ONZIN deficiency attenuates contact hypersensitivity responses in mice

Julie G Ledford¹, Martina Kovarova², Leigh A Jania¹, MyTrang Nguyen¹ and Beverly H Koller¹,²

ONZIN is abundantly expressed in immune cells of both the myeloid and lymphoid lineage. Expression by lymphoid cells has been reported to further increase after cutaneous exposure of mice to antigens and haptens capable of inducing contact hypersensitivity (CHS), suggesting that ONZIN has a critical role in this response. Here, we report that indeed ONZIN-deficient mice develop attenuated CHS to a number of different haptens. Dampered CHS responses correlated with a significant reduction in pro-inflammatory IL-6 at the challenge site in ONZIN-deficient animals, compared with wild-type controls. Together the study of these animals indicates that loss of ONZIN impacts the effector phase of the CHS response through the regulation of pro-inflammatory factors.

Keywords: cell trafficking; contact hypersensitivity; inflammation; ONZIN

ONZIN is a small cysteine-rich cytoplasmic protein expressed at high levels in both cells of the immune system and in many epithelia, particularly of the intestinal and respiratory tracts.¹ Promoter levels in both cells of the immune system and in many epithelia, ONZIN is a small cysteine-rich cytoplasmic protein expressed at high levels in both cells of the immune system and in many epithelia, particularly of the intestinal and respiratory tracts.¹ Promoter analysis suggests that the expression of this gene is subject to regulation during immune responses (unpublished data) and genome-wide expression studies have identified increased levels of ONZIN in inflammatory lesions. Despite these findings, relatively little progress has been made in assigning a role for ONZIN in specific immune responses. Recent studies have shown that ONZIN has an important function in the innate immune response to bacterial infections. Studies from our lab have demonstrated that expression of ONZIN by neutrophils is necessary for optimal intracellular killing of several common pathogens.¹ However, an independent function in adaptive immune responses is suggested by expression of ONZIN in cells orchestrating this response: T cells and dendritic cells (DCs). Subtractive hybridization using complementary DNA obtained from monocyte-derived DCs versus plasmacytoid-derived DCs identified Onzin as a gene that is differentially expressed between these two populations of human DCs.² Whereas ONZIN expression was relatively high in plasmacytoid DCs, only minimal levels of expression were observed in monocyte-derived DCs.² This differential expression is of particular interest as plasmacytoid DCs have been observed to differentiate into mature DCs capable of priming CD4⁺ T cells toward either Th1 or Th2 responses depending on the activation stimuli. In an independent complementary DNA microarray analysis, Onzin was identified as a gene highly upregulated in draining auricular lymph node tissue following a primary exposure to the contact allergen 2,4-Dinitrofluorobenzene (DNFB).³ Onzin was maximally expressed 48 h after exposure and represented one of the most upregulated genes observed in the lymph tissue by transcriptional profiling. Although these experimental data suggested the importance of ONZIN in this DC/T-cell interaction, a defined role for ONZIN in this process has not established.

Contact hypersensitivity (CHS), is a type of DTH reaction, caused by repeated epicutaneous exposure to a known contact allergen (reviewed in Black⁴). CHS can be induced experimentally in mice by topical application of sensitizing agents, such as Oxazalone or DNFB. The resulting response mimics the reactions observed in groups of people sensitive to poison ivy, various drugs and industrial or household chemicals. CHS develops through several distinct steps, which remain the focus of much research. The first phase, sensitization, occurs after an initial epicutaneous allergen exposure and typically little to no symptoms of exposure are evident at this point. In the elicitation phase, allergen-specific memory T cells residing within the lymphatics are activated upon reexposure by the same allergen. An immune response is provoked at the site of the second encounter resulting in a local inflammatory reaction characterized clinically as allergic contact dermatitis.⁵

Animal models of CHS often use haptens, which are non-proteinaceous substances that themselves do not elicit antibody formation but act through altering endogenous proteins that result in their immunogenicity. During the different phases of CHS, several cell types have been identified as key players during a hypersensitivity reaction. First, Langerhans cells (LC), a type of DC that resides in the epidermis, grasp the hapten at the primary site of contact and migrate to draining lymph nodes (LNs). Once there, the resident naïve T cells are primed

¹Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and ²Department of Medicine, Division of Pulmonary and Critical Care, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
Correspondence: Dr BH Koller, Department of Genetics, University of North Carolina at Chapel Hill, 120 Mason Farm Road, Chapel Hill, NC 27599, USA.
E-mail: treawouns@aol.com
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ORIGINAL ARTICLE
against the hapten through a T-cell–LC interaction. Reexposure of the same hapten at a distant secondary site, leads to an inflammatory response with a cellular infiltrate containing a wide variety of immune cells: macrophages, neutrophils and T cells.

In this study, we utilized Onzin−/− mice in order to elucidate the role(s) of ONZIN in these cellular-mediated inflammatory diseases and immune responses. Using this model, we demonstrate that not only does ONZIN expression increase during this response but also its expression is necessary for normal CHS responses in mice.

