Published in final edited form as:

J Allergy Clin Immunol. 2015 February ; 135(2): 477–487. doi:10.1016/j.jaci.2014.07.003.

Eosinophil-dependent skin innervation and itching following contact toxicant exposure in mice

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Abstract

Background—Contact toxicant reactions are accompanied by localized skin inflammation and concomitant increases in site-specific itch responses. The role(s) of eosinophils in these reactions is poorly understood. However, previous studies have suggested that localized eosinophil-nerve interactions at sites of inflammation significantly alter tissue innervation.

Objective—To define a potential mechanistic link between eosinophils and neurosensory responses in the skin leading to itching.

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The authors have no conflicting financial interests.

These funding sources had no involvement in study design, data collection (including analysis and interpretation), the writing of the manuscript, or the decision to submit for publication.

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Methods—BALB/cJ mice were exposed to different contact toxicants, identifying trimellitic anhydride (TMA) for further study on the basis of inducing a robust eosinophilia accompanied by degranulation. Subsequent studies using TMA were performed with wild type vs. eosinophil-deficient *PHIL* mice, assessing edematous responses, remodeling events such as sensory nerve innervation of the skin, and induced pathophysiological responses (i.e., itching).

Results—Exposure to TMA, but not dinitrofluorobenzene (DNFB), resulted in a robust eosinophil skin infiltrate accompanied by significant levels of degranulation. Follow-up studies using TMA with wild type vs. eosinophil-deficient *PHIL* mice showed that the induced edematous responses and histopathology were, in part, causatively linked with the presence of eosinophils. Significantly, these data also demonstrated that eosinophil-mediated events correlated with a significant increase in substance P content of the cutaneous nerves and an accompanying increase in itching, both of which were abolished in the absence of eosinophils.

Conclusions—Eosinophil-mediated events following TMA contact toxicant reactions increase skin sensory nerve substance P and, in turn, increase itching responses. Thus, eosinophil-nerve interactions provide a potential mechanistic link between eosinophil-mediated events and neurosensory responses following exposure to some contact toxicants.

Keywords

contact hypersensitivity; eosinophil-deficient; sensory nerve; degranulation

INTRODUCTION

Chemically-induced contact hypersensitivity responses induce inflammatory skin reactions with typical clinical features characterized by toxicant-specific inflammatory cell infiltrates (see for example ^{1, 2}), poorly defined erythematous and edematous events, and skin remodeling (e.g., dermal thickening) in chronic settings ³. These contact responses, together with related diseases such as allergic contact sensitivities and atopic dermatitis, are typically linked with self-perpetuating scratch-itch cycles and common skin conditions associated with 7–10% of the population ⁴. The underlying immunobiology of these responses is complex and also likely includes both genetic and environmental factors that affect immune responsiveness ⁵, skin barrier function(s) ⁶, and the context of toxicant/allergen exposure ⁶.

The specific character of the inflammatory responses to contact toxicant exposure defines the immune responses, and, in turn, the symptoms/pathologies linked with a specific toxicant. Mixed Th1/Th17 and Th2 cellular responses characterized by the production of IL-2, IFN- γ , and IL-17 are prevalent responses linked with the inflammation and injury associated with many toxicant contact hypersensitivity reactions see for example ⁷. For example, epicutaneous sensitization by haptens such as dinitrofluorobenzene (DNFB ⁸), dinitrochlorobenzene (DNCB ⁹), or certain metals (e.g., Nickel ¹⁰) induce contact hypersensitivity reactions characterized by acute inflammatory and Th1 responses that are accompanied by skin inflammatory infiltrates often dominated by activated T lymphocytes, monocytes, and increased numbers of neutrophils. In contrast, other toxicants such as trimellitic anhydride (TMA ¹¹) represent non-classical contact allergens that elicit the production of Th2 cytokines such as IL-4, IL-5, and IL-13. This cytokine profile is also

linked with acquired humoral immune responses leading to antigen-specific IgE-immunoglobulin production similar to those associated with atopic dermatitis and contact allergic dermatitis ¹². Moreover, in contrast to DNFB or DNCB mediated toxicant hypersensitivity reactions, TMA-induced Th2 contact responses invariably include a robust eosinophilia ¹¹. The contribution of these skin-infiltrating eosinophils to immune/inflammatory cascades, disease pathology, and ultimately symptoms, is often speculative (reviewed in ¹³).

