Elevated Epidermal Ornithine Decarboxylase Activity Suppresses Contact Hypersensitivity

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Previous reports have shown that elevated polyamine biosynthesis is sufficient to promote skin tumorigenesis in susceptible mouse strains. We hypothesized that increased activity of epidermal ornithine decarboxylase (ODC), a key regulatory enzyme in polyamine biosynthesis, may suppress the cutaneous immune response in addition to stimulating proliferation. Using an ODCER transgenic mouse model in which ODC is targeted to the epidermis, we examined the effect of ODC overexpression in keratinocytes on a classic contact hypersensitivity (CHS) response. Compared with normal littermate mice, ODCER transgenic mice showed reduced ear swelling, reduced neutrophil infiltration, and decreased migration of fluorescein isothiocyanate-loaded dendritic cells (DCs) to draining lymph nodes following hapten elicitation of CHS. In addition, elevated epidermal ODC activity suppressed the levels of cytokines keratinocyte-derived chemokine, monocyte chemoattractant protein-1, interleukin-6 (IL-6), and IL-10. Adoptive transfer of lymphocytes from sensitized ODCER transgenic or normal littermate mice to naive ODCER transgenic or wild-type mice indicated that elevated epidermal ODC activity suppresses both the sensitization and elicitation phases of CHS. The specific ODC inhibitor, α-difluoromethylornithine, abrogated all suppressive effects of ODC in CHS reactions. Collectively, these data suggest that the immunosuppression promoted by increased epidermal ODC is mediated by a reduction in cytokine levels, which suppresses DC migration and reduces immune cell infiltration to the site of hapten application.

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INTRODUCTION

Elevated polyamine biosynthesis is found in normal tissue remodeling such as wound healing, and in pathogenic conditions such as cancer (Tabor and Tabor, 1984; Pegg, 1986, 1988). Polyamines, including putrescine, spermidine, and spermine, are some of the major cations present in all cells. Ornithine decarboxylase (ODC), the initial rate-limiting enzyme involved in polyamine biosynthesis, is responsible for converting L-ornithine to putrescine. The induction of ODC is a hallmark of tumor promoting activity (O'Brien, 1976; DiGiovanni, 1992), and targeted overexpression of ODC in the epidermis has been shown to promote carcinogenesis in susceptible mouse strains (Megosh *et al.*, 1995; O'Brien *et al.*, 1997; Lan *et al.*, 2005). The tumor promoting activity of agents, such as 12-O-tetradecanoylphorbol 13-acetate, is known to depend on both inflammatory processes and hyperplasia (DiGiovanni, 1992). However, although chronic inflammation can contribute to tumor development, cancer immunoediting by integrated innate and adaptive immune responses can either eliminate tumors or promote tumor outgrowth. Early studies demonstrated the association between the tumor promoting activity of 12-O-tetradecanoylphorbol 13-acetate and its analogs with their ability to inhibit contact hypersensitivity (CHS) responses in mice (Halliday *et al.*, 1988; Kodari *et al.*, 1991). Subsequent studies have lent further support to the notion that immunosuppressive/immunomodulating properties of 12-O-tetradecanoylphorbol 13-acetate have a relevant role in tumor promotion (Coussens and Werb, 2002; Swann *et al.*, 2008). It is not clear what overexpression of ODC in the epidermis does to influence cutaneous immune reactions, in addition to promoting cellular proliferation and skin carcinogenesis.

Although it is well known that polyamines are essential for cellular proliferation, little is known about their role in modulating immune responses *in vivo*. Moreover, because keratinocytes have an important role in modulating the immune response, it is important to understand how elevated polyamine biosynthesis in these cells may affect cutaneous immune function. *In vitro* studies have shown that spermine and spermidine inhibit lymphocyte proliferation and interleukin-2 (IL-2) production through the reactive oxidation species generated by polyamine catabolic oxidases; they have also been shown to decrease macrophage-mediated tumoricidal activity, neutrophil locomotion, and NK cell activity, which is IL-2 dependent (Labib and Tomasi, 1981; Ferrante *et al.*, 1986; Chamaillard *et al.*, 1993, 1997). These

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Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; DFMO α-difluoromethylornithine; EBD, Evans blue dye; FITC, fluorescein isothiocyanate; IL-6, Interleukin-6; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1; ODC, ornithine decarboxylase; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol 13-acetate

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studies suggest that increased polyamine biosynthesis in the microenvironment generally suppresses the function of innate immunity. However, no studies have investigated the effect of increased polyamine biosynthesis on an adaptive T-cell-mediated immune response.

CHS, a classic delayed-type hypersensitivity reaction to topically applied haptens, is a dendritic cell (DC)-dependent, T-cell driven, cytokine-mediated skin inflammation (Eisen *et al.*, 1952; Xu *et al.*, 1996). The mechanism of CHS can be separated into two distinct phases, sensitization and elicitation. During the sensitization phase, hapten binds covalently to cell-associated or extracellular proteins in the skin to form a hapten-carrier complex that is taken up by DCs that migrate into T-cell-rich areas of skin-draining lymph nodes to present hapten-MHC complexes to naive T cells (Kripke *et al.*, 1990). The T cells subsequently become activated and clonally proliferate. The elicitation phase of CHS is initiated by exposure of the same hapten to the skin. This subsequent challenge with hapten results in an activation of hapten-specific T cells and their recruitment to the site of contact. $CD8^+$ T cells that produce proinflammatory cytokines such as IFN-γ, IL-1, IL-6, and keratinocyte-derived chemokine (KC) are the predominant effector cells that mediate CHS (Xu *et al.*, 1996). It is thought that induction of IL-4- and IL-10-producing $CD4^+$ T cells provides a protective role by dampening response and preventing exuberant immunopathology during the CHS response (Xu *et al.*, 1996).

