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IL-17 Amplifies Human Contact Hypersensitivity by Licensing Hapten Nonspecific Th1 Cells to Kill Autologous Keratinocytes

Davide Pennino,* Kilian Eyerich,* Claudia Scarponi,* Teresa Carbone,* Stefanie Eyerich,† Francesca Nasorri,* Simone Garcovich,‡ Claudia Traidl-Hoffmann,† Cristina Albanesi,* and Andrea Cavani*

Th17 is a newly identified lineage of effector T cells involved in autoimmunity and immune responses to pathogens. We demonstrate in this study the pathogenic role of IL-17–producing CD4$^+$ T lymphocytes in allergic contact dermatitis (ACD) to skin-applied chemicals. IL-17$^+$ T cells infiltrate ACD reactions and predominantly distribute at the site of heavy spongiosis. Skin IL-17$^+$ T cells were functionally and phenotypically heterogeneous: although pure Th17 prevailed in ACD skin, hapten responsiveness was restricted to Th1/IL-17 (IFN-γ$^+$IL-17$^+$) and Th0/IL-17 (IFN-γ$^+$IL-17$^+$IL-4$^+$) fractions, and to lesser extent Th2/IL-17 cells. In the IFN-γ–dominated ACD environment, IL-17–releasing T cells affect immune function of keratinocytes by promoting CXCL8, IL-6, and HBD-2 production. In addition, compared with Th1, supernatants from Th1/IL-17 T cells were much more efficient in inducing ICAM-1 expression on keratinocytes and keratinocyte–T cell adhesiveness in vitro. As a consequence, exposure to combined IFN-γ and IL-17 rendered keratinocytes susceptible to ICAM-1–dependent Ag nonspecific T cell killing. Thus, IL-17 efficiently amplifies the allergic reaction by rendering virtually all of the T lymphocytes recruited at the site of skin inflammation capable to directly contribute to tissue damage. ***The Journal of Immunology*****, 2010, 184: 000–000.**

Allergic contact dermatitis (ACD) is a worldwide prevalent disease that results from an unbalanced immune response to small-molecular, highly reactive chemicals—the haptens (1, 2). These substances contacting the skin induce in susceptible individuals a specific immune response. The ensuing effector phase—clinically apparent as acute eczema—depends on the expansion and rapid migration of chemical-reactive CD8$^+$ T cytotoxic 1 and effector CD4$^+$ Th1 cells, possibly as a consequence of an impaired function of CD25$^+$CD4$^+$ regulatory T cells and IL-10–releasing T regulatory 1 lymphocytes (3–5). It has been demonstrated in murine models of contact hypersensitivity (CHS) that CD8$^+$ and CD4$^+$ T cells have distinct functions, the former being pathogenic and the latter being predominantly regulatory (6). However, the relative contribution of these subsets is much less defined in human beings. Expansion of nickel-specific cytotoxic CD8$^+$ T cells appears to be critical for the development of nickel contact allergy. However, the intensity of the inflammatory reaction is mostly controlled by effector CD4$^+$ T cells, which outnumber CD8$^+$ T lymphocytes in CHS skin and release proinflammatory lymphokines affecting the immune function of resident cells. In particular, IFN-γ and TNF-α promote the release of cytokines and chemokines and the induction of MHC class II and ICAM-1 expression in keratinocytes (7). Thus, T cell–keratinocyte interactions are critical for the full expression of the inflammatory reaction.

Alongside IFN-γ, other cytokines, such as IL-4 and IL-17, are actively released by subsets of skin-infiltrating chemical-reactive T lymphocytes and may modulate ACD responses (8). IL-17A (IL-17) and IL-17F are coreleased by a well-defined subpopulation of effector CD4$^+$ T lymphocytes, named Th17 cells (9–11). Th17 cells have been linked to a variety of autoimmune diseases, including murine experimental encephalomyelitis and collagen-induced arthritis (12–15). In addition, Th17 appears to be involved in protective immune responses to several pathogens, such as *Candida albicans* (16–18). More recently, evidence has been provided by us and others that IL-17 is also involved in the regulation of allergic skin diseases, such as atopic dermatitis, and in the pathogenesis of skin immunomediated skin conditions, such as psoriasis (19–21). In mice, naive T cell maturation toward the Th1 or Th17 phenotype appears mutually exclusive and under the reciprocal control of dendritic cell-derived IL-12 or IL-23. However, evidence exists that IL-17 and IFN-γ are coexpressed by a relevant number of human T lymphocytes isolated from both peripheral blood and from inflamed tissues, such as gut and skin (8, 22–24).

