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Alon Y. Hershko, Nicolas Charles, Ana Olivera, Damiana Alvarez-Errico and Juan Rivera

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Cutting Edge: Persistence of Increased Mast Cell Numbers in Tissues Links Dermatitis to Enhanced Airway Disease in a Mouse Model of Atopy

Alon Y. Hershko,¹ Nicolas Charles, Ana Olivera, Damiana Alvarez-Errico, and Juan Rivera

The development of chronic allergic dermatitis in early life has been associated with increased onset and severity of allergic asthma later in life. However, the mechanisms linking these two diseases are poorly understood. In this study, we report that the development of oxazolone-induced chronic allergic dermatitis, in a mouse model, caused enhanced OVA-induced allergic asthma after the resolution of the former disease. Our findings show that oxazolone-induced dermatitis caused a marked increase in tissue mast cells, which persisted long after the resolution of this disease. Subsequent OVA sensitization and airway challenge of mice that had recovered from dermatitis resulted in increased allergic airway hyperreactivity. The findings demonstrate that the accumulation of mast cells during dermatitis has the detrimental effect of increasing allergic airway hypersensitivity. Importantly, our findings also show that exposure to a given allergen can modify the immune response to an unrelated allergen. *The Journal of Immunology*, 2012, 188: 000–000.

Atopy is a common phenomenon characterized by a predisposition to produce specific IgE Abs in response to common allergens (1). This predilection occurs primarily in children and is associated with atopic dermatitis (AD), asthma, and rhinoconjunctivitis, collectively known as the atopic diseases. Anaphylaxis also has been described as a related feature, as food anaphylaxis is a more common sequela in children with eczema and children with asthma are at risk for severe episodes of anaphylaxis regardless of the trigger (1). The natural history of these atopic manifestations follows a typical pattern in which AD predates the development of respiratory allergy, a process referred to as the atopic march. It has been suggested that AD is an “entry point” for subsequent allergic disease (2). This hypothesis is supported by the finding that severe AD patients develop exaggerated airway responses to methacholine challenge and

airway allergy (3–5). This concept raises the fundamental question of the possible causative role that AD may play in the development of allergic airway inflammation. An answer to this question would be expected to have significant clinical implications. For example, if the atopic march reflects causation, then the recently reported increase in AD might indicate an impending rise in the prevalence of asthma (6, 7). Furthermore, such a link implies that an aggressive, as opposed to symptomatic, approach to the treatment of AD may halt the atopic march, thus preventing future asthma.

A common conclusion drawn from multiple studies is that the skin can be the site of potent allergic sensitization. This is particularly true after the disruption of skin integrity by mechanical means (8) or by the induction of allergic, AD-like dermatitis. An elegant example is provided in a study in which OVA was applied epicutaneously to tape-stripped skin to induce an allergic skin response (9). Subsequent challenge of these mice by the inhalation of a single dose of OVA caused a considerably greater response to the inhaled allergen than that in mice sensitized with saline.

In the current study, we explored the mechanisms contributing to the progression of the atopic march. Given that the early development of chronic allergic dermatitis and the later development of allergic asthma are not necessarily linked by a shared allergen, we chose to test the link between these two diseases using two different allergens. In this study, we found that upon the resolution of oxazolone-induced AD in mice, OVA sensitization and challenge caused increased airway responses. This was attributed to increased numbers of mast cells (MCs) in the tissues of OVA-challenged mice after the resolution of AD. The findings show that the increase in MCs in chronic allergic dermatitis can have a detrimental effect by promoting subsequent allergic airway hyperreactivity.

Materials and Methods

Animals

BALB/c, C57BL/6, and mast cell-deficient *Kit^{W^{sh}/W^{sh}}* mice (C57BL/6) were obtained or came from our colonies at Taconic Farms. Age- and gender-matched animals were used in all of the individual experiments. Mice were

Molecular Immunology Section, Laboratory of Molecular Immunogenetics, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892

¹Current address: Department of Medicine, Meir Medical Center, Kfar Saba, Israel.

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Address correspondence and reprint requests to Dr. Juan Rivera, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Building 10, Room 13C103, Bethesda, MD 20892-1930. E-mail address: juan_rivera@nih.gov

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Abbreviations used in this article: AD, atopic dermatitis; i.d., intradermal; MC, mast cell.

maintained and used in accordance with National Institutes of Health guidelines, and the animal study proposals (A010-04-03) were approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases Animal Care and Use Committee.

