HHS Public Access

Author manuscript

Immunol Cell Biol. Author manuscript; available in PMC 2013 February 01.

Published in final edited form as:

Immunol Cell Biol. 2012 August ; 90(7): 733–742. doi:10.1038/icb.2011.107.

ONZIN Deficiency Attenuates Contact Hypersensitivity Responses in Mice

Julie G. Ledford[1], **Martina Kovarova**[2], **Leigh A. Jania**[1], **MyTrang Nguyen**[1], and **Beverly H. Koller**[1,2]

[1]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

[2]Department of Medicine, Division of Pulmonary and Critical Care, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Abstract

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, suggesting that ONZIN plays a critical role in this response. Here, we report that indeed ONZIN-deficient mice develop attenuated CHS to a number of different haptens. Dampened CHS responses correlated with a significant reduction in pro-inflammatory IL-6 at the challenge site in ONZIN-deficient animals compared to 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

INTRODUCTION

ONZIN is a small cysteine-rich cytoplasmic protein expressed at high levels in both cells of the immune system and in many epithelia, particularly that 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 Promoter analysis suggests that the expression of this gene is subject to regulation during immune responses (unpublished data) and genome wide expression studies have identified increased levels of ONZIN in inflammatory lesions.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

To whom correspondence should be addressed: Beverly H. Koller, treawouns@aol.com, 120 Mason Farm Road, Chapel Hill, NC 27599, Phone: (919) 962-2153, Fax: (919) 843-4682.

The authors have no financial interests to disclose.

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 cDNA 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 dendritic cells, only minimal levels of expression were observed in monocyte-derived DCs[2]. This differential expression is of particular interest as plasmacytoid dendritic cells have been observed to differentiate into mature dendritic cells capable of priming $CD4^+$ T cells toward either Th1 or Th2 responses depending on the activation stimuli. In an independent cDNA microarray analysis, *Onzin* was identified as a gene highly up-regulated in draining auricular lymph node tissue following a primary exposure to the contact allergen DNFB[3]. *Onzin* was maximally expressed 48 hours after exposure and represented one of the most up-regulated genes observed in the lymph tissue by transcriptional profiling. While this 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[4]). 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 re-exposure 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 results 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 dendritic cell 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 against the hapten through a T cell-LC interaction. Re-exposure 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 contact hypersensitivity responses in mice.

RESULTS

ONZIN expression in draining auricular lymph nodes

Previous studies indicate that ONZIN expression in draining auricular nodes is increased after a single exposure to the hapten DNFB[3]. 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 LNs associated with either the oxazalone treated ear or the untreated ear were collected 24 and 48 hours after challenge. Protein lysates were prepared and analyzed by SDS-PAGE 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 oxazalone treated ears at both time points. Lysates prepared from the LNs associated with both naïve 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 oxazalone. Wild type and C57BL/6 *Onzin*$^{-/-}$ animals were sensitized to oxazalone by a topical application to the shaved abdomen. The mice were challenged after five days by application of the sensitizing agent to the left pinna, while the right was treated with vehicle. The response to oxazalone was assessed using a number of criteria including the difference in the weight of the tissue biopsies obtained from the vehicle and hapten treated ears. As expect a treatment of sensitized WT C57BL/6 mice with oxazalone resulted in a robust increase the weight of the tissue biopsies 12 hours after challenge (figure 2A). This increase in weight was more pronounced 24 hours after treatment with impending resolution of the response evident 48 hours 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 hours 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 hours 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 oxazalone. While 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

challenges with the hapten, oxazalone. Tissues biopsies were obtained and the amount of myloperoxidase (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 increases 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 oxazalone 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 i.v. immediately prior to application of AA to the left ear. The acute response to AA was first analyzed by collecting and weighing 8 mm ear discs. Intense ear swelling was observed within 1 hour 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).

DNFB induced CHS in ONZIN Deficient mice

A number of haptens, in addition to oxazalone, have been described that are capable of eliciting contact hypersensitivity. As small differences in the pathophysiology of the immune response to different haptens 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 hematopoetic 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[1]. 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 four 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 CHS response (figure 4A, **WT:WT**). However, as expected the response was attenuated in the *Onzin*$^{-/-}$ mice reconstituted with *Onzin*$^{-/-}$ bone marrow (figure 4A, **KO:KO**).

If ONZIN deficient hematopoetic cells are the primary mediators of the diminished hypersensitivity response, then the mice lacking ONZIN but reconstituted with wild type bone marrow should display a 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*$^{-/-}$ BM demonstrated a significantly attenuated response to oxazalone ($p<0.01$ compared to wild-type mice with wild-type marrow) (figure 4A, **KO:WT**).

Neutrophil depletion does not affect contact hypersensitivity to oxazalone

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[6]. 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, oxazaolone 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 to 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 Langerhans cells (LCs) are induced to migrate from the skin and accumulate as dendritic cells (DCs) in the draining lymph node where they interact with allergen-specific T cells[7]. 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 dendritic cells. FITC, unlike most haptens, is fluorescent. This feature aids in identification and trafficking studies of cells that have taken up the antigen[7]. 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 dendritic cells were collected from the draining lymph nodes 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 H^3 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 lymph node cells