RESULTS

ONZIN expression in draining auricular LN

Previous studies indicate that ONZIN expression in draining auricular nodes is increased after a single exposure to the hapten DNFB. To determine whether this increase was limited to the sensitization phase of the response and/or the response to this specific hapten, C57BL/6 Onzin−/− and wild-type mice were sensitized to oxazolone and challenged 5 days later. The draining auricular LN associated with either the oxazolone-treated ear or the untreated ear were collected 24 and 48 h after challenge. Protein lysates were prepared and analyzed by SDS–polyacrylamide gel electrophoresis and western using an ONZIN-specific antibody. As shown in Figure 1a, ONZIN expression was dramatically increased in lysates prepared from the draining auricular LN associated with the oxazolone-treated ears at both time points. Lysates prepared from the LN associated with both naive and the vehicle-treated ear had comparable levels of ONZIN expression as demonstrated by western analysis (Figure 1a). Densitometry analysis of westerns containing multiple LN lysates from each sample type was used to quantify relative levels of ONZIN expression (Figure 1b).

Delayed-type hypersensitivity in ONZIN-deficient mice

We next examined a possible role for ONZIN in the CHS reaction induced by oxazolone. Wild-type and C57BL/6 Onzin−/− animals were sensitized to oxazolone by a topical application to the shaved abdomen. The mice were challenged after 5 days by application of the sensitizing agent to the left pinna, whereas the right was treated with vehicle. The response to oxazolone was assayed using a number of criteria including the difference in the weight of the tissue biopsies obtained from the vehicle and hapten-treated ears. As expected, a treatment of sensitized wild-type (WT) C57BL/6 mice with oxazolone resulted in a robust increase in the weight of the tissue biopsies 12 h after challenge (Figure 2a). This increase in weight was more pronounced 24 h after treatment with impending resolution of the response evident 48 h after exposure. As shown in Figure 2a, the response of the ONZIN-deficient mice was attenuated at all time points examined, and this difference achieved statistical significance at both 24 and 48 h after challenge.

Change in the weight of tissue after induction of the immune response reflects increases in cellularity, increased blood flow to the tissue and edema formation. We therefore determined which of these parameters were attenuated in the ONZIN-deficient animals. CHS was induced as described above and animals and tissues collected 24 h after challenge. This tissue was fixed, stained and the number of immune cells present determined by microscopy. As expected, low numbers of resident immune cells could be identified in the sections obtained from the vehicle-treated pinna (Figure 2b). In contrast, leukocytes were abundant in the wild-type tissue treated with oxazolone. Although leukocytes could also be easily detected in the sections from the Onzin−/− mice, the number of these cells was significantly lower (Figure 2c).

Neutrophils, easily identified based on morphological criteria, comprised a high percentage of cells present in the inflamed wild-type tissue. Few neutrophils appeared to be present in tissue from similarly treated Onzin−/− mice. To verify this, mice were again sensitized and challenged with the hapten, oxazolone. Tissue biopsies were obtained and the amount of myeloperoxidase (MPO), an enzyme specific to neutrophils, present in the sample determined. Consistent with the histological observation, the amount of MPO was reduced to about 50% of that observed in the wild-type animals (Figure 2d). We next determined whether the attenuated cellularity observed in the tissue of the Onzin−/− mice was paralleled by a decrease in edema formation in response to challenge with hapten. Mice were treated with Evans Blue, a dye that binds to serum proteins. Because of this binding, extravasation of serum proteins into the tissue is paralleled by increased levels of Evans Blue measured in tissue, and is therefore, indicative of vascular permeability due to the induced inflammation. Treatment of wild-type mice with hapten resulted in a measurable increase in serum protein present in the tissue biopsies: this was reduced by about 50% in the Onzin−/− mice (Figure 2e).

To determine if the decreased vascular permeability to oxazolone challenge is indicative of a generalized defect in the ability of the Onzin−/− mice to respond to cutaneous inflammatory insults by altering vascular permeability and recruit circulating leukocytes, we examined these mice in an arachidonic acid (AA)-induced acute model of cutaneous inflammation. WT and Onzin−/− mice received 0.5% Evans blue dye intravenously immediately prior to application of AA to the left ear. The acute response to AA was first analyzed by

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Figure 1 ONZIN expression in draining auricular LN after challenge with Oxazolone. (a) Either 24 or 48 h after Oxazolone challenge, Evans blue dye was injected into the ear of C57B1/6 mice. The auricular LN, visualized by the blue dye, associated with the treated ear (+) or the untreated ear (−) were collected and protein lysate prepared. Naïve Onzin+/+ (WT) and Onzin−/− (KO) LN were collected from mice of the same genetic background. Protein lysates were prepared and analyzed by SDS–polyacrylamide gel electrophoresis and western using an ONZIN-specific antibody. 10 μg total protein was loaded for each sample and β-actin was used as a loading control. (b) The relative level of ONZIN expression as shown by western analysis was quantified using Scion Image software and was adjusted relative to the loading control, β-actin. n=4,4 and *P<0.05, **P<0.01.
collecting and weighing 8-mm ear discs. Intense ear swelling was observed within 1 h of challenge, with ear-weight changes of greater than 10 mg (Figure 2f). However, there was no significant difference in ear-weight change between the wild-type and Onzin−/− animals (Figure 2f). Extravasation of plasma proteins into tissue was also quantified by extraction of Evan’s blue dye followed by analysis of the extracts. Vascular permeability due to AA challenge did not differ between the Onzin−/− and control animals (Figure 2g).

