Chemical Activation of Innate and Specific Immunity in Contact Dermatitis

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Recent reports have suggested that chemical-induced allergic contact dermatitis may not be a traditional type IV hypersensitivity, in part due to the dual irritant and antigenic properties of sensitizing chemicals. In order to investigate the contribution of these properties to the molecular and cellular mechanism underlying allergic contact dermatitis, we evaluated oxazolone-induced changes in cell populations and cytokine production in the dermis of transgenic mice with impaired innate immunity (the FcγR subunit knockout mouse), and absent specific immunity (the athymic mouse), and the appropriate B6,129F2 and C57BL/6 control mice. Oxazolone and croton oil were applied in a single sensitizing dose, or in sensitizing and challenge doses, and the dermal response was evaluated by immunohistochemistry. In the wild type mice, with or without sensitization to oxazolone or croton oil, we observed mixed Th1/Th2 cytokine production and both $CD4^+$ and $CD8^+$ T lymphocytes; however, the neutrophil was the predominant cell in the dermis, even 72 h after final chemical application. Athymic mice displayed a similar neutrophil response with moderate Th1/Th2 cytokine production, and FcγR subunit knockout mice exhibited very mild dermatitis when treated with either oxazolone or croton oil. These results provide support for the hypothesis that allergic contact dermatitis is not a classic delayed type hypersensitivity, demonstrate the importance of the interaction between the irritant and antigenic properties of sensitizing chemicals in the development of allergic contact dermatitis, and suggest that the irritant effect of chemicals may be mediated through the cutaneous innate immune system. ***Key words: cytokine/innate immunity inflammation/specific immunity.***
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Irritant and allergic contact dermatitis are cutaneous responses to chemical exposures that utilize similar cellular and molecular pathways to achieve the same compensatory outcome, the re-establishment of dermal homeostasis (Brasch *et al*, 1992). Allergic contact dermatitis has been considered a conventional type IV hypersensitivity, characterized by $CD4^+$ T lymphocyte infiltration and Th1 cytokine production (Grabbe *et al*, 1996). Several distinct features of chemically induced contact hypersensitivity (CHS), however, suggest that it is different from classic type IV hypersensitivity. First, the stimulating agents are chemical haptens, not peptides or bacteria, and the pathogenesis is induced, in part, by the distinct physical characteristics of the chemical. Previous studies have also shown that elicitation of CHS is dose dependent and involves relatively high concentrations of chemical, whereas the classic peptide-induced delayed type hypersensitivity can be elicited by very low concentrations of antigen and does not exhibit strict dose dependence once threshold concentrations have been surpassed (Rycroft *et al*, 1995). Furthermore, most known chemical allergens possess both sensitizing and irritant properties. The irritant property is thought to contribute significantly to the elicitation of dermatitis and to be responsible for the concentration dependence of the response (Grabbe *et al*, 1996). Additionally, the ear swelling response resulting from coadministration of irritants or proinflammatory cytokines with low doses of sensitizers is not equivalent to the response generated by two applications of the sensitizing chemical, thus suggesting that the irritant property of sensitizers may be qualitatively different from the proinflammatory signal provided by irritants (Grabbe *et al*, 1996). Although the study did not explore the molecular or cellular basis for the nonantigenic effects of sensitizers, these observations confirmed the importance of the interplay between irritancy and antigenicity in development of contact dermatitis and differentiated the chemical-induced contact dermatitis from the traditional delayed type hypersensitivity response to peptide antigen.

It has been proposed, from studies in other animal models and organ systems, that activation of innate immunity is required for activation of specific immunity and that the specific immune response receives information about the antigen and the type of antigen response to be induced from the innate immune system (Medzhitov and Janeway, 1997; Ezekowitz and Hoffman, 1998). The innate immune response is a nonspecific defense mechanism through which invariant molecular patterns of infectious agents are recognized. Receptors of the innate immune system are found on professional antigen-presenting cells and on epithelial cells, and recognize structurally diverse protein families. Thus, a limited repertoire of receptors, which includes the Fc receptor (FcR) family, is able to provide widespread protection to the host.

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Abbreviations: AOO, acetone:olive oil; CHS, contact hypersensitivity; FcγR, Fc receptor gamma subunit; OXA, oxazolone; PBMC, peripheral blood mononuclear cells.

Activation of the innate defense mechanism induces the expression of endogenous effector molecules, proinflammatory cytokines such as interleukin-12 (IL-12) and IL-4, and chemokines. These cytokines and chemokines, in turn, may contribute to elicitation of the adaptive immune response, thus providing a link between the nonclonal and clonal immune mechanisms.

Chemicals that induce contact hypersensitivity are, for the most part, highly reactive haptens that combine with endogenous proteins to form a complete antigen. Although these molecular characteristics do not meet the current criteria for activation of the innate immune system, chemical stimulation of innate immunity may provide the irritant response necessary for the development of cutaneous hypersensitivity.

Therefore, we hypothesize that the inflammatory response induced by chemicals differs from the proinflammatory response in classic type IV hypersensitivity and that chemical irritancy activates the innate immune system, an activation step necessary for development of specific immunity in the skin. To address this hypothesis, we first conducted an *in situ* study of the inflammatory response to the sensitizer oxazolone (OXA) and the irritant croton oil in the dermis, the site of inflammatory cell infiltration. To explore the possibility that chemical irritancy activates the innate immune system and that activation of innate immunity is required for development of cutaneous specific immunity, we extended this study to transgenic mouse models with impaired innate immunity, FcγR subunit knockout (FcγR KO) mouse, and absent specific immunity, the athymic mouse.

The γ subunit of the FcR is an essential component of the high affinity receptor for IgE, FcεRI, which is expressed on mast cells and basophils, and the IgG-specific FcγRIII, which are found on most effector cells of the immune response. The γ subunit is required for FcR assembly and cell surface expression, and for FcR signal transduction. Consequently, the FcγR KO mouse has dysfunctional mast cells that are unable to mount an allergic response, phagocytic cells that have impaired antibody-dependent phagocytic capability, and diminished neutrophil function. The γ subunit is also associated with the T cell receptor-CD3 complex; however, thymic and peripheral T lymphocytes from these mice are phenotypically normal and present in normal CD4 to CD8 ratios with normal T cell αβ expression. These knockout mice are unable to produce a type I or type II hypersensitivity and have a diminished inflammatory response. Thus knockout of the FcγR subunit permits us to evaluate the cutaneous response to chemical in the absence of a functional innate immune response (Takai *et al*, 1994), and the athymic mouse has intact innate immunity but no T-lymphocyte-specific immune response (Grabbe *et al*, 1993). To test the innate and specific responses to chemical in these mouse models and their respective wild type control mice, we challenged mice with OXA or croton oil on the ear, with and without sensitization on the abdomen, and compared cellular infiltrates and cytokine production in the dermis.

MATERIALS AND METHODS

Mice Homozygotic FcγR KO mice and their control, the B6.129F2 mice, the athymic C57BL/6-nu/nu homozygote and euthymic heterozygote controls, C57BL/6-nu/+ (Jackson Laboratory, Bar Harbor, ME), 4–6 wk of age, were housed five per cage, maintained on a 12 h light/dark cycle and provided with food and water *ad libitum* according to ALAC guidelines.

Reagents OXA (4-ethoxymethylene-2-phenyloxazo-5-one) and PHA-M (phytohemmagglutinin-M) were obtained from Sigma (St. Louis, MO), croton oil was obtained from Advance Scientific (Fort Lauderdale, FL), and tetanus toxoid from the Massachusetts Biolab (Boston, MA). Antibodies against IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and RANTES were obtained from R&D Systems (Minneapolis, MN). Antibodies against CD3, CD4, CD8, Ly6, and Mac-1 were obtained from PharMingen (San Diego, CA). Biotinylated rabbit antirat IgG, goat antirabbit IgG, and rabbit antigoat IgG secondary antibodies and ABC Elite Kit were purchased from Vector Laboratory (Burlingame, CA). Normal rat, goat, and rabbit IgG were obtained from Sigma.