Our studies defining the potential role(s) of eosinophil mediated activities during inflammatory diseases have recently identified a previously underappreciated link between eosinophils and the nerves that innervate the skin in biopsies from patients with atopic dermatitis ¹⁴. We were able to extend these observations *in vivo* using a keratinocyte-specific IL-5 overexpressing transgenic line of mice that elicits an eosinophilic dermatitis. In both cases, the induced dermal eosinophilia occurring in these mice correlated with a corresponding increase in nerve axon length and branching. We also co-cultured eosinophils with dorsal root ganglia cells (i.e., sensory neurons) and showed that this alone increased axon growth. Provocatively, these studies further demonstrated that eosinophil-mediated effects on nerves were complex and occurred through the secretion of one or more factors beyond obvious explanations such as the release of nerve growth factor ¹⁴.

The potential consequences of eosinophil-nerve interactions in the skin were examined in this report of chemically-induced contact toxicant reactions using an array of eosinophil-specific reagents and mouse models we have developed and characterized. In particular, we showed that eosinophil infiltration and eosinophil degranulation within the skin are prominent features of TMA contact responses but not DNFB contact hypersensitivity reactions. We further demonstrated that edematous inflammatory responses following TMA exposure were dependent on the presence of the induced eosinophil infiltrate as were remodeling events linked with dermal innervation by sensory nerves. Significantly, TMA-induced pathophysiological responses such as itch were reduced in eosinophil deficient mice exposed to TMA, suggesting that eosinophil mediated effects on dermal sensory nerve innervation may represent a mechanistic link between eosinophils and TMA-induced itching.

MATERIALS AND METHODS

Mice

All studies were performed with 6–14 week old mice on a BALB/cJ background. Eosinophil-deficient **PHIL** mice ¹⁵ were bred in-house, continually backcrossing to BALB/cJ mice (n>15 generations). BALB/cJ control mice were purchased directly from Jackson Laboratories (Jackson Research Laboratories, Bar Harbor, ME). Mice in these studies were maintained in ventilated micro-isolator cages housed in the specific pathogen-free animal facility at the Mayo Clinic in Arizona. All protocols and studies involving animals were performed in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

Induction of toxicant contact responses to Dinitrofluorobenzene (DNFB) and Trimellitic Anhydride (TMA) exposure

Dinitrofluorobenzene (DNFB) and Trimellitic anhydride (TMA) sensitization and exposure were performed as described in ⁸ and ¹¹, respectively.

Assessments of toxicant-induced ear swelling and collection of biopsies for histology

The region of the ear below the first cartilage ridge was measured using a Digimatic Caliper (Mitutoyo Corporation Aurora, IL) as an inflammatory marker of TMA toxicant contact exposure. The change in ear thickness in these studies was determined by calculating the absolute increase in thickness of experimental and control ears (day 15) from the average thickness measured at baseline (day 6).

Assessments of TMA-mediated skin histopathology: Structural tissue changes, eosinophil infiltration/degranulation as well as collagen deposition/fibrosis

Ear biopsies were collected and processed for histopathology and immunohistochemistry using eosinophil-specific antibodies as described previously ^{16, 17}. Six transverse serial sections across the midline of the anterior (A)-posterior (P) axis of each ear were obtained and numbered 1, 2, 3, 4, 5, and 6 (A-P). Slide 3 of each set was stained with Hematoxylin-Eosin (H&E; general histological assessments). Slides 1 and 2 were used for immunohistochemistry with an eosinophil-specific mouse anti-mouse EPX monoclonal antibody (**EPX-mAb**, Slide 2) and a negative isotype control antibody (Slide 1) as previously described ¹⁸. Evaluation of tissue infiltrating eosinophils and eosinophil degranulation were evaluated as described in earlier studies ^{18, 19} with modifications noted in this report. Slides 4 and 5 were reserved for qualitative and quantitative assessments of remodeling events, staining Slide 4 with Masson's Trichrome (MT; collagen deposition/fibrosis) and Slide 5 with Picrosirius Red (PSR; collagen deposition/fibrosis), respectively. Quantitative morphometric assessments of subepithelial fibrosis occurring in each experimental cohort of mice were determined from Picrosirius Red stained ear sections evaluated under polarized light as described previously ²⁰. Slide 6 was used for additional histology evaluations, including assessments of mast cell numbers and tryptase release by activated mast cells ²¹.

Imaging of epidermal ear innervation

Immunofluorescence Staining - Epidermal nerve imaging was performed on whole mounts of micro-dissected skin obtained from the base of control (vehicle-alone treated) and toxicant exposed ears. The concave half of the ear was initially separated from the convex half and intervening cartilage using sharp micro-dissection scissors. The base of the concave half of ear skin was manually plucked free of large hairs using forceps. Afterwards, a rectangular strip of skin that encompassed the full width of the tissue section was isolated, extending from the base of the ear up 1cm. The tissue was then flipped epidermis-side down and secured with dissecting pins for removal of deeper dermis layers using forceps under a dissecting microscope. This processed epidermal biopsy was then submerged and fixed overnight in Zamboni's Fixative (American MasterTech Scientific Inc., Lodi, CA) at 4°C. Following fixation, the ears were removed and placed in a 40µm cell strainer and washed

extensively (20mL) with 1x PBS until the wash buffer became clear, losing the yellow hue associated with Zamboni's Fixative.

