d. MC reconstitution (n = 5). Statistical analysis was done by a two-way ANOVA test (A, D, and E) or by a two-tailed Student's t test (B). Results are means $\pm$ SEM. ***p < 0.001. See also Figure S3.

manner. Analysis of oxazolone-specific IgE in the spleen prior to, midcourse, and during the full course of disease revealed a time-dependent increase that mirrored the increase of MCs in the spleen (Figure 4B). To assess whether IgE was important for the increased numbers of MCs in the spleen, we challenged the IgE-deficient (*Igh-7^{-/-}*) mice with oxazolone in the presence of active or inactive (anti-CD25-treated) IL-2R. As shown in Figure 4C, accumulation of MCs in the spleen was absent in challenged *Igh-7^{-/-}* mice and the administration of anti-CD25 did not substantially change MC numbers, demonstrating that IgE production was required for MC accumulation in the spleen. Moreover, oxazolone challenge of *Igh-7^{-/-}* mice resulted in

Figure 4. MC Recruitment and Disease Suppression Is IgE Dependent

(A) Comparison between MC recruitment to the spleen (n = 6) and oxazolone-specific (Oxaz sp) IgE levels in the serum (n = 4) of C57BL/6 mice treated with oxazolone. Dermatitis was induced after presensitization treatment of mice with either control IgG or anti-CD25. (B) Kinetics of IgE and MC accumulation in the spleens of BALB/c mice, as measured on days 0, 17, and 27 of oxazolone dermatitis (n = 4). (C) MC numbers in the spleens of control (acetone) or oxazolone-challenged IgE-deficient (*Igh-7^{-/-}*) in the absence (IgG only) or in the presence of anti-CD25 (n = 5). (D) Disease course in *Igh-7^{-/-}* mice, as compared to C57BL/6 wild-type controls (*Igh-7^{+/+}*) (n = 5). (E) Quantitation of MCs in the ears of the mice in (D). Three independent experiments were performed. Statistical analysis was by a two-tailed Student's t test (A, C, and E) or by two-way ANOVA (D). Results are means $\pm$ SEM. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S4.

more severe eczematous dermatitis than seen in *Igh-7^{+/+}* mice (Figure 4D). However, unlike the spleen, MC accumulation in the ear was equal in both *Igh-7^{+/+}* and *Igh-7^{-/-}* mice (Figure 4E), suggesting that MC numbers in the ears are not associated with disease suppression. Collectively, the findings show that the IgE-dependent recruitment of MCs to the spleen is associated with disease suppression.

Chronic Oxazolone-Challenged *Kit^{W-sh/W-sh}* Mice Are Hypersensitive to IL-2R Blockade

Given the importance of IL-2 in regulating the severity of the dermatitis, we investigated whether IL-2 function might be altered in *Kit^{W-sh/W-sh}* mice. Treg cells are highly dependent on IL-2 and analysis of cells from the spleen, LN, and ear of unchallenged *Kit^{W-sh/W-sh}* mice revealed no significant difference in the

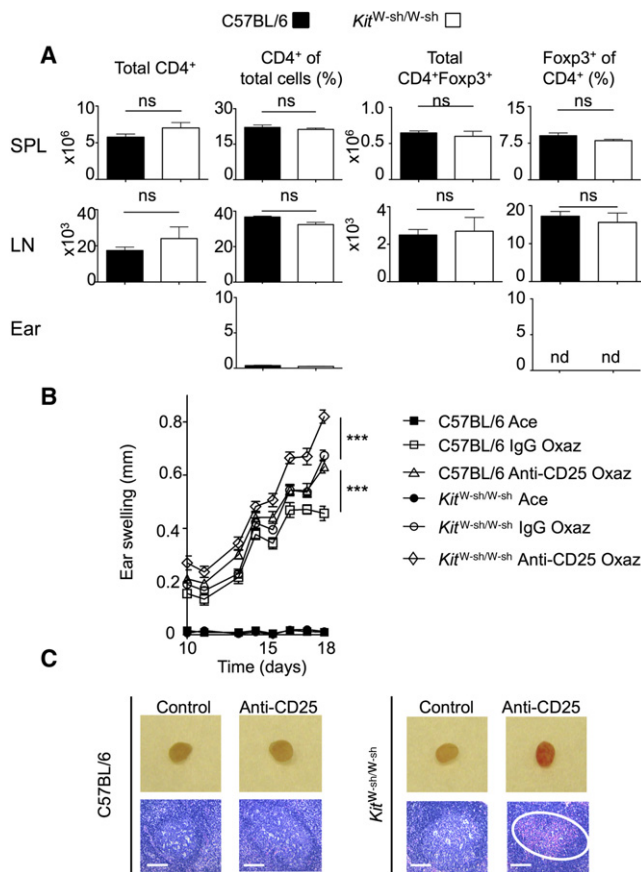


Figure 5. MCs Cooperate with Treg Cells in Disease Suppression
(A) Flow cytometric analysis of total cell populations extracted from tissues and stained for CD4 and Foxp3 (SPL, spleen; LN, cervical lymph node). Statistical analysis was by a two-tailed Student's *t* test. ns, not significant; nd, not detectable.
(B) Ear swelling in C57BL/6 and *Kit^{W-sh/W-sh}* treated with oxazolone after pre-sensitization injection with either control IgG or anti-CD25 (*n* = 5). Data are presented as mean ± SEM; statistical significance was determined by a two-way ANOVA, ****p* < 0.01.
(C) Representative draining lymph nodes from mice with oxazolone dermatitis and corresponding H&E-stained sections. The circle indicates hemorrhage within the lymph node. Scale bars represent 50 μm.
See also Figure S5.

total CD4⁺ cells or in the proportion of CD4⁺Foxp3⁺ Treg cells when compared to unchallenged WT control mice (Figure 5A). Few CD4⁺ cells were found in the ears of unchallenged control and *Kit^{W-sh/W-sh}* mice and Treg cells were not detectable in the ear (Figure 5A). This observation indicated that Treg cell homeostasis in naive animals is independent of MCs, implying no defect in IL-2 homeostasis. However, this did not rule out the possibility that under conditions of repeated oxazolone challenges, MCs are required to maintain adequate IL-2 production. In order to assess the extent of IL-2 function in the absence of MCs, *Kit^{W-sh/W-sh}* and control WT mice were injected with anti-CD25 and subsequently challenged with oxazolone. As shown in Figure 5B, WT mice treated with anti-CD25 showed a strikingly similar profile of disease intensity to MC-deficient *Kit^{W-sh/W-sh}* mice. However, when *Kit^{W-sh/W-sh}* mice were treated with anti-

CD25 and were subsequently challenged with oxazolone, an exaggerated disease was observed when compared to the *Kit^{W-sh/W-sh}* mice injected with control IgG or control WT mice injected with anti-CD25. Occasionally, the cervical draining LNs in this group of mice became hemorrhagic and necrotic (Figure 5C), reflecting the physiological severity of the inflammatory response. Thus, although MCs do not appear to be required for Treg cell homeostasis in naive animals, at the chronic stage of dermatitis, MCs appear to support IL-2-mediated Treg cell responses. To assess this possibility, we quantitated the numbers of Foxp3⁺ cells in the ear skin of oxazolone-challenged *Kit^{W-sh/W-sh}* mice or MC-reconstituted *Kit^{W-sh/W-sh}* mice. Whereas no Foxp3⁺ cells were observed in the ear skin of unchallenged *Kit^{W-sh/W-sh}* mice (Figure 5A), oxazolone challenge led to recruitment of Foxp3⁺ cells in the ear skin and reconstitution of *Kit^{W-sh/W-sh}* mice with MCs increased the numbers of Treg cells at this site (Figure S7A). These findings argue that MCs contribute to Treg cell-mediated suppression of chronic allergic dermatitis at a late stage of disease.

