

IL-23 and T_H17-mediated inflammation in human allergic contact dermatitis

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Background: IL-17-producing T_H (T_H17) cells are key mediators of chronic inflammation in mice. Recent studies have implicated T_H17-mediated inflammation in the pathogenesis of human autoimmune diseases; however, the involvement of T_H17 cells in allergic disorders remains largely elusive.

Objective: To investigate T_H17-mediated inflammation in human beings with allergic contact dermatitis; in particular, the innate response of keratinocytes to contact allergen, the induction of allergen-specific T_H17 cells, and the presence of T_H17-related effector cells in inflamed skin.

Methods: Human keratinocytes were stimulated with nickel *in vitro* followed by measurements of IL-23 and IL-12 production by quantitative PCR and ELISA. Allergen-specific memory T cells from the blood of individuals with nickel allergy and healthy controls were identified and characterized by using a short-term *ex vivo* assay. Nickel patch test lesions and normal skin were analyzed for the expression of T_H17-related cells and molecules by using immunohistochemistry.

Results: Keratinocytes were found to produce IL-23, but no detectable IL-12, in a response to nickel stimulation. Memory T cells isolated from peripheral blood of individuals with nickel allergy, but not healthy controls, contained T_H17 and T_H1 cells proliferating in response to nickel-pulsed DCs. Inflamed skin of nickel-challenged allergic individuals contained infiltrating neutrophils and cells expressing IL-17, IL-22, CCR6, and IL-22R.

Conclusion: Our results demonstrate the involvement of T_H17-mediated immunopathology in human allergic contact dermatitis, including both innate and adaptive immune responses to contact allergens. (J Allergy Clin Immunol 2008;122:122-130.)

Key words: Allergic contact dermatitis, contact hypersensitivity, inflammation, keratinocytes, T_H17 cells, IL-23

Abbreviations used

ACD: Allergic contact dermatitis
BrdU: 5-Bromo-2-deoxyuridine
CCL: Chemokine ligand
CHS: Contact hypersensitivity
DC: Dendritic cell

Allergic contact dermatitis (ACD) is one of the most common skin diseases, with great socioeconomic consequences for patients.^{1,2} ACD is the clinical manifestation of contact hypersensitivity (CHS) after sensitization and encounter with low-molecular-weight contact allergens (haptens). Sensitization to contact allergens is caused by uptake of allergen by antigen-presenting cells in the skin (Langerhans cells or dermal dendritic cells) followed by priming of allergen-specific T cells in the draining lymph nodes. The manifestation of ACD has been ascribed to activation of CD8⁺ cytotoxic T cells and IFN- γ -producing CD4⁺ T_H1 cells, which cause tissue pathology by destroying haptenized keratinocytes and stimulate IFN- γ -mediated immune activation.³⁻⁶

Recently, a novel IL-17-producing CD4⁺ T_H (T_H17) cell subset has been described in both human beings and mice.⁷⁻⁹ IL-17 targets epithelial cells, endothelial cells, and macrophages, and stimulates the expression of proinflammatory cytokines and chemokines, which mobilize and activate neutrophils in the inflamed tissues.¹⁰ Furthermore, IL-22 and IL-17, which are coexpressed by T_H17 cells, may cooperate to recruit other inflammatory cells across the endothelium during initiation of the immune response.^{11,12} A critical role of IL-17 in the inflammatory process has been demonstrated by impaired cellular and humoral immune responses caused by impaired antigen sensitization in IL-17 knockout mice.¹³ Proliferation and cytokine production by T_H17 cells are driven by IL-23 produced by the innate immune system.¹⁴ Accumulating experimental work has provided evidence for the importance of the IL-23/IL-17 axis in the development and progression of autoimmune disease, previously primary thought to be driven by T_H1-mediated inflammation.¹⁵⁻¹⁹ In line with these findings, studies of autoimmunity in human beings show increased IL-23 and IL-17, as well as the presence of T_H17 cells in inflamed tissue correlating to the expression of disease.²⁰⁻²³

Studies have recently indicated a crucial role for IL-17 and T_H17 cells in eliciting murine ACD. IL-17-deficient mice have shown impaired CHS responses to contact allergens.¹³ Furthermore, allergen-specific IL-17-producing CD4⁺ and CD8⁺ T cells have been shown to develop in sensitized mice.²⁴ Interestingly, treatment of these sensitized mice with IL-17-neutralizing

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antibodies reduced immunopathology by inhibiting immune cellular recruitment of T cells, monocytes/macrophages, and granulocytes in challenged tissues. Although earlier studies have indicated that IL-17 production takes place in nickel-specific T_H1 and T_H2 clones isolated from individuals with allergy,^{25,26} the role of T_H17 -mediated inflammation in human ACD remains largely unknown.