The diminished response of the *Onzin*$^{-/-}$ mice could reflect a defect in sensitization to antigen or failure to respond to antigen upon challenge. To distinguish between these two possibilities wild type and *Onzin*$^{-/-}$ mice were only sensitized with oxazalone and the draining axillary and inguinal LNs were collected. A single cell suspension isolated from the sensitized mice was then injected into naïve 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 PMNs, antigen presenting cells, T and B cells of lymph nodes of the *Onzin*$^{-/-}$ and wild type mice (figure 5A,B). If the decreased DTH response observed in the ONZIN deficient mice is due to impairment in sensitization to antigen, including the migration of Langerhan cells, then the transfer of T cells isolated from the sensitized lymph nodes 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, **WT:WT**). A similar response was observed when wild type mice received cells from sensitized *Onzin*$^{-/-}$ mice (**KO:WT**). In contrast, the response of naïve *Onzin*$^{-/-}$ mice that received wild type sensitized cells remained significantly attenuated after challenge with oxazalone 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 lymph nodes. Cells were isolated 24 hrs post challenge from draining nodes and stained with antibodies specific for various leukocyte populations. FACs analysis revealed no significant difference in the relative number of CD4+ or CD8+ T cells, B cells, macrophages, neutrophils or dendritic cells between the WT and *Onzin*$^{-/-}$ mice (figure 5D). Dendritic cells from draining lymph node expressed comparable level of GR-1, Mac-1 and MHC II suggesting similar maturity and/or activation of this population in WT and *Onzin*$^{-/-}$ mice[8] (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 six hours 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 ELISA (protein). This analysis revealed a significant difference between the *Onzin*$^{-/-}$ and wild type animals in the expression of IL-6 and CXCL1 (figure 6A,B). As expected the mRNA levels for both IL-6 and CXCL1 were increased in biopsies from the antigen exposed tissue compared to 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 challenges 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). There were no significant differences in IL-2, IL-4, IL-17e, CCR6, CCR7 and IL-12 at the timepoints tested (figure 6E–J).

DISCUSSION

ONZIN was first identified in a screen of LIF regulated genes in the mouse uterus over 10 years ago[9]. Despite this, remarkably little is known regarding the function of this relatively abundant protein. Early reports, however, showed that ONZIN was highly induced in lymph nodes draining regions of the skin exposed to contact allergens[3]. 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 expression in virtually all the immune populations that play a critical role in orchestrating the response to contact allergens[1].

We found that, consistent with the elevated expression of ONZIIN 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[1–3]. ONZIN is not ubiquitously expressed and while 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[1]. 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 to those reconstituted with WT marrow.

Although the expression of ONZIN in the various dendritic cell populations of the skin is not know, ONZIN expression by plasmacytoid dendritic cells has been documented[2]. This suggests the possibility that loss of ONZIN could impact the sensitization phase of the DTH response by altering the functioning of dendritic cells, either their ability to capture antigen or their ability to migrate to draining nodes for antigen presentation. Multiple populations of epidermal and dermal dendritic cells have been identified in the mouse. One of these, the epidermal dendritic cell population is radiation resistant and therefore is expected to be of host origin in the bone marrow chimeras[10,11]. 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 play a regulatory role in the DTH response and that other dendritic cell populations derived from circulating precursors, present in both the dermis and epidermis, play a critical role in antigen capture and presentation to T cells[12]. This function of dendritic cells can be evaluated by using the florescent 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 dendritic cells to the lymph nodes 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 mixed lymphocyte reaction. 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 allogenic T cells, again indicating that loss of this protein did not interfere with presentation of antigen to naïve T cells. The expansion of T cells in response to allogenic stimuli was also not altered by the loss of ONZIN. While clearly this does not rule out possible defects in the T cells response to presentation of chemical allergens *in vivo*, it does suggest that the fundamental ability of antigen presenting cells to elicit T cells 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 naïve wild type animals. The immune response elicited on contact to oxazlone did not differ in these animals from the response observed in naïve animals reconstituted with cells collected from the LN of similarly treated wild type animals.

The ability of LN cells from KO mice to transfer sensitivity to naïve animals, together with the studies of the DTH response in the bone marrow chimeras, indicates that the defect in the hematopoietic cell function does not reflect a deficit in the function of the hematopoietic cells during sensitization, but rather their functioning as effector cells during challenge. Upon re-exposure 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*$^{-/-}$lymph node cells to transfer sensitivity to naïve animals, makes it unlikely that the attenuated response in the ONZIN null animals reflect a deficit in the recruitment of T cells to the skin after re-exposure 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 response to signals initiated by the recruited T cells. An attractive candidate population is the neutrophil, cells which express high levels of ONZIN[1] and have been reported to drive the effector phase of the DTH response[13,14]. Additionally, in *Onzin*$^{-/-}$ mice we found decreased levels of CXCL1, a major neutrophil recruiting chemokine, in the challenge site as compared to 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[13]. 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 to 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 re-exposure 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 to 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[1]. These cells accumulate in the skin after re-exposure to antigen and once activated produce a number of chemokines, many of which have been shown to contribute the magnitude of the DTH response: MIP-1α, IL-6, IL-10, IL-12, MCP-1 and CXCL1[15]. We found no differences in the levels of the majority of these cytokines/chemokines, including IL-2, IL-4, IL-17e, IL-12, CCR7 and CCR6 in the tissue after antigen challenge. A notable exception however was IL-6. RNA expression at 6 hrs and protein expression by 16 hrs were significantly elevated in WT challenged mice. In contrast, IL-6 RNA and protein levels remained low at both of these timepoints, at similar levels to the vehicle treated ears. Interestingly, studies in

IL-6$^{-/-}$ mice show that induction of contact hypersensitivity is significantly reduced when IL-6 is absent and that IL-6 appears to be more important for the later phases of contact hypersensitivity induced by oxazalone[16]. 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. Since IL-6 levels are attenuated in challenged *Onzin*$^{-/-}$ mice and since IL-6 is known to act as a proinflammatory cytokine that recruits other cells and is also involved in vascular cell adhesion molecule regulation[17], 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 (PLSCR1)[18], 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[19–21]. There is also evidence that PLSCR1 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 PLSCR1 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 re-exposure to antigen. Contact hypersensitivity 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 twenty 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 re-exposure 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 backrossed to both C57BL/6 and BALB/c backgrounds for 12 generations. *Onzin*$^{+/-}$ mice from the twelfth generation backross 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 Institutes 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.