Treatment paradigms Sensitization is defined as application of chemical to the abdomen and challenge is defined as application of chemical to the dorsum of the ear. To sensitize and challenge naïve mice, 50 μl of 0.5% OXA in a 4:1 acetone:olive oil (AOO) vehicle was applied to the shaved abdomen on day 1, and 20 μl was applied to the dorsal side of the right ear on day 6. These mice are designated OXA/OXA in the figures. Mice that were "challenged only" received 50 μl of AOO on the abdomen on day 1 and 20 μl of 0.5% OXA on the dorsal side of the ear on day 6 and are designated AOO/OXA. Irritant dermatitis was induced with application of 50 μl of AOO on the abdomen on day 1 and 25 μl of 0.5% croton oil on the dorsal side of the ear on day 6. These mice are designated AOO/CO. To evaluate the effect of the vehicle, AOO (4:1) was applied to the abdomen on day 1 and to the dorsal side of the ear on day 6, and this treatment group is labeled AOO/AOO. Chemically treated ears were harvested 24, 48, or 72 h after the final chemical application.

To test T cell functionality of FcγR KO mice, B6,129F2 mice and FcγR KO mice received 30 μg tetanus toxoid by intraperitoneal injection on day 1 and day 4. Mice were sacrificed by CO_2 inhalation, and whole blood was collected by cardiac puncture on day 7.

Immunohistochemistry Harvested ears were prefixed in 4%–8% paraformaldehyde for 4–8 h and then snap-frozen in liquid nitrogen. The tissues were either stored at −80°C for later use or immediately embedded in OCT embedding medium (Sigma). Cryostat sections (5 μm) were cut at −20°C and collected on slides. The sections were air-dried and then fixed using a graded series of acetone solutions in water (60%, 70%, 80%, and 90% acetone, 3 min per solution). The sections were either air-dried overnight and then stored at −20°C or rehydrated for immunostaining.

An avidin-biotin-peroxidase complex (ABC) method was used for the immunohistologic staining of the frozen sections. Briefly, primary antibodies were diluted to 1–10 μg per ml in freshly prepared 2% casine solution. Normal goat, rabbit, and rat IgG were diluted in the same way as primary antibody preparation. These normal IgG and tissue sections from normal untreated mice were used as negative controls. Sections were first incubated with 2% casine solution for 2 h before addition of primary antibodies. After the primary antibody incubation for at least 2 h, the endogenous peroxidase activity was inactivated by 5% hydrogen peroxide in 0.1% sodium azide for 10 min. Secondary antibodies were prepared in 2% casine solution at a concentration of 1–10 μg per ml and incubated with section slides for at least 2 h. Then the avidin-biotin-peroxidase complex was prepared and used according to the ABC kit protocol. After a 2 h incubation, the peroxidase reaction was developed in 0.05% 3′3′-diaminobenzidine tetrahydrochloride and the sections were counterstained with Gill's hematoxylin no. 3 (Sigma).

Ear swelling response Baseline ear thickness measurements were made prior to the application of chemical, and the ear swelling response to chemical was measured 24 and 72 h after chemical was applied to the ear. Measurements were made with a spring-loaded caliper (Mitutoyo 20–1, Kawasaki, Kanagawa, Japan). Changes in the ear thickness were calculated as described by Back and Groth (1983): namely (thickness of ear at 24 or 72 h after challenge on the ear) minus (baseline ear thickness measured on day 0) divided by (baseline ear thickness measured on day 0) multiplied by 100.

Inflammatory index The number of neutrophils in each experimental condition was assessed by histologic analysis of the nuclear morphology and correlated to immunohistochemical analysis. For cytokine-producing cells and inflammatory cells expressing phenotypic markers, the inflammatory index is the mean ± SD of the total number of positively stained cells in three noncontiguous 40× microscopic fields.

Lymphocyte proliferation assay T lymphocyte proliferation was measured by ^{3}H-thymidine (^{3}H-TdR) incorporation. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated against a Ficoll Hypaque gradient, rinsed three times in 1 × phosphate-buffered saline and resuspended in RPMI 1640 containing 2 mM L-glutamine, 10% (vol/vol) iron-supplemented calf serum, 100 U per ml penicillin, and 100 mg per ml streptomycin at 1×10^6 cells per ml under standard mammalian tissue culture conditions. PBMCs were plated in quadruplicate and stimulated with PHA (1 mg per ml, n = 5 mice), anti-CD3 antibody (2.5 mg per ml, n = 5), or tetanus toxoid (0.1 and 10 μg per ml, n = 8). After 48, 72, or 96 h in culture, cells were pulsed with 0.5 mCi ^{3}H-TdR for 4 h, harvested onto glass fiber filters, and counted in a liquid scintillation counter. The cpm for each set of quadruplicates were averaged. The stimulation index, or fold increase in T cell proliferation, was calculated at each time point as the average cpm for each experimental condition divided by the average cpm for unstimulated cells.

Assessment of epidermal cell functionality To test activation of cells in the epidermis of B6.129F2 and FcγR KO mice, 1% OXA was applied to the dorsal side of each ear. Two hours after chemical application, ears were harvested, the dorsal side was incubated in 1 × phosphate-buffered saline containing 0.02% ethylenediamine tetraacetic acid at 37°C for 30 min, and the epidermis was separated from the dermis. Ears (n = 8 per group) were pooled and homogenized in 400 μl RPMI 1640.

Enzyme-linked immunosorbent assay (ELISA) The concentration of TNF-α and IL-1β in tissue homogenates was measured in duplicate with commercially available, solid-phase, two-site ELISA (R&D Systems) performed according to the manufacturer's directions. Reported sensitivities for the ELISA were 4.4 pg per ml for TNF-α and 3.0 pg per ml for IL-1β.

RESULTS

OXA is widely used as a sensitizer for the initiation and elicitation of CHS. Most studies have examined the effects of OXA following challenge, frequently in the lymph node or the epidermis, and less is known about its inflammatory properties in the dermis.

To examine the irritant and the allergic effects of OXA in the dermis, we treated B6.129F2 mice with OXA or AOO on the abdomen on day 1. Five days later, all mice were challenged on the ear with OXA. The effect of the vehicle, AOO, was examined in mice who received AOO on the abdomen and the ear. Twenty-four hours post challenge, cytokine production and cellular infiltration in the dermis were evaluated by immunohistochemistry. To confirm the development of a cutaneous response to chemical, the ear swelling response was measured 24 and 72 h after challenge.

B6,129F2 mice treated with AOO on the abdomen and on the dorsum of the ear displayed no increase in the number of infiltrating inflammatory cells or cytokine-producing cells in the dermis (**Figs 1**, **2**) and no increase in ear thickness. In contrast, B6,129F2 mice sensitized with either 0.5% OXA or AOO on the abdomen and challenged with 0.5% OXA on the ear had a significant increase in the number of infiltrating cells at 24 and 72 h post challenge. The majority of the infiltrating cells were neutrophils, although significant increases in $CD4^+$ and $CD8^+$ T lymphocytes were evident (**Figs 1**, **2**). Furthermore, we observed an increase in the number of cells producing the proinflammatory cytokines IL-1β and TNF-α, the Th1 cytokines IL-2 and IFN-γ, and the Th2 cytokines IL-4, IL-10, and IL-13 (**Figs 2**, **3**). Although a mixed Th1 and Th2 cytokine profile was consistently identified, the IL-12-producing cells were observed only in small numbers. Interestingly, neutrophils remained the dominant infiltrating cell 72 h after challenge; however, there was a significant increase in the number of $CD4^+$ and $CD8^+$ T lymphocytes at this time point (**Figs 1**, **2**). These data demonstrate that, in the dermis, the cellular and cytokine responses to OXA are not dependent upon a sensitizing dose of OXA and thus are not consistent with the typical type IV hypersensitivity that requires a sensitizing dose of antigen and, after challenge, involves a $CD4^+$/Th1 T cell population. Our results are consistent with observations of mixed Th1 and Th2 cytokine production in the draining lymph nodes of mice treated with 0.5% dinitrofluorobenzene or 1% OXA (Xu *et al*, 1996) and with previous studies in which the neutrophil was identified as the predominant cell population up to 24 h after picryl chloride