MC IL-2 Production Supports Treg Cell-Mediated Disease Suppression

Given that MCs were necessary to promote disease suppression and caused increased Treg cell numbers at the site of inflammation, we examined the hypothesis that MCs could mediate suppression by providing IL-2 to control the balance between Treg cells and activated T cells in the context of oxazolone-induced chronic dermatitis. FcεRI stimulation of MCs induced significant levels (50–100 pg/ml) of IL-2 secretion in both BMMCs and peritoneal-derived cultured MCs (PDMCs) (Figure 6A). PMA + ionomycin stimulation was also a potent inducer of MC IL-2 secretion, demonstrating that MCs have the capacity to produce copious amounts of IL-2. To confirm these findings in vivo, we analyzed expression of IL-2 in splenic MCs from mice with dermatitis by flow cytometry (Figure 6B). Importantly, immunofluorescent staining (Figure 6C) of spleen sections from i.v. reconstituted *Kit^{W-sh/W-sh}* mice, via either *Il2^{+/+}* or *Il2^{-/-}* MCs, formally demonstrated the in situ production of IL-2 by MCs in the spleen (Figure 6C). Further quantitative analysis revealed that the total numbers of splenic MCs producing IL-2 was markedly increased during the course of disease (Figure 6D). In contrast, the numbers of splenic basophils (CD117⁺FcεRI⁺CD49b⁺) decreased upon oxazolone challenge (data not shown), ruling out these cells as a confounding factor in the accumulation of MCs in the spleen.

To directly assess the role of splenic MC IL-2 production in controlling the severity of disease *Kit^{W-sh/W-sh}* mice, reconstituted i.v. with MCs from *Il2^{-/-}* or from WT littermates were challenged with oxazolone. In contrast to the mice carrying WT MCs, the loss of IL-2 production by MCs resulted in an increased severity of disease (Figure 7A). We hypothesized that this may be due to a compromised balance of activated T cells to Treg cells relative to the general CD4⁺ response. Analysis of cells from tissues of mice with dermatitis disclosed no difference in CD4⁺, CD4⁺Foxp3⁺, and the activated- to regulatory-T cell ratio (Tact cell:Treg cell), in the spleen and lymph node, for WT mice or *Kit^{W-sh/W-sh}* mice carrying IL-2-producing or -deficient MCs (Figure 7B). In mice with *Il2^{-/-}* MCs, there was also no difference in the proportion of CD4⁺ T cells in the ear. Nonetheless, a decrease

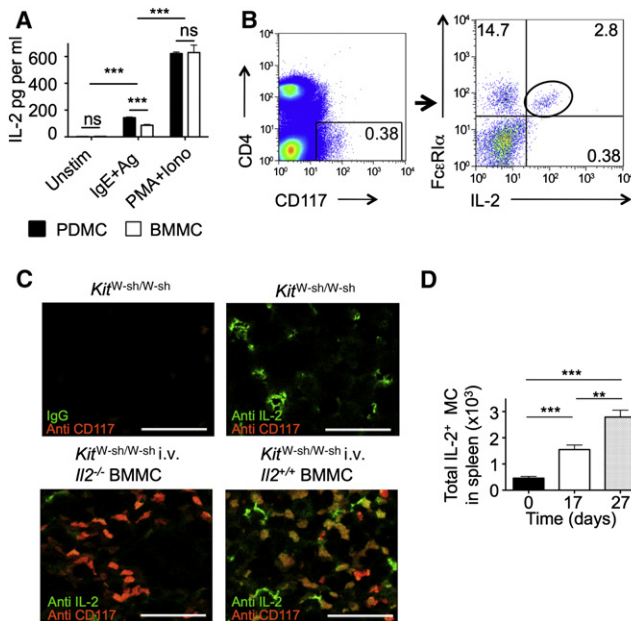


Figure 6. Oxazolone Dermatitis Increases MC-IL-2 Production In Vivo

(A) Quantitation of IL-2 production by in vitro stimulation of peritoneal- and bone-marrow-derived MC (PDMC and BMMC, respectively) after FcεRI or PMA + ionomycin stimulation. IL-2 was measured by ELISA.

(B) Flow cytometric detection of IL-2⁺ MCs in the spleen of BALB/c mice with oxazolone dermatitis. This strain was preferred for cytometry over C57BL/6 because of the higher numbers of splenic MCs.

(C) Immunofluorescent detection of IL-2 production in *Kit*^{W-sh/W-sh} mice reconstituted (i.v.) with BMMCs from *Il2*^{+/+} and *Il2*^{-/-} mice. Double staining was done with anti-IL-2 (green) and anti-CD117 (Kit) (red), as indicated. Scale bars represent 30 μm.

(D) Total numbers of IL-2⁺ MCs was determined in spleens harvested from BALB/c mice on days 0, 17, and 27 of oxazolone-induced dermatitis (n = 4). Data are means ± SEM and significance was determined by a two-tailed Student's t test (A) or by two-way ANOVA (D); **p < 0.01; ***p < 0.001. See also Figure S6.

in the percent of CD4⁺Foxp3⁺ cells relative to total CD4⁺ cells was observed with a significant (p < 0.05) increase in the ratio between activated (CD4⁺CD5⁺CD69⁺) T cells and Treg cells (Figure 7B). Taken together, these data indicate that, in the absence of MC IL-2, Treg cell expansion is sluggish compared to T cell activation and CD4⁺ T cell proliferation, thus creating an imbalance between the regulatory and effector responses. Histological analysis confirmed comparable engraftment in the spleens of the recipients of *Il2*^{+/+} and *Il2*^{-/-} MCs (Figure S7B). These findings demonstrate a previously unappreciated role for MCs, and their IL-2-producing ability, in controlling the appropriate ratio of activated T cells:Treg cells at the site of inflammation during chronic allergic dermatitis.

DISCUSSION

There is increasing evidence of the importance of MC-T cell crosstalk in regulating the immune response. MC effector activity is suppressed by Treg cells (Gri et al., 2008) and Treg cells can also use MCs as mediators of immune suppression

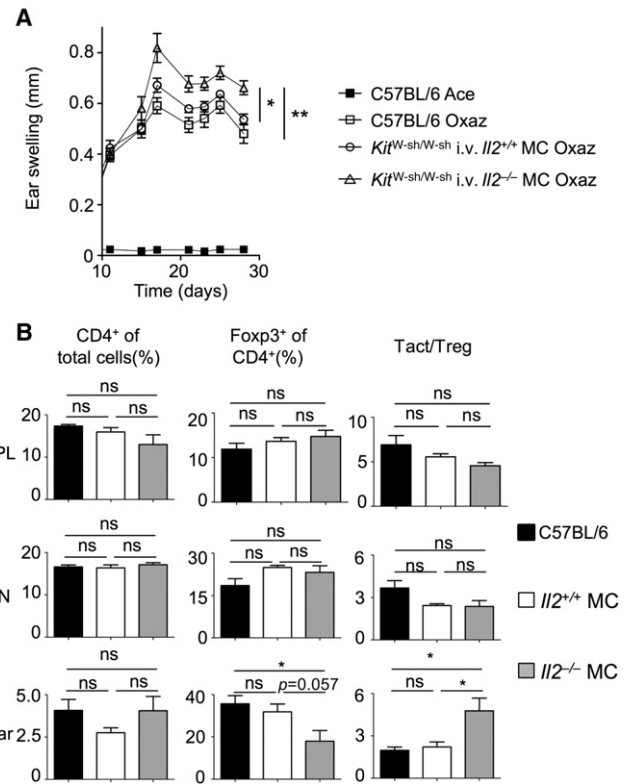


Figure 7. Splenic MC IL-2 Production Controls the Ratio of Activated T Cells:Treg Cells and Is Needed for Late-Stage Suppression of Dermatitis

(A) Ear swelling in C57BL/6 mice or *Kit*^{W-sh/W-sh} mice reconstituted with *Il2*^{+/+} or *Il2*^{-/-} MCs via an i.v. injection (n = 5).