The current study addresses the question whether T_H17 mediated inflammation is involved in the immunopathology of human ACD. We found that keratinocytes produce IL-23 in response to nickel, thus indicating keratinocytes as a source of dermal IL-23 with the potential to drive T_H17 -mediated skin inflammation in response to contact allergens. Furthermore, individuals with nickel allergy are found to have allergen-specific T_H17 cells in the peripheral blood, demonstrating that T_H17 cells are primed during the contact allergic sensitization of human beings. Finally, by using fluorescence immunohistochemistry, we demonstrate infiltration of neutrophils and cells expressing IL-17, IL-22, CCR6, and IL-22R in ACD induced by nickel. These results support the involvement of T_H17 -mediated inflammation in human ACD.

METHODS

Patients and health controls

In the study of peripheral blood allergen-specific T_H17 cells, 6 individuals with nickel allergy with previous positive skin patch test results were enrolled. Six healthy individuals without allergy were enrolled as controls. Neither individuals with allergy nor control individuals received any prescribed systemic medication for the treatment of allergic disease or had diagnosed autoimmune disorders. For the immunohistochemical study of allergen-inflamed skin, 3 patients with nickel allergy with a patch test reaction graded at least ++ according to the European contact dermatitis scoring system²⁷ and 2 controls with a negative nickel patch test were enrolled. All participants gave informed consent, and the study was approved by the local medical ethics committee (no. KA-20060109).

Materials and reagents

A list of materials and reagents used in this study is found in this article's Methods in the Online Repository at www.jacionline.org.

Keratinocyte IL-23 production

Immortalized human keratinocytes (HaCaT cells) were seeded in 6-well plates (2.5×10^5 cells/well). After 48 hours of culture, cells were stimulated in triplicates with or without 50 $\mu\text{g/mL}$ NiCl_2 for another 4 hours before RNA extraction and qPCR analysis of IL-12p40, IL-23p19, and IL-12p35 expression.

Primary keratinocytes were seeded in 96-well plates (10^4 cells/well) for 48 hours followed by NiCl_2 stimulation (0, 1, 5, and 10 $\mu\text{g/mL}$) for 24 hours. IL-23 and IL-12 heterodimer protein production was measured by using commercial ELISA kits (eBioscience, San Diego, Calif) according to the manufacturer's recommendations. To allow low-level protein detection, keratinocyte supernatants and cell-lysates of 3 wells were pooled and incubated serially during the antigen-capture step of the ELISA.

Quantitative PCR analysis

Quantitative PCR was performed as described in the Methods section in the Online Repository.

Cell culture and flow cytometry

Cell culturing and flow cytometry were performed as described in the Methods section in the Online Repository.

Assaying allergen-specific T cells

Monocyte derived dendritic cells (DCs) were generated from adhered PBMCs by incubating with 1000 U/mL GM-CSF (Berlex, Richmond, Calif) and 250 U/mL IL-4 (CellGenix, Freiburg, Germany) for 7 days with cytokine refreshment at day 5 as described previously.²⁸ DC phenotype ($\text{CD14}^+ \text{CD1a}^+$) and purity (>95 %) were confirmed routinely by flow cytometry. $\text{CD4}^+ \text{CD45RO}^+$ memory T cells were enriched from PBMCs by as described in the Methods section in the Online Repository. DCs were pulsed with 5 $\mu\text{g/mL}$ NiCl_2 (Sigma, Broendby, Denmark) in serum-free RPMI medium or medium alone for 20 minutes at 37°C and washed 3 times. Memory T cells and autologous DCs were cocultured 10:1 in the presence of 10 $\mu\text{mol/L}$ 5-bromo-2-deoxyuridine (BrdU) for 5 days.

Allergen-specific memory T cells were determined as the proliferating T_H cells identified by intracellular BrdU staining and flow cytometry using a commercial flow kit according to the manufacturer's instructions (BD Biosciences, Broendby, Denmark). The T_H17 ($\text{IL-17}^+ \text{IFN}\gamma^-$) or T_H1 ($\text{IFN}\gamma^+ \text{IL-17}^-$) phenotype of proliferating memory T cells was determined by intracellular cytokine stainings as described in the Methods section in the Online Repository. The allergen-specific response was calculated as the fraction of cytokine producing memory T cells responding to nickel-pulsed DCs compared with unpulsed DCs (background).