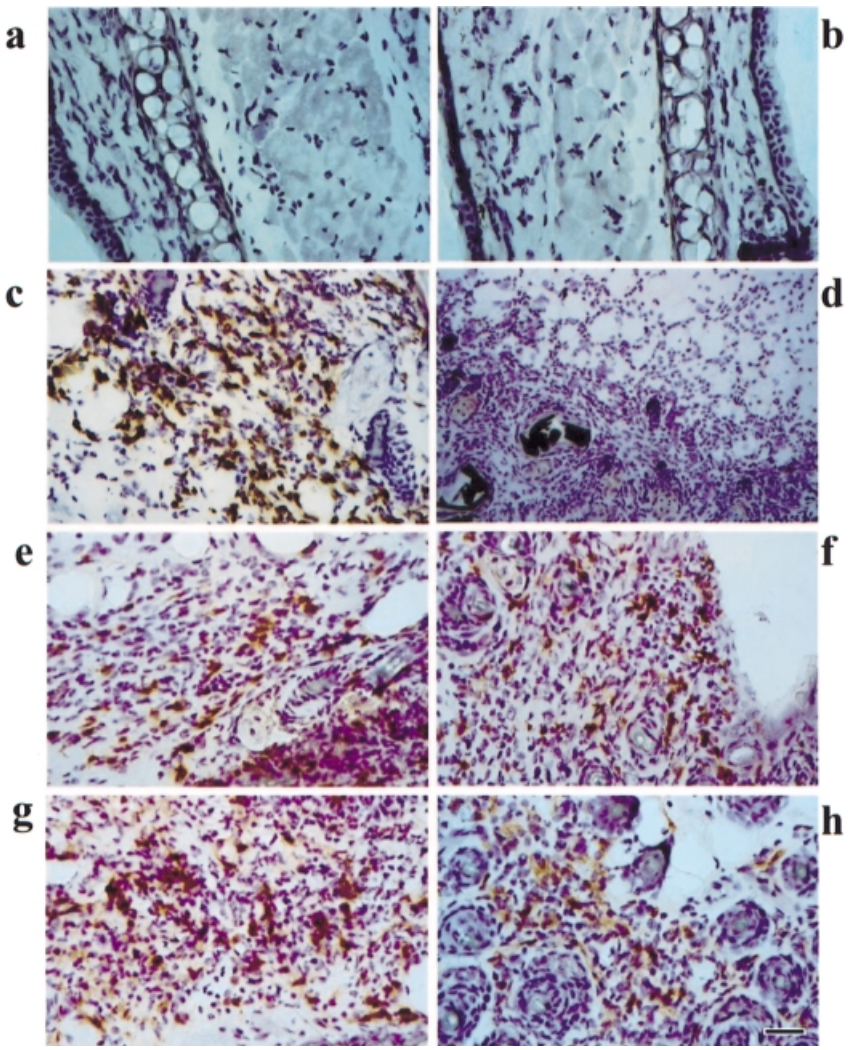

Figure 1. The $CD4^+$ and $CD8^+$ T lymphocyte populations are both present in the dermis at 24 and 72 h after challenge. Untreated (*a*) and vehicle (AOO/AOO) treated (*b*) B6.129F2 (n = 5 per group) mice have few inflammatory cells in the dermis. Mice sensitized on the abdomen and then challenged on the ear with 0.5% OXA (OXA/OXA) display marked increases in neutrophils at 24 h (*c*), $CD4^+$ T lymphocytes at 24 h (*e*) and 72 h (*g*), and $CD8^+$ T lymphocytes at 24 h (*f*) and 72 h (*h*). Normal rat serum provided the negative control for the primary antibodies. *Scale bar*: 35 μm.

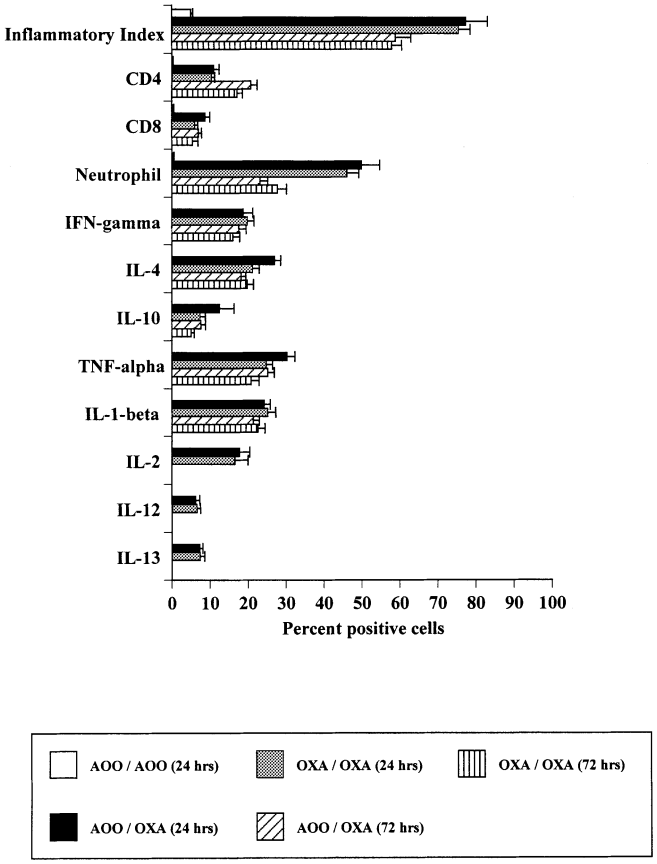

Figure 2. A mixed Th1/Th2 cytokine profile and dominant neutrophil infiltration were observed in B6.129F2 mice. The cellular and cytokine profiles observed in mice sensitized on the abdomen with AOO and challenged on the ear with 0.5% OXA (AOO/OXA) match those observed in nonsensitized mice challenged on the ear with 0.5% OXA (AOO/OXA) at 24 and 72 h. The number of cells expressing antibody-identified phenotypic markers in three noncontiguous 40× microscopic fields were counted and are presented as mean ± SEM.

challenge (Roupe and Ridell, 1979; Back and Groth, 1983). At 48–72 h after picryl chloride challenge, however, lymphocytes and eosinophils were reported to be the predominant infiltrating cells (Back and Groth, 1983). In our study, the lymphocyte population increased at 72 h, but the neutrophil remained the predominant cell population. This finding was verified by histologic analysis of the nuclear morphology of the neutrophils as well as immunohistochemical staining with antibody directed against Ly6 antigen.

To determine if chronic chemical exposure shifted the dermal response pattern, B6.129F2 mice were sensitized on the abdomen once and challenged on the ear three times at 10 d intervals. Twenty-four hours after the last chemical application, we observed a mixed Th1 and Th2 cytokine production and a cellular infiltrate composed predominantly of neutrophils, although significant increases in $CD4^+$ and $CD8^+$ T lymphocytes were also evident (**Fig 4**). The same cytokine and cellular pattern was also observed in mice sensitized with 1% and 3% OXA (data not shown).

The ear swelling response confirmed the inflammatory property of 0.5% OXA that has been reported previously by Grabbe and colleagues to occur following a single application of 0.5% OXA to the ear; however, the response is larger in B6.129F2 mice than reported for BALB/C mice (Grabbe *et al*, 1996). Mice sensitized with AOO and challenged with OXA displayed a 19% increase in ear thickness 24 h after challenge that declined to 12% at 72 h. Mice sensitized and challenged with OXA had a 35% and 28% increase at 24 h and 72 h, respectively (**Fig 5**). Consideration of these ear swelling data in combination with our immunohistochemical data demonstrating similar patterns of cellular infiltration and cytokine production in mice sensitized and challenged, and challenged only, may indicate differences in the vascular response underlying the development of the edema rather than the immunologic response, a hypothesis proposed by Back and Groth (1983).