Here, we examined whether ODC overexpression targeted to the epidermis influences the CHS response to the classic contact sensitizer, oxazolone. Using transgenic mouse models in which ODC is targeted to the epidermis, we show that elevated epidermal ODC potently suppresses a hapten-induced contact allergic response.

RESULTS

Increased ODC activity suppresses CHS

The contact sensitizer, oxazolone, was used to evaluate the hypersensitivity response in ODCER and normal littermate mice. Oxazolone-sensitized normal FVB mice exhibited a robust CHS response with significant ear swelling on subsequent challenge with oxazolone (Figure 1a). The response was maximal at 24 hours and began to return to baseline within 72 hours. In contrast, ODCER mice showed little or no response when challenged with oxazolone during the same time course. To evaluate changes in vascular permeability associated with adaptive inflammatory responses, a modified Miles assay was performed (Kunstfeld *et al.*, 2004). Challenge of previously sensitized normal mice with oxazolone led to a significant increase in dye extravasation (Figure 1b). The amount of dye present in the ears of ODCER mice was reduced by about 45%. No difference in dye extravasation was observed when a nonspecific irritant, sodium dodecyl sulfate, was used. These data indicate that the diminished ear swelling response during the CHS reaction is due to a specific repression of an inducible reaction but not due to the innate response to general irritation.

Single-cell suspensions were prepared from ears 24 hours after elicitation. Cells were analyzed by flow cytometry for the presence of leukocytes on the basis of CD11b and Gr-1 staining. A significant number of $CD11b^+$ $Gr\text{-}1^{Hi}$ cells, a phenotype indicative for neutrophils, was present in the ears of normal FVB mice following oxazolone elicitation; they were drastically reduced in oxazolone-challenged ears of ODCER transgenic mice (Figure 1c). No change was observed in the percentage of $CD11b^+Gr\text{-}1^{Lo/Mod}$ cells between ODCER and normal littermate mice at the same time point ($7.4 \pm 1.96\%$ vs. $7.5 \pm 1.8\%$, respectively). The vehicle control-treated ears from both ODCER and normal FVB mice had no leukocyte infiltration. Immunohistochemical staining of oxazolone-challenged ears from normal mice exhibited strong immunoreactivity when stained with a neutrophil-specific antibody (Figure 1d). In contrast, oxazolone-challenged ears obtained from ODCER mice showed reduced neutrophil infiltration. Together, these data suggest an immunosuppressive role for increased epidermal ODC activity during oxazolone elicitation of CHS.

Elevated epidermal ODC activity suppresses the sensitization and elicitation phase of CHS

To show the dependence of CHS suppression on epidermal polyamine biosynthesis, a specific inhibitor of ODC activity, α-difluoromethylornithine (DFMO), was used. To determine the effect of elevated ODC activity on the sensitization phase of the CHS response, ODCER mice were treated with 4-hydroxytamoxifen for 2 weeks and subsequently sensitized to oxazolone. Around 5 days later, normal and ODCER mice were placed on DFMO for 4 days. The mice were elicited on day 9 after sensitization and the ear swelling response was determined (Figure 2a). Elevated ODC activity during the sensitization phase inhibited the CHS response. To determine the effect of ODC overexpression during the elicitation phase of the CHS response, ODCER mice were sensitized to oxazolone, while on DFMO, to inhibit any basal ODC activity, and before 4-hydroxytamoxifen induction of enzyme activity. Around 3 days after sensitization, DFMO treatment was withdrawn and epidermal ODC activity was induced by topical application of 4-hydroxytamoxifen (Lan *et al.*, 2005). After 4 days of ODC induction, the mice were challenged with oxazolone. Elevated ODC activity during the elicitation phase was capable of suppressing oxazolone-induced CHS (Figure 2b). To reverse the ODC-mediated immunosuppression of CHS response to oxazolone, K6/ODC mice were administered 1% DFMO through their drinking water for the duration of the CHS protocol (Figure 2c). K6/ODC transgenic mice treated with DFMO showed a significant CHS response to oxazolone, similar to the level observed in non-treated wild-type littermates. There was a slight decrease in ear swelling in normal mice administered DFMO; however, the response was still significantly greater than in K6/ODC transgenic mice given tap water. These data demonstrate that elevated epidermal ODC activity is sufficient to suppress either the sensitization phase or the elicitation phase of CHS. As the CHS response is dependent on both the sensitization and elicitation phases, DFMO inhibition of ODC activity is only effective in reversing the suppression when administered throughout the CHS protocol.