In this study, we investigated the expression of IL-17 in ACD, and we functionally characterized IL-17–releasing T cell subsets involved in the immune reaction. We show that Th1/IL-17 T cells infiltrating ACD amplify the immune responses to haptens by inducing chemokine and cytokine release from keratinocytes and by intensifying the ICAM-1–dependent keratinocyte-T cell interaction, thus promoting nonspecific T cell-induced keratinocyte apoptosis.

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Abbreviations used in this paper: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; HS, human serum; Tcc, T cell clone; TdR, thymidine deoxyribose.

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Materials and Methods

Patients

Peripheral blood or 4-mm skin biopsies, or both, were obtained from seven ACD patients. Keratinocyte primary cultures from suction blisters were obtained from three ACD patients. Diagnosis was confirmed by clinical history and by positive patch tests to $NiSO_4$ ($n = 3$), fragrance mix ($n = 1$), thiuram ($n = 1$), and cobalt ($n = 1$). Blood and skin samples were obtained after informed written consent according to the Declaration of Helsinki with regard to scientific use and upon approval of the ethical committee of the Istituto Dermopatico dell'Immacolata, Rome, Italy.

Culture medium, reagents, and Abs

T cell lines were cultured in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Lonza, Basel, Switzerland) (complete RPMI) plus 5% human serum (HS) (Sigma-Aldrich, St. Louis, MO). T cell clones (Tccs) were cultured in complete RPMI plus 5% HS and 10% FBS (Hyclone, Logan, UT). Keratinocytes were grown in keratinocyte modified medium (Lonza) or in supplemented Ham's F12 and DMEM (Biochrom, Berlin, Germany) medium, as previously described (8).

For cell culture, the following cytokines were used: recombinant human IFN-γ, TNF-α, and IL-17 were from R&D Systems (Minneapolis, MN), and IL-2 was from Novartis Pharmaceuticals (East Hanover, NJ). Mouse PE-, allophycocyanin-, or FITC-conjugated mAb anti-human CD4 (SK3, IgG_1), CD8 (SK1, IgG_1), HLA-DR (L243, IgG_1), HLA A, B, C (G46-2.6G, IgG_1), CD69 (L78, IgG_1), IFN-γ (B27, IgG_1), and TNF-α (Mab11 IgG_1) were from BD Biosciences (San Diego, CA), and mouse anti–IL-22 (142928, IgG_1), IL-4 (3007.11, IgG_1), CD3 (UCHT-1, IgG_1), and CD54 (BBIG, IgG_1) were from R&D Systems. Mouse Alexa Fluor 647- and PE-conjugated anti-human IL17A (eBio64DEC17, IgG_1) (4S.B3, IgG_1) was from Biolegend (San Diego, CA). Mouse IgG isotype controls were purchased from BD Biosciences.

Immunohistochemistry

Noninvolved skin and positive patch tests to 5% $NiSO_4$ applied on the backs of two sensitized patients punch biopsied at 24, 48, 72, and 96 h were paraffin-embedded. Five-micrometer sections, pretreated with hydrogen peroxide in PBS, were incubated in a pH 6 epitope retrieval solution (Dako, Carpinteria, CA) and subsequently incubated with goat anti-human IL-17 (R&D Systems) or rabbit anti-human CD3 (Dako), or both, followed by a biotinylated secondary Ab. Streptavidin peroxidase was added for 10 min to the samples. In single staining immunohistochemistry, IL-17 was detected by using a 3-amino-9-ethyl-carbazole (red; Dako) as a substrate. In double staining, IL-17 was detected by using alkaline phosphates (blue; Vector Laboratories, Burlingame, CA), whereas CD3 was detected by using 3-amino-9-ethyl-carbazole complex substrate solutions. Slides were counterstained with hematoxylin. Immunohistochemistry results were quantified by two independent analyzers. Positive cells were counted in 20 casual photographic fields for each condition considered.