Mixed lymphocyte reaction

One-way mixed lymphocyte reaction (MLR) was performed using splenocytes from wild type controls and ONZIN deficient mice as described previously[22]. Briefly, single-cell suspensions of responder splenocytes (collected from C57BL/6mice) 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 rpm for 5 minutes at 4° C and resuspended in 5 ml of RBC lysis buffer (0.144 M NH_4Cl, 1 mM $KHCO_3$ in dH_20). RBCs were lysed for 10 minutes at 4° C and the remaining cells were washed twice with 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 (4×10^5) in a total of 180 μl. Cells were cultured in a 5% CO_2 humidified incubator at 37°C for 48 hours. After 48 hours of incubation, cells were pulsed with 20 μl of 0.5 μCi ^{3}H-thymidine per well for the final 18 hours of culture. The cells were harvested onto a glass fiber filtermat, and the amount of ^{3}H-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.

Spenocytes for the mixed lymphocyte reaction were isolated as follows: cells isolated from spleen were pelleted at 1300 rpm for 5 minutes at 4° C and resuspended in 5 ml of RBC lysis buffer (0.144 M NH_4Cl, 1 mM $KHCO_3$ in dH_20). RBCs were lysed for 10 minutes at 4° C and the remaining cells were washed twice with 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.

Contact hypersensitivity to Oxazalone

Wild type and Onzin$^{-/-}$ C57BL/6 mice were anesthetized with 2,2,2-tribromoethanol and the plane of anesthesis determined by pinching the mouse toe. Mice were sensitized by topical application of 50 μl of 3% oxazalone (4-ethoxymethylene-2-phenyloxazol-5-one; Sigma) dissolved in 100% ethanol to all four paw pads as well as the shaved abdomen. After 5 days, a hypersensitivity reaction was elicited by topical application of 10 μl of 1% oxalazone to the left pinna, while 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 hours post oxazalone challenge with 1% Evans blue dye (10 ml of dye solution/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 hours, 8 mm discs collected and wet weight determined. Each ear disc was incubated in 1 ml formamide at 55° C for 24 hours. 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 25×10^6 cells were adoptively transferred into recipient mice. Mice were immediately challenged with 1% oxazalone. Ear weight was determined 24 hours post challenge as described above.

Contact hypersensitivity to DNFB

Onzin$^{+/+}$ and *Onzin*$^{-/-}$ 129.B6 F1 hybrids were sensitized with 25 μl of 0.5% DNFB (2,4-Dinitrofluorobenzene; 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) twice, 3 hours 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 (Gibco) and cellular debris was removed by filtering through a miracloth (Calbiochem). Cells were resuspended in PBS and each mouse received 2.5×10^7 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 protocol[23] modified from Bradley *et al*[24]. 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 Langerhan cells

Mice were anesthetized with 2,2,2-tribromoethanol and the plane of anesthesis 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:dibutylpthalate) to the shaved abdomen. The axillary and inguinal lymph nodes were collected 24 hours after sensitization. Cell surface staining of FITC$^+$ CD11c$^+$ (APC-labeled Cd11c, BD Pharmingen) Langerhan cells were assayed as described previously[25].

Inflammatory responses induced by Arachidonic Acid

129/SvEv *Onzin*$^{+/+}$ and *Onzin*$^{-/-}$ mice were injected i.v. with 0.5% Evans blue dye as describe 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, while the right ear received vehicle. After 1 hr 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 for24 hours 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 hours after challenge, Evans blue dye was injected into the ear in order to locate the draining auricular lymph nodes associated with each ear. Auricular lymph nodes 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-Bee, Tel-Test. Inc) according to manufacturer protocol. RNA (5–10 μg) was reverse transcribed and cDNA was amplified with Taqman PCR Universal Master Mix (Applied Biosystems) 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 phosphate buffer saline (PBS) containing protease inhibitors [2 mM phenylmethylsulfonyl fluoride and Complete Mini protease inhibitor cocktail tablet (Roche)] for 20 min at 4° C follow by sonication for 10s. 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 ELISA (Duo set, R&D system).

Flow cytometry

Draining auricular lymph nodes were collected from challenged mice and the cellular composition was analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Antibodies from BD Pharmingen: APC-labeled GR-1 for neutrophils; PE-labeled Mac-1 for macrophages; APC-labeled CD11c for dendritic cells; PE-labeled B220 for B cells; and APC-labeled CD3, FITC-labeled CD4, PE-labeled CD8 for T cells. FITC labeled GR-1, PE-labeled Mac-1 and PE-labeled MHCII (Miltenyi Biotec) were used for analysis of expression of various markers of DC maturation/activation.

Acknowledgments

This work was supported by an NIH grant HL076790 to Beverly H. Koller.