(B) FACS analysis of total cell populations from tissues of *Kit*^{W-sh/W-sh} mice after i.v. injection with either *Il2*^{+/+} or *Il2*^{-/-} MCs and induction of oxazolone dermatitis (day 30). Activated T (Tact) cells are CD4⁺CD5⁺CD69⁺; regulatory T (Treg) cells are CD4⁺Foxp3⁺.

Data are means ± SEM and significance was determined by two-way ANOVA (A) and a two-tailed Student's t test (B); *p < 0.05; **p < 0.01. See also Figure S7.

(Lu et al., 2006). MCs have also been implicated in promoting CD4⁺ and CD8⁺ T cell expansion (Piconese et al., 2009; Stelekati et al., 2009). Herein, we describe a regulatory mechanism in which secretion of IL-2 by MCs suppresses chronic allergic dermatitis by enhancing the proportion of Treg cells at the site of inflammation and decreasing the fraction of activated T cells at this site.

It has been reported that MCs can either promote (Biedermann et al., 2000; Bryce et al., 2005; Dudeck et al., 2011; Suto et al., 2006) or dampen (Depinay et al., 2006; Grimbaldston et al., 2007; Hart et al., 1998) contact hypersensitivity, the latter being mediated primarily through MC production of IL-10. Dudeck and coworkers recently reported (Dudeck et al., 2011) that MCs are key promoters of contact hypersensitivity and not suppressors of this response. They induced contact hypersensitivity in a manner similar to that reported in the Grimbaldston study (Grimbaldston et al., 2007), using a mouse model (*Mcp5-Cre⁺iDTR⁺*) in which connective tissue type MCs are selectively depleted. Their findings showed that the absence of

connective tissue type MCs impaired migration of DCs to the lymph nodes and caused a marked reduction in contact hypersensitivity. They proposed that previous studies in mast cell-deficient mice (*Kit^{W-sh/W-sh}* or *Kit^{W/W-v}*) showing MC-protective effects were flawed because of potential immunological abnormalities in these mice. Although it is clear that *Kit^{W-sh/W-sh}* or *Kit^{W/W-v}* mice have various abnormalities (Grimbaldeston et al., 2005; Wolters et al., 2005), such overt dismissal for the role of MCs as suppressors should be cautioned by the fact that the particular disease model, mouse strain or genetics, route of sensitization and challenge, and strength of stimulus can all influence the immune response. In agreement with Dudeck et al. (2011), we (data not shown) and others (Norman et al., 2008; Suto et al., 2006) have seen that MCs can promote acute contact hypersensitivity in *Kit^{W-sh/W-sh}* or *Kit^{W/W-v}* mice in models where strength of the stimulus may be modest. Interestingly, Norman et al. (2008) showed that induction of contact hypersensitivity by a weak versus strong stimulus was a determinant of whether MCs induce or suppress disease, respectively. In our study, we utilize a model that differs from that used by Grimbaldeston et al. (2005) and Dudeck et al. (2011) in that repeated challenges of oxazolone on the ear were done for a period of approximately 28 days. This chronic model shows many of the characteristics of human AD (Leung et al., 2004) including the presence of both Th1 and Th2 cell cytokines. Our finding of MC-mediated suppression is consistent with the results of Norman et al. (2008) where a strong stimulus induced both Th1 and Th2 cell cytokines and resulted in a protective role for MCs. In addition, our findings also show that MCs can act to cause suppression from tissues (spleen) distal to the site of inflammation. Thus, one cannot exclude that the presence of mucosal MCs, which are seemingly not depleted in the diphtheria toxin-induced *Mcpt5-Cre⁺iDTR⁺* mice, could also influence observed responses.

Our deduction that the spleen represents a site involved in MC regulatory function is based on several findings. Splenectomized mice had a diminished inflammatory response, and anti-CD25 treatment of these mice failed to exacerbate disease. This demonstrated the contribution of the spleen in oxazolone-induced inflammation and showed that the effect of anti-CD25 was dependent on the spleen. The effect of anti-CD25 treatment was independent of MCs as shown by the fact that oxazolone-induced dermatitis was markedly exacerbated in the MC-deficient *Kit^{W-sh/W-sh}* mice. Intravenous MC reconstitution of *Kit^{W-sh/W-sh}* mice, which causes engraftment of the spleen, restored regulation of disease despite no engraftment of MCs in ear pinna. It is important to note that i.v. reconstitution of *Kit^{W-sh/W-sh}* mice with MCs engrafts several other sites (Grimbaldeston et al., 2005; Wolters et al., 2005), including LNs (a key site of MC-T cell interactions; Gri et al., 2008; Piconese et al., 2009), and therefore these sites may be contributory. Migration of MCs to the spleen was dependent on IgE accumulation in the spleen and mirrored both the increased production of IL-2 by MCs in the spleen and the suppression of disease. The precise mechanism underlying the increase of MCs in the spleen has yet to be elucidated. It does not appear to involve FcεRI, because mice deficient in FcεRIβ (and thus unable to express FcεRI) showed increased numbers of MCs in the spleen after oxazolone challenge (data not shown). However, antibody blockade of CD23 in-

hibited the increase of splenic MCs (data not shown), suggesting the possible involvement of this low-affinity IgE receptor. IgE engagement of CD23 on monocytes (which infiltrate the spleen upon oxazolone challenge; Sugiura et al., 2010) has been shown to induce secretion of CCL3 (Ezeamuzie et al., 2009), a potent chemoattractant for MCs. Collectively, these findings demonstrate a connection between the spleen and the skin in modulating chronic allergic dermatitis, and the putative trafficking of Treg cells between the two sites warrants further investigation. It should be noted that the spleen has also been shown to be essential for the induction of antigen-specific allergic diarrhea because of mobilization of primed splenic CD4⁺ T cells to the large intestine (Kurashima et al., 2007). Moreover, migration of MCs to the spleen is a well-described phenomenon: acute models of DNFB (Wang et al., 1998) and peptidoglycan (Matsui and Nishikawa, 2006) dermatitis and a model of helminth infection (Friend et al., 2000) showed increased numbers of MCs in the spleen. MC-mediated regulation of disease severity at a location distal to the site of inflammation was also shown in a mouse model of experimental autoimmune encephalomyelitis, where MCs exacerbated disease after adoptive transfer without repopulating the inflammatory site, i.e., the central nervous system (Tanzola et al., 2003). Thus, we propose that MC migration to the spleen may be important beyond allergic skin disease.

Localization of Treg cell-MC interactions to a site such as the spleen may provide an explanation of the observation that exogenous administration of IL-2 in postsensitized mice did not modify disease course, whereas MC-derived IL-2 alleviates chronic disease. We hypothesize that a systemic bolus of IL-2, while disease is ongoing, may stimulate both regulatory and effector T cells that express the IL-2R, thus failing to alter the course or severity of disease. Conversely, a systemic bolus prior to disease would target those cells constitutively expressing the IL-2R, which are primarily Treg cells (Malek and Bayer, 2004), and cause amplification of their activity, thus suppressing the disease. The production of IL-2 by MCs at a distal lymphoid organ may serve a similar role in compartmentalizing the effect of IL-2 during the disease course. Our data do not define the numbers of MCs required in order to maintain normal regulation in dermatitis. In fact, comparable degrees of regulation were obtained in C57BL/6 as in *Kit^{W-sh/W-sh}* mice after i.d. or i.v. injection of the latter with MCs. Although in C57BL/6 mice MC numbers in the spleen (per 20 mm²) were in the range of 5–10, i.d. reconstituted *Kit^{W-sh/W-sh}* showed 4- to 5-fold greater numbers and for i.v. reconstituted *Kit^{W-sh/W-sh}* several hundreds (Grimbaldeston et al., 2005; data not shown). Thus, the absolute numbers of MCs migrating to the spleen does not appear to affect the onset or extent of the suppressive regulatory effect on disease. We also theorize that in the early phase of dermatitis (or throughout disease course in the case of *Kit^{W-sh/W-sh}* mice), IL-2 is provided by cells other than MCs. This is supported by the finding of a small population of splenic CD4⁺ T cells (approximately 0.8%) that stain positive for IL-2 in naive WT mice (data not shown). However, unlike MCs, this population of T cells did not expand during disease course. In addition, we detected IL-2-producing cells in the spleens of non-MC-reconstituted *Kit^{W-sh/W-sh}* mice with dermatitis. We cannot formally exclude that some of the observed responses in *Kit^{W-sh/W-sh}* mice might result from other described abnormalities in these mice beyond

the MC compartment (Wolters et al., 2005). Nonetheless, one must conclude from the MC reconstitution of *Kit^{W-sh/W-sh}* mice that IL-2 expression in MCs is vital for late-stage disease suppression, but that it is not the rate-limiting factor in initiating regulatory control.