Immunostaining tissue whole mounts was performed using extensive tissue permeabilization, long antibody incubation, and specific washing steps as adapted from our previously described methodology²². Specifically, we avoided dehydration, tissue embedding/sectioning, and two-dimensional image analysis in order to minimize known distortion artifacts of nerve length, nerve surface area, and nerve volume. Following fixation and washing, whole-mount immunofluorescence staining was performed at 4°C. Ear whole-mounts were placed on a charged microscope slide with an aqueous mounting medium (Vectorlabs, Burlingame, CA). Fragments of 1mm glass coverslips were used to secure temporarily the ear whole-mounts in an epidermis-side down configuration before mounting. The 1mm coverslip fragments were removed and the whole-mounts were quickly secured with a 22x22x1.5mm coverslip that was weighed down by 100g weights prior to sealing with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). Substance P expressing nerves were stained via the same protocol using a rat-anti substance P (BD Pharmingen (clone NC1), San Jose, CA) primary antibody and a goat anti-rat 555 (Invitrogen, Carlsbad, CA) secondary antibody.

Quantification of Nerve Structural Characteristics - For large field-of-view images, we developed diffusion filtering methods to reduce image noise and enhance nerve edges as previously described²². Measures of epithelial nerve branching and three-dimensional length were derived from nerve maps using commercially-available image processing software (Imaris Bitplane, Zurich, Switzerland). Mean intensity of substance P staining nerves, including epidermal nerve length and frequency of branching, was calculated and expressed as the ratio of measurements in TMA-exposed ears relative to vehicle-alone treated contralateral ears from the same mouse.

Assessment and quantification of itching in response to TMA exposure

A time-lapsed video based strategy was developed to quantify the level of itching that resulted as a consequence of induced inflammatory responses. This pathophysiological measurement was compiled from video observations of groups of mice on the night prior to the conclusion of the TMA exposure protocol between protocol days 14 and 15. Specifically, groups of mice (typically 4 wild types and 4 eosinophil-deficient *PHIL*) were housed individually side-by-side in separate sectioned areas of a container and are filmed from above in minimal lighting (2–3 lumens) over the course of 6–8 hours. The raw digital video is screened to identify 8 non-overlapping 15 minute segments during the filming period where the mice are alert and display evidence of activity. Video clips of individual mice (15 minutes each) were created from the raw digital data and given a virtual label number of 1 through 8. Three of the video segments were randomly selected and scored by 3 investigators blinded to the mouse genotypes and the exposure of each ear, counting itching-events associated with either the left or right ear. In these studies, 100 total itching events were counted from each of the available video segments (i.e., tallying the itching events from both the left and right ears of a given mouse). An itching event is defined as a unique and deliberate scratching of the ear in question using either the front or hind paws. For

clarity, “face-washing” and scratching events that were directed near but not at an ear were not counted as ear-specific itching events. To insure uniformity of counting and minimize investigator to investigator variability, training video segments scored by experienced investigators who were not evaluators in this study were recorded and used as training tools for naïve evaluators. The study evaluators then viewed and scored three video segments recorded for a given study corresponding to each of the mice in a study; each investigator scoring 100 itching events. Itching event scores associated with TMA exposure for each mouse within a given genotype (i.e., wild type vs. *PHIL*) were assessed as fold-increase of ear itching events over the contralateral control ear of that mouse. The data are expressed as the mean $\pm$ SEM derived from these evaluations.

RESULTS

Toxicant contact responses to trimellitic anhydride (TMA), and not dinitrofluorobenzene (DNFB), are accompanied by a robust tissue eosinophilia

The inflammatory skin responses in mice utilizing the non-classical allergen toxicant trimellitic anhydride (TMA¹¹) were compared specifically to contact hypersensitivity reactions elicited by dinitrofluorobenzene (DNFB⁸) for their ability to induce a concomitant tissue eosinophilia. As shown in Figure 1, despite the differences associated with induced immune responses⁹ these models share logistically similar strategies (overlaid on model-specific timelines). These similarities include a sensitization phase of toxicant exposure to a shaved region of a dorsal flank prior to the application of toxicant to the same mouse later in the protocol to the experimental (left) ear (control contralateral right ears were exposed to vehicle alone (olive oil:acetone (4:1))). In this comparison, ear tissue biopsies recovered from mice exposed to either toxicant were subjected to immunohistochemistry using a monoclonal antibody recognizing the unique eosinophil granule protein eosinophil peroxidase (*EPX-mAb*¹⁸). These data confirm data from earlier studies (see for example¹¹) suggesting the more Th2-like character of immune responses linked with exposure to TMA relative to DNFB. Specifically, whereas DNFB challenge of previously exposed mice was accompanied by only a nominal dermal eosinophil infiltrate, a robust tissue eosinophilia was easily identified by specific antibody staining in the area of skin receiving a secondary exposure to TMA (Figure 2).