Figure 2 Delayed-type hypersensitivity response in Onzin−/− mice in response to Oxazalone. (a) Increase of ear swelling in Onzin+/+ and Onzin−/− C57BL/6 mice from Oxazalone-induced DTH response at different time points after challenge. n=5,5 at 6 h; n=6,6 at 12 h; n=15,15 at 24 h; n=30,29 at 48 h. *P, **P<0.001. (b) Histology of ear tissue 24 h after challenge with Oxazalone or vehicle was visualized by hematoxylin-eosin staining at 4× magnification. (c) Total infiltrating cells per field were enumerated from a blinded set of histology sections of oxazalone- and vehicle-challenged ears using Scion Image. n=8,10 and *P<0.001. (d) Lysates were prepared of Oxazalone- or vehicle-challenged ear skin harvested 24 h post challenge. MPO concentrations were measured from the ear lysates. n=5,6 for vehicle and Oxazalone and is representative of two independent experiments. *P<0.05. (e) Vascular permeability in response to Oxazalone was measured by injecting Evan’s blue dye intravenously into Oxazalone-sensitized/challenged mice 2 h prior to the 24 h harvest time point. Exudative dye in the ear skin was extracted for 24 h and the absorbance at 610 nm was determined. n=5,5 and is representative of two independent experiments. *P<0.01. (f) Onzin+/+ and Onzin−/− mice received 0.5% Evan’s blue dye intravenously immediately prior to application of AA (2 mg in acetone) to the left ear. The right ear received acetone alone. After 1 h, ears were collected and discs were cut for analysis. The wet weight difference between the treated and untreated ears of each animal was calculated as an indicator of tissue edema. (g) Extravasation of plasma proteins into tissue was quantified by extraction of dye with formamide and spectrophotometric analysis of extracts at 610 nm. The difference between the A610 for the left and right ears of each mouse is indicated. n=5,5.
Onzin is expressed at high levels in neutrophils and previous studies have indicated that, at least under some experimental conditions, these cells contribute to the tissue inflammation characteristic of CHS. To determine whether loss of ONZIN might attenuate CHS through a function in this cell type, we first determined whether we could measure a contribution of neutrophils to this response under our experimental conditions. Towards this end, oxazaloned-sensitized mice were depleted of mature neutrophils by treatment with anti-Gr-1 antiserum prior to challenge with oxazalone. To our surprise, no significant difference was observed in the DTH response to either oxazalone or DNFB in these neutrophil-depleted animals compared with control animals (Figure 4b). The successful elimination of the neutrophil population was verified by measurement of MPO levels in the ear tissue after treatment with oxazalone (Figure 4c) and by induction of peritonitis in a parallel group of animals (data not shown). Thus in this model, neutrophils do not contribute significantly to the effector phase of the response, making it unlikely that loss of ONZIN expression in neutrophils contributes to the diminished CHS observed in the Onzin/−/− animals.

Antigen acquisition, migration and presentation in Onzin/−/− mice
After epicutaneous exposure to allergen, epidermal LCs are induced to migrate from the skin and accumulate as DCs in the draining LN, where they interact with allergen-specific T cells. We next sought to determine if the attenuated hypersensitivity responses observed in ONZIN-deficient mice is due to a defect in the sensitization phase, such as a failure in antigen acquisition or migration by DCs. Fluoroscein isothiocyanate (FITC), unlike most haptens, is fluorescent. This feature aids in identification and trafficking studies of cells that have taken up the antigen. After epicutaneous sensitization with the fluorescent antigen, FITC, cells from draining LNs were isolated and analyzed by flow cytometry. Equivalent percentages of FITC+ CD11c+ double-positive DCs were collected from the draining LNs of wild-type and Onzin/−/− mice (Figure 4d). Additionally, there were no differences in the percentage of FITC+ cells from the CD11c+ gated subset (Figure 4e) indicating that equivalent proportions of DCs were migrating to the inguinal LNs from the skin after topical application of FITC.

To further examine the impact of loss of ONZIN expression on antigen presentation and resultant T-cell responses, we examined the capacity of ONZIN-deficient cells to respond to stimulation in a mixed-lymphocyte reaction (MLR). Splenocyte suspensions were prepared from age-matched WT and Onzin/−/− C57BL/6 mice and proliferation was stimulated upon co-culture with WT BALB/c-irradiated cells. Cultured splenocytes from both ONZIN-deficient and control mice demonstrated equivalent levels of proliferation to the allogenic stimulator cells, as shown by H3 incorporation (Figure 4f). An additional MLR experiment was conducted to determine if differences in the proliferative response would be observed by utilizing Onzin/−/− BALB/c irradiated cells as stimulators. The responder cell population was isolated from WT and Onzin/−/− C57BL/6 splenocytes. Both WT and Onzin/−/− cells were induced to proliferate by the Onzin/−/− allogenic stimulator cells (Figure 4g).