References

1. Ledford JG, Kovarova M, Koller BH. Impaired host defense in mice lacking ONZIN. J Immunol. 2007; 178:5132–5143. [PubMed: 17404296]
2. Rissoan MC, et al. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. Blood. 2002; 100:3295–3303. [PubMed: 12384430]
3. Betts CJ, et al. Assessment of glycosylation-dependent cell adhesion molecule 1 as a correlate of allergen-stimulated lymph node activation. Toxicology. 2003; 185:103–117. [PubMed: 12505449]
4. Black CA. Delayed type hypersensitivity: current theories with an historic perspective. Dermatol Online J. 1999; 5:7. [PubMed: 10673450]
5. Grabbe S, Schwarz T. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. Immunol Today. 1998; 19:37–44. [PubMed: 9465487]
6. Dilulio NA, et al. Groalpha-mediated recruitment of neutrophils is required for elicitation of contact hypersensitivity. Eur J Immunol. 1999; 29:3485–3495. [PubMed: 10556803]
7. Cumberbatch M, Illingworth I, Kimber I. Antigen-bearing dendritic cells in the draining lymph nodes of contact sensitized mice: cluster formation with lymphocytes. Immunology. 1991; 74:139–145. [PubMed: 1937567]
8. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol. 2002; 2:151–161. [PubMed: 11913066]
9. Sherwin JR, Sharkey AM, Smith SK. Identification of LIF regulated genes in the mouse uterus. Direct submission to NCBI. 2000 AF263458.
10. Granstein RD, Lowy A, Greene MI. Epidermal antigen-presenting cells in activation of suppression: identification of a new functional type of ultraviolet radiation-resistant epidermal cell. J Immunol. 1984; 132:563–565. [PubMed: 6228590]
11. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity. 2005; 23:611–620. [PubMed: 16356859]
12. Ginhoux F, et al. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. J Exp Med. 2007; 204:3133–3146. [PubMed: 18086862]
13. Engeman T, Gorbachev AV, Kish DD, Fairchild RL. The intensity of neutrophil infiltration controls the number of antigen-primed CD8 T cells recruited into cutaneous antigen challenge sites. J Leukoc Biol. 2004; 76:941–949. [PubMed: 15328335]
14. Molesworth-Kenyon SJ, Oakes JE, Lausch RN. A novel role for neutrophils as a source of T cell-recruiting chemokines IP-10 and Mig during the DTH response to HSV-1 antigen. J Leukoc Biol. 2005; 77:552–559. [PubMed: 15629884]
15. Pastore S, Mascia F, Mariotti F, Dattilo C, Girolomoni G. Chemokine networks in inflammatory skin diseases. Eur J Dermatol. 2004; 14:203–208. [PubMed: 15319150]
16. Hope JC, Campbell F, Hopkins SJ. Deficiency of IL-2 or IL-6 reduces lymphocyte proliferation, but only IL-6 deficiency decreases the contact hypersensitivity response. Eur J Immunol. 2000; 30:197–203. [PubMed: 10602041]
17. Eugster HP, Frei K, Kopf M, Lassmann H, Fontana A. IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. Eur J Immunol. 1998; 28:2178–2187. [PubMed: 9692887]
18. Li Y, Rogulski K, Zhou Q, Sims PJ, Prochownik EV. The negative c-Myc target onzin affects proliferation and apoptosis via its obligate interaction with phospholipid scramblase 1. Mol Cell Biol. 2006; 26:3401–3413. [PubMed: 16611984]
19. Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta. 1999; 1439:317–330. [PubMed: 10446420]
20. Sims PJ, Wiedmer T. Unraveling the mysteries of phospholipid scrambling. Thromb Haemost. 2001; 86:266–275. [PubMed: 11487015]

21. Sun J, Nanjundan M, Pike LJ, Wiedmer T, Sims PJ. Plasma membrane phospholipid scramblase 1 is enriched in lipid rafts and interacts with the epidermal growth factor receptor. Biochemistry. 2002; 41:6338–6345. [PubMed: 12009895]

22. Nataraj C, et al. Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. J Clin Invest. 1999; 104:1693–1701. [PubMed: 10606623]

23. Goulet JL, Snouwaert JN, Latour AM, Coffman TM, Koller BH. Altered inflammatory responses in leukotriene-deficient mice. Proc Natl Acad Sci U S A. 1994; 91:12852–12856. [PubMed: 7809134]

24. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol. 1982; 78:206–209. [PubMed: 6276474]

25. Nakae S, et al. IL-1 alpha, but not IL-1 beta, is required for contact-allergen-specific T cell activation during the sensitization phase in contact hypersensitivity. Int Immunol. 2001; 13:1471–1478. [PubMed: 11717188]

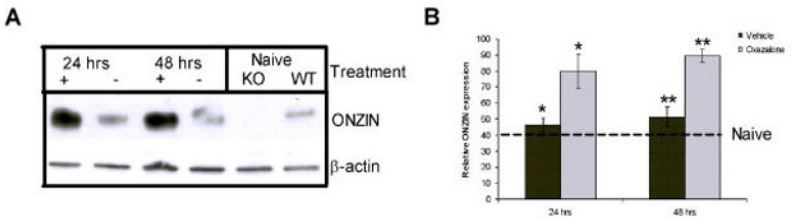

Figure 1. ONZIN expression in draining auricular lymph nodes after challenge with Oxazalone

A, Either 24 or 48 hours after Oxazalone challenge, Evans blue dye was injected into the ear of C57Bl/6 mice. The auricular lymph nodes, visualized by the blue dye, associated with the treated ear (+) or the untreated ear (−) were collected and protein lysate prepared. Naive *Onzin*$^{+/+}$ (WT) and *Onzin*$^{-/-}$ (KO) lymph nodes were collected from mice of the same genetic background. Protein lysates were prepared and analyzed by SDS-western and visualized with 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<.05, **p<.01.