To determine if a similar *in situ* response occurred following application of irritant chemicals, we applied AOO to the abdomen of mice on day 1 and 0.5% or 1% croton oil to the ear on day 6. Twenty-four hours after croton oil treatment, immunohistochemical analysis revealed a pattern of cellular infiltrates and cytokine production comparable to that induced by OXA (**Fig 6**). We observed significantly more $CD4^+$ T lymphocytes, however, than were observed with OXA application. Increases in the ear thickness

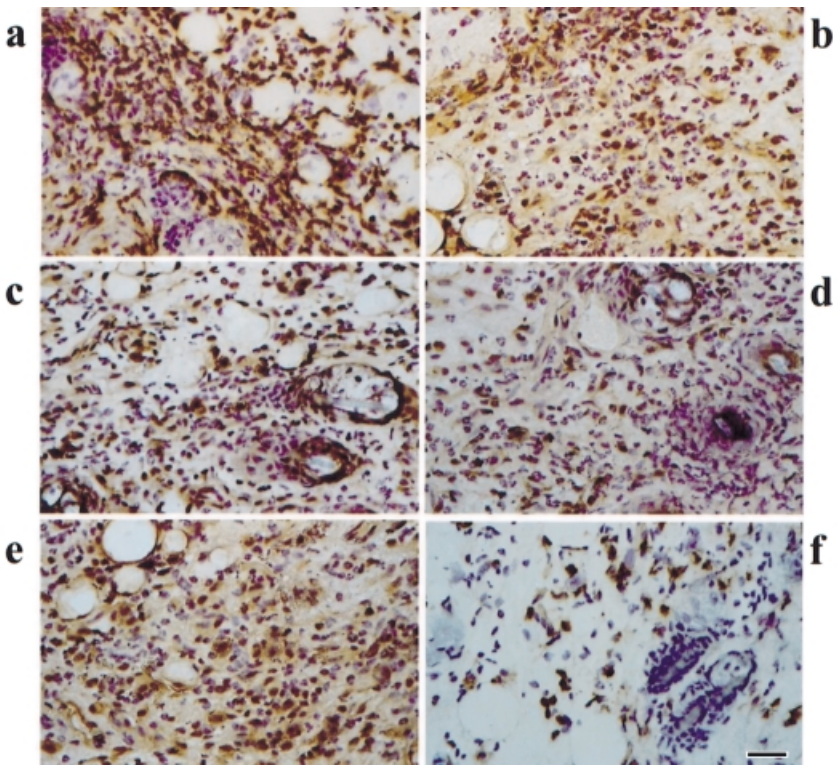

Figure 3. A mixed Th1/Th2 cytokine profile is maintained 72 h after chemical challenge. B6.129F2 mice (n = 5 per group) were sensitized on the abdomen and challenged on the ear with 0.5% OXA as described in *Materials and Methods*. The presence of TNF-α (*a*), IL-1β (*b*), IL-4 (*c*), IL-10 (*d*), IFN-γ (*e*), and IL-2 (*f*) producing cells was readily observed. *Scale bar*: 35 μm.

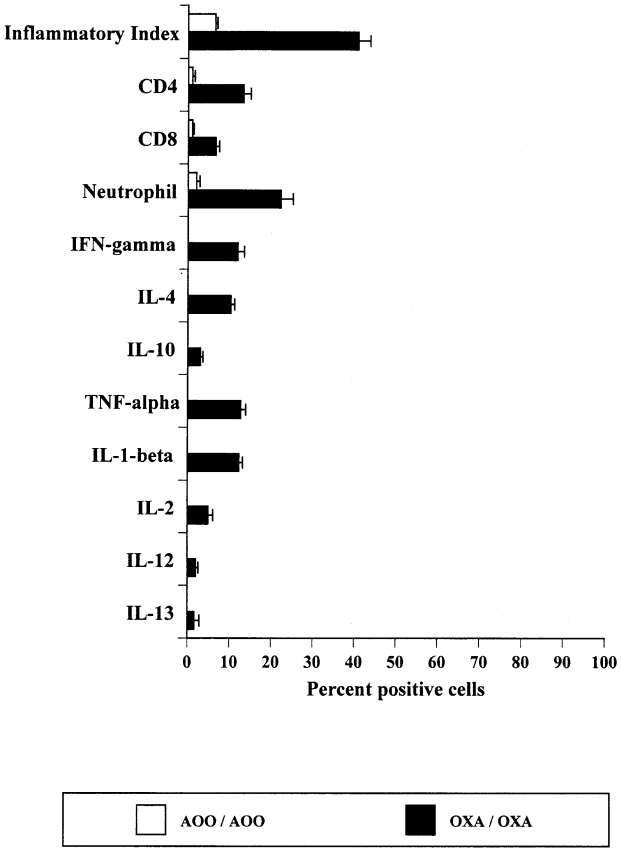

Figure 4. Repeated chemical challenge does not alter skin inflammatory response. B6.129F2 mice sensitized and challenged with 0.5% OXA every 10 d for 30 d (OXA/OXA). The number of cells expressing antibody-identified phenotypic markers 24 h after final chemical application. Data are presented as mean ± SEM for three noncontiguous 40× microscopic fields.

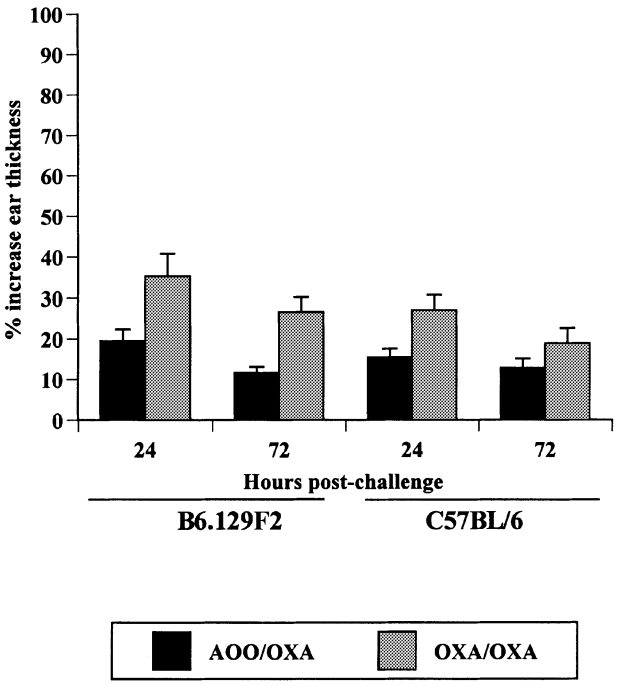

Figure 5. Ear swelling response was induced by 0.5% OXA in nonsensitized mice. Ear swelling response induced by 0.5% OXA in nonsensitized (AOO/OXA) and sensitized (OXA/OXA) B6.129F2 and C57BL/6 mice. Changes in the ear thickness were calculated as described in *Materials and Methods*. Data are presented as the mean ± SEM.

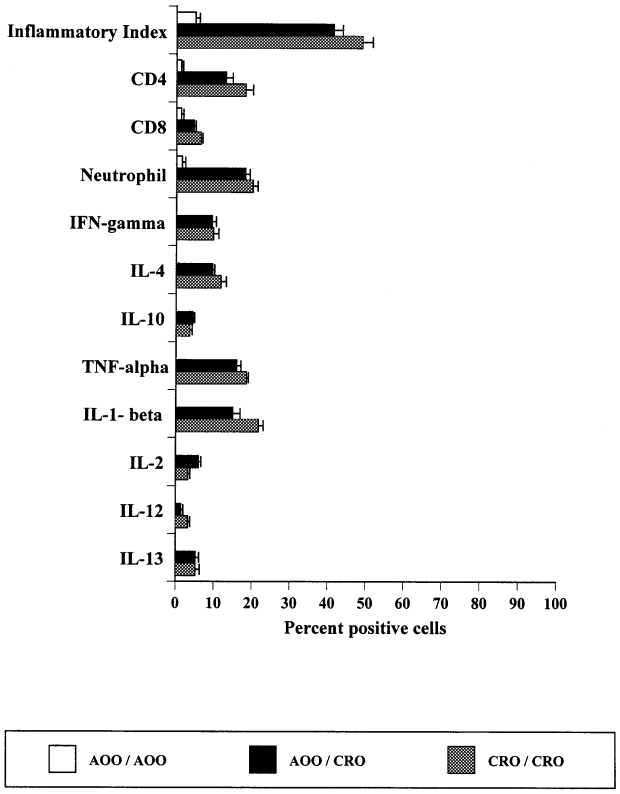

Figure 6. Croton-oil-induced inflammation produces a similar cellular and cytokine profile as that observed in OXA sensitized and challenged B6.129F2 mice 24 h after chemical application. Data are presented as mean ± SEM for three noncontiguous 40× microscopic fields.

stimulated by croton oil were similar to those obtained for mice sensitized with AOO and challenged with OXA, 28% and 12% at 24 h and 72 h, respectively.