Adoptive transfer of sensitized LN cells
The diminished response of the Onzin/−/− mice could reflect a defect in sensitization to antigen or failure to respond to antigen upon

![Graph](image)

**Figure 3** Impaired delayed-type hypersensitivity response in Onzin/−/− mice to DNFB challenge. Increase of ear swelling in Onzin+/+ and Onzin/−/− 129.B6 F1 hybrid mice from DNFB induced DTH response 24 and 48 h after the second challenge. n=5,5 at 24 h and n=13,15 at 48 h. """"**P<0.01.

DNFB-induced CHS in ONZIN-deficient mice
A number of hapten, in addition to oxazalone, have been described that are capable of eliciting CHS. As small differences in the pathophysiology of the immune response to different hapten have been noted, we next determined whether the attenuated response of the Onzin/−/− mice was generally observed in CHS by treating Onzin/−/− and wild-type animals with DNFB. In general, the response of the mice to DNFB was less robust than the responses elicited by oxazalone. However, again we observed a decreased CHS DNFB-induced reaction in the animals lacking ONZIN (Figure 3).

Oxazalone sensitization and challenge post bone marrow transplant
To further define the mechanism by which ONZIN contributes to CHS response, we asked whether expression of ONZIN, by hematopoietic cells specifically was required for optimal response to oxazalone. ONZIN is expressed by some epithelial cell populations, including those of the digestive and respiratory tracts and at low levels in the skin. To address which cell population ONZIN is interacting with in this response, bone marrow chimeras were generated in which the loss of ONZIN expression was limited either to somatic cells or to cells of hematopoietic origin. In addition, two control populations were generated, the first consisting of wild-type mice reconstituted with wild-type marrow, and the second consisting of Onzin/−/− mice reconstituted with Onzin+/− marrow. All mice were exposed to a lethal dose of irradiation prior to receiving the donor marrow and allowed to recover for 4 weeks prior to sensitization with the hapten. A robust response was observed in the wild-type population of mice reconstituted with wild-type marrow, indicating this experimental protocol did not interfere with the ability of the mice to mount a robust response to oxazalone similar to that of wild-type animals. Indeed, oxazalone-provoked tissue inflammation in these animals was not significantly different from that observed in the wild-type mice that received wild-type bone marrow (Figure 4a, WT:KO). Conversely, wild-type irradiated mice reconstituted with Onzin/−/− bone marrow demonstrated a significantly attenuated response to oxazalone (P<0.01 compared with wild-type mice with wild-type marrow) (Figure 4a, KO:WT).
challenges. To distinguish between these two possibilities, wild-type and Onzin−/− mice were only sensitized with oxazolone, and the draining axillary and inguinal LNs were collected. A single-cell suspension isolated from the sensitized mice was then injected into naive WT and Onzin−/− recipients. A sample of each population was analyzed by flow cytometry to verify that there was no difference in the cellular composition, including polymorphonuclear leukocytes, antigen-presenting cells, T and B cells of LNs of the wild-type animals should normalize the response. As expected, C57BL/6 WT mice that received sensitized leukocytes from WT mice showed a robust immune response upon their first exposure to the hapten (Figure 5c). A similar response was observed when wild-type mice received cells from sensitized Onzin−/− mice (KO:WT). In contrast, the response of naive Onzin−/− mice that received wild-type sensitized cells remained significantly attenuated after challenge with oxazolone suggesting a defect in the effector/elicitation phase of the response (Figure 5c).

To further characterize the effector/elicitation phase of the DTH response induced by oxazolone in WT and Onzin−/− mice, we analyzed the cellular composition of the auricular-draining LNs. Cells were isolated 24 h post challenge from draining nodes and stained with antibodies specific for various leukocyte populations. Fluorescence-activated cell sorting (FACS) analysis revealed no significant difference in the relative number of CD4+ or CD8+ T cells, B cells, macrophages, neutrophils or DCs between the WT and Onzin−/− mice (Figure 5d). DCs from draining LN expressed comparable levels of Gr-1, Mac-1 and MHC II similar to wild-type mice suggesting similar maturity and/or activation of this population in WT and Onzin−/− mice (Figure 5e).

To further define differences and similarities in the response of Onzin−/− and wild-type mice during the challenge phase of the DTH response, we profiled the expression of cytokines at the site of antigen exposure. Sensitized mice were challenged with oxazolone and 6 h later tissue was collected from the control and the antigen-treated ears. RNA or protein was prepared and the expression of CXCL1, IL-6, IL-2, IL-4, IL-17e, IL-12, CCR7 and CCR6 in the control and challenged tissue evaluated by real-time PCR (RNA) or enzyme-linked immunosorbent assay (protein). This analysis revealed a significant
The difference between the Onzin−/− and wild-type animals in the expression of IL-6 and CXCL1 (Figures 6a and b). As expected, the mRNA levels for both IL-6 and CXCL1 were increased in biopsies from the antigen-exposed tissue compared with control tissue. However, this increase was significantly blunted in the Onzin−/− mice. To verify these findings, we determined whether a corresponding difference in the level of IL-6 and CXCL1 could be detected in protein extracts prepared from the tissue after challenge. Consistent with our RNA analysis, the levels of both CXCL1 and IL-6 were elevated in the antigen-challenged tissue of WT mice. Again the elevation in IL-6 observed in tissue from wild-type animals was significantly blunted in the Onzin−/− mice, similar to that of vehicle controls (Figure 6c).