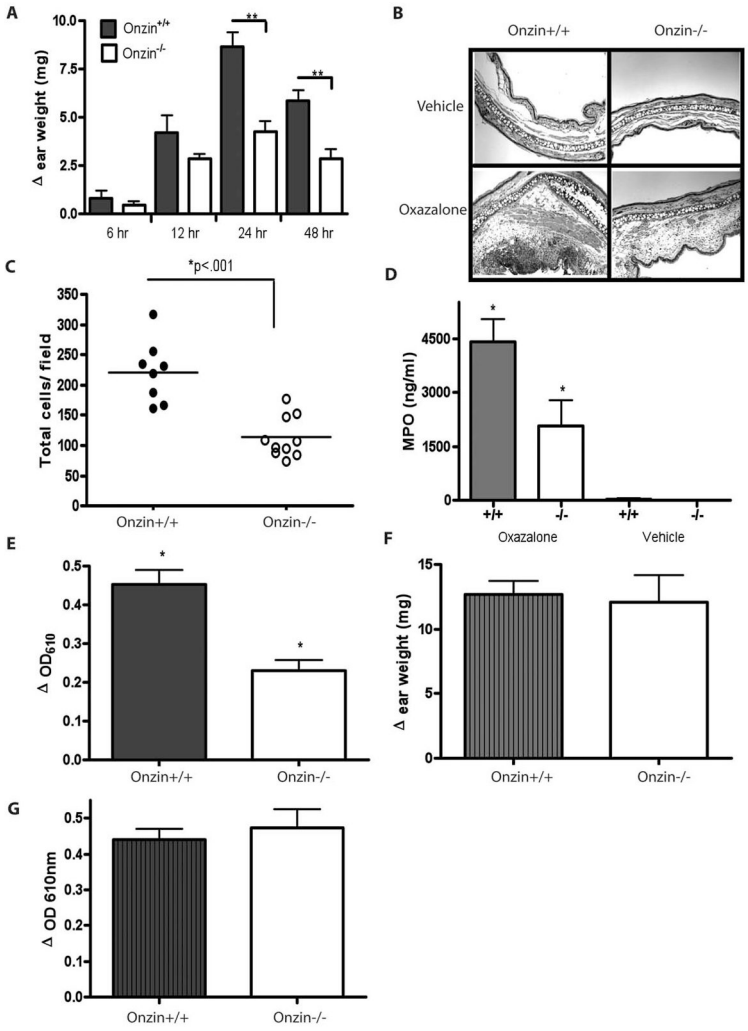

Figure 2. Delayed-type hypersensitivity response in *Onzin*$^{-/-}$ mice in response to Oxazalone

A, Increase of ear swelling in *Onzin*$^{+/+}$ and *Onzin*$^{-/-}$ C57Bl/6 mice from Oxazalone induced DTH response at different timepoints after challenge. n=5,5 at 6 hrs; n=6,6 at 12 hrs; n=15,15 at 24 hrs; n=30,29 at 48 hrs. *p, **p<.001. **B**, Histology of ear tissue 24 hrs after challenge with Oxazalone or vehicle was visualized by Hematoxylin-eosin staining at 4x 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<.001. **D,** Lysates were prepared of Oxazalone (O) or vehicle (V) challenged ear skin harvested 24 hrs 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<.05. **E**, Vascular permeability in response to Oxazalone was measured by injecting Evans blue dye i.v. into Oxazalone sensitized/challenged mice 2 hrs prior to the 24 hr harvest time point. Exudative dye in the ear skin was extracted for 24 hrs and the absorbance at 610 nm was determined. N=5,5 and is representative of two independent experiments. *p<.01. **F,** *Onzin*$^{+/+}$ and *Onzin*$^{-/-}$ mice received 0.5% Evans blue dye i.v. immediately prior to application of AA (2 mg in acetone) to the left ear. The right ear received acetone

alone. After 1 hr, 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 A_{610} for the left and right ears of each mouse is indicated. n=5,5.