Eosinophils contribute to the inflammatory responses and histopathologies associated with TMA induced toxicant contact responses

The dependence of endpoint inflammatory measures linked with the observed eosinophilia associated with the non-classical allergic responses of TMA exposure was assessed by comparing the induced responses occurring in cohorts of wild type vs. eosinophil-deficient *PHIL* mice. The contribution of eosinophils to these induced histopathologies was immediately evident in Hematoxylin-Eosin stained sections derived from each group of mice (Figure 3(A)). These data showed that in the absence of eosinophils, TMA challenge of previously sensitized mice led to smaller inflammatory cell infiltrates. Cell differential assessments demonstrated that the smaller inflammatory cell infiltrate in *PHIL* mice was variable and resulted not only from the loss of eosinophils relative to wild type animals but also decreases in each of the other prominent cell infiltrates (i.e., lymphocytes and mast

cells) that occurred following TMA challenge. This lower cell infiltrate was also accompanied in some cases by a demonstrable decrease in the activation of these proinflammatory cells such as a decrease in mast cell degranulation (Supplementary Figure 1). In addition, histopathological evidence of reduced epithelial hypertrophy/hyperplasia, tissue edema, and structural changes linked with remodeling events were observed in TMA challenged *PHIL* mice relative to wild type animals. Indeed, quantitative assessment of collagen-deposition (Picrosirius Red stained tissue sections (Figure 3(B)) in response to TMA challenge demonstrated that tissue fibrosis was significantly reduced relative to TMA challenged wild type animals (Figure 3(C)). Gross morphological consequences of the eosinophil-dependent remodeling events occurring in response to TMA were determined with a caliper measuring changes in ear thickness as a consequence of TMA challenge exposure (control ears were treated with vehicle alone). These data showed that both wild type and *PHIL* mice each displayed significant increases in ear thickness relative to vehicle-alone treated contralateral ears (Figure 4). However, detailed comparisons of TMA treated wild type vs. *PHIL* ears showed that ear thickness was significantly reduced in the absence of eosinophils, suggesting that the presence of these granulocytes contributes to the induced inflammatory responses associated with this model (Figure 4).

TMA-induced toxicant contact responses result in both a robust eosinophil infiltrate and significant levels of eosinophil degranulation

EPX-mAb based immunohistochemistry was used to quantify the extent of TMA-induced eosinophil infiltration and the magnitude of tissue degranulation in wild type mice relative to controls (i.e., contralateral vehicle-alone treated ear). Representative *EPX-mAb* stained tissue sections from TMA treated wild type vs. *PHIL* mice (Figure 5(A)) showed that in contrast to the eosinophil-deficient character of *PHIL* mice, wild type animals displayed a significant increase in tissue infiltrating eosinophils. These data were used to identify the maximum foci within the biopsy at 400X, 0.29mm² field-of-view. When quantified using a comparative numerical scale this increase was shown to be >4-fold higher relative to contralateral ears of the same mice (Table 1). Similar detailed assessments of the eosinophil degranulation accompanying TMA challenge of sensitized mice showed that this toxicant contact reaction also resulted in a spectrum of events from no evidence of eosinophil degranulation (Level 1) to evidence of increasing eosinophil degranulation (Level 2 and Level 3) that ends at Level 4 – extensive granule protein release and deposition within the extracellular matrix (Figure 5(B)). More importantly, this degranulation was quantitatively higher in TMA challenged mice relative to contralateral vehicle-alone treated ears from the same mice (Table 1). As we have demonstrated in earlier studies^{18, 19}, our numerical assessments of both eosinophil infiltration and degranulation can be used to develop an algorithm (Eosinophil Activity Index (**EAI**)) representative of eosinophil-mediated activities in a given biopsy. These assessments (Figure 5(C)) provide a robust and discriminatory histopathologic metric of TMA-induced toxicant contact reactions in wild type mice.