Similarly, the accumulation of CXCL1 in the challenge site was attenuated in the ONZIN-deficient mice, however, in this case the difference between the level of this chemokine in the wild-type and Onzin−/− mice did not achieve statistical significance (Figure 6d).

DISCUSSION
ONZIN was first identified in a screen of LIF-regulated genes in the mouse uterus over 10 years ago. Despite this, remarkably little is known regarding the function of this relatively abundant protein. Early reports, however, showed that ONZIN was highly induced in LNs draining regions of the skin exposed to contact allergens. This raised the possibility that ONZIN might be essential for mounting a response to these chemicals. Certainly this possibility needed to be considered given the demonstration that ONZIN is expressed in virtually all the immune populations that have a critical role in orchestrating the response to contact allergens.
We found that, consistent with the elevated expression of ONZIN in draining nodes after contact with chemical allergen, mice lacking ONZIN showed an impaired response to two commonly studied chemical allergens, oxalozone and DNFB. Loss of ONZIN affected a number of different parameters used to evaluate the pathogenesis of the response to these agents, including recruitment of immune cells and edema formation upon secondary contact with the chemical. The attenuation of the response to these chemical allergens did not reflect a universal deficit in the ability of the mice to respond to topical immune challenges, as the immune response to heightened production of leukotrienes in the skin resulted in expected increases in vascular permeability, extravasation of serum proteins and recruitment of neutrophils in ONZIN-deficient mice.

Extensive knowledge concerning the expression of ONZIN has been amassed from various studies. ONZIN is not ubiquitously expressed and whereas it is found at high levels in most immune cells, with high levels observed in cells of myeloid lineage and lymphocytes, its expression is not limited to hematopoetic cells. High levels can also be observed in epithelial cells, particularly in the lung and intestinal tract and ONZIN expression can be detected in the skin. However, our experiments show that it is the expression of ONZIN by bone marrow-derived cells that appears to be critical in DTH as mice reconstituted with ONZIN-deficient bone marrow showed an impaired response to oxazolone compared with those reconstituted with WT marrow.

Although the expression of ONZIN in the various DC populations of the skin is not known, ONZIN expression by plasmacytoid DCs has been documented. This suggests the possibility that loss of ONZIN could impact the sensitization phase of the DTH response by altering the functioning of DCs, either their ability to capture antigen or their

Figure 6 Expression of cytokines in mouse ears after challenge with oxazolone. Wild-type and Onzin−/− mice were sensitized and challenged with 1% oxazolone or vehicle. Vehicle- and oxazalone-treated ears were harvested either 6 or 16 h post challenge and RNA or proteins were isolated for analysis. Quantitative analysis of (a) IL-6 and (b) CXCL1 RNA expression by RT-PCR. Quantitative analysis of protein for presence of (c) IL-6 and (d) CXCL1 as measured by enzyme-linked immunosorbent assay. n=5, *P<0.05. Quantitative analysis of IL-2 (e), IL-4 (f), IL-17e (g), CCR6 (h), CCR7 (i) by RT-PCR 6 h post challenge and IL-12 (j) by enzyme-linked immunosorbent assay 16 h post challenge.
ability to migrate to draining nodes for antigen presentation. Multiple populations of epidermal and dermal DCs have been identified in the mouse. One of these, the epidermal DC population is radiation resistant, and therefore, is expected to be of host origin in the bone marrow chimera. This makes it unlikely that a deficit in these cells results in the impaired DTH, as the response is normalized when ONZIN-/- mice are reconstituted with wild-type bone marrow. However, some studies suggest that this population of cells may primarily have a regulatory role in the DTH response and that other DC populations derived from circulating precursors, present in both the dermis and epidermis, have a critical role in antigen capture and presentation to T cells. This function of DCs can be evaluated by using the fluorescent chemical antigen FITC. The uptake of this fluorescent antigen facilitates the detection of cells that have trafficked from the skin to the draining nodes after exposure. Based on these criteria, we could detect no deficit in antigen capture and subsequent trafficking of DCs to the LNs in the Onzin-/- animals.

To further assess a possible impact of loss of ONZIN on antigen presentation and subsequent T-cell proliferation, we evaluated the ability of ONZIN-deficient cells to either stimulate or respond in a MLR. To do this, the ONZIN null allele was move to the H2D genetic background by several consecutive crosses to BALB/c mice. This provided cells differing at the MHC from the cogenic C57BL/6 line. However, no difference was observed in the ability of ONZIN-deficient splenocytes to stimulate proliferation of allogeneic T cells, again indicating that loss of this protein did not interfere with presentation of antigen to naive T cells. The expansion of T cells in response to allogenic stimuli was also not altered by the loss of ONZIN. Although clearly this does not rule out possible defects in the T cell's response to presentation of chemical allergens in vivo, it does suggest that the fundamental ability of antigen-presenting cells to elicit T cell's responses is not disturbed by loss of ONZIN.