Fluorescence immunohistochemistry

Three individuals with nickel allergy and 2 healthy controls were patch-tested with nickel or vehicle for 48 hours followed by punch biopsies from vehicle and nickel-challenged skin at 72 hours. Biopsies were frozen in liquid nitrogen and embedded in optimal cutting temperature medium (Tissue Tech; Sakura Finetechnical, Tokyo, Japan). Frozen tissue sections 4 μm were cut and then fixed in acetone. IL-17 and IL-22 stainings were performed on tissues sections of biopsies fixed in 4% paraformaldehyde and permeabilized by using 0.1% saponin. Primary antibodies were incubated overnight at 4°C. IL-17, IL-22, and IL-22R stainings were amplified by using biotin-conjugated swine antgoat antibodies (SouthernBiotech, Birmingham, Ala). Detection of antibody stained cells was performed by using Alexa Fluor 488/546 conjugated streptavidin or secondary antibodies specific for the primary antibodies used (Molecular Probes, Invitrogen, Taastrup, Denmark). No nonspecific binding of secondary antibodies or streptavidin was observed in the absence of primary antibodies or the use of isotype control antibodies on skin tissue sections. Representative pictures of immunohistochemical stainings were obtained using a Zeiss Axiopt epifluorescence microscope (Carl Zeiss, Göttingen, Germany). Neutrophil infiltration and cells expressing IL-17, IL-22, or IL-22R was quantified per microscope field of view by taking 8 random pictures across the stained tissues sections.

Statistical analysis

Statistical analysis was performed as described in the Methods section in the Online Repository.

RESULTS

Keratinocytes produce IL-23 in response to nickel

Keratinocytes represents the most abundant cell type in the human epidermis and play an important role in mediating cutaneous immune responses.^{29,30} Keratinocytes have been shown to be activated and produce proinflammatory cytokines and chemokines in response to contact allergens.^{31,32} Furthermore, keratinocytes have the capacity to modulate T_H cell responses to contact allergens in ACD via costimulatory molecules.³³ Thus we hypothesized that keratinocytes would produce IL-23 in response to contact allergens, and hence have the ability to promote T_H17 -mediated skin inflammation in ACD. IL-23 and IL-12 are members of the same cytokine family and share the

IL-12p40 subunit in combination with the IL-23p19 and IL-12p35 subunits, respectively.³⁴ To address our hypothesis, we first analyzed the expression of IL-12p40, IL-23p19, and IL-12p35 relative to the GAPDH housekeeping gene in unstimulated immortalized keratinocytes by quantitative PCR. IL-23p19 and IL-12p35 were found to be constitutively expressed, whereas IL-12p40 was barely detectable, making IL-12p40 rate limiting in the production of IL-23 and IL-12 (Fig 1, A). When immortalized keratinocytes were stimulated with nickel, IL-12p40 expression was induced (Fig 1, B). We investigated whether the mRNA messages were translated into IL-23 and IL-12 protein by establishing primary cultures of keratinocytes isolated from skin of healthy donors. The cells were stimulated with increasing concentrations of nickel followed by IL-23 and IL-12 production measurements by ELISA. We found that primary keratinocyte cultures produced IL-23 in response to nickel (Fig 2, A). The production of IL-23 by keratinocytes was found to correlate significantly with the nickel stimulation in a concentration-dependent manner ($P < .05$, Pearson product-moment correlation). On the contrary, no IL-12 production by keratinocytes could be detected in response to nickel using ELISA with a detection-limit of 4 pg/mL (data not shown). These results demonstrate that primary keratinocytes produce IL-23 rather than IL-12 in response to a contact allergen.

IL-23 stimulates proliferation of T_H17 and T_H1 memory T cells

IL-23 has been shown to stimulate IL-17 production and clonal expansion of T_H17 cells in the mouse,^{7,8} whereas in the human system, IL-23 stimulates IL-17 and IFN- γ production in cultures of CD4⁺CD45RO⁺ memory T cells.³⁴ This indicates that IL-23 stimulates both T_H17 and T_H1-related cytokine production by human T cells. To elucidate further the role of IL-23 on activated human memory T cells, we purified CD4⁺CD45RO⁺ memory T cells from the peripheral blood of healthy controls, and stimulated the cells polyclonally in the presence or absence of recombinant IL-23. After stimulation, we determined the number of T cells with T_H17 (IL-17⁺IFN- γ ⁻) or T_H1 (IFN- γ ⁺IL-17⁻) phenotype by flow cytometry. It was found that IL-23 enhanced memory T-cell proliferation by almost doubling the number of T_H17 and T_H1 cells (Fig 2, B).

Altogether, the results suggest that IL-23 derived from keratinocytes in response to nickel have the potential to mediate intradermal expansion of both T_H17 and T_H1 cells in human ACD.