It has been established that, in naïve mice, T lymphocytes specific for any single antigen constitute only a small proportion of the T cell population, and that, without sensitization, no specific immune response to a particular determinant can be demonstrated (Marrack and Kappler, 1986). These observations, coupled with our data showing that 0.5% OXA induced similar cellular and cytokine profiles in the presence and absence of OXA sensitization, suggest that the response we observed in the dermis does not require OXA-specific T cells. To address the hypothesis that the irritant property of OXA is sufficient to initiate skin inflammation in the absence of T lymphocytes, we applied our chemical treatment paradigm to the athymic mouse, a murine model with a severely limited T lymphocyte population and hence no specific T lymphocyte immunity. We observed a strong inflammatory cell infiltration in the skin, approximately 70% of the inflammatory index measured in the wild type C57BL/6 mice (**Figs 7**, **8**). Few $CD4^+$ or $CD8^+$ T cells were identified, and the majority of the infiltrating cells were neutrophils. Additionally, athymic mice receiving sensitizing and challenge applications of 0.5% OXA displayed a larger influx of neutrophils than mice sensitized on the abdomen with AOO and challenged on the ear with OXA. Few cytokine-producing cells, for the proinflammatory cytokines IL-1β and TNF-α, or for IL-2, IL-4, IL-10, IL-13, or IFN-γ, were observed. Athymic mice also displayed an ear swelling response comparable to that observed in C57BL/6 mice for the AOO sensitization, OXA challenge treatment paradigm. The pinnae thickness in athymic mice increased 11% and 3% at 24 h and 72 h, respectively, and in C57BL/6 mice those measures were 15% and 13%. Sensitization and challenge with OXA increased ear thickness 27% and 19% for the same time points in the control mice, but only 18% and 8% in the knockout mice. In contrast, the ear swelling

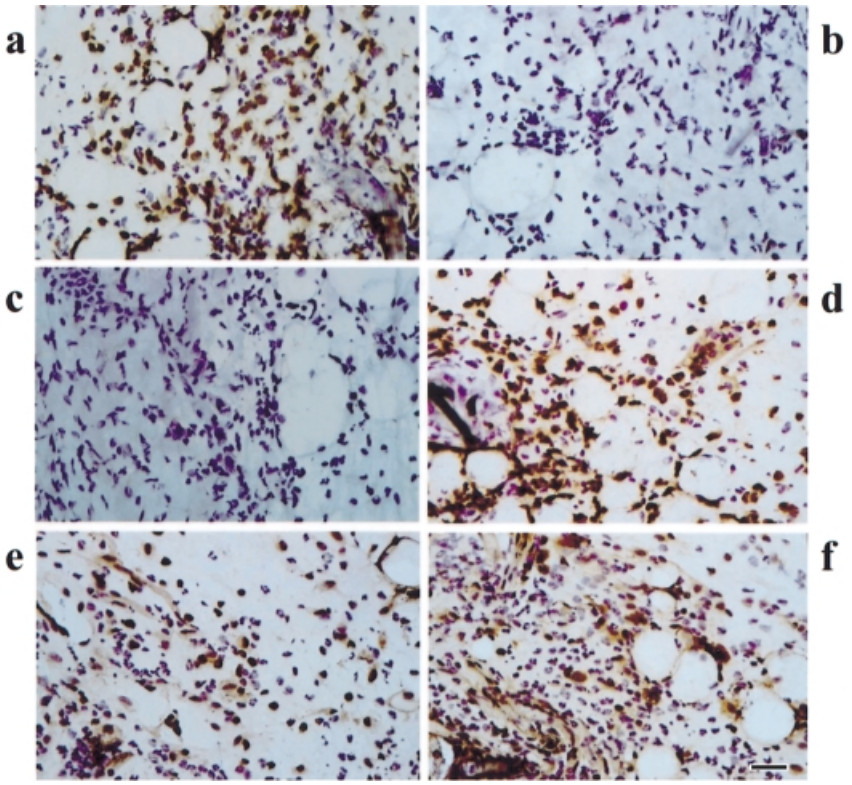

Figure 7. A neutrophil-dominant dermal inflammation was observed in nonsensitized athymic mice challenged on the ear with 0.5% OXA (AOO/OXA). A strong neutrophil infiltration (*a*), but no $CD4^+$ (*b*) and $CD8^+$ (*c*) T lymphocytes are observed 24 h after chemical application. Cells producing TNF-α (*d*), IL-4 (*e*), and IFN-γ (*f*) were consistently identified. *Scale bar*: 35 μm.

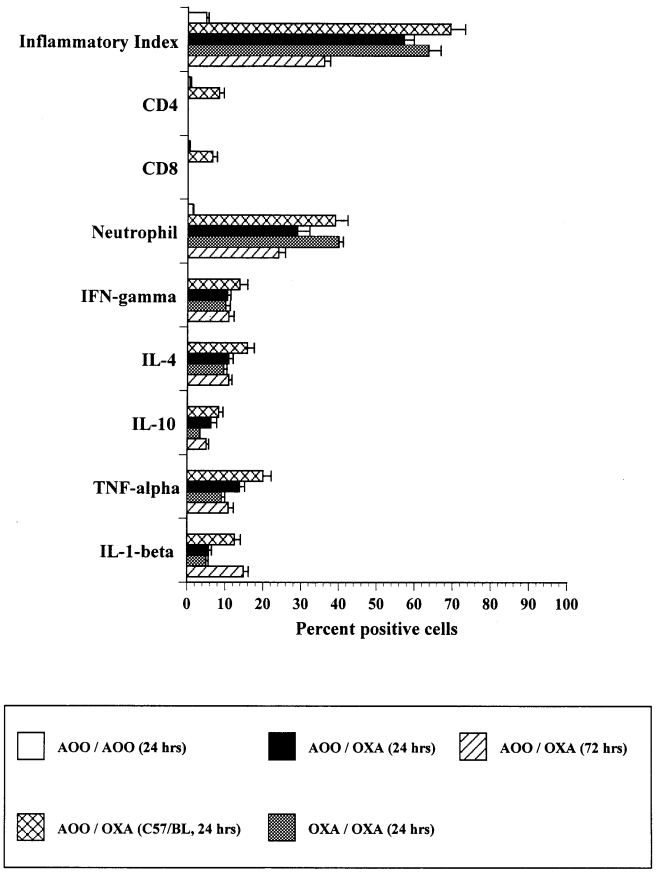

Figure 8. The dermal response to 0.5% OXA is comparable in sensitized and nonsensitized athymic mice. The cellular and cytokine profiles observed in sensitized (OXA/OXA) or nonsensitized (AOO/OXA) athymic mice match those observed in nonsensitized C57BL/6 control mice challenged on the ear with 0.5% OXA (AOO/OXA). Data are presented as mean ± SEM for three noncontiguous 40× microscopic fields.

response to croton oil was similar for both athymic mice and C57BL/6 mice: 24% and 21%, respectively, at 24 h, and 16% and 18%, respectively, at 72 h. These findings demonstrate that, in C57BL/6 mice, OXA-induced contact dermatitis is able to develop in the absence of a T lymphocyte response, but that T lymphocytes contribute to development of a full inflammatory response. Our results do not exclude the role of specific T cells in initiation, elicitation, and maintenance of CHS, but indicate the importance of the irritant property of OXA in CHS and suggest that the irritant property of OXA is sufficient to initiate skin inflammation.