Although our results indicate that Treg cells and MCs exert comparable effects in suppressing the response to repeated oxazolone challenge, the overall impact on immune homeostasis differs greatly. This is exemplified by comparing the severe phenotype resulting from Treg cell deficiency in *Foxp3^{sf}* mice (Brunkow et al., 2001) and the mild phenotype associated with the absence of MCs in *Kit^{W-sh/W-sh}* mice (Grimbaldeston et al., 2005). Whereas *Foxp3^{sf}* mice have a life span of 16–25 days, *Kit^{W-sh/W-sh}* mice have a nearly normal life span. *Foxp3^{sf}* mice can develop a spontaneous and severe eczematous skin disease (which made them unsuitable for our studies), whereas *Kit^{W-sh/W-sh}* mice may show mild skin lesions in their late life. In fact, the data argue that Treg cells do not require MCs to suppress disease at early stages (prior to the fourth or fifth challenge); rather, their role appears to be in providing help to Treg cells once disease has reached almost maximal severity. This view is supported by the findings that inactivation of Treg cells increases the numbers of splenic MCs in the spleen and that consequent IL-2 production supports Treg cell numbers at the site of inflammation.

In summary, we propose that MC-Treg cell communication during the course of oxazolone-induced chronic allergic dermatitis is vital to disease regulation. Our data argue that the spleen plays a role in the inflammatory process and serves as a site where MCs are recruited as second line regulators in an IgE-dependent manner. By producing IL-2, MCs serve to maintain the Treg:Teff cell ratio at the site of inflammation, which is manifested by control of disease severity. Thus, the findings show that MCs play an overall protective role in late stages of chronic allergic dermatitis and suggest that failure of MC function in this disease leads to increased severity.

EXPERIMENTAL PROCEDURES

Mice

For the majority of experiments, littermate and aged-matched (8–16 week or as indicated) WT mice of C57BL/6 background were used with *Kit^{W-sh/W-sh}*, *Il2^{-/-}*, and *Igh-7^{-/-}* mice. WT BALB/c mice were used as indicated. All mice were purchased from Taconic Farms and the Jackson Laboratories. Splenectomized C57BL/6 mice and normal controls were purchased from the Jackson Laboratories. Mice were maintained and used in accordance with National Institutes of Health (NIH) guidelines and National Institute of Arthritis and Musculoskeletal and Skin Diseases-approved animal study proposal A007-03-01.

Oxazolone-Induced Dermatitis

Mice were sensitized on day 0 by application of 15 ml 1% oxazolone (Aldrich) in acetone on both aspects of both ears (total of 60 ml per challenge per mouse). From day 7, mice were challenged in the same way with 0.5% oxazolone, three times per week for up to a total of ten challenges. Under conditions where disease was considerably exaggerated, the protocol was shortened. In control mice, treatment was performed with acetone only. Ear thickness was measured and recorded daily with a dial thickness gauge (Mitutoyo).

In Vivo Administration of Cytokines and Antibodies

Anti-mouse CD25 (clone B6.1), anti-mouse IL-2, and rat IgG1 were purchased from BD Bioscience and IL-2 from PeproTec, Inc. Anti-CD25 or IgG1 isotype control were injected intraperitoneally (i.p.) at doses of 300 mg per injection,

either on day -1 (presensitization) or day 6 (postsensitization). Immune complexes were prepared by incubating 1 mg anti-IL-2 with 2 mg IL-2 at 4°C overnight with rotation. This solution was given i.p. in two separate doses either on days -2 and -1 (presensitization) or on days 6 and 7 (postsensitization).

Histological Staining

Tissue samples were collected at the end of each experiment and placed in formalin solution, 10% neutral buffered (Sigma-Aldrich). Embedding, sectioning, H&E, and toluidine blue staining were done by American Histolabs. Immunohistochemical analysis for IL-2-CD117(Kit) was done on formalin-fixed paraffin-embedded tissues. Antigen retrieval was performed by a microwave oven in either Tris-EDTA buffer (pH 9) or citrate-based antigen unmasking solution (Vector Laboratories). Binding of an anti-Kit (Abcam) was detected with an Alexa Fluor 568-conjugated secondary antibody. Detection of IL-2 was with Alexa Fluor 488-conjugated IL-2 antibody (Santa Cruz, 5 mg/mL) or for control a similarly conjugated normal IgG. Stained sections were mounted with an anti-fade medium (Vector Laboratories) and analyzed by confocal laser scanning microscopy (LSM 510 Meta, Carl Zeiss, Germany).

Cytokine and IgE Titers

Tissues were homogenized in 700 ml PBS supplemented with complete protease inhibitor cocktail tablets, 1 tablet/50 ml PBS (Roche Diagnostics). After 10 min centrifugation at 14,000 × g (4°C), the supernatant was collected and kept at -80°C. Total protein concentration was measured with Bio-Rad DC Protein Assay (Bio-Rad Laboratories) and cytokines titers were detected in 1:5 diluted homogenate samples with ExcelArray Mouse Inflammation Array I (Pierce). Total IgE titers in tissue homogenates and in sera were determined with mouse IgE ELISA quantitation kit (Bethyl Laboratories, Inc). Oxazolone-specific IgE was measured in a semiquantitative ELISA. For coating, 2% oxazolone was prepared in ethanol and added to 2% bovine serum albumin (BSA) (MP Biomedicals) at a ratio of 3:50. The oxazolone/BSA mixture was diluted 1:10 in carbonate buffer and used for coating overnight at 4°C. Blocking was performed with 10% fetal bovine serum (FBS) (GIBCO) in PBS for 3 hr at room temperature. Sera was diluted 1:10 in buffer prior to use. Buffers, plates, antibodies, and HRP substrate were from Bethyl Laboratories, Inc. For in vitro IL-2 detection, MCs were suspended at 5 × 10⁶ cells per ml. Activation was induced with anti-dinitrophenyl-specific IgE and DNP-HSA (Ag) or with 40 nM PMA (phorbol 12-myristate 13-acetate; Sigma) and 400 nM ionomycin (Calbiochem) for 6 hr at 37°C. The supernatant was analyzed with mouse IL-2 ELISA (eBioscience).

Generation of MCs and Reconstitution of Mice

Bone marrow-derived cultured MCs were generated as previously described (Gonzalez-Espinosa et al., 2003). Differentiation of MCs was confirmed by FACS analysis with c-Kit and FcεRI as markers. Cells were used when cultures were more than 95% Kit⁺FcεRI⁺. MCs were injected at a concentration of 2 × 10⁶ or 10⁷ cells (as indicated) in 200 ml RPMI either to the ear skin or into the tail vein of 6- to 8-week-old male *Kit^{W-sh/W-sh}* recipients. Engraftment was allowed for a minimum of 6 or 10 weeks depending on the method of reconstitution (i.d. or i.v., respectively). Subsequently, mice were subjected to oxazolone sensitization and challenge, after which reconstitution in all mice was confirmed by toluidine blue staining of tissue sections.