Oxazolone-induced dermatitis

Mice were sensitized on day 0 by the application of 15 μ l of 1% oxazolone (Sigma-Aldrich) in acetone on both aspects of both ears. Starting from day 7, mice were challenged in the same manner with 0.5% oxazolone, three times per week, for up to 10 challenges. For control mice, treatment was performed with acetone only. Ear thickness was measured by a dial thickness gauge (Mitutoyo).

Histological staining

At the end of each experiment, mice were euthanized by CO₂ inhalation, and tissues were collected and placed in 10% neutral buffered formalin. Lungs were inflated with 2 ml formalin for 1 h before harvest and then left in formalin overnight with rotation to ensure fixation. Embedding, sectioning, and staining were done at American Histolabs.

Flow cytometry

Splenocytes were prepared and stained as described previously (10). Flow cytometry acquisitions were done with FACSCalibur (BD Biosciences) and CellQuest software. Analysis was with FlowJo software (Treestar).

Passive systemic anaphylaxis

Anaphylaxis was induced as described previously (11). Briefly, mice were sensitized by i.v. injection of 3 μ g DNP-specific IgE (H1-DNP- ϵ -26.82, 0.2 ml). Twenty-four hours later, they were challenged with 250 μ g DNP₃₆-human serum albumin. Measurement of body temperature was performed using an implantable electronic transponder as described (11).

Generation of MCs and reconstitution of Kit^{W-sh/W-sh} mice

Suspended bone marrow cells from C57BL/6 mice were cultured for 4 wk in the presence of stem cell factor and IL-3 (20 ng/ml) (PeproTech), as described previously (12, 13). Differentiation of MCs was confirmed by cytometric analysis using c-Kit and Fc ϵ RI as markers. MCs were injected to both ear pinnae at a concentration of 10⁷ cells/ml and a total volume of 120 μ l. Engraftment was allowed for a minimum of 6 wk. After disease induction (dermatitis and/or asthma), engraftment was confirmed by toluidine blue staining of tissue sections.

Induction and measurement of allergic airway reactivity

For sensitization, mice were injected i.p. with 50 μ g OVA (Sigma-Aldrich) in a volume of 0.1 ml on days 0, 3, and 7. Starting from day 12, they were challenged intranasally with 20 μ g OVA in 30 μ l PBS under isoflurane anesthesia (Baxter). Challenges were administered once per week for 9 wk. Starting from 24 h after the final OVA challenge, bronchial reactivity was determined. Mice were anesthetized by i.p. administration of 300 μ l ketamine/xylazine mixture (1 ml ketamine [100 mg/ml], 0.5 ml xylazine [20 mg/ml], and 8.5 ml PBS). A 19-gauge blunt-end needle was inserted into the trachea, and the animals then were ventilated mechanically and challenged with increasing doses of aerosolized methacholine using flexiVent (Scireq Scientific Respiratory Equipment).

Statistical analysis

For a comparison between two populations, two-tailed Student *t* tests were performed. Airway responses to methacholine were compared using a two-way ANOVA test. Data are presented as mean $\pm$ SEM. Statistical analysis was done with Prism software (GraphPad Software).

Results and Discussion

Increased asthma in mice after recovery from oxazolone-induced dermatitis

AD-like disease was elicited by repeated exposures of the ear skin of mice to oxazolone. Disease course could be divided into three phases (Fig. 1A): 1) sensitization, which is a critical step in determining the intensity of subsequent inflammation; 2) acute inflammation manifested as continuously increasing ear swelling; 3) chronic inflammation, starting from approximately the fifth challenge onward in which ear thickness