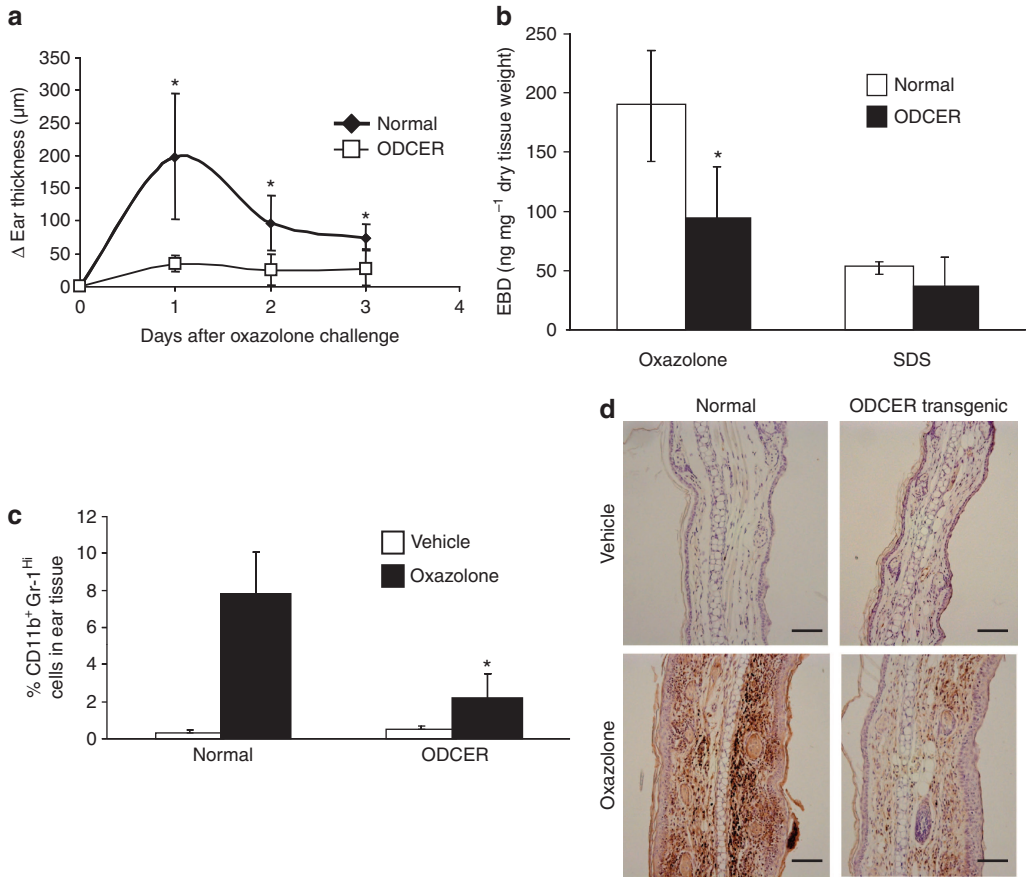

Figure 1. Elevated epidermal ODC activity suppresses oxazolone-induced contact hypersensitivity. (**a**) Hapten-specific ear swelling was measured at 24-hour intervals for 3 days after elicitation. The results represent the mean swelling response ± SD. (**b**) Vascular permeability was analyzed using a modified Miles assay 24 hours after oxazolone elicitation or 4 hours after sodium dodecyl sulfate treatment. The graph represents the average amount of extravasated Evans blue dye (EBD) ± SD (**c**) Single-cell suspensions of ear tissue were stained 24 hours after elicitation for the infiltration of neutrophils. The graph represents the average percentage of positive events per group ± SD. (**d**) Immunohistochemistry of vehicle treated and oxazolone-challenged ear tissue from ODCER and normal littermate mice stained with an antineutrophil antibody. Scale bar = 50 μm. *$P \leqslant 0.05$ vs. normal group.

Direct injection of immune effectors into the ear, but not intravenous adoptive transfer, restores CHS in ODCER mice

To determine the effect of elevated epidermal ODC activity on the ability to adoptively transfer CHS, donor lymphocytes were obtained from oxazolone-sensitized ODCER transgenic mice and their normal littermates. Recipient naive mice, either ODCER or normal littermates, received 2×10^7 cells intravenously. Normal recipient mice receiving cells from normal sensitized mice showed oxazolone-induced ear swelling 24 hours after challenge (Figure 3a). In contrast, ODCER recipients of normal donor lymphocytes showed no hapten-driven ear swelling. No significant ear swelling was observed in either group of recipient mice, ODCER or normal littermates, which received ODCER donor lymphocytes. These data corroborate findings from our studies with DFMO, indicating that elevated epidermal ODC activity suppresses both the sensitization and elicitation phases of the CHS response.

A number of investigations have demonstrated that a lack of CHS response may be overcome by directly delivering effector cells to the site of elicitation (Biedermann *et al.*, 2000; Engeman *et al.*, 2004; Grimbaldeston *et al.*, 2007). The decreased immune cell infiltration into the elicited ears of ODCER transgenic mice was addressed by intradermally injecting lymphocytes isolated from the auricular draining lymph nodes of elicited normal or ODCER mice. Intradermal injection of 1×10^6 lymphocytes from ODCER mice was sufficient to restore the CHS response in sensitized ODCER mice (Figure 3b). These data, in contrast to the adoptive transfer data using naive recipients, demonstrate that lymphocytes from ODCER transgenic mice are capable of mediating a hypersensitivity response when delivered directly to the challenged site.