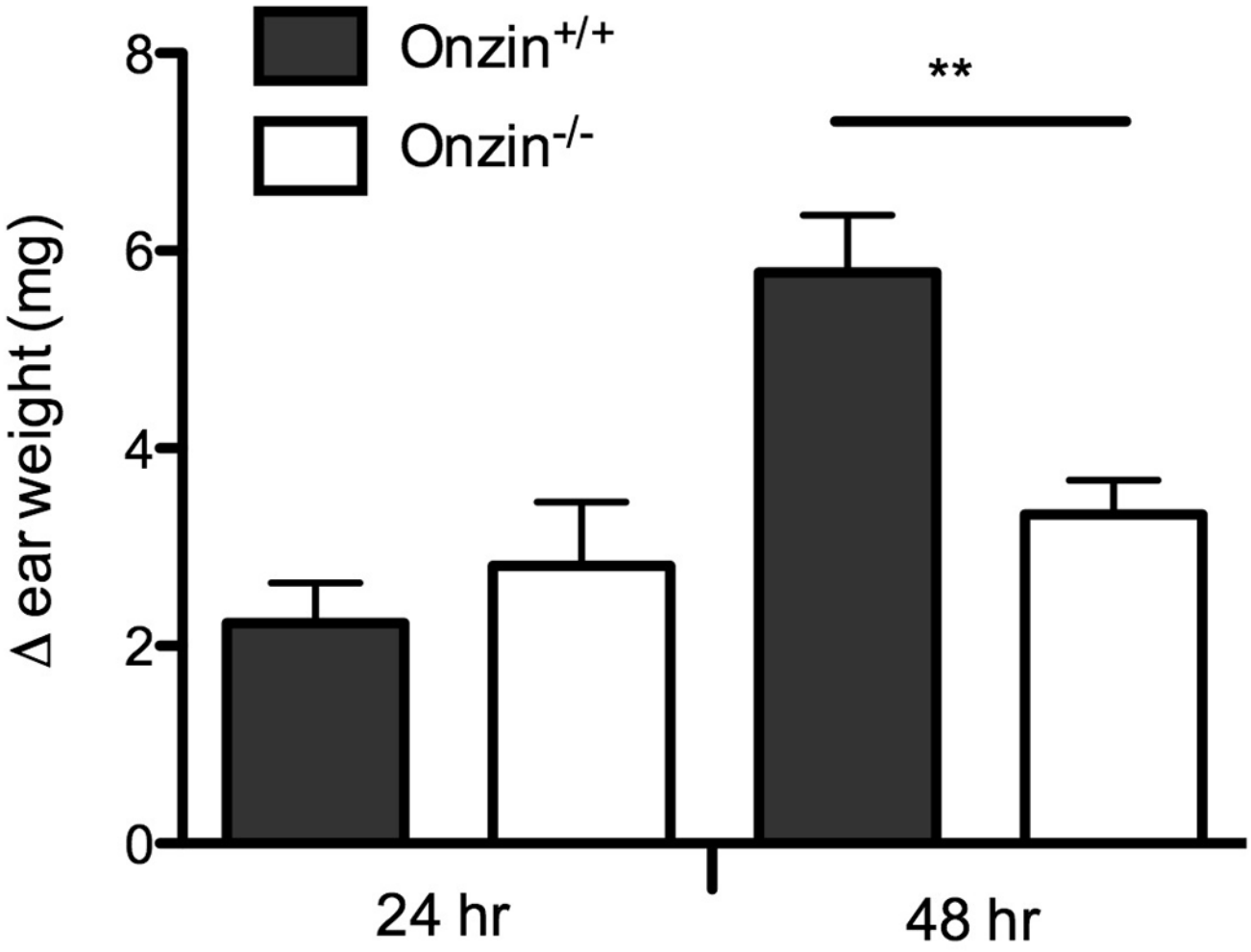

Figure 3. Impaired delayed-type hypersensitivity response in *Onzin*$^{-/-}$ mice to DNFB challenge

Increase of ear swelling in *Onzin*$^{+/+}$ and *Onzin*$^{-/-}$ 129.B6 F1 hybrid mice from DNFB induced DTH response 24 and 48 hours after the second challenge. N=5,5 at 24 hrs and n=13,15 at 48 hrs. **p<.01.