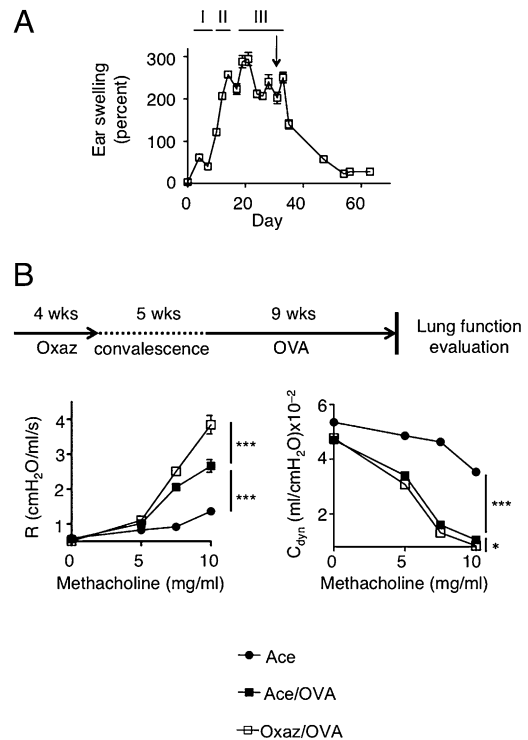


FIGURE 1. The postdermatitis state confers increased airway response to OVA. *A*, Ear-thickness measurements in BALB/c mice with chronic oxazolone-induced dermatitis. Mice were challenged up to day 30 and then allowed to convalesce. The arrow denotes the time of the last challenge. I, sensitization; II, acute swelling; III, chronic inflammation (plateau) (*n* = 5). *B*, Scheme of protocol and measurement of lung function in mice where OVA-induced airway reactivity was initiated 5 wk after the cessation of oxazolone challenges (*n* = 5). **p* = 0.014, ****p* < 0.0001. C_{dyn}, compliance; R, resistance.

reaches a plateau. After the withdrawal of oxazolone treatment (Fig. 1A, arrow), ear swelling gradually subsided and stabilized by 20 d postwithdrawal. As shown in Fig. 1B, chronic airway hyperreactivity was induced subsequently using a protocol of OVA challenges without adjuvant. In this model, MCs have been shown to be key participants and can considerably intensify the inflammatory response to OVA (14). When OVA sensitization was initiated 5 wk after the last oxazolone challenge (Fig. 1B) airway hyperreactivity was more severe, with increased resistance (Fig. 1B, left panel) and, to a lesser but nonetheless significant extent, reduced compliance (Fig. 1B, right panel). Having ruled out a direct effect of oxazolone dermatitis on lung function (Supplemental Fig. 1A), we hypothesized that it may enhance airway disease by increasing sensitization to OVA. However, OVA-specific IgE was not increased in the postdermatitis state (Supplemental Fig. 1B), despite the diverse cytokines induced by oxazolone (Supplemental Fig. 2A–C), which included IL-4 that is required for full development of dermatitis (Supplemental Fig. 2B).

We reported previously (15) that, during the course of oxazolone-induced chronic AD in mice, MCs become activated, migrate from the skin to the draining lymph nodes and spleen, and expand in the tissues. In this setting, MCs were found to suppress inflammation during the late stages of disease by producing IL-2 and supporting regulatory T cell function at the site of inflammation. In humans, it is well known that MC numbers increase in the affected skin of individuals with AD (16). Thus, we postulated that the ex-

acerbation of post-AD airway disease, in our mouse model, might be caused by increased numbers of tissue MCs. We evaluated MC numbers in the spleens of mice with dermatitis and found that their numbers were increased (data not shown), which is consistent with prior findings (15). The increased numbers of MCs in tissues remained during convalescence and after the challenge with OVA (Fig. 2*A*, 2*B*). Our previous study (15) demonstrated that during dermatitis a significant proportion of splenic MCs produced IL-2. In contrast, 5 wk postdermatitis, splenic MCs were no longer producing IL-2 (Fig. 2*C*), suggesting that these cells were no longer activated or could be nonresponsive. To test the latter possibility, we conducted a systemic anaphylactic challenge of dermatitis-induced mice 5 wk after convalescence. As shown in Fig. 2*D*, an enhanced anaphylactic response was observed for dermatitis-induced mice relative to that of those treated with solvent alone, and this enhanced response was still present after 5 wk of convalescence. This demonstrated that

MCs are not cleared readily from the tissues and remain fully capable of responding to a subsequent challenge. Moreover, examination of the spleens of mice challenged with oxazolone, allowed to convalesce, and subsequently challenged with OVA disclosed more MCs than in the spleens of mice challenged with OVA alone (Fig. 2*E*). This demonstrates that the development of dermatitis is the cause of the marked increase in tissue MCs seen in postdermatitis airway challenged mice.