Circulating nickel-specific T_H17 and T_H1 cells in individuals with allergy

If T_H17-mediated inflammation is involved in human ACD, allergen-specific IL-17-producing T_H17 cells would be primed during the sensitization phase of CHS. To address this question, we developed a novel assay to identify and characterize the phenotype of T cells responding to nickel. Monocyte-derived DCs were generated from PBMCs of individuals with and without nickel allergy. DCs were pulsed with or without nickel and cocultured with purified CD4⁺CD45RO⁺ memory T cells of the same donor. BrdU, a bromide-containing nucleic acid derivative, were added to the cultures to allow flow cytometry identification of allergen-specific T cells responding by proliferating (BrdU⁺ cells). Using this assay, a population of CD4⁺CD45RO⁺ IL-

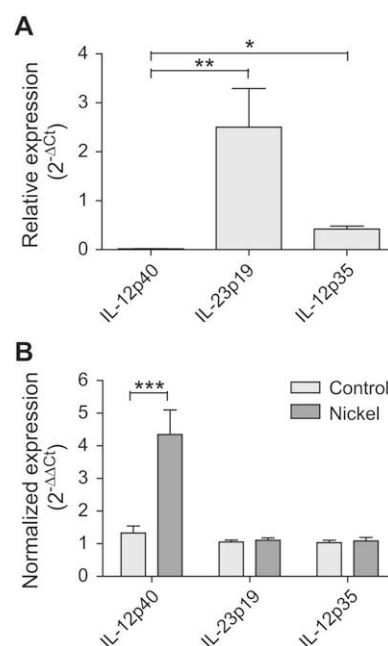


FIG 1. Expression of IL-12p40, IL-23p19, and IL-12p35 mRNA by unstimulated immortalized keratinocytes (A) and nickel-stimulated relative to unstimulated immortalized keratinocytes (B). The data shown are averages of 3 separate experiments. Error bars show SEM, and probability values between data groups are shown using asterisks (* $P < .05$; ** $P < .01$; *** $P < .001$).

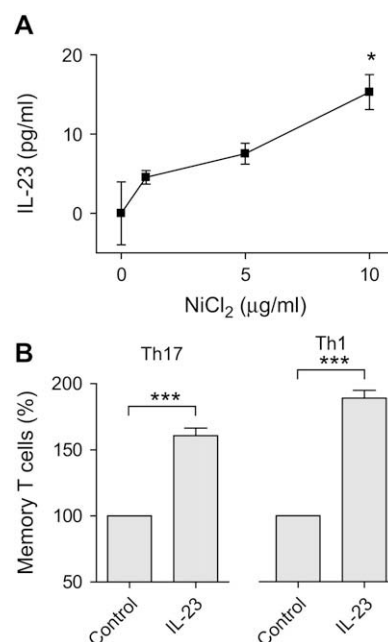


FIG 2. IL-23 protein production by primary keratinocytes after nickel stimulation (A). Expansion of T_H17 and T_H1 memory cells by IL-23 (B). The data shown are representative of 3 (A) or 2 (B) separate experiments using different health donors. Error bars show SEM, and probability values between data groups are shown using asterisks (* $P < .05$; *** $P < .001$).

17-producing (T_H17) cells proliferated in response to nickel-pulsed DCs. The proliferating allergen-specific T_H17 cells were identified only in individuals with allergy and not in controls without allergy (Fig 3, A). Because T_H1 responses to nickel are