Altered DC migration and fluorescein isothiocyanate (FITC)-induced CHS in ODCER transgenic mice

To track the migration of DCs *in vivo*, the ears of ODCER and normal littermate mice were painted with FITC; after 36 hours, draining lymph nodes were harvested and analyzed for the presence of $CD11c^+MHCII^+FITC^+$ DC by flow cytometry. The auricular draining lymph nodes from FITC-treated normal FVB mice showed a significantly greater number of $CD11c^+MHCII^+FITC^+$ cells (Figure 4a) when compared with ODCER mice. No difference in expression of costimulatory molecules, CD80 or CD86, was detected in

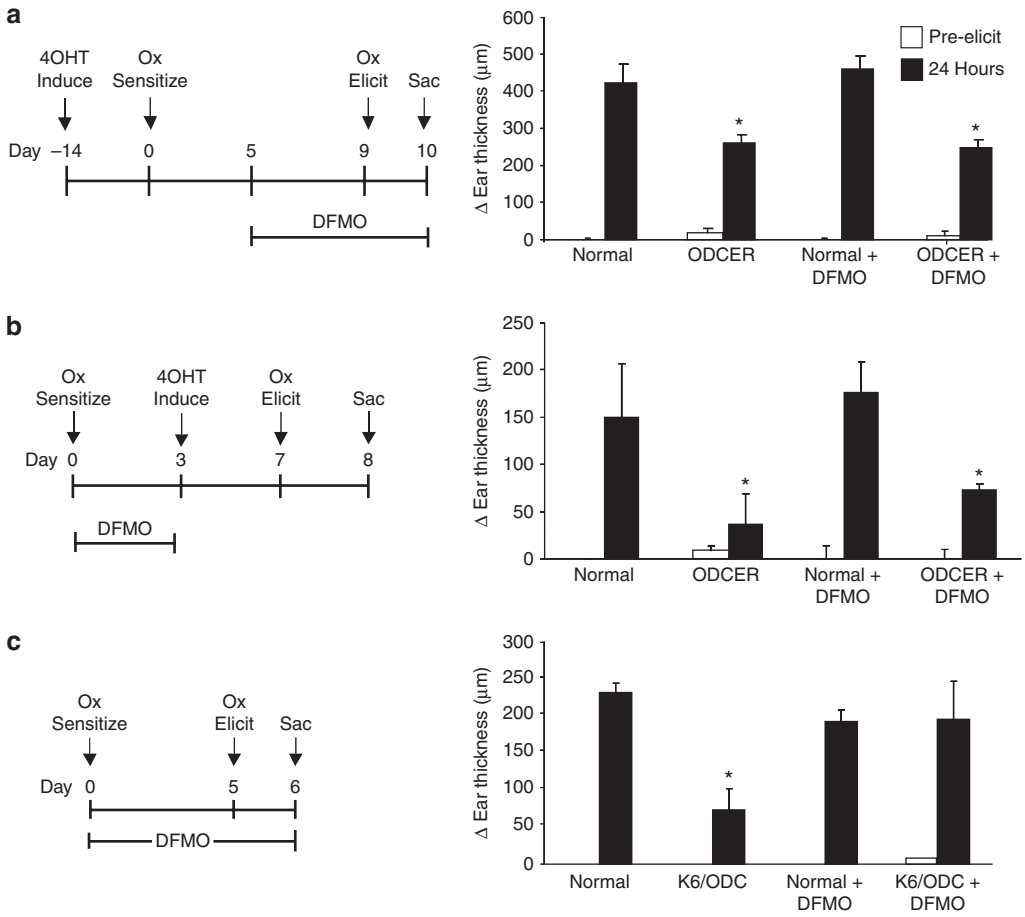

Figure 2. Elevated ODC suppresses the sensitization and elicitation phase of CHS. ODCER (**a** and **b**) or K6/ODC (**c**) were subjected to a CHS regimen using oxazolone, as illustrated by the time lines adjacent to each graph. During various stages of the CHS protocol, mice were administered 1% α-difluoromethylornithine (DFMO), the ODC-specific inhibitor, through their drinking water. (**a**) DFMO treatment was used to inhibit ODC activity during elicitation. (**b**) DFMO treatment for 3 days before 4OHT induction of epidermal ODC activity. (**c**) DFMO during the sensitization and elicitation phases of oxazolone-induced CHS in K6/ODC mice and normal littermates. Graphs show the change in ear swelling response 24 hours after elicitation ± SD. *$P \leqslant 0.05$ vs. the normal group. 4OHT, 4-hydroxytamoxifen.

FITC-positive DCs that had migrated to the draining lymph node on the hapten-treated side (data not shown). A similar number of $CD11c^+MHCII^+$ cells was present in the vehicle-treated skin and draining lymph nodes of ODCER mice when compared with normal littermate mice, indicating that the migratory defect was not due to a change in the number of resident DCs (data not shown). In conjunction with the increased numbers of FITC-positive cells, the intensity of FITC fluorescence in the migrated DC population was significantly greater in normal FVB mice when compared with ODCER mice (Figure 4b). In addition, ODCER mice showed a diminished ear swelling response when FITC was used as a contact sensitizer (Figure 4c). These data suggest that elevated ODC activity in the epidermis impairs DC migration during hapten sensitization and the inhibition of the CHS response is not hapten specific.

Elevated epidermal ODC activity suppresses inflammatory cytokines during sensitization

The ears of ODCER and normal littermate mice were sensitized to oxazolone; after 24 hours, the ears were removed, homogenized, and supernatants were analyzed for the presence of inflammatory cytokines by flow cytometry. A significant decrease in the levels of KC, monocyte chemoattractant protein-1 (MCP-1), and IL-6 was observed in ODCER mice 24 hours after sensitization with oxazolone (Figure 5a).