TMA challenge of previously sensitized wild type mice elicits an eosinophil-dependent increase in substance P-expressing nerve innervation

Our previous studies using transgenic mice constitutively expressing the eosinophil agonist cytokine IL-5 from keratinocytes showed that the cutaneous eosinophilia in these mice

correlated with significant increases in cutaneous innervation¹⁴. We therefore hypothesized that the loss of eosinophils in *PHIL* mice may attenuate similar responses in TMA exposed mice and provide a mechanistic link between eosinophils and neurosensory responses such as itching. We stained whole mounts of ears using an antibody to PGP 9.5 (a pan-neuronal marker) and an antibody to substance P to assess TMA-induced changes in dermal nerves and the potential role eosinophils may have in these changes (Figure 6(A)). Quantification of three dimensional reconstructions of these confocal images²² showed that innervation of the ear (i.e., PGP 9.5 staining) was equivalent in wild type vs. *PHIL* mice and did not increase in response to TMA exposure. However, while the presence of substance P-expressing sensory nerves was the same in the vehicle-alone treated contralateral ears of wild type and *PHIL* mice, TMA induced a 40% increase in these substance P-expressing nerves in wild type relative to similarly exposed eosinophil-deficient *PHIL* mice (Figure 6B).

Eosinophils modulate the level of localized itching events linked with TMA-induced inflammation

Our demonstration that the presence of eosinophils correlated with increased substance P containing nerves in the skin after TMA exposure suggested that eosinophil-deficient mice may display different neurosensory responses associated with TMA exposure relative to wild type animals. Time-lapse videography was performed on mice exposed to TMA during the evening hours between the last day of challenge (day 14) and the day of analysis (day 15). Video images were collected and ear-specific itching events following TMA challenge were quantified relative to itching events occurring in the vehicle-alone treated contralateral ears of each mouse (Figure 7). These data showed that similar to the reduction substance P innervation of the skin in *PHIL* mice (relative to wild type), the absence of eosinophils was correlated with a significant reduction in TMA-induced itching events.

DISCUSSION

Contact responses to various toxicants and/or allergens are accompanied by a spectrum of downstream immune/inflammatory reactions that focally elicit local T cell mediated events in the skin. In turn, this leads to accumulation of proinflammatory effector cells and concomitant histopathological and pathophysiological changes. Clearly, the translational interest in the character and extent of the induced immune responses in the skin is how the immunity surrounding toxicant/allergen exposure contributes to the pathologies and, in turn, the symptoms experienced by afflicted subjects. Scratching represents a near ubiquitous response whose origins, onset, and progression are poorly understood.

The diversity of the immune/inflammatory consequences of toxicant exposures was highlighted here in the eosinophilic inflammation that occurred only upon exposure to TMA and not DNFB. Specifically, earlier studies have shown that despite eliciting similar downstream endpoint pathologies and/or symptoms, the toxicant contact hypersensitivity responses elicited by the non-classical allergen TMA were characterized by Th2 polarized acquired immune responses that also included a robust eosinophil infiltrate¹¹. The TMA exposure studies presented in this report confirm and characterize in more detail the induced

local eosinophil infiltrate in response to this toxicant. Specifically, the TMA induced dermal eosinophilia was linked with degranulation and the release of eosinophil secondary granule proteins, an observation rare in mouse models of human diseases (reviewed in ²³). The potential importance of these eosinophil-mediated events as part of the complex mechanisms contributing to the inflammation observed in response to TMA were highlighted by our use of an eosinophil-deficient strain of mice. For example, in addition to the loss of eosinophil effector functions such as degranulation, the absence of eosinophils in TMA-treated *PHIL* mice led to decreases in the local accumulation of unique pro-inflammatory cell populations such as mast cells. Collectively, the loss of these eosinophil mediated events was also linked with decreases in TMA-induced edema (i.e., ear thickness) and other histopathological events such as tissue fibrosis, suggesting that eosinophils are necessary contributors to many of the inflammatory events following TMA exposure. However, it is also noteworthy that the current data do not identify a specific eosinophil-mediated mechanism(s) leading to these induced inflammatory changes and tissue remodeling events. That is, it remains possible that eosinophils are either directly contributing activities that promote inflammation/remodeling events (e.g., through the release of granule proteins ²⁴ or eosinophil-derived TGF β expression ^{25, 26}) or are doing so through downstream effects on resident cells (e.g., activation of skin keratinocytes ²⁷) and/or other pro-inflammatory cells such as the modulation of T cell activation/polarization ²⁸ as well as eosinophil mediated changes in local immune cascades leading to mast cell accumulation and activation.