Isolation of skin-infiltrating T cells and T cell cloning

Skin biopsies were minced with a scalpel and placed in culture in complete medium plus 60 U/ml IL-2. After 2–5 d, T cells emigrated from tissue samples were collected for phenotypic and functional characterization and for T cell cloning by limiting dilution (0.6 cells per well) in the presence of irradiated allogeneic feeder cells plus 1% PHA.

Flow cytometry analysis

Skin-derived lymphocytes were stimulated with PMA and ionomycin (Sigma-Aldrich) for 6 h in the presence of monensin and brefeldin (BD Bioscience), permeabilized with BD Cytofix/Cytoperm (BD Biosciences), and incubated with Abs toward surface markers and cytokines. Acquisition and analysis was done using a FACSAria (BD Biosciences).

T cell proliferation and activation assays

Tccs were cocultured with autologous monocytes in the presence or absence of 20 μg/ml $NiSO_4$ (Sigma-Aldrich) for 48 h. A total of 1 μCi/ml [^{3}H] thymidine deoxyribose (TdR) (Amersham Biosciences, Little Chalfont, U.K.) was added to the cultures for the last 8 h. Radioactivity incorporation was measured in a β counter. Results are given as mean cpm ± SD of triplicate cultures.

To assess the proliferation to nickel of T cell lines, skin-derived lymphocytes or peripheral blood $CD4^+CD25^-$ T cells, purified with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), were incubated with CFSE (Molecular Probes, Eugene, OR) prior to culture with autologous monocytes in the presence or absence of nickel. At day 5, the proliferating fraction was measured by flow cytometry. To evaluate the activation induced by nickel in Th17 subsets, PBMCs of nickel-allergic patients were isolated and cultured in RPMI plus 5% HS for 3 d in the absence or presence of 20 μg/ml $NiSO_4$. After immunomagnetic separation, the $CD4^+$ fraction was permeabilized with BD Cytofix/Cytoperm (BD Biosciences) and incubated with Abs toward surface markers and cytokines.

Keratinocyte cultures

Primary keratinocyte cultures were obtained from enzymatic digestions of the roofs of suction blisters by treatment with 0.05% trypsin/0.02% EDTA (Biochrom) for 30 min at 37°C. Keratinocytes were then expanded on mitomycin C-treated mouse fibroblast 3T3 cells. Experiments were performed using second passage keratinocytes cultured in keratinocyte modified medium and pulsed for 3 h in the absence of hydrocortisone with a combination of recombinant human IFN-γ, TNF-α, and IL-17 or with supernatants of Th1/IL-17 or Th1 Tccs activated for 48 h with plate-coated anti-CD3 plus soluble anti-CD28 Abs. Total RNA was extracted after 18 h by using TRIzol reagent (Invitrogen Italia, San Giuliano Milanese, Italy), whereas keratinocyte supernatant was collected at 48 h for cytokine and chemokine content determination.

ELISA

Cell culture supernatants were collected, filtered, and measured for their content of IFN-γ, IL-4, IL-17, IL-22, TNF-α, IL-6, and CCL5 by using ELISA DuoSet kits from R&D Systems; GM-CSF, CXCL10, CXCL8, and CCL2 were measured with BD OptEIA kits, whereas CCL20 release was measured by using the Ab pair MAB-360 and BAF-360 (all from BD Biosciences).

Real-time PCR analysis

Keratinocyte total RNA was reverse-transcribed into cDNA by using oligo (dT) primers and analyzed by real-time RT-PCR. Real-time PCR was performed using the SYBR Green PCR master mix or TaqMan PCR master mix (Applied Biosytems, Branchburg, NJ). The forward and reverse primers used for PCR were as follows: for Bcl-2 5′-ATGGGATCGTTGCCTTATGC-3′ and 5′-TCTACTTCCTCTGTGATGTTGTATTTTTT-3′; for Bcl-xl 5′-GGATACTTTTGTGGAACTCTATGGG-3′ and 5′-CGGTTGAAGCGTTCCTGG-3′; for CCL5 5′-CTACTGCCCTCTGCGCTCC-3′ and 5′-TGGTGTCCGAGGAATATGGG-3′; for β-actin 5′-CATCGAGCACGGCATCGTCA-3′ and 5′-TAGCACAGCCTGGATAGCAAC-3 as an endogenous control. HBD-3 and LL-37 mRNA were analyzed by using specific PCR primers and probes (25) and normalized to 18S rRNA expression (TaqMan Gene Expression Assay HS99999901 from Applied Biosystems). Quantification of mRNA expression was performed using the method described by Schmittigen et al. (26). Real-time PCR was conducted in duplicate for each sample, and the procedure was performed in three independent experiments.