Next, lymphocytes isolated from the draining lymph nodes 24 hours after elicitation were stimulated *ex vivo* and analyzed for cytokine expression. Following 18 hours of *ex vivo* stimulation, lymphocytes isolated from normal mice showed significantly greater levels of IL-10 and IL-6 in comparison with lymphocytes from ODCER mice (Figure 5b).

Primary keratinocytes were isolated from neonatal K6/ODC transgenic and normal littermate mice to analyze the contribution of cytokine expression from keratinocytes. Conditioned media were collected from primary keratinocytes after 36 hours in culture and subsequently analyzed for the levels of cytokines. A significant amount of MCP-1 was present in the conditioned media of normal primary keratinocyte cultures in comparison with K6/ODC primary keratinocyte cultures (Figure 5c). Together with the whole

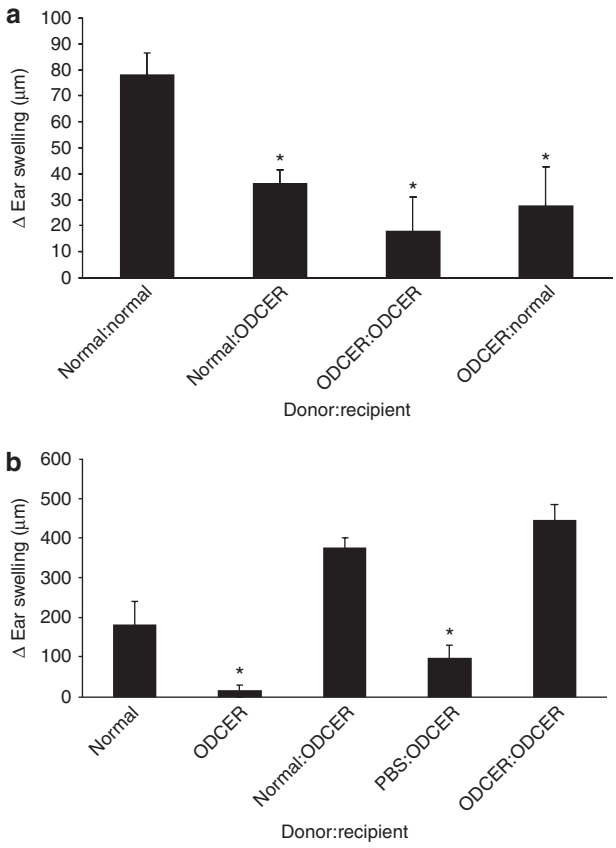

Figure 3. Intradermal injection of oxazolone-sensitized lymphocytes restores CHS response in ODCER mice. (**a**) Donor lymphocytes from sensitized normal or ODCER mice were adoptively transferred intravenously into naive ODCER or normal littermate recipients as indicated. At 12 hours after transfer, the recipient mice were elicited on the right ear with oxazolone. At 24 hours after elicitation, the CHS response was determined by measuring ear thickness. (**b**) Lymphocytes from oxazolone-elicited draining lymph nodes of donor mice were harvested and injected intradermally into sensitized recipient mice. Immediately after injection, the ears were elicited with oxazolone. The CHS response was determined 24 hours after elicitation. Graphs represent the average ear swelling response ± SD. *$P \leqslant 0.05$ compared with (**a**) the normal:normal group and (**b**) the normal group.

tissue and lymphocyte cytokine data, this result indicates that elevated epidermal ODC activity promotes reduced cytokine secretion by different cell types involved in host response to hapten stimulation.

DISCUSSION

This study shows that elevated ODC activity in keratinocytes suppresses a classic hapten-induced CHS immune response that is dependent on cytokine expression following hapten application. The central role of cytokines during this cutaneous immune response to reactive haptens has been demonstrated in a number of studies. For instance, MCP-1-deficient mice have a normal ear swelling response but diminished immune cell infiltration during the elicitation phase of CHS (Lu *et al.*, 1998). Mice treated with a neutralizing antibody against KC/CXCL1 exhibit an impaired CHS response (Dilulio *et al.*, 1999). In addition, mice lacking the KC receptor, CXCR2, have an impaired CHS response that is directly attributable to reduced neutrophil infiltration (Cattani *et al.*, 2006). Moreover, IL-6-deficient mice fail to respond to hapten challenge because of impaired DC migration (Nishimura *et al.*, 1999). In this study, elevated epidermal ODC activity leads to decreased levels of MCP-1, KC/CXCL1, and IL-6 in the ears of oxazolone-treated mice, and primary cultures of ODC overexpressing keratinocytes express less MCP-1. During the elicitation phase, fewer neutrophils are present in the ears of ODCER mice when compared with their normal littermates. Finally, hapten-stimulated DC migration to regional lymph nodes is impaired in mice with elevated epidermal ODC activity. Collectively, these data demonstrate that elevated ODC activity in keratinocytes prevents the expression of key cytokines required for immune cell recruitment and DC migration following hapten stimulation.