Perhaps the strongest evidence demonstrating that the attenuated DTH response of the Onzin-/- mice does not reflect a defect in sensitization comes from our demonstration that cells collected from LN of oxalozone-sensitized ONZIN-deficient mice can transfer sensitivity to this antigen to naive wild-type animals. The immune response elicited on contact to oxazalone did not differ in these animals during challenge. Upon reexposure to antigen, CD4+ T cells interact with local antigen-presenting cells and induce local inflammation characterized by production of chemokines and cytokines, and thus, leading to recruitment of additional populations of leukocytes, including neutrophils. The ability of Onzin-/- LN cells to transfer sensitivity to naive animals, makes it unlikely that the attenuated response in the ONZIN-null animals reflect a deficit in the recruitment of the hematopoietic cells during sensitization, but rather their functioning as effector cells during challenge. Upon reexposure to antigen, CD4+ T cells interact with local antigen-presenting cells and induce local inflammation characterized by production of chemokines and cytokines, and thus, leading to recruitment of additional populations of leukocytes, including neutrophils. The ability of Onzin-/- LN cells to transfer sensitivity to naive animals, makes it unlikely that the attenuated response in the ONZIN-null animals reflect a deficit in the recruitment of the T cells to the skin after reexposure to the antigen. Rather, it suggests that loss of ONZIN leads to a deficit in the functioning of an additional population(s) of effector leukocytes, which responds to signals initiated by the recruited T cells. An attractive candidate population is the neutrophil, cells which express high levels of ONZIN and have been reported to drive the effector phase of the DTH response. Additionally, in Onzin-/- mice we found decreased levels of CXCL1, a major neutrophil-recruiting chemokine, in the challenge site as compared with WT mice, which further supported of a possible neutrophil-mediated contribution to the attenuated DTH response in Onzin-/- mice.

Neutrophils have been reported to contribute to the DTH response, and consistent with this, we observe neutrophils in the inflamed skin of sensitized mice after antigen challenge. Neutrophil influx in the site of antigen challenge was easily quantified by measuring levels of the neutrophil-specific enzyme myeloperoxidase in tissue homogenates. Previous studies have reported that neutrophils contribute to the effector phase of the DTH. In these studies, DTH is carried out in animal in which neutrophils population have been depleted by treatment with anti-Ly-6G antibody. Given this, it was reasonable to assume that if neutrophils contribute to the DTH response and expression of ONZIN is essential for the normal function of the neutrophils during the challenge phase of DTH, the impaired response of Onzin-/- animals compared with wild-type animals will not be apparent in neutrophil-depleted mice. However, despite the observance of neutrophils in the tissue challenged with oxazalone, we were unable to demonstrate a contribution of these cells to a measurable change in the inflammatory response upon reexposure to antigen. No difference was observed in the extent of tissue swelling and increase in tissue weight in the antibody-treated (neutrophil-depleted) animals compared with control. Our inability to detect a measurable contribution of neutrophils to oxazalone-induced DTH makes it unlikely that ONZIN expression in these cells contributes to the response.

Similar to neutrophils, macrophages express high levels of ONZIN as well. These cells accumulate in the skin after reexposure to antigen and once activated produce a number of chemokines, many of which has been shown to contribute the magnitude of the DTH response: MIP-1α, IL-6, IL-10, IL-12, MCP-1 and CXCCL1. We found no differences in the numbers of the majority of these cytokines/chemokines, including IL-2, IL-4, IL-17a, IL-12, CCR7 and CCR6 in the tissue after antigen challenge. A notable exception however was IL-6. RNA expression at 6h and protein expression by 16h were significantly elevated in WT-challenged mice. In contrast, IL-6 RNA and protein levels remained low at both of these time points, at similar levels to the vehicle-treated ears. Interestingly, studies in IL-6-/- mice show that induction of CHS is significantly reduced when IL-6 is absent and that IL-6 appears to be more important for the later phases of CHS induced by oxazalone. These findings are in agreement with our study showing that the role of ONZIN during a DTH response is during the elicitation phase and not during the sensitization phase. As IL-6 levels are attenuated in challenged Onzin-/- mice, and as, IL-6 is known to act as a proinflammatory cytokine that recruits other cells and is also involved in vascular cell adhesion molecule regulation, further studies examining the role of ONZIN is regulating IL-6 production from various cell types should be explored.

Additionally, ONZIN has been shown to bind phospholipid scramblase-1, an endofacial membrane protein that has been suggested to mediate the bidirectional movement of plasma membrane phospholipids in response to high levels of cytosolic calcium, injury or apoptotic insult. There is also evidence that phospholipid scramblase-1 interacts with a number of molecules involved in cytokine signaling. Decreased cytokines or chemokines secreted from immune cells, such as IL-6, through ONZIN's interaction and binding with phospholipid scramblase-1 may be a mechanism by which ONZIN is involved in regulating the elicitation phase during CHS reactions and should be explored in more detail in future experiments.