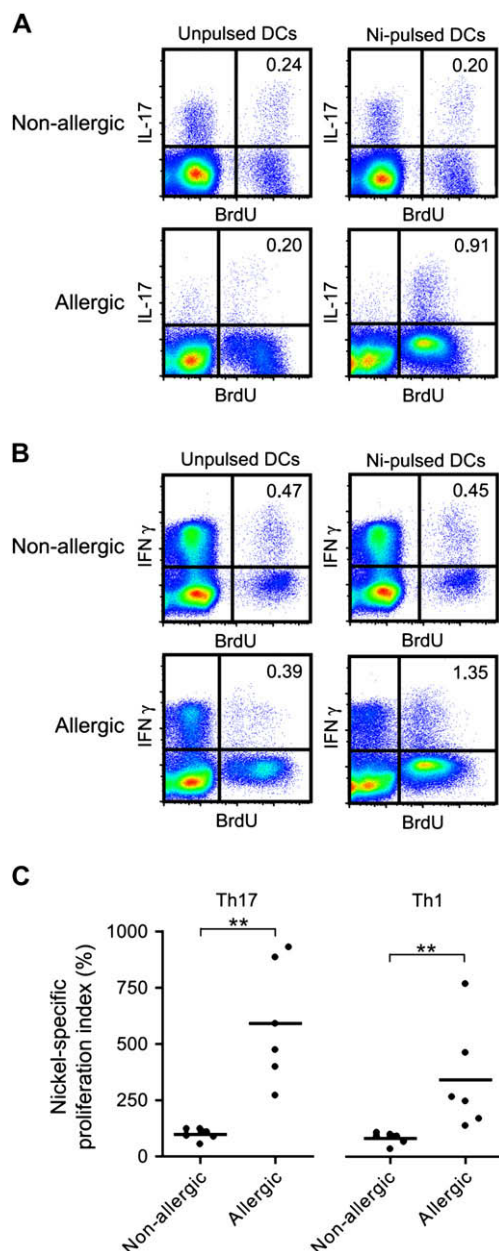


FIG 3. Memory $CD4^+CD45RO^+$ T cells from donors with and without nickel allergy were cocultured with autologous unpulsed or nickel-pulsed DCs and BrdU. The phenotype of responding T cells ($BrdU^+$) was determined by flow cytometry. Representative data from 1 of 6 donors with allergy and 6 donors without allergy (**A and B**). The nickel-specific response by T_H17 or T_H1 cells was summarized (**C**). $^{**}P < .01$.

described in human beings, the presence allergen-specific T_H1 cells were analyzed as a positive control. It was confirmed that a population of nickel-specific IFN- γ -producing (T_H1) cells were identified only in individuals with allergy (Fig 3, B).

Finally, the flow-cytometry data of 6 individuals with allergy and 6 individuals without allergy were summarized by calculating a T_H17 and T_H1 nickel-specific response index as the fraction of proliferating cytokine-producing memory T cells responding to nickel compared with a control condition (nickel-pulsed vs unpulsed DCs; Fig 3, C). These results show that only individuals with allergy have nickel-specific T_H17 and T_H1 cells in the peripheral blood. Thus, the results demonstrate that allergen-

specific T_H17 cells are primed during contact allergen sensitization in human beings and may play a role in eliciting ACD.

T_H17 -mediated skin inflammation in response to nickel

Having demonstrated the development of allergen-specific T_H17 cells in individuals with allergy and ACD, we investigated the *in situ* presence of T_H17 -mediated skin inflammation on challenge with a contact allergen. To this end, 3 individuals with allergy and 2 individuals without allergy were challenged with nickel or vehicle. Punch biopsies were obtained from challenged skin after 72 hours for fluorescence immunohistochemical analysis. Using standard hematoxylin/eosin staining, the nickel-challenged area of individuals with allergy showed heavy leukocyte infiltration and intraepidermal vesicle formation compared with vehicle-challenged skin or skin biopsies from individuals without allergy (Fig 4, A; data not shown).

Work using knockout mice have demonstrated a critical role of CCR6 in mediating CHS responses to contact allergens.³⁵ Interestingly, CCR6 is expressed by all human peripheral blood T_H17 cells and a subset of T_H1 cells.⁹ To address further the role for CCR6 in human ACD, we performed fluorescence immunohistochemical analysis of skin biopsies stained for CD4 and CCR6. It was found that nickel-challenged skin of individuals with allergy contained $CD4^+CCR6^+$ T cells (Fig 4, B), suggesting that T_H17 -mediated inflammation plays a role during ACD elicitation via CCR6.

Activated T_H17 cells produce effector cytokines that mediate different inflammatory processes during the immune response. Among these are the T_H17 lineage-specific IL-17 and IL-22 cytokines. To address further the *in situ* involvement of T_H17 cells, we investigated the expression of these cytokines in ACD-inflamed skin of individuals with allergy by using fluorescence immunohistochemistry. It was found that nickel-challenged skin of individuals with allergy contained cells expressing IL-17 and IL-22 (Fig 4, C-E). These results indicate that T_H17 cells infiltrate the skin in human ACD and may participate in the inflammatory process via the IL-23–IL-17/22 axis.

IL-17 plays a central role in the recruitment and activation of neutrophils and may collaborate with IL-22 in promoting tissue recruitment of inflammatory cells.^{10–12} To investigate whether T_H17 cells mediate inflammation via the IL-17 and IL-22 cytokines in human ACD, we analyzed the infiltration of neutrophils and IL-22R expression in the skin biopsies. Using fluorescence immunohistochemical staining for neutrophil elastase, we found that neutrophils infiltrated nickel-challenged skin of individuals with allergy (Fig 4, F and H). Similarly, IL-22R expression was found only in the nickel-challenged skin of individuals with allergy (Fig 4, G and I), suggesting a role for IL-22 in the inflammatory process of ACD. Neither nickel nor vehicle-challenged skin of individuals without allergy showed inflammation, $CCR6^+CD4^+$ T cells, IL-17, IL-22, IL-22R expression, or neutrophil recruitment (data not shown). These findings support that T_H17 cells and mechanisms of IL-17 and IL-22-mediated inflammation are involved in the elicitation of human ACD.

DISCUSSION

This study is the first to provide evidence for the involvement of allergen-specific T_H17 -mediated inflammation in human allergic contact dermatitis.