The induction of CHS is dependent on DC and T-cell activation. The central role of dermal dendritic and Langerhans cells in the elicitation of this cutaneous immune response to reactive haptens has been demonstrated in a number of studies. Ablation or inhibition of migration of these resident antigen presenting cells leads to suppression/attenuation of the CHS response (Bennett *et al.*, 2005; Wang *et al.*, 2009). In contrast, increased mobilization of DC in response to haptens, as in IL-10 knockout mice, has been shown to exacerbate the CHS response (Wang *et al.*, 1999). DC migration and function are dependent on cross-talk with epidermal keratinocytes as well. For instance, the interaction of keratinocytes and DC can modulate the host response to contact sensitizers to prevent deleterious inflammatory reactions (Kim *et al.*, 2009). We have shown that elevated epidermal ODC activity inhibits the migration of DC to local lymph nodes after topical application of FITC. Oxazolone-sensitized lymphocytes from ODCER mice fail to elicit a CHS response when adoptively transferred to naive normal littermate mice. Moreover, lymphocytes from hapten-sensitized normal mice are unable to elicit a CHS response in naive ODCER mice on adoptive transfer. However, the overall competency of lymphocytes does not seem to be altered, given that intradermal injection of primed lymphocytes into sensitized ODCER transgenic recipients restores the CHS response. These data collectively point to a diminished DC function mediated by elevated epidermal ODC activity. Studies are underway to investigate mechanisms by which elevated epidermal ODC activity suppresses the effective priming of $CD8^+$ T cells following hapten challenge and during skin tumorigenesis.

Polyamines have been shown to have immunomodulatory effects on immune cells (Chamaillard *et al.*, 1993, 1997), and treatment with exogenous spermine has a protective role in a murine model of bacterial sepsis characterized by a reduction in the peritoneal and serum levels of IL-6, KC, and MCP-1 (Zhu *et al.*, 2009). Following ODC enzyme induction, elevated levels of putrescine are observed in the epidermis of ODCER mice with no detectable change in spermine or spermidine (Lan *et al.*, 2005). In this study, elevated epidermal ODC activity suppresses the expression of IL-6,

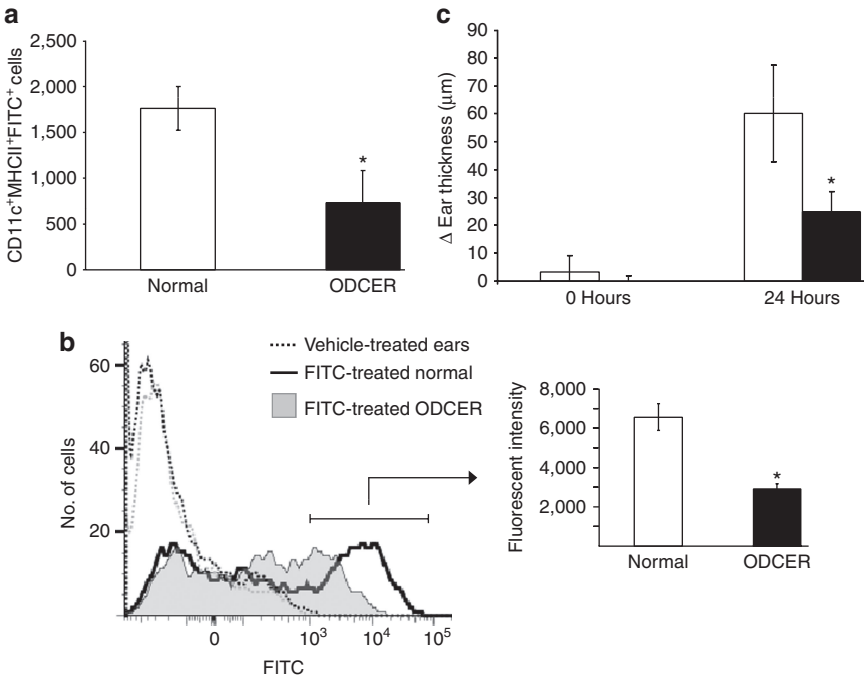

Figure 4. FITC-induced DC migration and CHS response in ODCER mice. FITC was used as a contact sensitizer to track DC migration and induce CHS. (**a**) Draining lymph nodes were harvested 36 hours after topical FITC sensitization and the number of $CD11c^+MHCII^+FITC^+$ cells was determined by flow cytometry. The graph shows the average number of $FITC^+$ DC/DLN/group ± SD. (**b**) Histogram overlay showing the intensity of FITC fluorescence in migrated DCs in the DLN. The average FITC fluorescence for migrated $CD11c^+MHCII^+$ cells with background fluorescence gated out ± SD is plotted on the right; (**c**) FITC was used as a contact sensitizer and to elicit a CHS response. The graphs show the mean ear swelling response ± SD. $*P \leqslant 0.05$.

MCP-1, and KC, as was reported in the bacterial sepsis model. This finding suggests that high levels of putrescine present in the epidermis of ODCER mice are sufficient to suppress the inflammatory response to contact sensitizers.

The current study has demonstrated an immunosuppressive role for elevated epidermal ODC activity during a cutaneous hypersensitivity response in ODC transgenic mice. Our data suggest that this suppressive effect on a hapten-induced CHS response is dependent, at least in part, on effects of ODC-overexpressing keratinocytes on antigen presenting DC function that is essential for T-cell activation in this adaptive immune response. Immunosuppressive responses are quite beneficial when suppressing the inflammatory response to environmental stimuli. However, the suppressive environment may provide a setting for immune escape of developing cutaneous tumors.