In summary, findings presented here show that loss of ONZIN impairs the ability of mice to mount a DTH response. This does not reflect a deficit in the ability of the Onzin-/- mice to become sensitized to chemical antigens, rather, the defect appears to reflect
impaired effector cell function after reexposure to antigen. CHS is one of the most intensively investigated immune responses in vivo and is regarded as a prototype of T cell-mediated delayed-type hypersensitivity reactions. Virtually all of the mouse lines generated over the past 20 years that carry mutations in immune-related genes have been evaluated in models of CHS and DTH. In the majority of cases, the change in the response of the mutant lines is associated with impaired sensitization to allergen. Few studies have identified genes critical only to the immune response elicited on reexposure to antigen. Identification of molecules such as ONZIN that contribute to this aspect of DTH is important, as these molecules likely provide more desirable targets for therapeutic intervention in the many diseases modeled by CHS and DTH.

METHODS

Experimental mice

Mice deficient in Onzin were generated as previously described.1 129/SvEv Onzin+/− mice were backcrossed to both C57BL/6 and BALB/c backgrounds for 12 generations. Onzin+/− mice from the twelfth generation backcross were intercrossed and the resultant mouse colonies formed from Onzin+/− and Onzin−/− progeny. F1 129/B6−/− mice were generated by the intercross of 129/SvEv−/− mice and C57BL/6−/− mice, which were derived from intercross of N12 C57BL/6−/−. Wild-type congenic F1 mice were generated by intercross of the corresponding wild-type littermates. All studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill.

MLR

One-way MLR was performed using splenocytes from wild-type controls and ONZIN-deficient mice as described previously.2 Briefly, single-cell suspensions of responder splenocytes (collected from C57BL/6 mice) were reconstituted at various concentrations and were mixed with irradiated stimulator splenocytes (isolated from BALB/c mice) at the indicated ratios. Cells in suspension were pelleted at 1300 r.p.m. for 5 min at 4 °C and resuspended in 5 ml of red blood cell lysis buffer (0.144M NH4Cl, 1 mM KHCO3 in dH2O). Red blood cells were lysed for 10 min at 4 °C and the remaining cells were washed twice with phosphate-buffered saline (PBS). Splenocytes were then resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 μM BME, 0.08 U/ml penicillin, 0.04 mg/ml gentamicin, 2 mM l-glutamine and 10 mM HEPES. Stimulator cells were irradiated at 2400 rads. Responder cells (90 μl at various concentrations) were mixed with irradiated stimulator splenocytes (4 × 105) in a total of 180 μl. Cells were cultured in a 5% CO2 humidified incubator at 37 °C for 48 h. After 48 h of incubation, cells were pulsed with 20 μl of 0.5 μCi [3H]-thymidine per well for the final 18 h of culture. The cells were harvested onto a glass fiber filtermat, and the amount of [3H]-thymidine incorporated in cells was assessed by Betaplate scintillation counting. Values are expressed as specific counts per minute, which are calculated from counts in wells with responders alone subtracted from counts in wells with responders and stimulators. Within each experiment, individual conditions were examined in quadruplicate samples. Splenocytes for the MLR were isolated as follows: cells isolated from spleen were pelleted at 1300 r.p.m. for 5 min at 4 °C, and resuspended in 5 ml of red blood cell lysis buffer (0.144M NH4Cl, 1 mM KHCO3 in dH2O). Red blood cells were lysed by for 10 min at 4 °C and the remaining cells were washed twice with phosphate-buffered saline (PBS). Splenocytes were then resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 μM BME, 0.08 U/ml penicillin, 0.04 mg/ml gentamicin, 2 mM l-glutamine, and 10 mM HEPES and used for the MLR.

CHS to Oxazalone

Wild-type and Onzin−/− C57BL/6 mice were anesthetized with 2,2,2-trichloroethanol and the plane of anesthesia determined by pinching the mouse toe. Mice were sensitized by topical application of 10 μl of 1% oxazalone to the left pinna, whereas the right pinna was unchallenged. At the time points, examined mice were euthanized, ears collected and an 8-mm diameter disc of tissue was obtained from the center of each ear for analysis. The differences in weight between experimental (left) and control (right) ears were compared for mice of each genotype. Sections of experimental ears were fixed in formalin and stained with hematoxylin and eosin for analysis. Digital pictures were captured on a Nikon Microphot-FXA microscope at 4X magnification. Individual fields from each image were analyzed for total cellular content in Scion Image program.

For vascular permeability measurements, sensitized mice were injected 22 h post oxazalone challenge with 1% Evans blue dye (10 ml of dye solution per kg of body weight) that was dissolved in PBS and filtered through a 0.2 μm nitrocellulose membrane. Ears were harvested after an additional 2 h, 8-mm discs collected and wet weight determined. Each ear disc was incubated in 1 ml formamide at 55 °C for 24 h. Extravasation of Evans blue dye was quantified by spectrophotometric analysis of the formamide extracts at 610 nm.