Because we found previously (15) that MCs dampen chronic AD, we asked whether MCs can function as regulatory and effector cells in dermatitis and airway disease, respectively. MC-deficient (*Kit^{W-sh/W-sh}*) and appropriate control C57BL/6 mice (colony established from F₁ wild-type progeny of a *Kit^{W-sh/+}* mating) were subjected to oxazolone-induced dermatitis. Control C57BL/6 mice demonstrated milder inflammation relative to that of *Kit^{W-sh/W-sh}* mice, which is consistent with our prior results (15). In a separate experiment, induction of airway disease revealed increased airway hyperreactivity in control C57BL/6 mice when compared

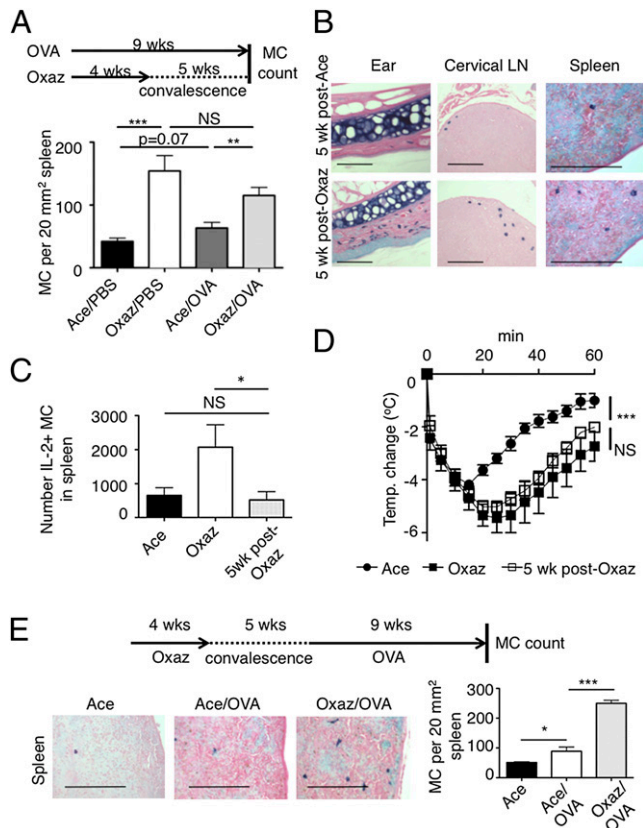


FIGURE 2. Long-term persistence of increased MC numbers and enhanced systemic anaphylaxis in postdermatitis mice. *A*, Protocol scheme and measurement of MC numbers in BALB/c mice after 5 wk of postdermatitis convalescence and concomitant 9 wk of OVA treatment, as shown ($n = 5$). $^{**}p = 0.0039$, $^{***}p = 0.0006$. *B*, Toluidine blue staining for MCs in the indicated tissues, 5 wk after the cessation of either vehicle (acetone) or oxazolone. Scale bars, 150 μ m. *C*, Numbers of IL-2-producing (IL-2⁺) MCs in the spleens of mice with dermatitis (Oxaz) or after recovery (5wk post-Oxaz). $^{*}p = 0.03$. *D*, Passive systemic anaphylaxis in mice with oxazolone-induced dermatitis or after their recovery (postdermatitis). After challenge, body temperature was recorded using a s.c. probe ($n = 5$). $^{***}p < 0.0001$. *E*, Representative picture (left panel) of toluidine blue-stained MCs in the spleens of mice after sequential oxazolone dermatitis and OVA airway hyperreactivity induction, solvent (Ace) challenge only, or OVA airway hyperreactivity induction only. Quantification of MC numbers in spleens ($n = 5$). Scale bars, 150 μ m. $^{*}p = 0.017$, $^{***}p < 0.0001$.