Our earlier studies using a keratinocyte IL-5 overexpressing transgenic mouse model showed that the eosinophil infiltration of the skin in these models correlated with greatly enhanced innervation of the skin ¹⁴. These studies further showed *in vitro* that eosinophils promoted growth and branching of cultured dorsal root ganglion neurons. Significantly, these interactions did not require cell-cell contact nor were eosinophils simply secreting obvious candidate molecules such as nerve growth factor ¹⁴. Provocatively, the current report demonstrated that TMA induced increases in substance P-expressing innervation of the skin were lost in the absence of eosinophils as were TMA-induced increases in itching. This concomitant loss of TMA-induced substance P-expressing innervation of the skin and itching thus suggests a mechanistic link between resident eosinophils and itching. In this hypothesis, inflammatory skin exposures elicit the accumulation of eosinophils and the activation of these granulocytes may either directly promote the growth and branching of nerves in affected skin lesions (as suggested by our *in vitro* studies) and/or promote skin innervation through downstream effects on resident cells within the skin or the accumulation/activation of other pro-inflammatory cells. The specific eosinophil-mediated mechanisms eliciting substance P-expressing nerve growth and branching (and, in turn, the itching events linked with TMA-induced inflammatory skin reactions) remain unresolved and will clearly be the target of future studies.

These studies of contact toxicant exposure in the mouse have potentially significant clinical implications. Itch is a prominent feature of many forms of dermatitis, and is frequently severe, compromising quality of life. For example, a study of the effects of allergic skin disease on quality of life in children found decreases similar to those in children with asthma, and renal disease ¹⁴. Scratching typically worsens the condition of the skin, making

control of itch important both for relief of this unpleasant symptom and for dampening of the inflammation associated with dermatitis. Nonetheless, despite its clinical importance the neurophysiology of itch remains comparatively unresolved relative to insights associated with pain. However, several similarities have been noted. Cutaneous nerve number and tachykinin content of the skin leads to increases in the perception of itch in dermatitis patients ^{29, 30}. A similar phenomenon is well studied in inflammatory pain. Increased tachykinin expression and, in particular, expression of tachykinins in mechanosensitive sensory A fibers, which normally do not express tachykinins leads to “allodynia” where stimuli that would normally be perceived as touch are now perceived as pain ³¹. The corresponding process where touch stimuli are perceived as itch is referred to as “allokinesis” and although the neural changes involved are not as well characterized, both growth of cutaneous nerves and increased tachykinin expression have been observed in both human skin biopsies ^{32, 33} and mouse models of dermatitis ³⁴. Moreover, the use of Aprepitant, an antagonist at the Substance P receptor NK1, was effective in relieving itch in a pilot study ³⁵, and a similar antagonist, DNK333, is currently in clinical trials for the relief of itch in patients with atopic dermatitis (available at clinicaltrials.gov/ct2/show/NCT01033097). Our study suggests that new therapeutic strategies aimed at eosinophil-nerve interactions (either direct interactions or through 3rd party cells) may also be effective in eosinophilic skin diseases.

There is no reason *a priori* that our hypothesis linking eosinophil-nerve interactions to itching events is limited to the inflammation induced by chemical toxicants and/or delayed hypersensitivity responses in general. Indeed, the underlying mechanisms suggested here appear to lack such specificity and thus may have relevancy in more common conditions linked with eosinophils and/or the deposition of eosinophil secondary granules such as allergic contact dermatitis and atopic dermatitis ³⁶ (reviewed in ¹³). As such, the significance of eosinophil-nerve interactions may be far greater than previously realized and represented an underappreciated target for novel therapeutic approaches addressing contact dermatitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The performance of these studies, including data analysis and manuscript preparation, was supported by resources from the Mayo Foundation and a grant from the United States National Institutes of Health [JL (HL065228, RR0109709), NAL ((HL058723), DBJ (HL113023), ADF (ES017592 and ES014601) and JL and DBJ (AR061567)].

The authors wish to thank the Mayo Clinic in Arizona Veterinarian Pathologist, Dr. Ron Mahler for his valuable assistance evaluating ear biopsies following toxicant contact exposure. We also wish to thank members of Lee Laboratories who provided assistance in data collection, the necessary organization/infrastructure needed to complete the studies presented, and the review of various drafts of this manuscript. We also wish to acknowledge the invaluable assistance of the Mayo Clinic Arizona medical graphic artist, Marv Ruona, and the excellent administrative support provided to Lee Laboratories by Linda Mardel and Shirley (“Charlie”) Kern.

Abbreviations

TMA	Trimellitic anhydride (TMA)
DNFB	Dinitrofluorobenzene (DNFB)
DNCB	Dinitrochlorobenzene (DNCB 9)
PHIL	Eosinophil-deficient transgenic mice
SEM	Standard error of the mean
EPX-mAb	Eosinophil peroxidase-specific monoclonal antibody
EAI	Eosinophil Activity Index
PGP 9.5	Pan-neuronal antibody maker

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Key Messages

- Eosinophils differentially accumulate at the sites of exposure to some but not all chemical toxicants
- Eosinophils contribute to the magnitude of inflammation occurring in response to exposures with a non-classical allergen toxicant
- Eosinophil-dependent increases in skin innervation accompany exposure to a non-classical allergen toxicant
- Eosinophils accumulating in the skin are required for the induced itching responses that occur following non-classical allergen toxicant exposure

Figure 1. Toxicant contact exposure protocols to either Dinitrofluorobenzene (DNFB) or Trimellitic Anhydride (TMA)

Experimental and control mice are sensitized by exposure to (A) **DNFB** or (B) **TMA** and subsequently toxicant challenged (left ear) in the time frames described with the contralateral right ear of each mouse challenged using vehicle alone ((**DNFB**) olive oil:acetone (3:1) or (**TMA**) olive oil:acetone (4:1)) to provide a negative controls for exposure.