Keratinocyte apoptosis and T cell-mediated cytotoxicity

Keratinocyte cultures were treated for 48 h with recombinant cytokines (200 U IFN-γ; 50 ng/ml TNF-α; 50 ng/ml IL-17) or Tcc supernatants. Apoptosis was evaluated by determining the caspase 3 and 7 content (Caspases 3 & 7 FLICA kit, ImmunoChemistry Technologies, Bloomington, MN) and analyzed by flow cytometry.

T cell-mediated killing of autologous keratinocytes or EBV-transformed B cell lines (B-LCL) were evaluated by measuring DNA fragmentation by the [^{3}H]TdR release assay, as previously described (27). Briefly, target cells were preincubated with 2 μCi/ml [^{3}H]thymidine 12–14 h prior to coculture with effector T cells. Th1- and Th1/IL-17–mediated killing were compared by incubating 10^5 T cells with 10^4 B-LCL cells or 48 h cytokine-conditioned keratinocytes for 6 h in the presence or absence of 20 μg/ml nickel. Blocking experiments were performed by incubating keratinocytes with 10 μg/ml anti-CD54 prior to cocultures with effector T cells.

Keratinocyte–T cell adhesion assays

Subconfluent keratinocytes seeded in culture slides (BD Biosciences) were stimulated with IFN-γ alone or in combination with IL-17 for 48 h before addition of 5×10^5 CFSE-stained nickel-specific autologous T cells clones. Cocultures were performed in the presence or absence of 20 μg/ml nickel. In blocking experiments, keratinocytes were incubated 1 h with 10 μg/ml anti-CD54 prior to cocultures with effector T cells. After 6 h of incubation, cocultures were extensively washed in PBS, fixed in 4%

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paraformaldehyde, and counterstained with hematoxylin. T cells that adhered to keratinocytes were counted in 20 casual fields for each condition, as fluorescent dots using a fluorescent microscope (Zeiss, Oberkochen, Germany), and average T cell number per square millimeter ± SD was calculated

Statistical analysis

Statistical analysis was done using Student *t* test. Statistically significant differences were defined as $p < 0.05$.

Results

CD3⁺/IL-17⁺ in ACD skin correlates with the extent of the inflammatory reaction

Epicutaneous application of 5% $NiSO_4$ in petrolatum onto the backs of nickel-allergic individuals (patch test) induces an ACD reaction. To investigate the expression of IL-17 and the distribution of IL-17$^+$ T cells during ongoing reactions, we performed immunohistochemical studies on skin biopsies of noninvolved skin and 24, 48, 72, and 96 h $NiSO_4$ positive patch tests performed on two allergic donors. Sparse IL-17$^+$ cells were already detected in normal noninflamed skin (average number of positive cells per area = 9 ± 1.4) (Fig. 1*A*). At 24 h, IL-17 did not significantly increase (average number of positive cells per area = 20 ± 10) (Fig. 1*B*, 1*H*) whether a noticeable increment was detected up to 48 (average number of positive cells per area = 40 ± 7) and 72 h (average number of positive cells per area = 46 ± 12), when positive cells could be also observed in the papillary dermis (Fig. 1*C*, 1*D*, 1*H*). Interestingly, at 96 h, we observed numerous intraepidermal IL-17$^+$ cells, mostly distributed at the site of epidermal spongiosis and microvesiculation (average number of T cells per area = 64 ± 27) (Fig. 1*E*, 1*H*). CD3$^+$/IL-17$^+$ cells include almost the totality of IL-17–producing cells and represent ~14% of the total CD3$^+$ cells (average number of CD3$^+$ cells per area = 241 ± 13; number of IL-17$^+$ cells per area = 42 ± 11; number of CD3$^+$/IL-17$^+$ cells per area = 35 ± 12) (Fig. 1*F*, 1*G*). In general, the presence of IL-17$^+$ cells reflected the course of the inflammatory reaction. In the epidermis, IL-17$^+$ cells were particularly located at the site of intense tissue damage (Fig. 1*E* and *inset*).