MATERIALS AND METHODS

Mice

The mouse lines used for this study were (1) ODCER transgenic mice, in which the suprabasal expression of ODC activity is driven by the human involucrin promoter upstream of the murine ODC gene fused to a modified estrogen receptor binding domain (Lan *et al.*, 2005), and (2) K6/ODC transgenic mice, in which the keratin-6 promoter targets constitutive expression of ODC to the outer root sheath of the hair follicle (Megosh *et al.*, 1995). All mice used for these experiments were on the FVB background. In all experiments using inducible ODCER mice, both ODCER mice and their normal littermates were topically treated daily on their shaved dorsal skin with 4-hydroxytamoxifen ($0.1\,mg\,100\,\mu l^{-1}$ ethanol) beginning at 6 weeks of age to induce ODC activity (Lan *et al.*, 2005). All animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research. Where indicated, mice were administered 1% DFMO (w/v) in their drinking water. All experiments were repeated using at least three mice per group.

CHS to oxazolone

Mice were sensitized on their shaved abdomen (50 ul) and hind footpads (5 ul) with 3% oxazolone (Sigma, St Louis, MO). Around 5 days later, sensitized mice were anesthetized with isoflurane, each ear was measured using a dial thickness gauge (Fowler, A&M Industrial Supply, Rahway, NJ), and then the right ear was topically challenged with 20 μl of 1% oxazolone, with 10 μl on each side. The left ear was treated with vehicle alone, 100% ethanol, in a similar manner. The increase in ear thickness was calculated by subtracting left (vehicle) ear thickness from right (treated) ear thickness.

Preparation of single-cell suspensions from the ear

The ears were excised and digested by incubation with 0.3% collagenase per 0.1% hyaluronidase solution at 37 °C for 1.5 hours, followed by gentle trituration. The cell suspension was passed through a nylon mesh filter, the cells pelleted, and then suspended in fluorescent-activated cell sorting buffer for flow cytometric analysis.

Vascular permeability assessment with Evans blue dye

Around 2 hours before challenge with 1% oxazolone or 10% sodium dodecyl sulfate on the ears, mice were injected intravenously with

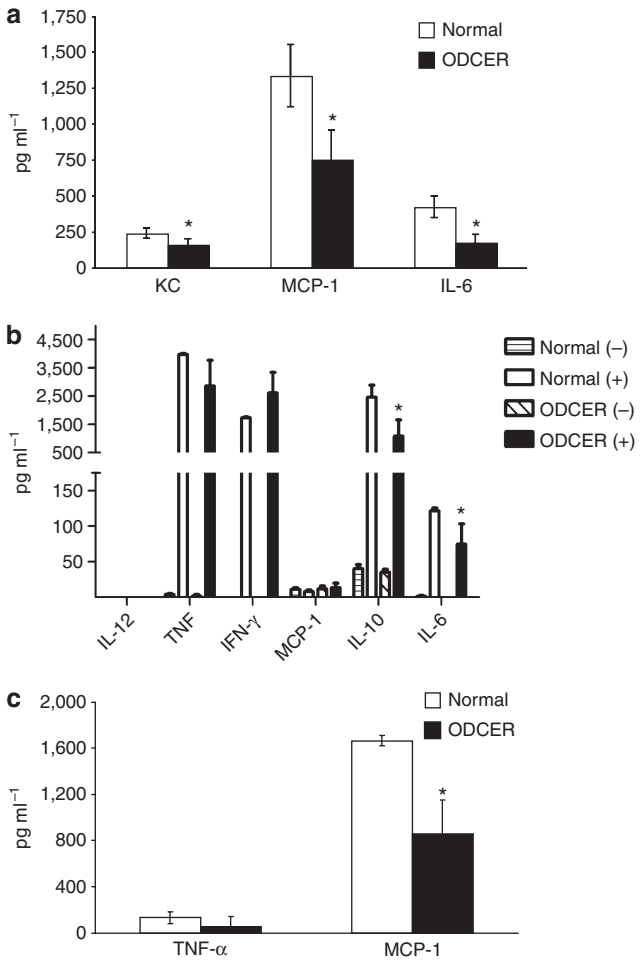

Figure 5. Reduced cytokine levels in ODCER transgenic mice after sensitization with oxazolone. The levels of indicated proinflammatory cytokines were measured using cytometric bead assays on (**a**) whole-tissue homogenates of oxazolone-sensitized ear skin from normal and ODCER mice 24 hours after application, (**b**) cell culture supernatants from 1×10^6 lymphocytes from auricular lymph nodes isolated 24 hours after elicitation and cultured *ex vivo* for 18 hours without (–) or with (+) PMA and ionomycin, and (**c**) conditioned medium from primary keratinocyte cultures from neonatal K6/ODC mice or normal littermate skin after 36 hours in culture. The graph represents the average pg/ml of each ligand ± SD. *$P \leqslant 0.05$ vs. normal.

100 µl of 5 mg ml^{-1} Evans blue dye (Sigma) in phosphate-buffered saline. At the peak of inflammation, that is, at either 24 hours (Oxazolone) or 4 hours (sodium dodecyl sulfate) (Mizumoto *et al.*, 2003), mice were killed and ears were removed, dried for 6 hours at 56 °C, weighed, minced, and the dye was extracted with 1 ml of formamide for 24 hours at 55 °C. Samples were filtered to remove tissue debris and OD_{620} was measured to assess the content of the extravasated dye on the basis of a standard curve of Evans blue dye. Vascular leakage specific for CHS was determined as the content of Evans blue dye per milligram of dried tissue found in the oxazolone or sodium dodecyl sulfate-painted ear (induced) minus the background value determined for the control ear of the same animal (spontaneous).