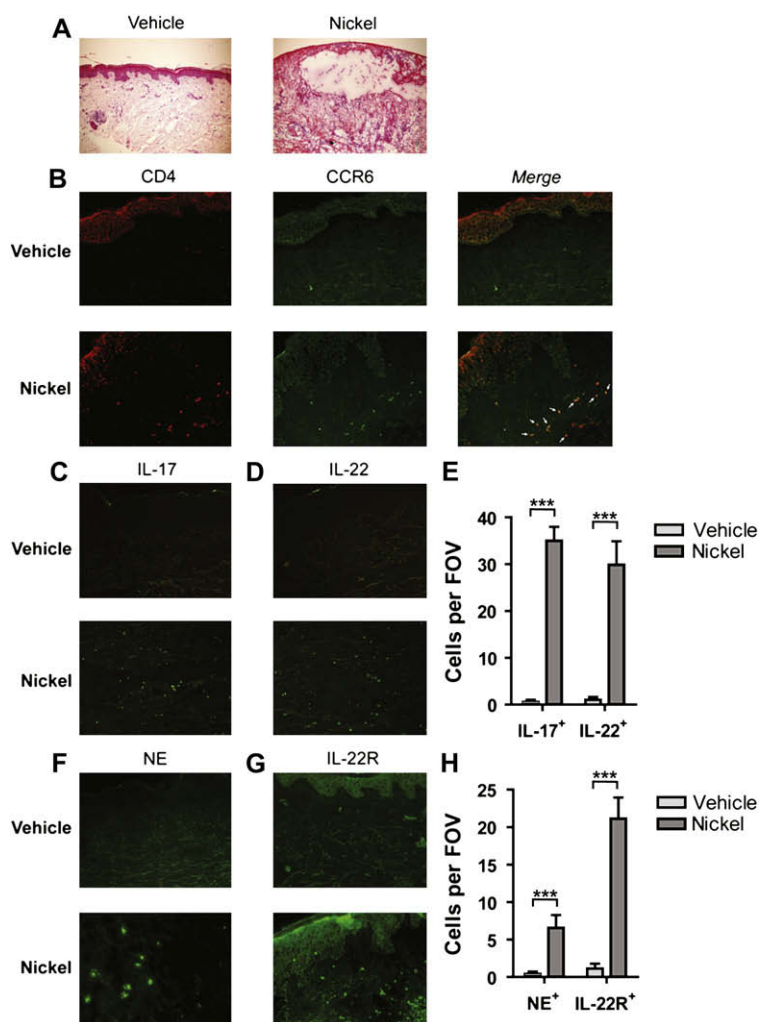


FIG 4. Biopsies from individual with allergy challenged with nickel or vehicle (A). CD4⁺CCR6⁺ cells (yellow) identified by CD4 (red) and CCR6 (green) coexpression (B). Cells expressing IL-17 (C), IL-22 (D), IL-22R (G), or neutrophil elastase (NE) (F). Fluorescent cells from 8 random pictures (E and H). Representative data from 1 of 3 donors with allergy. Skin sections at $\times 44$ or $\times 88$ magnification. FOV, Field of view. Error bars show SEM, and probability values between data groups are shown using asterisks (*** $P < .001$).

We found that keratinocytes induced IL-12p40 mRNA expression in response to nickel. This is in line with an earlier study demonstrating IL-12p40 mRNA expression by keratinocytes in response to various contact allergens but not chemical irritants.³⁶ Our results demonstrate, however, that keratinocytes have the potential to produce IL-23 rather than IL-12 in response to contact allergens *in vivo* because of high constitutive expression of IL-23p19. Indeed, we found that keratinocytes released IL-23, but no detectable IL-12, in response to nickel in a concentration-dependent manner. Furthermore, we demonstrated that IL-23 stimulated the clonal expansion of human T_H17 cells. Combined, these results indicate that keratinocytes have the potential to mediate intradermal T_H17 expansion during the elicitation of ACD. Previous studies have shown that keratinocytes can affect T_H cell responses to contact allergens via differential expression of costimulatory molecules.³³ This indicates close interaction between keratinocytes and T cells in the skin during ACD, where cytokine-mediated signaling also plays a role in the inflammatory process. Indeed, keratinocyte-derived IL-23 has been shown to affect cutaneous immune responses using transgenic mice constitutively

expressing IL-12p40 in keratinocytes. Interestingly, these mice were found to produce IL-23 but not IL-12,³⁷ which is in line with our findings. As a consequence, the transgenic mice showed increased cutaneous immunity by enhanced skin graft rejection, proinflammatory cytokine production, and spontaneous development of eczema characterized by inflammatory infiltrates.³⁸ Thus, these studies provide direct experimental evidence for keratinocyte-derived IL-23 in modulating skin immune responses. Although we found relatively low IL-23 production by keratinocytes, and others have demonstrated approximate 10-fold lower IL-23 production by keratinocyte compared with DCs in response to the same microbial stimuli,³⁹ the abundance of keratinocytes in the skin may contribute significantly to the overall IL-23 expression.