Flow Cytometric Analysis

For determination of cells in the skin, ears were removed from mice and split into dorsal and ventral halves. The skin was placed in 2 ml digestion buffer containing 0.1 mg/ml DNase I and 0.2 mg/ml Liberase TL (Roche Diagnostics) diluted in 1% FBS in RPMI (GIBCO). After a 1 hr incubation at 37°C, ears were placed into a 70 mm cell strainer (BD Biosciences) and mashed through the mesh. The strainers were washed with 2 ml of 1% FBS, 2 mM EDTA in PBS (GIBCO). Detection of cell surface markers and intracellular staining were performed as previously described (Charles et al., 2009). Flow cytometry acquisitions were done with a FACSCalibur (BD Biosciences) and Cell Quest software. Analysis was with FlowJo software (Treestar, Inc.). For IL-2 intracellular staining, cells were fixed with 3.7% paraformaldehyde solution in PBS, permeabilized in PBS, 0.1% BSA, 0.1% saponin, and stained in the same

buffer with fluorescently tagged IL-2 antibody. Foxp3 expression in CD4⁺ T cells was determined by Foxp3 staining (eBioscience).

Statistical Analysis

For comparisons between two populations, two-tailed Student's *t* tests were performed. Disease course was analyzed with a two-way ANOVA test. *p* values less than 0.05 were considered statistically significant and the following designation used: **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Statistical analysis was performed with Prism software (GraphPad Software, Inc.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.immuni.2011.07.013.

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Supplemental Information

Mast Cell Interleukin-2 Production Contributes to Suppression of Chronic Allergic Dermatitis

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Inventory

Seven Supplemental Figures

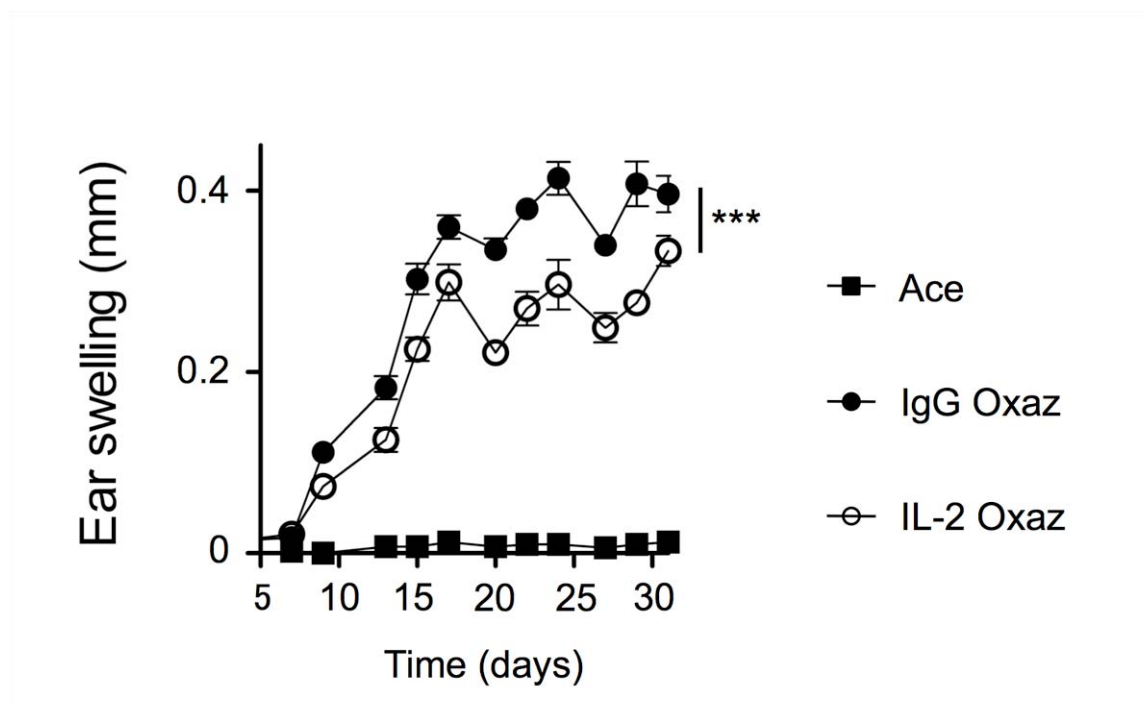


Figure S1, related to Figure 1. Injection of an IL-2 bolus alleviates dermatitis.

Disease course in WT mice treated with either control IgG or IL-2/anti-IL2 immune complexes, pre-sensitization on days -1 and 0 ($n = 4$). The experiment was performed twice. Statistical analysis was by two-way ANOVA. Results represent mean $\pm$ SEM. *** $p < 0.001$.