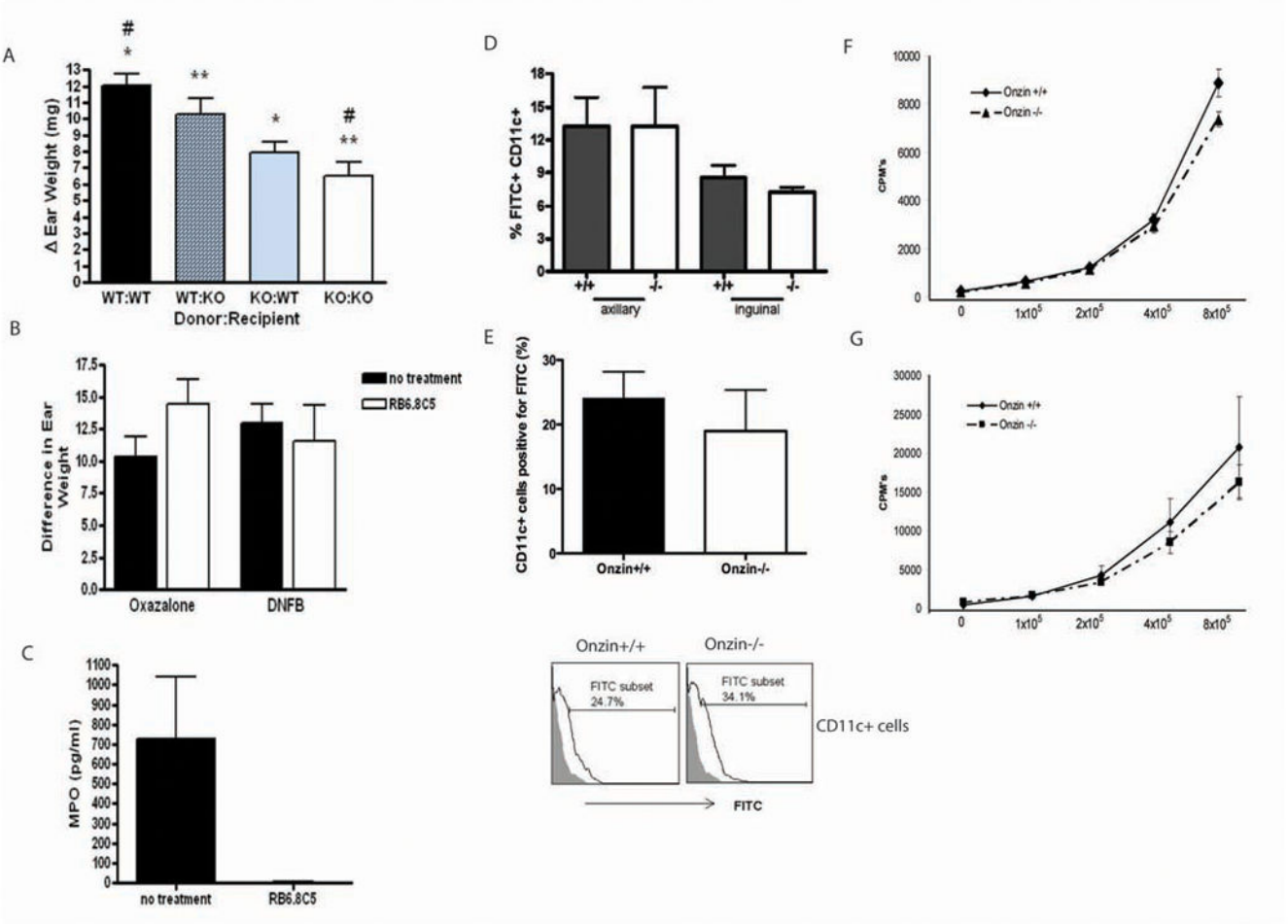

Figure 4. Cellular involvement in DTH response

A, Wild type and *Onzin*$^{-/-}$ mice were lethally irradiated and reconstituted with either 2.5 x 10^7 BMCs from *Onzin*$^{+/+}$ or *Onzin*$^{-/-}$ mice. Mice were allowed to recover for 4 weeks and then were sensitized and challenged with Oxazalone as described previously. n=21,22,21,19 and is combination of three independent experiments. *p<.01 (WT:WT versus KO:WT), **p<.05 (WT:KO versus KO:KO), #p<0.001 (WT:WT versus KO:KO). **B**, WT mice were depleted of PMNs by injections with the RB6.8C5 antibody prior to sensitization and challenge with oxazalone. Ear tissue was harvested and changes in ear weight were measured in comparison with vehicle treated ears from the same mouse. **C,** The absence of PMNs was verified in the oxazalone challenged ear tissue from mice receiving the RB6.8C5 antibody versus those receiving no antibody treatment by assessment of MPO in the ear lysates. **D**, The ability of epithelial cells to acquire antigen and migrate to draining lymph nodes was determined by FACs analysis. *Onzin*$^{+/+}$ and *Onzin*$^{-/-}$ mice were epicutaneously sensitized with 0.5% FITC and 24 hrs later axillary and inguinal lymph nodes were harvested, cells collected and incubated with CD11c antibody. The percentages of FITC+ CD11c+ cells contained within the total sensitized LN cell population are shown in D. **E**, The percentage of CD11c^{+} gated cells (versus isotype control) that are also positive for FITC expression (versus unstained cells) from the inguinal LN. n=5,5. **F**, Differing numbers

of responder cells, as indicated on the X-axis, were co-cultured with 4×10^5 irradiated stimulator cells. Proliferation of the responder cells was determined by addition of ^3H 18 hrs prior to harvest of the cells. Suspensions of responder splenocytes were harvested from *Onzin*$^{+/+}$ and *Onzin*$^{-/-}$ C57Bl/6 mice and irradiated stimulator splenocytes were collected from *Onzin*$^{+/+}$ BALB/c mice. N=5,5. **G,** Suspensions of responder splenocytes were obtained from *Onzin*$^{+/+}$ and *Onzin*−/− C57Bl/6 mice and irradiated stimulator splenocytes were collected from *Onzin*$^{-/-}$BALB/c mice. N=5,5.

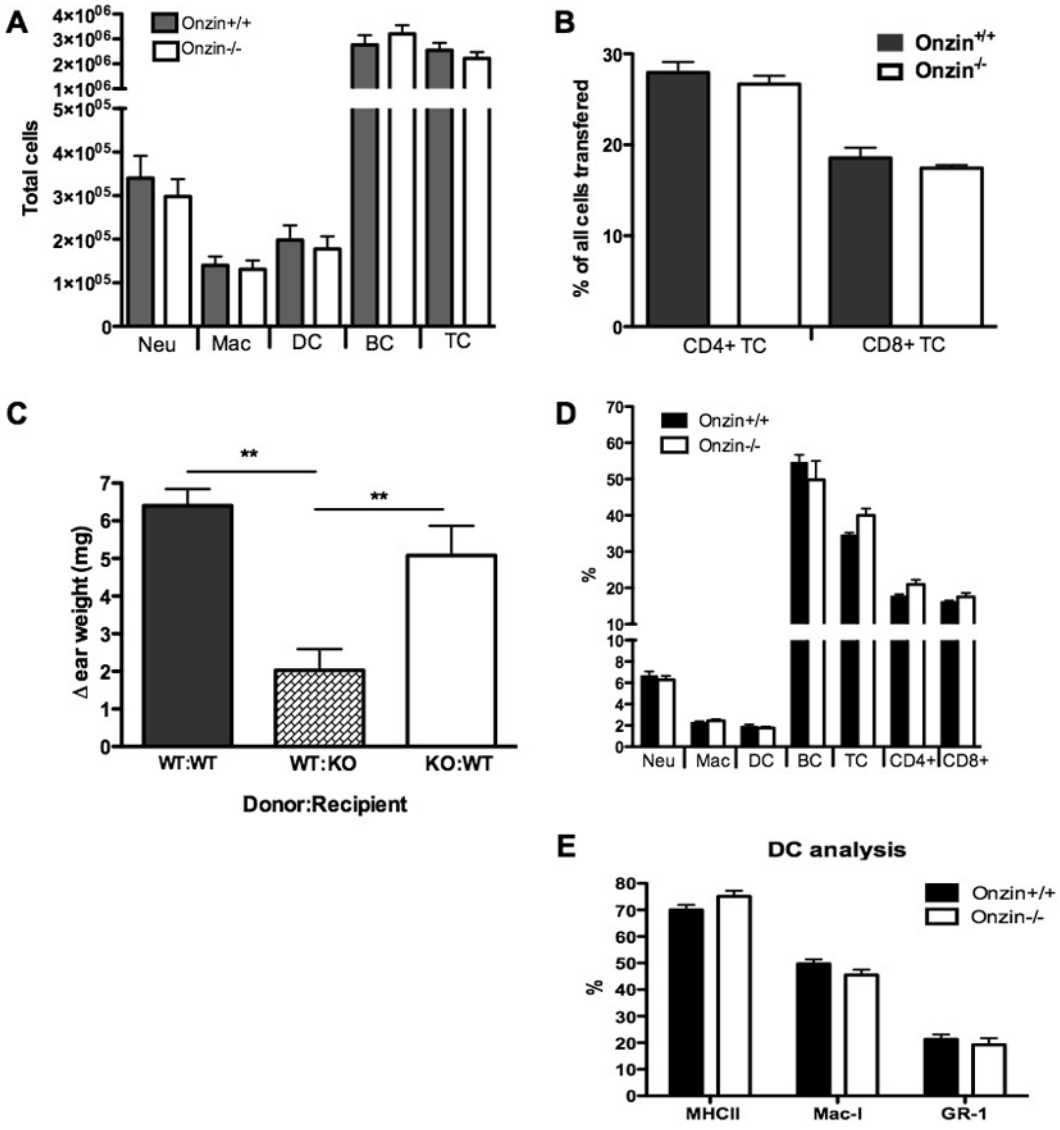

Figure 5. Response to Oxazalone in wild type and *Onzin*$^{-/-}$ mice after sensitized cellular transplants