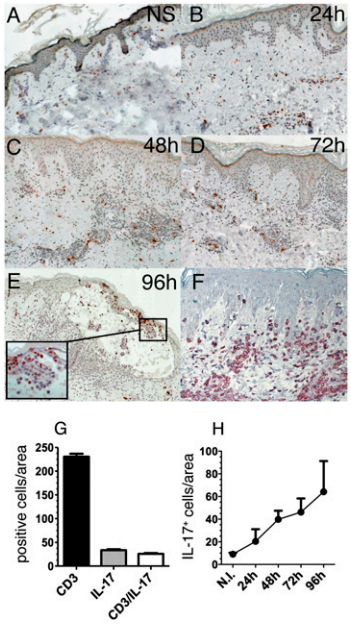

FIGURE 1. CD3$^+$ cells produce IL-17 during ACD reactions. Four-millimeter skin biopsies were fixed in formalin and stained with anti–IL-17 only or double stained with anti–IL-17 and anti-CD3 as described in *Materials and Methods*. Representative IL-17 staining in (*A*) uninvolved skin from allergic donor is compared with ACD reaction to nickel at (*B*) 24 h, (*C*) 48 h, (*D*) 72 h, and (*E* and *inset*) 96 h. Single staining with 3-amino-9-ethylcarbazole, counterstained with hematoxylin. Original magnification ×100; *inset* ×200. Representative double staining of CD3 (3-amino-9-ethylcarbazole, red) and IL-17 (alkaline phosphatase substrate kit III, blue) in a 48 h patch test. Original magnification ×100. (*F*). Quantification of (*G*) CD3$^+$/IL-17$^+$ cells in 48 h patch tests and (*H*) IL-17$^+$ cells during time courses of ACD reactions was performed measuring positive stained cells on randomly acquired photographic fields obtained from two distinct patients.

Skin T lymphocytes infiltrating ACD reactions contain distinct IL-17⁺ T cell subpopulations

T cells lines were prepared from skin biopsies from five allergic donors (one patch test to nickel and four cases of acute spontaneous ACD to nickel, cobalt, thiuram, and fragrances, respectively) and characterized phenotypically and functionally.

As previously described, the microenvironment in ACD to haptens is dominated by IFN-γ. Herein, we confirm this finding ex vivo showing that IFN-γ–releasing T cells are the most frequent infiltrating T cell population (22–42% of the total number of infiltrating T cells, as determined by intracellular staining upon activation with PMA/ionomycin (average ± SD = 35 ± 9%) (Fig. 2*A*, 2*E*). IL-17–releasing T cells ranged from 12 to 21% (average ± SD = 15 ± 3.7%) of total skin-infiltrating lymphocytes (Fig. 2*A*, 2*E*). The great majority of human skin IL-17$^+$ T cells belonged to the CD4$^+$ subset, whereas CD8$^+$IL-17$^+$ cells represented ~1% of skin T cells (Fig. 2*C*, 2*D*). The percentage of IL-4$^+$ T cells was substantially lower compared with that of IFN-γ$^+$ lymphocytes, ranging from 11 to 16% (average ± SD = 13 ± 3%). Among skin IL-17$^+$ T cells, four subsets could be identified on the basis of their cytokine profile: pure Th17, defined as IL-17$^+$IFN-γ$^-$IL-4$^-$ T lymphocytes, which represent >50% (average ± SD = 56 ± 5.5%) of the IL-17$^+$ T cells infiltrating ACD; IFN-γ$^+$IL-17$^+$ T cells (Th1/IL-17), which ranged from 42 to 18% (average ± SD = 25 ± 9.8%) of the total IL-17$^+$ T lymphocytes, and finally two minor subpopulations of IL-4$^+$ IL-17$^+$ T cells (Th2/IL-17, average ± SD = 8.2 ± 1.4%), and IL-17$^+$ IFN-γ$^+$IL-4$^+$ T lymphocytes (Th0/IL-17, average ± SD = 7.8 ± 1.9%) (Fig. 2*B*, 2*F*). Interestingly, IL-17 release was highly correlated with TNF-α production but not with that of IL-22 (Fig. 2*A*, 2*B*). Thus, skin IL-17$^+$ T cells are heterogeneous in terms of cytokine release and may differentially affect ACD expression. Additionally, although IL-17$^+$ CD8$^+$ T cells have been described to play a role in murine CHS, human skin-derived IL-17$^+$ T cells mostly belong to the CD4$^+$ T cell subset (Fig. 2*C*, 2*D*).