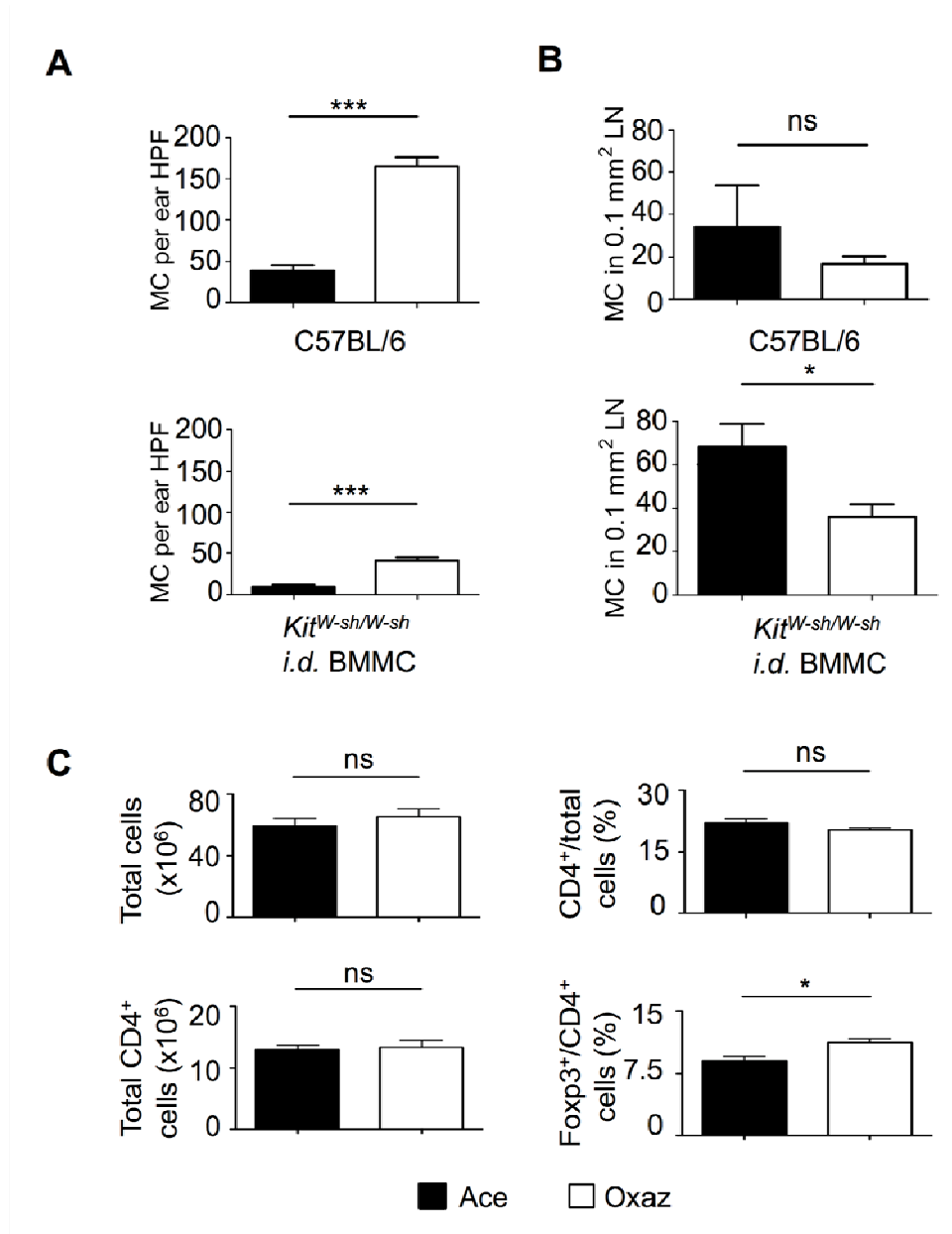


Figure S2 related to Figure 2. Quantitation of MCs in the ear of WT and *i.d.* reconstituted *Kit^{W-sh/W-sh}* mice and splenic CD4⁺ T cells following oxazolone-induced dermatitis. (A) Ears and (B) lymph nodes were collected from mice depicted in Figure 2. The corresponding histological sections were stained with toluidine blue and MCs were counted under the microscope. HPF, high power field, X200 magnification. (C) Spleens and draining lymph nodes were collected from C57BL/6 mice that had been fully treated with either acetone or oxazolone (i.e. sensitization and 10 challenges). Total cell content was determined and cells were stained with antibodies against CD4 and Foxp3 and analyzed by FACS (n = 4). Bars indicate mean $\pm$ SEM. Statistical significance was determined by a two-tailed student's *t* test. *, $p < 0.02$. **, $p < 0.01$; ***, $p < 0.001$.

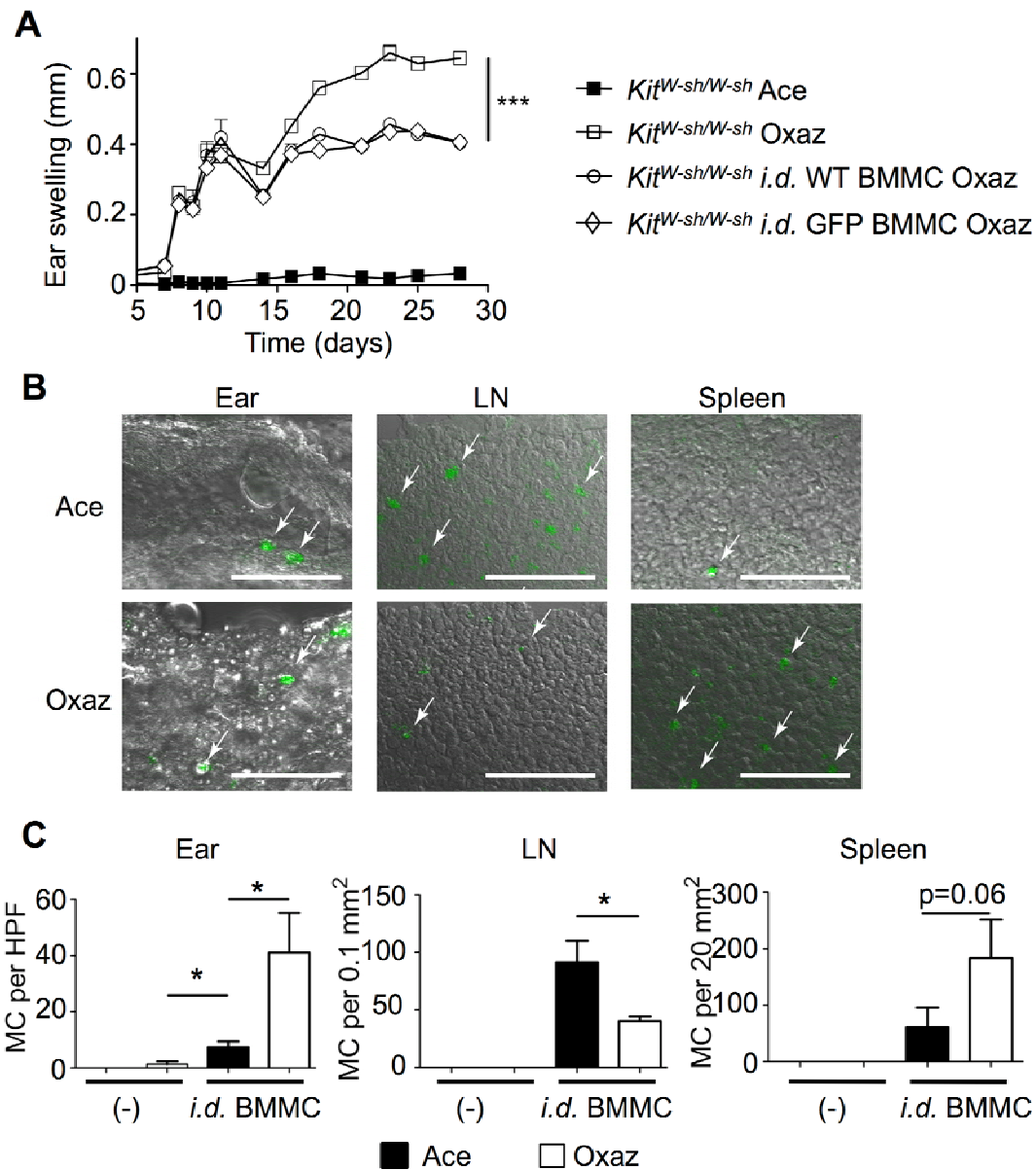


Figure S3 related to Figure 3. Disease suppression and MC distribution following i.d. reconstitution with small numbers of BMMC. Mice were i.d.-injected with 2×10^6 BMMCs (instead of 1×10^7 as presented in Figure 2) in order to examine the reproducibility of results under conditions used in previous studies (Grimbaldeston et al., 2007) (A) disease course during oxazolone treatment (n = 5). (B) Frozen tissue sections from $Kit^{W-sh/W-sh}$ mice i.d.-injected with GFP-expressing MCs were analyzed by confocal laser scanning microscopy. The white arrows indicate fluorescent MCs that had been introduced to the skin and were subsequently detected in the skin, the draining cervical lymph node and the spleen. (C) MC count in tissues harvested from non-injected (-) and i.d.-reconstituted $Kit^{W-sh/W-sh}$ mice that had been treated with either acetone or oxazolone, as indicated. HPF, high power field X200. Bar, 50 mm. Data are means $\pm$ SEM (A, Two way ANOVA; C, student's *t* test) *, $p < 0.05$; ***, $p < 0.001$.