To identify the pathway activated by the irritant property of OXA, we asked whether chemical activation of the innate immune response is required for development of CHS. For these studies, we employed FcγR KO mice, which have pleiotropic immune/inflammatory effector cell defects that result in dysfunctional mast cells, natural killer cells, and macrophages but normal populations of $CD4^+$ and $CD8^+$ T cells. Knockout mice sensitized with AOO on the abdomen and challenged with 0.5% OXA or 0.5% croton oil on the ear displayed no inflammatory response, or a very mild response, with the virtual absence of erythema and no increased ear thickness 24 and 72 h post treatment. A small number of $CD4^+$ and $CD8^+$ T lymphocytes and neutrophils were observed in the dermis, and no cytokine-producing cells were identified (**Figs 9**, **10**). Additional mice sensitized on the abdomen and challenged on the ear with 0.5% OXA displayed a similar response pattern, with a slight increase in the number of $CD4^+$ and $CD8^+$ T lymphocytes (**Fig 10**). These results suggest that the impairment of innate immune cells, as occurs with deletion of the FcγR subunit, prevents the development of contact dermatitis following application of a sensitizer or an irritant. Future experiments will identify the innate immune cells required for the development of skin inflammation.

To test the ability of the FcγR KO mice to mount an innate and a specific immune response, we examined T lymphocyte proliferation and cytokine production. Developers of the FcγR KO mouse model determined that the T lymphocytes in these mice are phenotypically normal and are present in the peripheral blood in the normal range (Takai *et al*, 1994). To exclude the possibility that deletion of the FcγR subunit nonspecifically impaired T

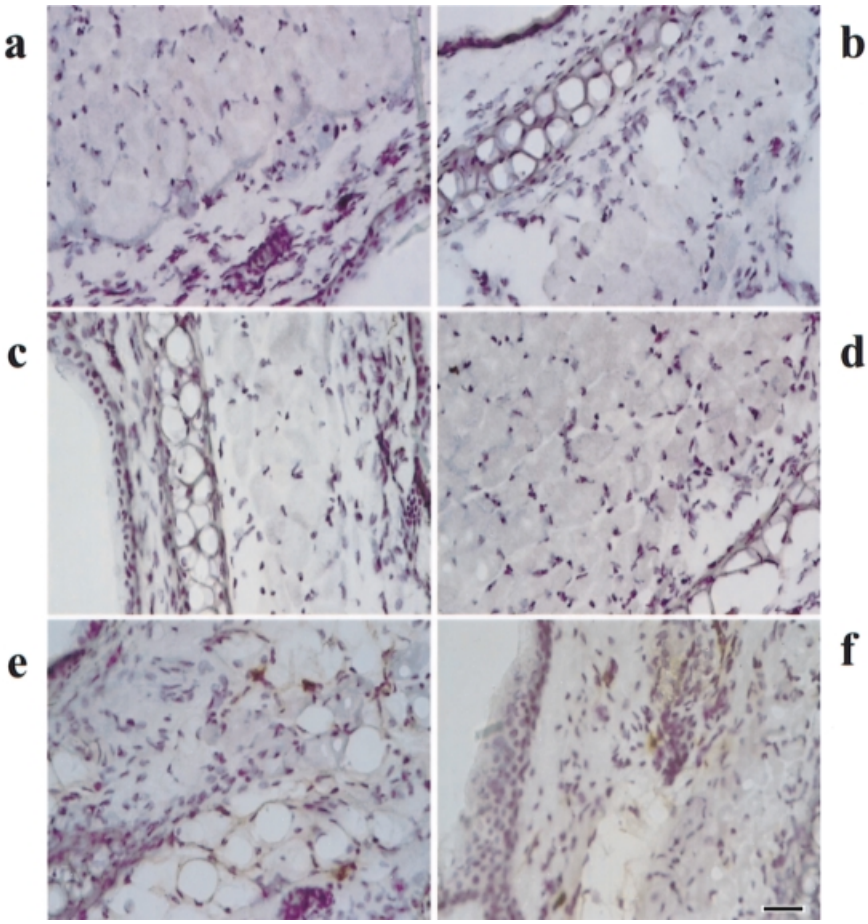

Figure 9. Mild inflammation was observed in FcγR KO mice sensitized with AOO and challenged with 0.5% OXA. No neutrophils (*a*), $CD4^+$ T lymphocytes (*b*), $CD8^+$ T lymphocytes (*c*), or TNF-α-producing cells (*d*) were identified 24 h after chemical treatment. A few neutrophils (*e*) and $CD4^+$ T lymphocytes (*f*) were identified at 72 h post chemical treatment. *Scale bar*: 35 μm.

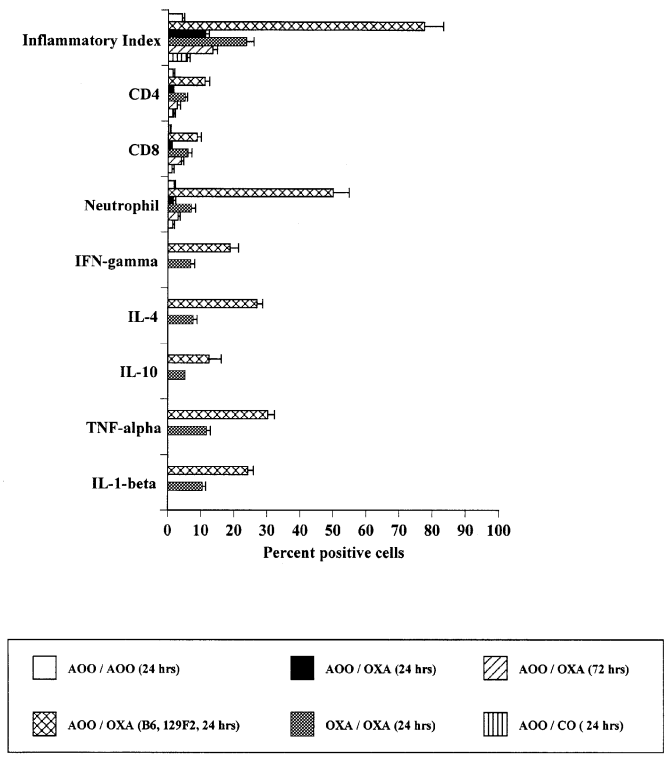

Figure 10. FcγR KO mice display significantly reduced dermal inflammation and cytokine-producing cells at 24 and 72 h after chemical challenge. FcγR KO mice were sensitized with AOO and challenged with OXA (AOO/OXA) or with croton oil (AOO/CO) or sensitized and challenged with 0.5% OXA (OXA/OXA). Data are presented as mean ± SEM for three noncontiguous 40× microscopic fields.

lymphocyte function, we compared first the proliferative capability of B6,129F2 and FcγR KO-derived PBMCs in response to anti-CD3 antibody and PHA. In response to anti-CD3 antibody, we measured 6861 cpm ^{3}H-TdR incorporation for B6,129F2 mice and 6180 cpm for FcγR KO mice, compared to 203 and 211 cpm for unstimulated cells. To confirm the ability of the T cells of FcγR KO mice to proliferate in response to protein, we sensitized and challenged B6,129F2 mice (n = 8) and knockout mice (n = 8) intraperitoneally with tetanus toxoid and measured tetanus-toxoid-stimulated T cell proliferation in PBMCs *in vitro* at 48 and 96 h. In B6,129F2 mice, 0.1 μg per ml tetanus toxoid stimulated a 2.7-fold increase in ^{3}H-TdR incorporation at 48 h, and 10 μg per ml stimulated a 4.6-fold increase at 96 h. In FcγR KO mice, tetanus toxoid stimulated a 1.9-fold increase and a 4.3-fold increase for the same doses of antigen after 48 and 96 h in culture. At 48 h, unstimulated PBMCs from KO mice incorporated 662 cpm of ^{3}H-TdR, whereas PBMCs stimulated with 10 μg per ml of tetanus toxoid incorporated 2818 cpm. These results, in combination with our anti-CD3 antibody data and PHA data, demonstrate normal T cell proliferative capability in the FcγR KO mice.