For adoptive transfer response, mice were sensitized with 3% oxazalone, as described above. On day 5, the axillary and inguinal LNs were collected, cells were flushed from LNs with a 30-gauge needle with PBS and single-cell suspensions prepared. Cells were washed (PBS), filtered and 25 × 106 cells were adoptively transferred into recipient mice. Mice were immediately challenged with 1% oxazalone. Ear weight was determined 24 h post challenge as described above.

CHS to DNFB

Onzin+/+ and Onzin−/− 129.B6. F1 hybrids were sensitized with 25 μl of 0.3% DNFB (Sigma) dissolved in acetone/olive oil (4:1) to the hind paws on days 0 and 1. On day 9, mice were challenged with 0.5% DNFB (10 μl) to the left pinna. Ear swelling was measured at the given time points after challenge by analyzing weight differences between 8-mm discs obtained from the treated and untreated ears.

Bone marrow transplantation

Onzin+/+ and Onzin−/− C57BL/6 mice were lethally irradiated with 1000 rad (Gamma cell; GC10 Exactor Cesium-137 irradiator, Best Theratronics, Ottawa, Ontario, Canada) twice, 3 h apart. Immediately following the second irradiation, mice were reconstituted with bone marrow cells isolated from donor mice. Bone marrow was harvested from femurs and tibias of donor mice by flushing with PBS (Life Technologies, Grand Island, NY, USA) and cellular debris was removed by filtering through a miracloth (Calbiochem, EMD Bioscience, San Diego, CA, USA). Cells were resuspended in PBS and each mouse received 2.5 × 107 cells in 0.2 ml through the tail vein.

MPO assay

Ear punches (8 mm diameter) were homogenized and analyzed according to a previously described protocol2 modified from Bradley et al.3 MPO present in the lysed cell supernatants was quantified by comparison with a standard curve derived from serial dilution of commercial MPO (Calbiochem).

Migration of LCs

Mice were anesthetized with 2,2,2-trichloroethanol and the plane of anesthesia determined by pinching the mouse toe. Onzin+/+ and Onzin−/− C57BL/6 mice were epicutaneously sensitized with 50 μl of 0.5% FITC (Sigma Aldrich; in 1:1 acetone/olubyl-phthalate) to the shaved abdomen. The axillary and inguinal LNs were collected 24 h after sensitization. Cell surface staining of FITC+ CD11c+ (APC-labeled Cd11c, BD Pharmingen, San Diego, CA, USA) LCs were assayed as described previously.

Inflammatory responses induced by AA

129/SvEv Onzin+/+ and Onzin−/− mice were injected intravenously with 0.5% Evans blue dye, as described above. The pinna of the left ear was coated with 20 μl of AA (Sigma; 100 mg/ml in acetone) to induce an inflammatory response, whereas the right ear received vehicle. After 1 h mice were euthanized, and an 8-mm diameter disc of tissue was obtained from the center of each ear for analysis. The wet weight of each ear biopsy was determined to assess edema.
formation. Each ear disc was then incubated in 1 ml formamide at 55 °C for 24 h and extravasation of Evans blue dye was quantified as described above. Data are expressed as mean differences between experimental and control ears for each animal.

Western analysis
Wild-type and ONZIN-deficient mice were sensitized and challenged with Oxazalone, as described above. Either 24 or 48 h after challenge, Evans blue dye was injected into the ear in order to locate the draining auricular LNs associated with each ear. Auricular LNs were collected and lysates prepared from naïve mice, those associated with the treated ear as well as those associated with the untreated ear. The ONZIN-specific antibody was produced as described previously.1

Isolation of RNA and proteins from ears of challenged mice
Frozen ears were homogenized mechanically by pounding into powder on dry ice. RNA was extracted from tissue using RNA isolation solvent (RNA-Beel, Tel-Test Inc, Friendswood, TX, USA), according to manufacturer protocol. RNA (5–10μg) was reverse transcribed, and complementary DNA was amplified with Taqman PCR Universal Master Mix (Applied Biosystems, Foster City, CA USA) using the Applied Biosystems 7900 HT Fast RT-PCR System. All samples were run in duplicate or triplicate and relative expression was determined by normalizing samples to either GAPDH or HPRT housekeeping genes. Protein was extracted from tissue in PBS containing protease inhibitors [2 μg phenylmethylsulfonyl fluoride and Complete Mini protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA)] for 20 min at 4 °C followed by sonication for 10 s. Homogenized samples were centrifuged for 20 min at 15,000 g and 4 °C. Supernatant was analyzed for CXCL1, IL-6 and IL-12 by enzyme-linked immunosorbent assay (Duoset, R&D System, Minneapolis, MN, USA).

Flow cytometry
Draining auricular LNs were collected from challenged mice and the cellular composition was analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). Antibodies from BD Pharmingen: APC-labeled GR-1 for neutrophils; PE-labeled Mac-1 for macrophages; APC-labeled CD11c for DCs; PE-labeled B220 for B cells and APC-labeled CD5, FITC-labeled CD4, PE-labeled CD8 for T cells. FITC-labeled GR-1, PE-labeled Mac-1 and PE-labeled MHCIIR (Miltenyi Biotec, Cambridge, MA, USA) were used for analysis of expression of various markers of DC maturation/activation.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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