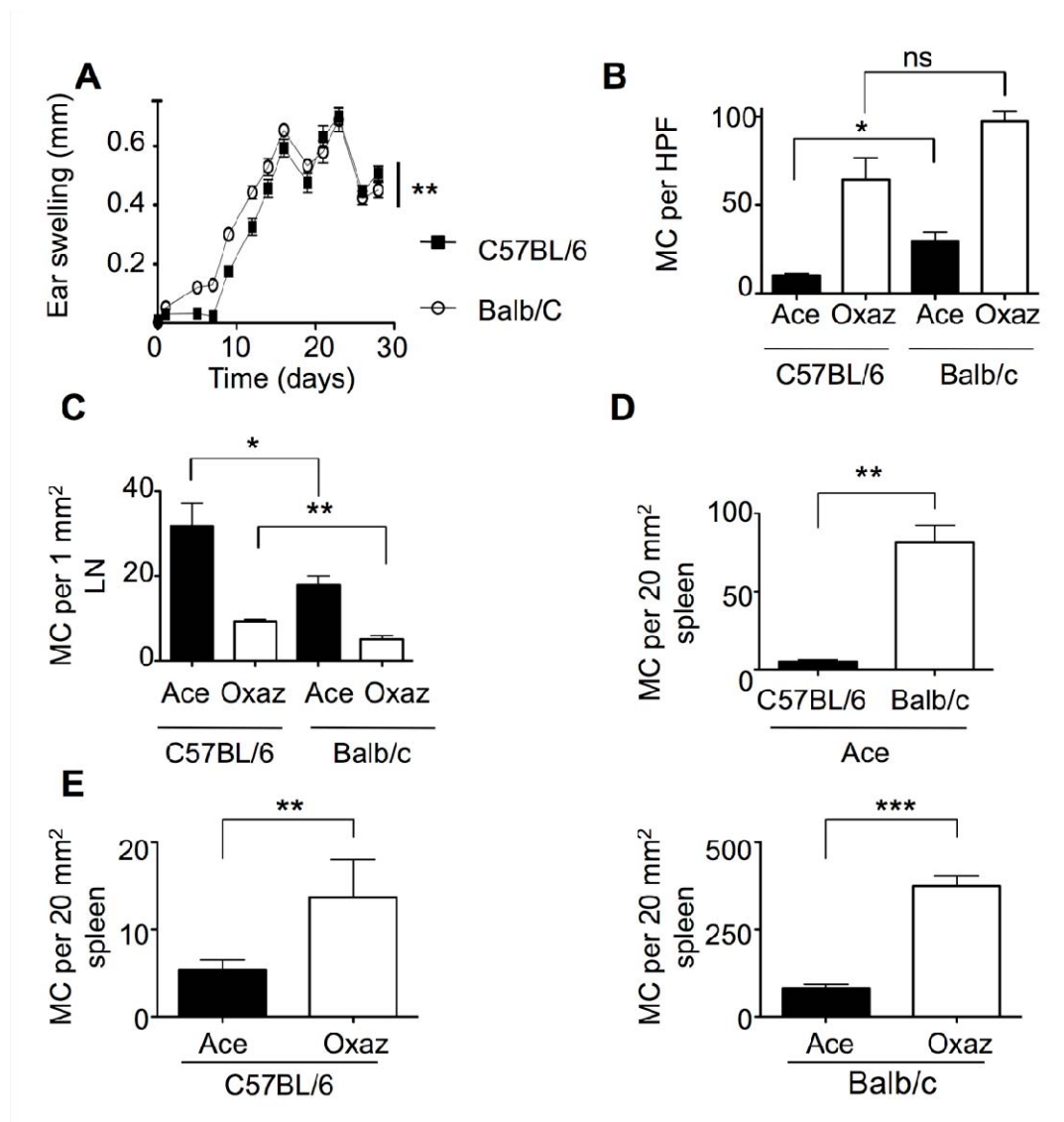


Figure S4 related to Figure 4. Comparison between C57BL/6 and Balb/c responses to oxazolone dermatitis. Age- and sex-matched C57BL/6 and Balb/c mice were subjected simultaneously to oxazolone treatment (n = 5). The two groups were compared for differences in: (A) ear swelling (B) MC in the ear (HPF=high power field, X 200) (C) MC in draining LN (D) baseline splenic MC (E) and post-induction spleens. Toluidine positive MCs were counted by microscopy. Data are means $\pm$ SEM (two-tailed student's *t* test). **p*<0.05; ***p*<0.01; ****p*<0.001.

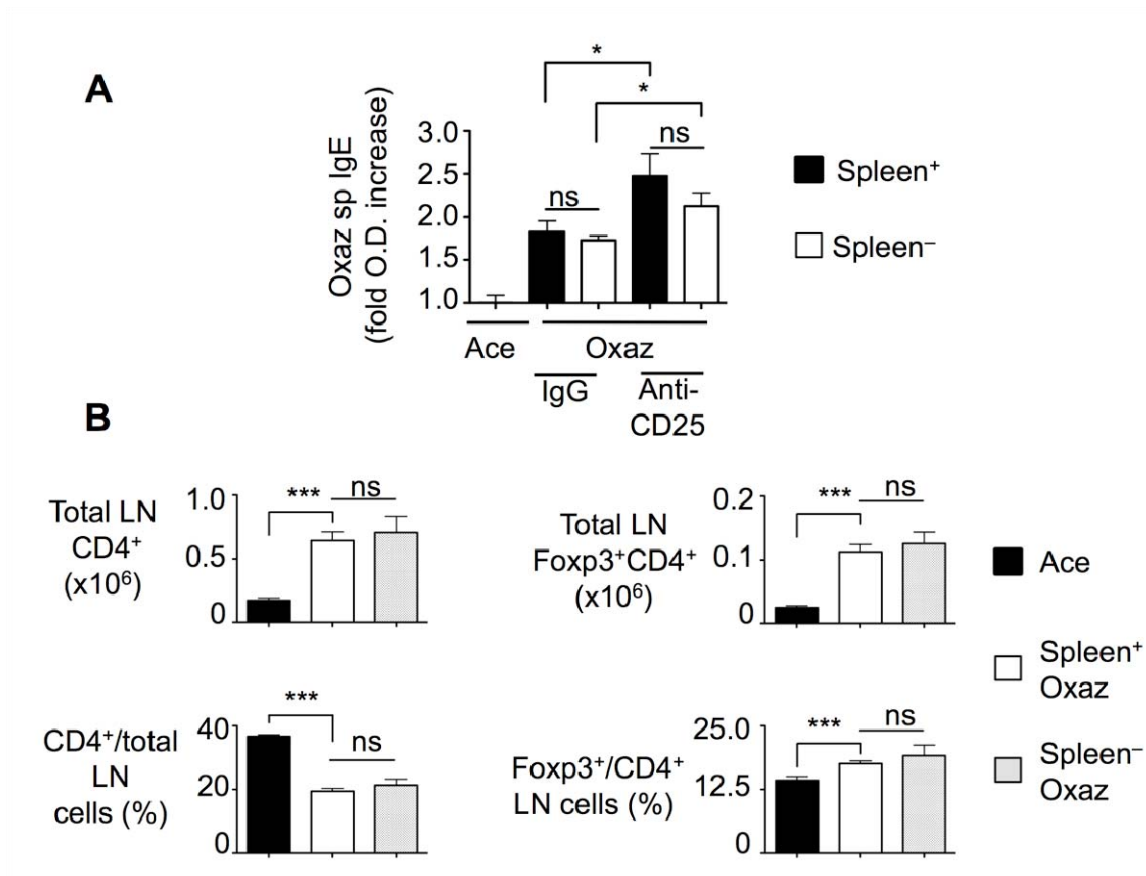


Figure S5 related to Figure 5. LN responses and oxazolone IgE levels in WT and splenectomized mice. C57BL/6 mice were splenectomized and subjected to oxazolone treatment, along with non-splenectomized littermates ($n = 5$). (A) Sera were collected from normal and splenectomized mice with dermatitis following pre-sensitization treatment with either control IgG or anti-CD25. IgE levels were determined by oxazolone-specific ELISA. (B) The draining cervical LNs were harvested and evaluated for CD4⁺ and Foxp3⁺ cells. Data are means $\pm$ SEM (two-tailed student's t test). * $p < 0.05$; *** $p < 0.001$.