A, Cells were flushed from oxazalone sensitized LNs, enumerated, and populations analyzed by flow cytometry. The percentage of each type, as obtained by flow cytometry, was multiplied by the total number of cells contained in each LN to determine the total cells of each type present in the LN. **B,** Populations of CD4+ and CD8+ T cells that were adoptively transferred were determined by cell surface antibody staining and flow cytometry analysis and were equivalent between WT and *Onzin*$^{-/-}$ mice. **C,** Wild type and *Onzin*$^{-/-}$ mice were sensitized with Oxazalone. Five days later, draining lymph nodes were harvested and 2.5 × 10^7 cells were adoptively transferred into wild type of *Onzin*$^{-/-}$ recipient mice. Mice were then immediately challenged with Oxazalone and ear swelling measured 24 hrs later. N=4,11,9 and is combination of two independent experiments. **p<.01. **D,** 24 hr after oxazolone challenge, Evans blue dye was injected to ear and auricular lymph nodes identify

by blue color were isolated. Cells from lymph node were isolated and analyzed by cell surface staining and FACS. (n=9,9). **E,** Analysis of CD11c+ dendritic cells from the draining nodes for expression of Mac-1, MHCII and GR-1. (n=6,6)

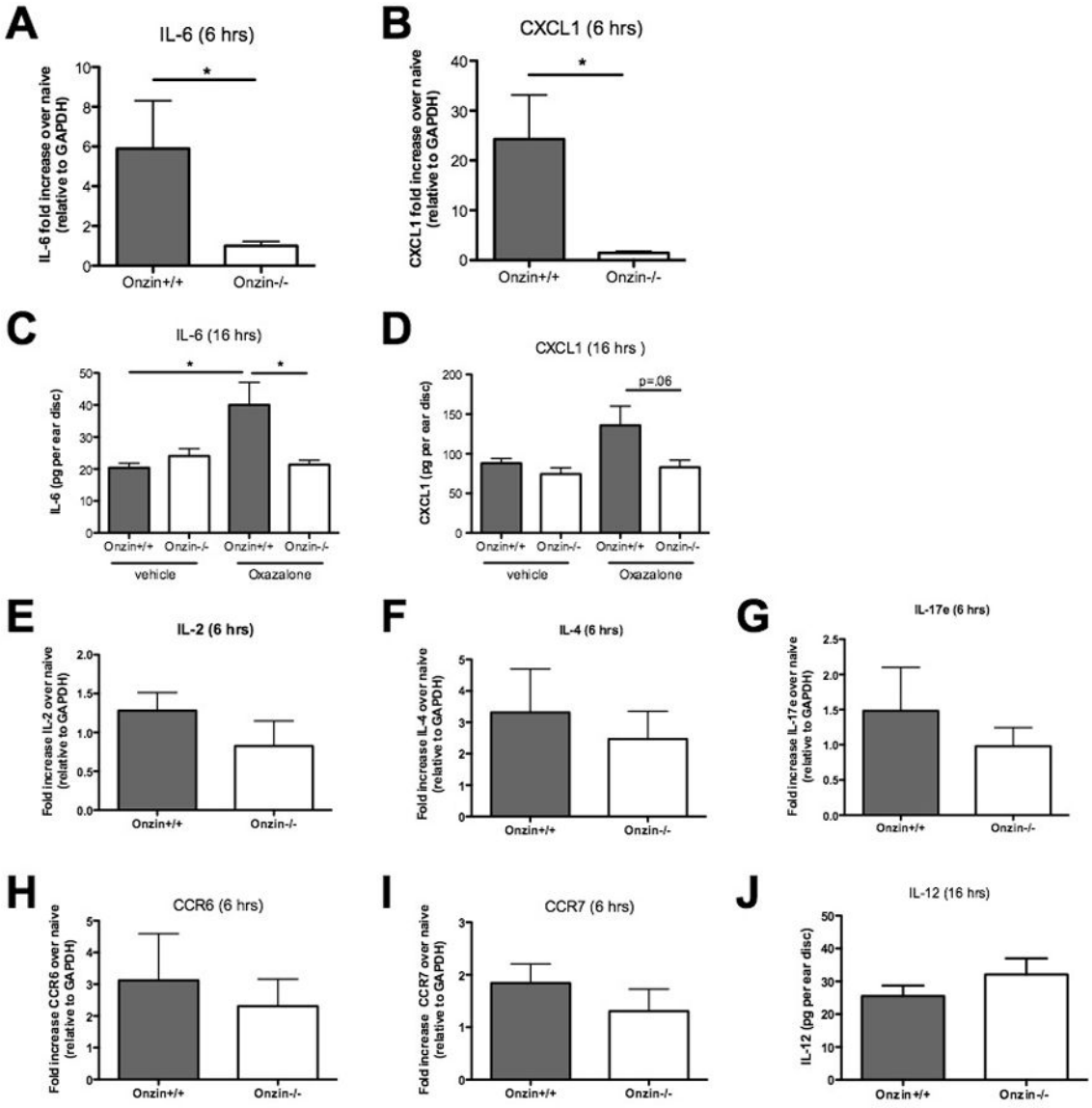

Figure 6. Expression of cytokines in mouse ears after challenge with oxazolone

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 hrs 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 ELISA. 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 hrs post challenge and IL-12 (**J**) by ELISA 16 hrs post challenge.