Figure 2. The toxicant contact skin reactions that follow exposure to Trimellitic Anhydride (TMA), but not dinitrofluorobenzene (DNFB), elicit a robust differential recruitment and/or accumulation of eosinophils

Infiltrating eosinophils in ear biopsies following exposure to TMA or DNFB were identified by immunohistochemistry using a rat anti-mouse MBP monoclonal antibody; negative control biopsies were derived from contralateral ears exposed to vehicle alone ((**DNFB**) olive oil:acetone (3:1) or (**TMA**) olive oil:acetone (4:1)). Scale bar = 100 μ m

Figure 3. Ear tissue biopsies demonstrate that eosinophil-mediated activities contribute to TMA-induced inflammatory and remodeling responses

(A) Hematoxylin-eosin stained sections of ear tissue biopsies from wild type and eosinophil-deficient *PHIL* mice exposed to TMA (negative controls are contralateral ears exposed to vehicle alone (olive oil:acetone (4:1))). Scale bar = 50μm. (B) Representative Picrosirius Red stained ear sections from Wild Type and age-matched (12 week postpartum) eosinophil-deficient *PHIL* mice (visualized under polarized light). E, epidermis; M, muscularis, CA, cartilage. Scale bar = 100μm. (C) Under polarized light, the sub-epidermal interstitial area within an entire ear biopsy from n = 5–9 mice/group were evaluated at a magnification of 200X – evaluating the area underlying 0.7mm linear length of epidermis. The extent and intensity of Picrosirius Red staining was quantified as the sum total (Σ) of pixel values (pixel number x average pixel intensity within the region surrounding the basement membrane of epidermis normalized to the total linear length of epidermis evaluated. n.s., not significant, *P<0.05

Figure 4. The remodeling events linked with eosinophils infiltrating the skin contribute to the increased ear thickness that occurs following exposure to TMA

Ear thickness (mm) of previously sensitized wild type (○) vs. eosinophil-deficient *PHIL* (●) mice challenged with TMA was measured on protocol day 15 using a micrometer as a surrogate marker for tissue edema; contralateral ears exposed to vehicle alone (olive oil:acetone (4:1)) were used as negative controls.

Figure 5. TMA-induced skin inflammatory responses lead not only to eosinophil accumulation but also to eosinophil degranulation and the release of secondary granule components within the interstitium underlying the epidermis

EPX-mAb based immunohistochemistry of ear biopsies ($n = 5/\text{cohort}$) demonstrated that a spectrum of eosinophil infiltration and degranulation levels occur in TMA-challenged wild type mice. (A) Representative moderate ($160\times$, 1.8mm^2 field-of-view) power photomicrographs were used to identify the maximum foci of eosinophil infiltration in each biopsy (Table 1). Scale bar = $100\mu\text{m}$. (B) Representative photomicrographs ($400\times$ *hpf* (0.29mm^2 field-of-view)) of each level of degranulation (Table 1) as determined by *EPX-mAb* based immunohistochemistry (**magenta staining cells and extracellular matrix regions**). Scale bar = $100\mu\text{m}$. (C) An algorithm entitled “Eosinophil Activity Index (EAI)”, representing both eosinophil tissue infiltration and eosinophil degranulation (Table 1), was used to quantify eosinophil-mediated activities in the skin of TMA-challenged wild type vs *PHIL* mice; negative controls are vehicle-alone treated ears (olive oil:acetone (4:1). * $P < 0.05$

Figure 6. TMA-induced inflammation elicits an eosinophil-dependent increase of sensory nerve innervation of the skin

(A) Representative confocal immunofluorescence images of Wild Type and eosinophil-deficient *PHIL* mice following challenge with TMA (control exposures were vehicle-alone (acetone:olive oil (4:1), identifying all dermal (PGP 9.5 (green)) and sensory dermal (substance P (red)) nerves. Scale bar = 100 μ m. (B) The quantification of the TMA-mediated increase in substance P expressing nerve density (relative to vehicle-alone treated contralateral ears) demonstrated that unlike total innervation, which did not change between wild type and *PHIL* ears treated with vehicle-alone (acetone:olive oil (4:1), the density of substance P expressing nerves increased (relative to contralateral ear treated with vehicle-alone) in TMA-treated wild type ears (n = 4) with ears from TMA-treated eosinophil-deficient *PHIL* mice displaying no increase in these nerves (n = 3). *P<0.05