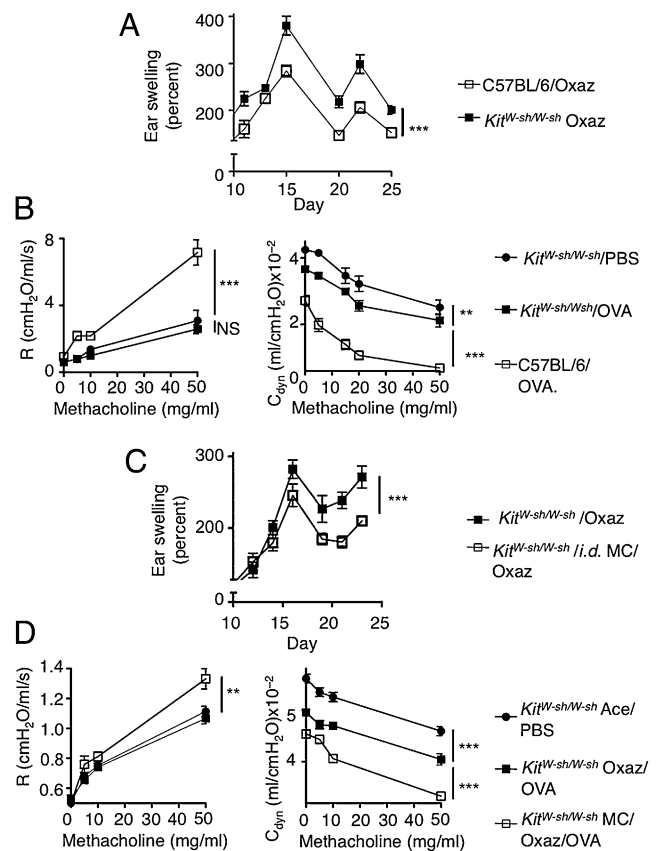


FIGURE 3. Introduction of MCs into the skin results in a dual effect of protection from dermatitis and exacerbated asthma. *A*, C57BL/6 and *Kit^{W-sh/W-sh}* mice were subjected to oxazolone challenges, and ear swelling responses were compared ($n = 5$). $^{***}p < 0.0001$. The graph shows the range of ear swelling (y-axis) with time (x-axis) previously shown (15) to be the point in the disease where MCs are involved. *B*, Concomitantly, OVA sensitization and challenge was started in littermates of each group, and lung function was assessed by a methacholine study ($n = 5$). $^{**}p = 0.0086$, $^{***}p < 0.0001$. *C*, *Kit^{W-sh/W-sh}* mice were injected i.d. with bone marrow-derived MCs. Six weeks later, mice were sensitized and subsequently challenged with oxazolone ($n = 7$). $^{***}p < 0.0001$. *D*, After 5 wk of recovery from dermatitis, sensitization to OVA was initiated in a manner similar to the experimental design depicted in Fig. 1*B*. At the end of asthma induction, lung functions were evaluated as in Fig. 3*B* ($n = 5$). $^{**}p = 0.0008$, $^{***}p < 0.0001$. C_{dyn} , compliance; R, resistance.