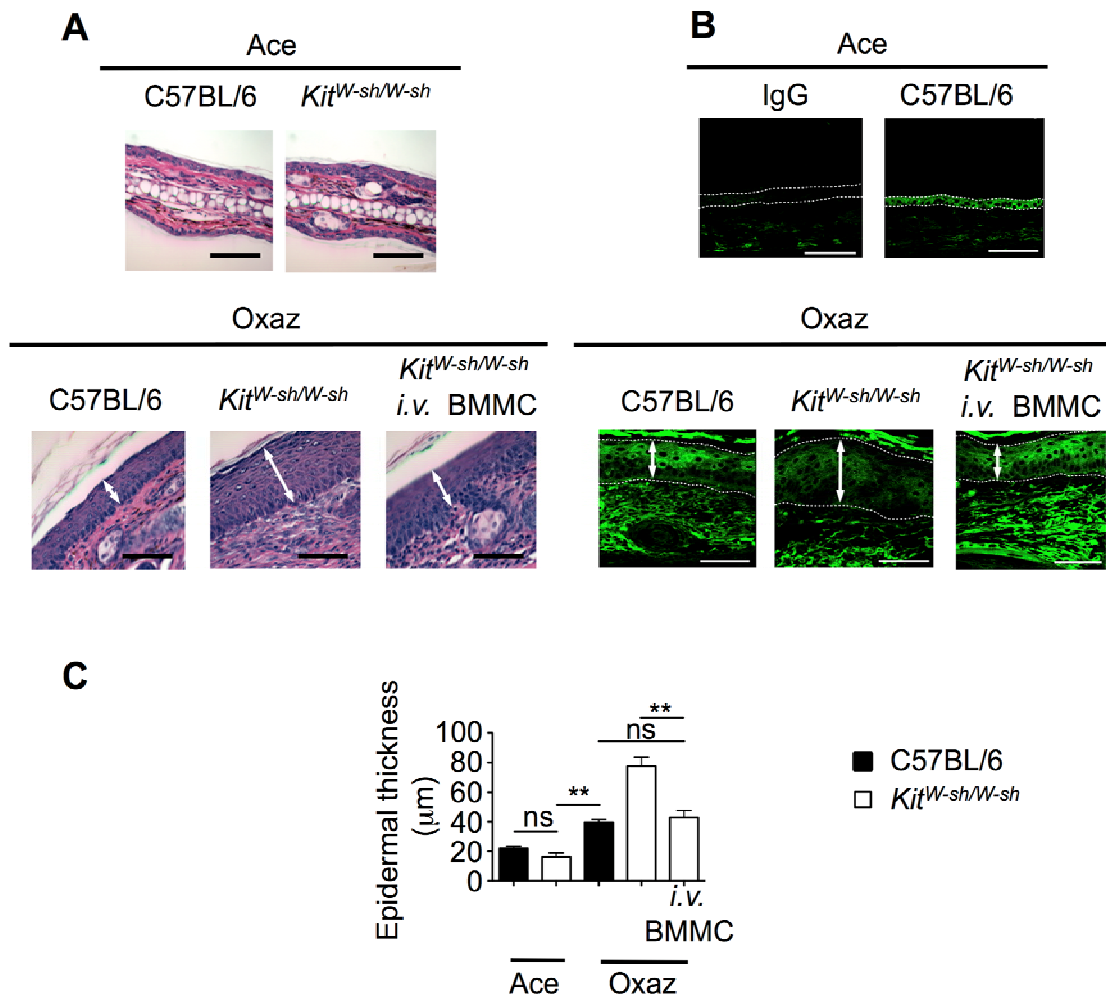


Figure S6 related to Figure 6. Epidermal hyperplasia is dampened in *i.v.* reconstituted *Kit^{W-sh/W-sh}* mice. Oxazolone dermatitis was induced in WT, *Kit^{W-sh/W-sh}* as well as in *i.v.* MC-reconstituted *Kit^{W-sh/W-sh}* mice. Ear swelling in this experiment is presented in Figure 3C. Following completion dermatitis induction, ears were collected, and tissue sections stained with (A) H&E and (B) pan-cytokeratin (bar=50mm). Representative ear sections are shown and white arrows indicate epidermal thickness. (C) Quantitation of epidermal thickness. Data is mean $\pm$ SEM and statistical significance was determined by two tailed student's *t* test. ***p*<0.01.

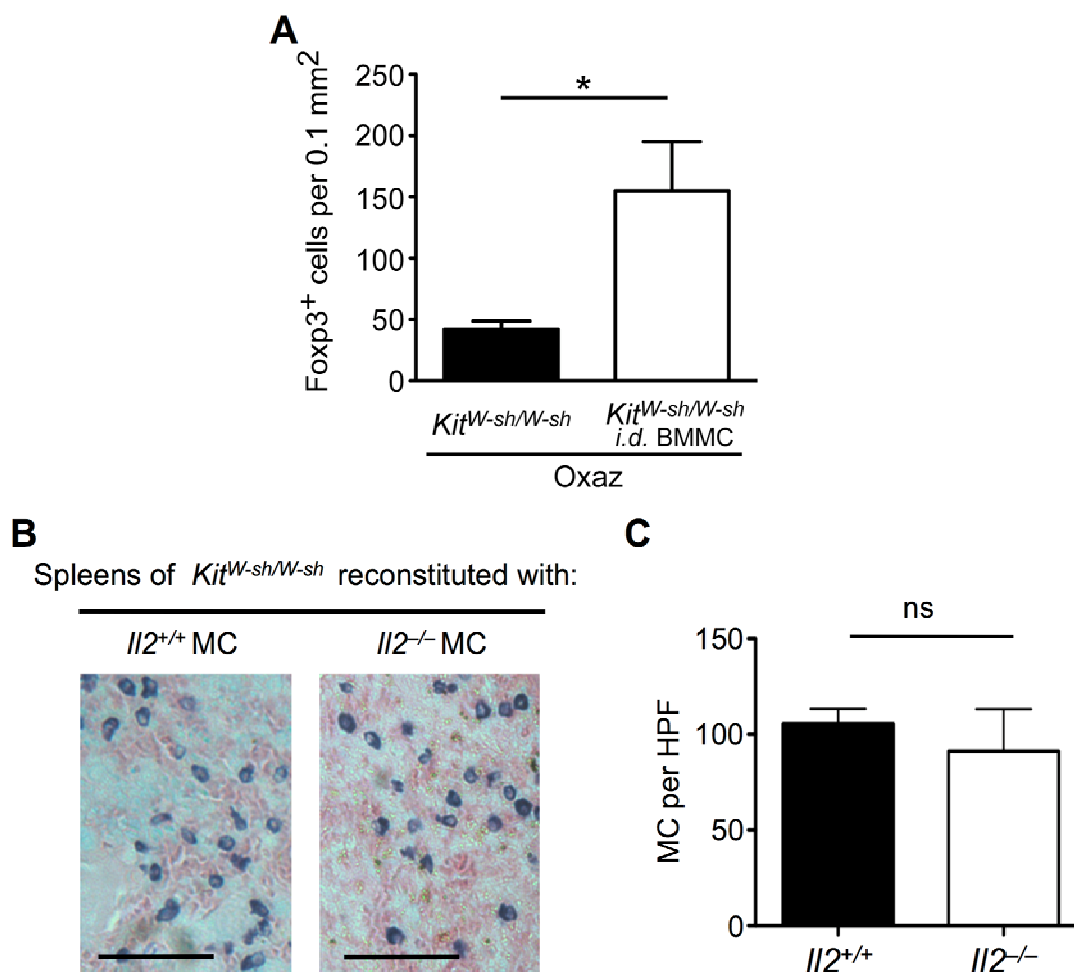


Figure S7 related to Figure 7. Number of Foxp3⁺ cells in ear skin of oxazolone challenged *i.d.* MC reconstituted and non-reconstituted *Kit*^{W-sh/W-sh} mice and engraftment efficiency following *i.v.* adoptive transfer of *Il-2*^{+/+} and *Il-2*^{-/-} BMMCs.. Oxazolone dermatitis was induced in *Kit*^{W-sh/W-sh} as well as in *i.d.* MC-reconstituted *Kit*^{W-sh/W-sh} mice. Ears were collected, and tissue sections were stained with CD4 and Foxp3. Foxp3 positive cells were counted by microscopy. (B and C) BMMCs (10 x 10⁶ per 200 ml) were injected to the tail vein of *Kit*^{W-sh/W-sh} and allowed 12 weeks for engraftment. Subsequently, oxazolone dermatitis was induced and tissues were collected. (B) Representative pictures from TB-stained spleen sections from *Kit*^{W-sh/W-sh} recipients of IL-2 sufficient or deficient mast cells (as indicated). Scale bar represents 50mm. (C) A summary of mast cell counts in the spleens of all recipient mice (n = 5), as determined by microscopy. ns, not significant. Data are means $\pm$ SEM (two-tailed student's *t* test). *p<0.05