To evaluate chemical activation of epidermal cells in the knockout mice, we stimulated epidermal sheets from B6,129F2 and FcγR KO mice for 2 h with 1% OXA under standard mammalian tissue culture conditions, and measured the concentrations of TNF-α, IL-1β, and KC in tissue homogenates. OXA stimulated an increase in both cytokines in epidermis derived from B6,129F2 mice (**Fig 11**). In contrast, we measured a lower concentration of TNF-α, only 30% of that measured in wild type mice, and no IL-1β or the murine chemokine KC in the FcγR KO mice. The reduced level of TNF-α and the absence of IL-1β and KC suggest impaired activation of the innate immune cells in the epidermis.

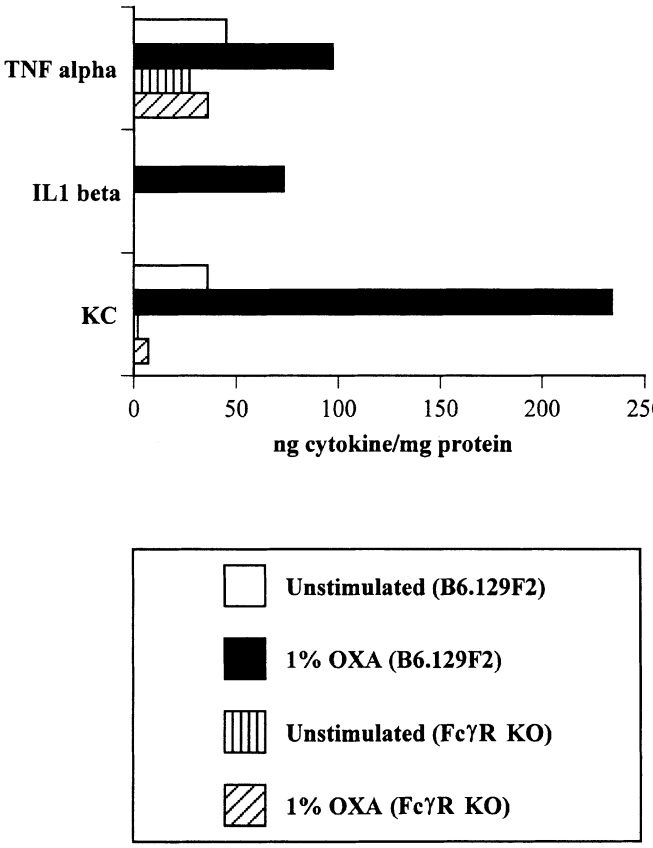

Figure 11. Epidermal sheets from FcγR KO mice were stimulated to produce a low concentration of TNF-α, but not IL-1β and KC. Two hours after application of 1% OXA on the dorsal side of each ear of FcγR KO mice and B6.129F2 mice, ears were harvested, and the dorsal epidermis was separated from dermis, as described in *Materials and Methods*, and homogenized for detection of cytokines by ELISA.

DISCUSSION

Data presented in this study provide support for the hypothesis that chemical-induced CHS is not a classic delayed type hypersensitivity, extend our understanding of the interaction between the irritant and antigenic properties of sensitizing chemicals in the development of allergic contact dermatitis, and suggest that the irritant effect of chemicals may be mediated through the cutaneous innate immune system.

Although chemical-induced CHS has been reported to be a $CD4^+$, Th1-dominant type IV hypersensitivity based primarily on data derived from lymph node studies (Hauser, 1990), Xu and colleagues have recently demonstrated that IL-4- and IL-10-producing $CD4^+$ T lymphocytes and IFN-γ-producing $CD8^+$ T lymphocytes are negative and positive regulators of chemical-induced ear swelling response (Xu *et al*, 1997). Our immunohistochemical data demonstrate a combined $CD4^+$ and $CD8^+$ T lymphocyte response in the dermis, concurrent production of Th1 and Th2 cytokines, and a predominantly neutrophil infiltrate, even 72 h after chemical challenge. These data are also consistent with reports that $CD8^+$ T lymphocytes are able to mediate contact hypersensitivity in major histocompatibility complex class II-deficient mice (Bouloc *et al*, 1998), that many cytokines, including IFN-γ, IL-2, IL-4, and IL-10, participate in the development of CHS (Kondo and Sauder, 1995), and that neutrophils accumulate at the site of epicutaneous chemical application (Roupe and Ridell, 1979; Wilmer *et al*, 1994). Our data also demonstrate that, *in vivo*, the cutaneous response to chemical in the dermis is not dependent on a sensitizing exposure to chemical and is similar for all conditions tested. We observed similar cellular and cytokine responses in the dermis of mice sensitized and challenged with OXA, challenged only with OXA, or challenged only with croton oil. These data demonstrate that, whereas the chemical response in the lymph node may be a $CD4^+$, Th1-type hypersensitivity, a mixed $CD4^+/CD8^+$, Th1/Th2 response occurs at the site of chemical application for at least 72 h post challenge. This mixed response underscores the nontraditional nature of cutaneous type IV hypersensitivity, attributable, at least in part, to the combined irritant and antigenic properties of OXA. Furthermore, persistence of the mixed Th1/Th2 cytokine pattern may partially explain the maintenance of the strong neutrophil response 72 h after final chemical application. Kunkel has postulated that cytokine-induced changes in chemokine production are important in the transition from an acute neutrophil-mediated response to a mononuclear-cell-mediated immune response (Kunkel, 1996). The persistence of the proinflammatory and Th1/Th2 cytokine pattern 72 h after challenge, in particular TNF-α, IL-1β, IL-4, and IL-10, may prolong the inflammatory response by providing mixed regulatory signals for chemokine production.

Additionally, our data are consistent with previous reports that found no significant differences in the cellular or cytokine responses to sensitizers and irritants (Brasch *et al*, 1992; Hoefakker *et al*, 1995) in epidermal sheets, epidermal cell suspensions (Kilgus *et al*, 1993; Hoefakker *et al*, 1995), and cultured Langerhans cells and keratinocytes (Wilmer *et al*, 1994; Kimber *et al*, 1995). There is no consensus, however, concerning discrimination between irritants and sensitizers based on the pattern of cytokine production, and there are reports that IL-6, IL-8, and IL-10 are produced in response to sensitizers only (Wilmer *et al*, 1994; Dearman *et al*, 1996).

Given the consistency of the cellular and cytokine response in the dermis under all treatment paradigms employed in this study, and the strong OXA-induced neutrophil influx in the dermis, we asked if the irritant, nonantigenic property of OXA activates innate immune cells and is required for the initiation of the specific immune response in the skin. Evidence from several laboratories has shown that the irritancy of chemicals is important in the development of cutaneous responses. First, OXA, trinitrochlorobenzene, dinitrofluorobenzene, and dinitrochlorobenzene, at concentrations commonly used for allergen challenge, induced significant ear swelling in nonsensitized mice (Back and Groth, 1983; Enk *et al*, 1993; Kondo *et al*, 1994; DiIulio *et al*, 1996; Grabbe *et al*, 1996; Xu *et al*, 1997). Second, mice sensitized and challenged with 0.5% trinitrofluorobenzene produced a complex cytokine messenger RNA profile that was not significantly different in mice that received only a challenge dose of chemical (Enk and Katz, 1992; Kondo *et al*, 1994).

To investigate the molecular and cellular basis for these direct, or irritant, effects of OXA, we utilized the athymic mouse that possesses intact innate immunity but no T lymphocyte immune response, and the FcγR KO mouse that has functional T lymphocytes and pleiotropic defects of innate immune effector cells (Takai *et al*, 1994). Data derived from our athymic mice demonstrate the strong irritant property of 0.5% OXA, as the inflammatory index was approximately 70% of that measured in the wild type mice. The strong neutrophil and cytokine response we observed, however, is T-lymphocyte-independent, and it is possible that the absence of T lymphocytes contributes to the diminution of the response. It has been reported, however, that reactive chemical compounds can nonspecifically activate keratinocytes and Langerhans cells in both *in vivo* and *in vitro* systems (Grabbe and Schwarz, 1998). In combination, these data support our hypothesis that chemical-induced irritancy and functional innate immunity are necessary for the development of cutaneous hypersensitivity. Our results from athymic mice do not negate the importance of T lymphocytes in the elicitation and maintenance of the cutaneous immune response to chemicals. Interaction between the innate and specific immune responses has been suggested by

Janeway and colleagues (Medzhitov and Janeway, 1997; Ezekowitz and Hoffman, 1998), and our athymic mouse data show that the irritant property of chemicals can activate a nonspecific, T-cell-independent skin inflammation that is necessary for the development of CHS.