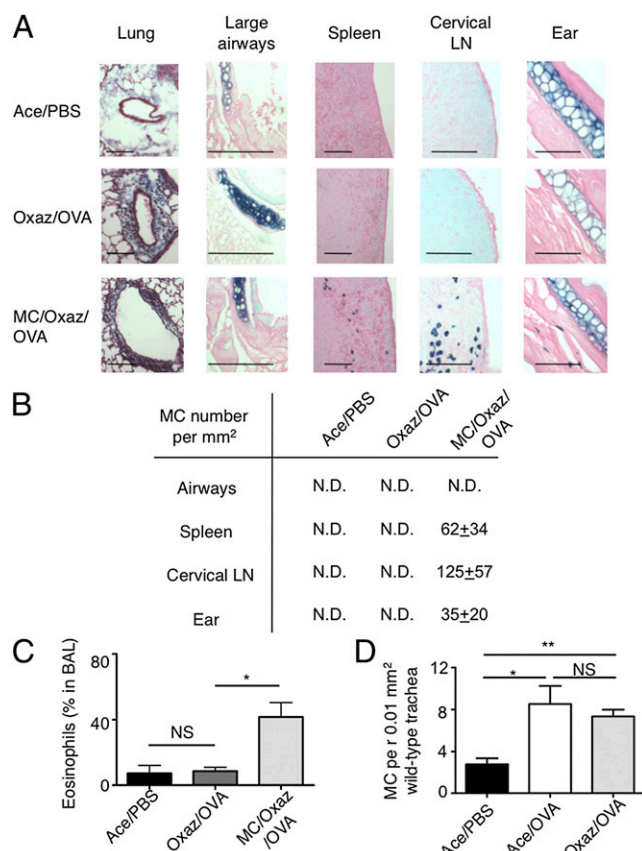


FIGURE 4. MC distribution in tissues after oxazolone dermatitis and OVA-induced asthma in i.d. reconstituted *Kit^{W-sh/W-sh}* mice. *A*, Tissues were harvested from the indicated three groups of mice and embedded in paraffin after the OVA challenge. Lung samples were stained with Masson's trichrome to demonstrate inflammatory infiltrate within peri-bronchiolar collagen. Large airways, spleens, cervical lymph nodes, and ear tissues were stained for MCs with toluidine blue. Scale bars, 100 μ m. *B*, Quantification of MCs in tissues of mice with asthma was done under a light microscope and is presented as mean $\pm$ SEM per mm². N.D., not detected. For comparison, the basal MC numbers in unchallenged i.d. reconstituted mice were: airways, N.D.; spleen, 4.5 ± 1.5 ; cervical lymph node, 218 ± 59 ; ear, 27 ± 6 ($n = 5$). *C*, Quantification of eosinophils in bronchoalveolar lavage (BAL) fluid of mice in (*A*, *B*) ($n = 3$). * $p = 0.02$. *D*, MCs in trachea tissue of wild-type BALB/c mice, enumerated after toluidine blue staining under a light microscope ($n = 5$). * $p = 0.016$, ** $p = 0.0011$.

with that in *Kit^{W-sh/W-sh}* mice (Fig. 3*B*). This suggests that MCs, which dampen chronic AD (15), conversely promote the induction of OVA-mediated airway hyperresponsiveness. To formally demonstrate that this transition from regulatory to effector MCs could occur in the same mouse, we performed an adoptive (intradermal [i.d.]) transfer of MCs in *Kit^{W-sh/W-sh}* mice, and afterward we induced oxazolone dermatitis in these mice as well as in non-reconstituted *Kit^{W-sh/W-sh}* mice. Disease induction was followed by 5 wk of convalescence and subsequent OVA-induced airway hyperactivity. As shown in Fig. 3*C*, MC-reconstituted *Kit^{W-sh/W-sh}* mice showed reduced inflammation during the late stages of oxazolone-induced dermatitis when compared with that in their non-reconstituted counterparts. No protection was observed at early stages of disease. In contrast, OVA-induced airway hyperactivity was increased markedly in MC-reconstituted *Kit^{W-sh/W-sh}* mice (Fig. 3*D*) when compared with that in non-reconstituted littermates. These findings

demonstrate that, although MCs play a protective role in one disease, they are conversely able to promote the severity of another disease. The findings are consistent with the view that induction of a Th2 response in one disease may well predispose one to another disease, as reported previously for *lyn^{-/-}* mice (10, 17). Examination of the tissues of MC-reconstituted *Kit^{W-sh/W-sh}* mice revealed that i.d. injected MCs not only engrafted the skin but also were found in cervical lymph nodes and the spleen (Fig. 4*A*, 4*B*), which is consistent with our previous findings (15). These dynamics were associated with increased inflammation, as judged by airway inflammation (Fig. 4*A*) and by increased numbers of eosinophils in the bronchoalveolar lavage fluid (Fig. 4*C*). Although some MCs could be found in the tracheae of wild-type mice (14) (Fig. 4*D*), we observed no MC engraftment in the lungs or large airways of MC-reconstituted mice (Fig. 4*A*, 4*B*) demonstrating that the dermatitis-induced effect on airway hyperactivity is mediated distal to the site of inflammation.

In summary, the work presented herein provides a murine model for the atopic march with several characteristics of the syndrome in humans. First, accumulation of MCs during chronic allergic dermatitis was observed, which is consistent with reports in human disease (16). Although in our model of oxazolone-induced AD the accumulation of MCs was protective (15), these cells also were able to enhance anaphylaxis as reported in some humans with AD (1). Second, post-dermatitis mice developed more severe airway disease, which is consistent with the well described observations of increased incidence and severity of asthma after AD (18). Finally, exposure to one allergen caused increased responsiveness to another allergen, suggesting potential epigenetic regulation, which is consistent with a role for the epigenome in increasing susceptibility and/or severity of allergic disease (19). Thus, the atopic model described herein provides a mechanistic link between the dermatitis-driven MC expansion and the increased risk and/or severity of asthma. It appears that, although the expansion of MCs in AD is beneficial at late stages of this disease, the cost is an increased risk for the development or severity of airway hyperactivity.

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Disclosures

The authors have no financial conflicts of interest.