If T_H17-mediated inflammation plays a role in human ACD, allergen-specific T_H17 cells would be expected to be primed during the sensitization of individuals with allergy. Most studies characterizing the development of allergen-specific T_H cells (T_H1/T_H2) in human ACD have analyzed the cytokine profiles of established T-cell lines by using ELISA. However, this approach has

uncertainties with regard to purity, allergen specificity, cellular phenotype, and lack of controls without allergy. Interestingly, IL-17 production has in earlier studies been reported by nickel-specific T-cell lines defined as T_H1 and T_H2 on the basis of IFN- γ and IL-4 production, respectively.^{25,26} However, it has not been established whether T_H17 (IL-17⁺IFN- γ ⁻) cells actually develop in human ACD. We established an assay to characterize the cytokine profile of CD4⁺CD45RO⁺ memory T cells that proliferated in response to allergen-pulsed DCs. Proliferating cells were identified by BrdU incorporation and characterized by using flow cytometry. In agreement with previous studies,⁶ we found allergen-specific T_H1 cells in the blood of sensitized individuals, confirming the assay's usability to characterize allergen-specific T cells. Using our assay, we were able to identify allergen-specific T_H17 cells in the memory T_H cell population of individuals with allergy, demonstrating the differentiation of IL-17-producing T_H17 cells during sensitization to contact allergens in human beings.

A number of recent studies have linked skin-derived IL-23 to dermal inflammation driven by T_H17 cells. It has been shown that IL-23 injection into mice skin activates T_H17 cells, which in turn mediate dermal inflammation and acanthosis.⁴⁰ Interestingly, the inflammation was partly mediated by the T_H17 -related cytokine IL-22, because IL-22 knockout mice showed reduced inflammation. Similarly, a recent study demonstrated IL-22-dependent T_H17 -mediated pathology in a mouse model of spontaneous psoriasis-like skin inflammation.⁴¹ This indicates that skin-derived IL-23 as well as IL-17 and IL-22 production by T_H17 cells play a central role in inflammatory skin diseases. By using immunohistochemistry, we found infiltrating cells expressing IL-17 and IL-22 in the inflamed skin of individuals with allergy challenged with a contact allergen. The result indicates that T_H17 cells play a role in eliciting ACD via the release of effector cytokines in the skin. This is supported by a previous study reporting IL-17 mRNA expression in ACD lesions but not normal skin.²⁶ To elucidate the involvement of T_H17 cell-related cytokines IL-17 and IL-22 in ACD, we analyzed the presence of inflammatory cells linked to these cytokines. Allergen-challenged skin of individuals with allergy contained infiltrating neutrophils and cells expressing IL-22R. This suggests the involvement of cytokines produced by T_H17 cells in the inflammatory response to contact allergens.

In addition to cytokines, chemokine networks play a crucial role in orchestrating inflammatory process. Interestingly, we found CCR6-expressing CD4 T cells in the inflamed skin of individuals with allergy. These cells may primarily be T_H17 because of the expression of CCR6 by all T_H17 cells, but only a subset of T_H1 cells.⁹ Furthermore, the expression of the CCR6 ligand chemokine ligand (CCL)-20 correlates to T_H17 but not T_H1 responses in inflamed tissues of human autoimmune disease.²⁰ Earlier studies have indicated mechanisms by which T_H17 cells may elicit inflammation in human ACD. CCR6 expressed by T cells has been associated with skin and gut migration via CCL20.⁴² Interestingly, CCL20 expression has been found to be rapidly induced, peaking at 3 hours in human skin after application of contact allergen, suggesting early T_H17 cell recruitment, which may initiate ACD.⁴³ IL-17 has been found to induce skin expression of chemokines CXCL9, CXCL10, and CXCL11,⁴⁴ a chemokine profile also associated with human ACD.⁴⁵ These chemokines attract CXCR3-positive T cells, which include both T_H1 and T_H17 memory T cells,⁹ as well as nickel-reactive CD4⁺CLA⁺ T cells from individuals with allergy.⁴⁶ These

studies indicate that early skin reactions in response to contact allergens lead to rapid T_H17 cell recruitment, which further enhances dermal inflammation because of elevated recruitment of allergen-specific T_H17 and T_H1 cells via IL-17 and IL-22-mediated mechanisms. In support of this, a recent study demonstrated T_H17 -mediated T-cell migration across human blood-brain barrier endothelial cells via IL-17 and IL-22,¹² and neutralizing IL-17 in sensitized mice reduced T-cell recruitment in contact allergen-challenged skin.²⁴

In summary, the data reported here demonstrate the involvement of T_H17 -mediated inflammation in human ACD. This finding has implications for the development of new treatments for this allergic skin disease. The focus could be to prevent the development of both T_H17 and T_H1 immune responses to contact allergens. Recent clinical trials using anti-IL-12p40 to inhibit T_H17 and T_H1 responses have reported efficacy in the treatment of psoriasis.⁴⁷ Our study underscores the possibility of using anti-IL-12p40 therapy in human beings with ACD.