In vivo DC migration and CHS to FITC

FITC was dissolved in dibutylphthalate:acetone (1:1) at 5 mg ml^{-1}. ODCER mice and their normal littermates received 20 µl of the FITC solution, 10 µl on the ventral and dorsal sides of the ear. After 36 hours, the mice were killed and draining lymph nodes were dissected to analyze for the presence of FITC-positive cells. Single-cell suspensions were prepared by incubating the lymph nodes in a 1% collagenase per 0.01 mg ml^{-1} DNase I solution at 37 °C for 30 minutes, followed by pressing the tissue through a nylon mesh filter. Migrating DCs were identified as CD11c$^+$MHCII$^+$FITC$^+$ cells using flow cytometry, and 5×10^5 events were acquired for each lymph node. For the CHS reaction, the abdomen was sensitized (400 µl) and the ears challenged (20 µl) 6 days later with FITC.

Cytokine bead array analysis

Naive ODCER and normal littermate mice were treated with a sensitizing dose of oxazolone on the right ear; the left ear received vehicle alone. After 24 hours, the mice were killed and the ears removed for cytokine analysis. Ear tissue was homogenized in phosphate-buffered saline with protease inhibitors and dithiothreitol. The debris was cleared by centrifugation and supernatants were analyzed using Cytometric Bead Array reagents (BD Biosciences, San Jose, CA) as per the manufacturer's protocol.

Draining lymph nodes from elicited ears were dissected free and single-cell suspensions were prepared for *ex vivo* stimulation with PMA and ionomycin. Lymphocytes, 1×10^6 ml^{-1}, were cultured in RPMI 1640 with or without TPA (50 ng ml^{-1}) and ionomycin (250 ng ml^{-1}) for 18 hours. The supernatants were cleared of debris by centrifugation and cytokine analysis was performed using flow cytometry as above. Keratinocyte cultures were prepared as described (Wei *et al.*, 2008).

Flow cytometry

Equal numbers of viable cells from total skin or draining lymph nodes were stained with combinations of the following: FITC-conjugated rat anti-Gr-1, (RB6-8C5, recognizing Ly-6C and Ly-6G antigens), PE-conjugated rat anti-CD11b (M1/70), PE-conjugated rat anti-MHC class II (M5/114.15.2IA), PerCP-Cy7-conjugated CD11c (N418), PE-Cy5-conjugated Armenian hamster anti-CD80 (clone 16-10A1), or APC-conjugated rat anti-CD86(clone Gl-1). All antibodies were from eBioscience (San Diego, CA). Stained cells were analyzed on a BD FACSCanto II cytometer using FACSDiva software (BD Biosciences) or FloJo7 software (Tree Star, Ashland, OR). Viable cells were gated on the basis of forward and side scatter profiles. A minimum of 10,000 events were analyzed for each condition.

Immunohistochemistry

Ears were fixed in 4% paraformaldehyde at room temperature, followed by embedding in paraffin. Skin sections were deparaffinized, hydrated, and then heated in 0.01 M sodium citrate buffer (pH 6.0). Sections were incubated with a rat antimouse neutrophil antibody recognizing the 7/4 antigen (Clone 7/4, Cedarlane, Burlington, NC), followed by treatment with the biotinylated secondary antibody and then with an avidin horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). A control section was treated in a similar manner, with the exception of incubation with the primary antibody. Immunoreactive

cells were localized by incubating sections with a chromagen solution containing diaminobenzidine and peroxide and then counterstaining with hematoxylin. Pictures were acquired using a Zeiss Axiophot microscope with a digital color camera and corresponding Axiocam software (Carl Zeiss, Oberkochen, Germany). All images were processed for printing using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Adoptive transfer of lymphocytes

Donor mice, both ODCER transgenic and normal littermates, were sensitized to oxazolone. After 5 days, peripheral lymph nodes and spleens were harvested, and single-cell suspensions were prepared by pressing the tissues through a nylon mesh filter with a syringe plunger. Red blood cells were removed by lysis in NH_4Cl buffer, and the remaining cells washed extensively in phosphate-buffered saline and labeled with carboxy fluorescein succimidyl ester. Naive recipient animals received 2×10^7 carboxy fluorescein succimidyl ester-labeled cells by intravenous injection through the retro-orbital sinus. The recipient mice were challenged on the right ear with 1% oxazolone 12 hours after adoptive transfer. The CHS response was monitored by determining the change in ear thickness 24 hours after oxazolone challenge. Equal numbers of carboxy fluorescein succimidyl ester-labeled cells were detected in the draining lymph nodes of all recipient mice.

For intradermal injections, auricular draining lymph nodes were isolated from elicited ODCER or normal littermate mice 24 hours after hapten challenge. Single-cell suspensions were prepared by pressing the tissue through a mesh filter. Cells were suspended at 2×10^7 cells per ml in sterile phosphate-buffered saline and 50 µl was injected into the pinna of the right ear of a sensitized recipient. Immediately after injection, the ears were challenged with 1% oxazolone. Ear measurements were determined 24 hours after elicitation.

Statistics

Student's *t*-test was used to evaluate the significance of the data. A *P*-value $\leqslant 0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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