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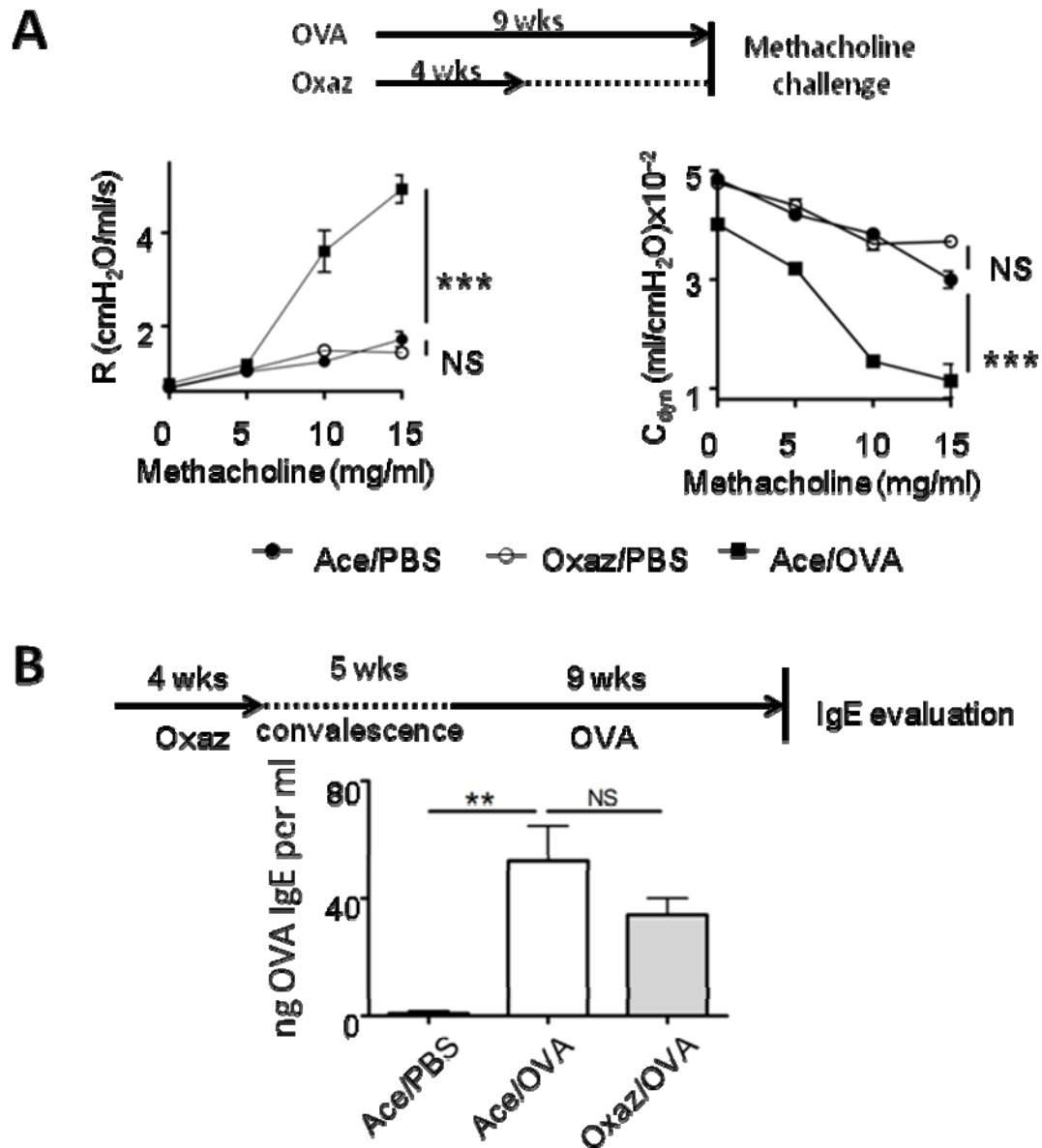


Figure S1: Oxazolone dermatitis does not affect lung function and does not enhance OVA sensitization. (A) As depicted in the top panel, lung functions were evaluated in vehicle-treated, 5 week post-dermatitis convalescence and OVA-treated Balb/c mice (R=resistance; C_{dyn}=compliance) (n=6). ***, p<0.0001; NS, not significant. (B) OVA specific IgE was measured by ELISA in mice whose lung functions are presented in Figure 1B (n=5). **, p=0.0016.