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Clinical implications: Novel therapies targeting T_H17 -mediated inflammation may be applicable for the treatment of contact dermatitis.

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METHODS

Materials and reagents

Dendritic cells and T cells were cultured in RPMI 1640 (Sigma, Broendby, Denmark) supplemented with 10% autologous heat-inactivated serum, 2 mmol/L L-glutamine (Sigma), 500 mIU/L penicillin (LEO Pharmaceuticals, Ballerup, Denmark), 500 mg/mL streptomycin (Merck, Whitehouse Station, NJ) and 20 μ mol/L 2-mercaptaethanol (Sigma). Immortalized human keratinocytes (HaCaT cells) were maintained in Dulbecco modified Eagle 1965 medium supplemented with 10% FCS (Invitrogen, Taastrup, Denmark), 2 mmol/L L-glutamine, 500 mIU/L penicillin, and 500 mg/mL streptomycin. Normal human keratinocytes (KCs) were isolated from normal skin and cultured in KC growth medium (KGM; Cambrex, Walkersville, MD) as described previously.^{E1}

All fluorochrome-conjugated and isotype-matched mAbs used for flow cytometry, except anti-IL-17 (eBioscience, San Diego, Calif), were obtained from BD Biosciences (Broendby, Denmark).

The following primary antibodies were used for immunohistochemistry: goat anti-hCD4, mouse anti-hCCR6 (clone 53103.1119), goat anti-hIL-17, goat anti-hIL-22, and goat anti-hIL-22R (all from R&D Systems, Minneapolis, Minn) and mouse anti-h neutrophil elastase (clone NP57; Dako, Glostrup, Denmark).

Quantitative PCR analysis

Total RNA was isolated from immortalized keratinocytes by using an RNeasy kit (Qiagen, Ballerup, Denmark) according to the manufacturer's instructions. RNA extractions were treated with 1 unit of DNase (Fermentas, Burlington, Ontario, Canada) for 30 minutes at 37°C. cDNA were synthesized first-strand from mRNA by poly-dT primers and Moloney Murine Leukemia Virus reverse transcriptase (Fermentas) by using a GeneAmp 9700 thermal cycler from Applied Biosystems (Naerum, Denmark). The amounts of IL-12p40, IL-12p35, IL-23p19, and GAPDH mRNA were determined by using TaqMan qPCR primers and probes (TAGC, Copenhagen, Denmark) as described previously.^{E2} Probes were conjugated with 6-FAM (dye) at the 5' end and black hole quencher 1 at the 3' end. All qPCR reactions included the standard curve for each of the genes analyzed, which was used to adjust sample Ct values. The amounts of IL-12p40, IL-12p35, and IL-23p19 mRNAs were calculated relative to the GAPDH housekeeping gene and normalized to control conditions by using the $2^{-\Delta\Delta C_t}$ method.^{E3} qPCR reactions were performed on an Applied

Biosystems PRISM 7700 thermal cycler and analyzed by using the manufacturer's software.

Cell culture and flow cytometry

CD4⁺CD45RO⁺ memory T cells were enriched from PBMCs by negative selection using a commercial kit for immunomagnetic cell sorting according to the manufacturer's instructions (Miltenyi Biotec, Bisley, Germany), and cell purity was routinely confirmed to be >95% by flow cytometry. Memory T cells were cultured 5:1 with anti-CD3/CD28-coated beads (Invitrogen) in the presence or absence of rhIL-23 for 72 hours (both from eBioscience). T-cell phenotype of T_H17 (IL-17⁺IFN- γ ⁻) and T_H1 (IFN- γ ⁺IL-17⁻) were determined by intracellular cytokine staining after restimulation with 10 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin in the presence of 3 μ mol/L monensin for 5 hours (all from Sigma). Flow cytometry analysis was performed as described.^{E4} Cells were analyzed on a FACSCalibur (Becton Dickinson, Broendby, Denmark) multicolor flow cytometer using Cellquest software.

Statistical analysis

Data were analyzed for statistical significance using the Mann-Whitney sum-rank test or the Student *t* test when normal distribution was ascertained. Trends in nickel concentration-dependent IL-23 production by KCs was tested by using Pearson product-moment correlation. Probability values less than .05 were considered statistically significant. Statistical analysis was performed by using the GraphPad Prism software package (GraphPad Software Inc, San Diego, Calif).

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