In FcγR KO mice, challenge with OXA induced a mild cutaneous response, approximately 20% of that observed in wild type mice, and sensitization plus challenge increased the cellular and cytokine responses to approximately 30%. We tested the functionality of T lymphocytes and epidermal cells and demonstrated that the FcγR KO T lymphocyte proliferative response matches that of the wild type mice and that epidermal cells, *in vitro*, can be stimulated to produce TNF-α. TNF-α is considered a primary cytokine capable of initiating an inflammatory response in the skin; however, this cytokine was unable to stimulate an inflammatory response in the knockout mice at the concentrations measured. Our study does not evaluate the possible impairment of Langerhans cell antigen presentation in the FcγR KO mice; however, Streilein and coworkers have shown that Langerhans cells are not required for the development of CHS (Streilein and Bergstresser, 1988; Grabbe and Schwarz, 1998). Furthermore, our athymic mouse data demonstrate that impairment of specific immunity minimally impacts development of the inflammatory response. The limited ability of the FcγR KO mice to mount an irritant response is probably not due to impairment of mast cell function, as studies in mast-cell-deficient mice have shown minimal alteration in the development of CHS (Askenase *et al*, 1983; Thomas and Schrader, 1983). Other *in vitro* studies have shown that haptens may directly induce nonspecific proinflammatory effects in immunocompetent cells, in the skin perhaps through the keratinocyte (Grabbe and Schwarz, 1998).

Takai and colleagues, developers of the FcγR KO mice, have documented unexpected pleiotropic effects for the γ subunit deletion and more pronounced functional defects than were predicted from *in vitro* reconstitution studies alone (Takai *et al*, 1994). It is possible that deletion of the FcγR signal-transducing subunit influences other signal transduction pathways, and the full effect of this deletion on innate and specific immune cell function will require additional clarification. Our results from FcγR KO mice, however, in combination with our findings from athymic mice, suggest that innate immune cells may be responsible for the T-cell-independent skin inflammation that is induced by the irritant property of chemicals, and that the subsequent interaction of chemical with the specific immune response may determine the outcome of CHS.

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REFERENCES

Askenase PW, Van Loveren H, Kraeuter-Kops S, *et al*: Defective elicitation of delayed-type hypersensitivity in W/Wv and SI/SId mast cell-deficient mice. *J Immunol* 131:2687–2694, 1983

Back O, Groth O: The cellular infiltrate of the contact sensitivity reaction to picryl chloride in the mouse. *Acta Dermatovener (Stockholm)* 63:304–307, 1983

Bouloc A, Cavani A, Katz SI: Contact hypersensitivity in MHC class II- deficient mice depends on CD8 T lymphocytes primed by immunostimulating Langerhans cells. *J Invest Dermatol* 111:44–49, 1998

Brasch J, Burgard J, Sterry W: Common pathogenetic pathways in allergic and irritant contact dermatitis. *J Invest Dermatol* 98:166–170, 1992

Dearman RJ, Basketter DA, Kimber I: Characterization of chemical allergens as a function of divergent cytokine secretion profiles induced in mice. *Toxicol Appl Pharmacol* 138:308–316, 1996

DiIulio NA, Xu H, Fairchild RL: Diversion of $CD4^+$ T cell development from regulatory T helper to effector T helper cells alters the contact hypersensitivity response. *Eur J Immunol* 150:3698–3704, 1996

Enk AH, Katz SI: Early molecular events in the induction phase of contact sensitivity. *Proc Natl Acad Sci U S A* 89:1398–1402, 1992

Enk AH, Angeloni VL, Udey MC, Katz SI: An essential role for Langerhans cell-derived Il-1β in the initiation of primary immune responses in skin. *Eur J Immunol* 26:2606–2612, 1993

Ezekowitz AB, Hoffman J: Innate immunity. The blossoming of innate immunity. *Curr Opin Immunol* 10:9–11, 1998

Grabbe S, Schwarz T: Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today* 19:37–44, 1998

Grabbe S, Gallo RL, Lindgren A, Granstein RD: Deficient antigen presentation by Langerhans cells from athymic (nu/nu) mice. Restoration with thymic transplantation or administration of cytokines. *J Immunol* 151:3430–3439, 1993

Grabbe S, Steinert M, Mahnke K, Schwartz A, Luger TA, Schwarz T: Dissection of antigenic and irritative effects of epicutaneously applied haptens in mice. Evidence that not the antigenic component but nonspecific proinflammatory effects of haptens determine the concentration-dependent elicitation of allergic contact dermatitis. *J Clin Invest* 98:1158–1164, 1996

Hauser C: Cultured epidermal Langerhans cells activate effector T cells for contact sensitivity. *J Invest Dermatol* 95:436–440, 1990

Hoefakker S, Caubo M, van't Erve EH, *et al*: *In vivo* cytokine profiles in allergic and irritant contact dermatitis. *Contact Dermatitis* 33:358–366, 1995

Kilgus O, Payer E, Schreiber S, Elbe A, Strohal R, Stingl G: *In vivo* cytokine expression in normal and perturbed murine skin – analysis by competitive quantitative polymerase chain reaction. *J Invest Dermatol* 100:674–680, 1993

Kimber I, Holliday MR, Dearman RJ: Cytokine regulation of chemical sensitization. *Toxicol Lett* 82–83:491–496, 1995

Kondo S, Sauder DN: Epidermal cytokines in allergic contact dermatitis. *J Am Acad Dermatol* 33:786–800, 1995

Kondo S, Pastore S, Shivji GM, McKenzie RC, Sauder DN: Characterization of epidermal cytokine profiles in sensitization and elicitation phases of allergic contact dermatitis as well as irritant contact dermatitis in mouse skin. *Lymphokine and Cytokine Research* 13:367–375, 1994

Kunkel SL: Th1- and Th2-type cytokines regulate chemokine expression. *Biol Signals* 5:197–202, 1996

Marrack P, Kappler J. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. In: Dixon FJ: ed. *Advances in Immunology* Vol 38, Orlando, FL: Academic Press, 1986:1–30

Medzhitov R, Janeway CA Jr: Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295–298, 1997

Roupe G, Ridell B: The cellular infiltrate in contact hypersensitivity to picryl chloride in the. mouse. *Acta Derm Venereol (Stockholm)* 59:191–195, 1979

Rycroft RJG, Menne T, Frosch PJ: eds. *Textbook of Contact Dermatitis* Berlin. Springer-Verlag, 1995

Streilein JW, Bergstresser PR: Genetic basis of ultraviolet-B effects on contact hypersensitivity. *Immunogenetics* 27:252–258, 1988

Takai T, Li M, Sylvestre D, Klans R, Ravetch JV: FcR gamma chain deletion results in pleiotropic effector cell defects. *Cell* 76:519–529, 1994

Thomas WR, Schrader JW: Delayed hypersensitivity in mast-cell-deficient mice. *J Immunol* 130:2565–2567, 1983

Wilmer JL, Burleson FG, Kayama F, Kanno J, Luster MI: Cytokine induction in human epidermal keratinocytes exposed to contact irritants and its relation to chemical-induced inflammation in mouse skin. *J Invest Dermatol* 102:915–922, 1994

Xu H, DiIulio A, Fairchild RL: T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon-gamma-producing (Tc1) effector $CD8^+$ T cells and interleukin (IL) 4/IL-10-producing (Th2) negative regulator $CD4^+$ T cells. *J Exp Med* 183:1001–1012, 1996

Xu H, Banerjee A, Dilulio NA, Fairchild RL: Development of effector $CD8^+$ T cells in contact hypersensitivity occurs independently of $CD4^+$ T cells. *J Immunol* 158:4721–4728, 1997