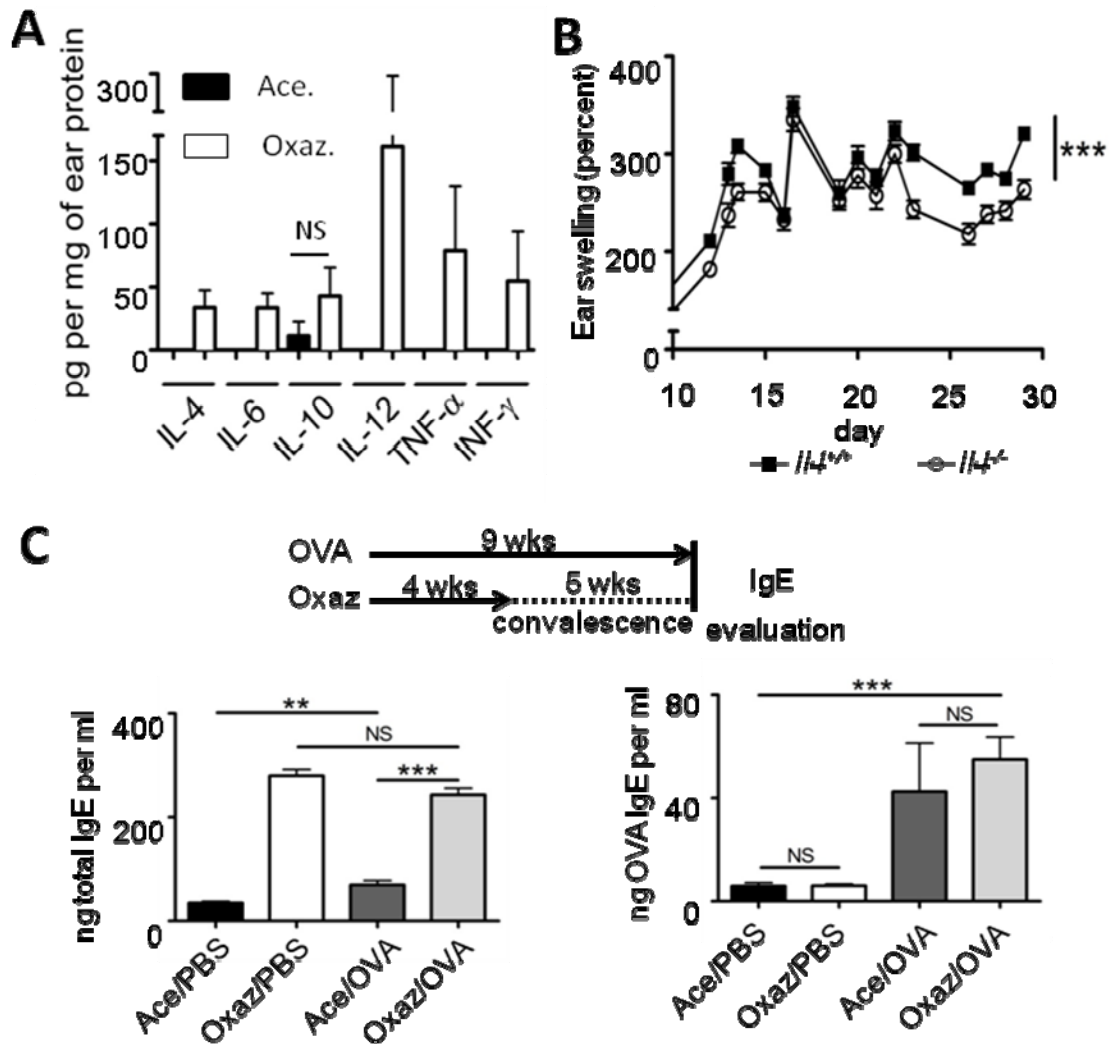


Figure S2: Dermatitis-induced IL-4 production does not enhance concomitant sensitization to OVA. (A) Ears were collected from acetone- and oxazolone-treated mice ($n=4$), homogenized in 700 μ l PBS and protease inhibitor and cytokines profiled by antibody arrays. (B) Comparison of ear swelling between wild type ($Il4^{+/+}$) and $Il4^{-/-}$ mice during induction of oxazolone dermatitis ($n=5$). ***, $p<0.0001$. (C) Mice were subjected to treatment with OVA (9 weeks) and oxazolone (4weeks) as depicted in the top panel. After 9 weeks, sera were collected from all mice and evaluated for total (bottom left) and OVA (bottom right) IgE ($n=6$). **, $p=0.0034$; ***, $p<0.0001$.