Figure 7. The induced itching occurring in response to TMA-mediated inflammation is an eosinophil-dependent phenomenon

TMA-mediated itching events were assessed from time-lapsed videography following the last TMA exposure (i.e., the night between protocol days 14 and 15). Quantification of itching events in wild type (○) vs. eosinophil-deficient *PHIL* (●) mice was assessed relative to contralateral control ear from the same mouse by three intra/inter-observer blinded investigators (n = 7–8 mice/group). These data showed that the induced itching associated with TMA-mediated inflammation was significantly lower in the absence of eosinophils (*PHIL*) compared to itching events occurring in wild type mice. *P<0.05

Eosinophil-associated Metrics in the Skin Following TMA-challenge of Wild Type vs. PHIL mice §

Table 1

Ear-challenge Exposure	Mouse Study Number	Eosinophil Infiltration in the Maximum Focus - E _{max} (Numerical Score) [†]	Level of Eosinophil Degranulation - E _{deg} (Numerical Score) [‡]	EAI Numerical Score (E _{max} x E _{deg})
TMA	1	63	2	8.0
	2	187	4	20.0
	3	23	2	4.0
	4	214	4	20.0
	5	147	4	20.0
	TMA-treated Wild Type Mice		3.2 ± 0.5 (3.2 ± 0.5)	14.4 ± 3.5
	6	5	3	3.0
	7	6	3	3.0
	8	15	3	3.0
	9	1	2	2.0
TMA	10	2	2	2.0
	Vehicle-treated Wild Type Mice		5.8 ± 2.5 (1.0 ± 0.0)	2.6 ± 0.2
	11	0	1	1.0
	12	0	1	1.0
	13	0	1	1.0
	14	0	1	1.0
	15	0	1	1.0
	TMA-treated PHIL Mice		0.0 ± 0.0 (1.0 ± 0.0)	1.0 ± 0.0
	16	0	1	1.0
	17	0	1	1.0
Vehicle	18	0	1	1.0
	19	0	1	1.0
	20	1	1	1.0
	Vehicle-treated PHIL Mice		0.2 ± 0.2 (1.0 ± 0.0)	1.0 ± 0.0
			1.0 ± 0.0 (1.0 ± 0.0)	1.0 ± 0.0

Serial sections from each biopsy were coded by histopathology laboratory personnel and in each case the middle (slide **3**) of six serial sections was stained with Hematoxylin - Eosin (H&E). Slides **1** and **2** of the series was subjected to immunohistochemical staining with a nonspecific isotype control and antibody and monoclonal antibody targeting eosinophil peroxidase (*EPX-mAb* ¹⁸), respectively. All evaluations were performed in an intra/inter-investigator blinded fashion. Summary values shown for each cohort are group mean averages $\pm$ SEM.

[‡] Numerical values were assigned to each level of eosinophil infiltration in the maximum foci of the biopsy (assessed as the average of five 400X *hpf* * (0.29mm² field-of-view)). The assigned levels are based on the distribution of the data spanning the observed levels in negative control contralateral ears to the highest levels of eosinophil infiltration observed in TMA-challenged mice. Eosinophil infiltration Level **1** represents TMA-challenged mice displaying the same variation of eosinophil numbers found in vehicle-treated contralateral ear tissue (i.e., 0–15 eosinophils/400X *hpf*). Eosinophil infiltration Level **2** were TMA-challenged mice that displayed an eosinophil infiltrate of 16–30 eosinophils/400X *hpf*. Eosinophil infiltration Level **3** are TMA-challenged mice ranging from 31–60 eosinophils/400X *hpf*. Eosinophil infiltration Level **4** are TMA-challenged mice ranging from 61–120 eosinophils/400X *hpf* and Eosinophil infiltration Level **5** are those TMA-challenged mice displaying extraordinarily high levels of eosinophil infiltrates of >120 eosinophils/400X *hpf*.

[¶] Numerical values were assigned to each level of eosinophil degranulation based the distribution of degranulation displayed by the data spanning levels similar to that observed in negative control contralateral ears to the highest levels of eosinophil infiltration observed in TMA-challenged mice. **Eosinophil degranulation level – 1:** No release of EPX; **Eosinophil degranulation level – 2:** Minimal level of EPX release (i.e., degranulation) around intact eosinophils; **Eosinophil degranulation level – 3:** Minimal level of EPX release (i.e., degranulation) around intact and fragmenting eosinophils; **Eosinophil degranulation level – 4:** Extensive and wide-spread extracellular matrix deposition of EPX.