Th1/IL-17, Th0/IL-17, and Th2/IL-17 but not Th17 cells are hapten-reactive

Skin-derived T cell lines from two nickel-allergic donors (one isolated from a positive patch test and one isolated from spontaneous nickel skin hypersensitivity) were cloned by limiting dilution, assayed for cytokine release by ELISA, and further confirmed by flow cytometry. A total of 320 CD4$^+$ Tccs were obtained and investigated for cytokine release (Fig. 3*A*); 71 Tccs released IL-17 upon PMA plus ionomycin activation. Among these, 25 Tccs displayed an IL-17$^+$IFN-γ$^-$IL-4$^-$ phenotype, 30 Tccs were IL-17$^+$IFN-γ$^+$IL-4$^-$, 11 Tccs were IL-17$^+$IFN-γ$^+$IL-4$^+$, and, finally, a minor fraction was characterized by a IL-17$^+$IFN-γ$^-$ IL-4$^+$ cytokine asset (Fig. 3*B*). Proliferation to Ag was assayed in the presence of autologous monocytes and 20 μg/ml $NiSO_4$. Seventy-two out of 320 Tccs displayed a proliferation index >5 in the presence of nickel (data not shown and Fig. 3*C*): of these, none belonged to the Th17 subset, 11 Tccs were Th1/IL-17, 4 Tccs were Th0/IL-17, and 2 Tccs displayed a Th2/IL-17 phenotype (Fig. 3*D*).

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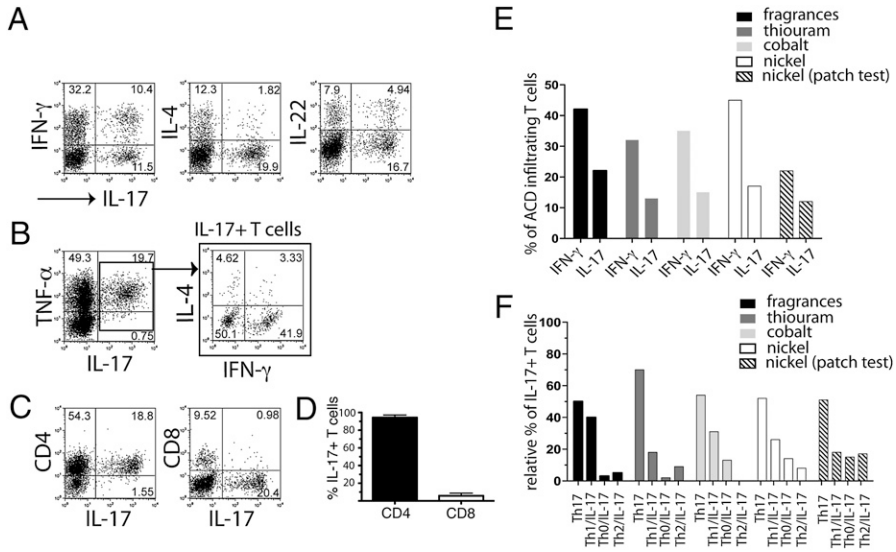

FIGURE 2. Distinct CD4$^+$ IL-17–producing T cell subpopulations are involved in ACD reactions. T Lymphocytes were isolated from acute ACD reactions to fragrances (1), thiuram (1), cobalt (1), and nickel (1) and from 48 h positive patch test to $NiSO_4$ (1). T cell lines isolated from acute ACD reaction to fragrances were characterized for (*A*, *B*) cytokine release by four-color FACS analysis upon PMA/ionomycin stimulation and (*C*) CD4/CD8 expression. The percentages of IL-17$^+$ T lymphocytes among CD4$^+$ and CD8$^+$ T cell subsets (*D*), the percentages of IFN-γ$^+$ and IL-17$^+$ T cells (*E*), and the relative distributions of the distinct IL-17–producing T cell subsets (*F*) as determined in five ACD T cell lines are shown.

The observation that only 22% of the Tccs isolated from skin biopsies were reactive to the causative Ag prompted us to investigate the frequency of specific T cells that infiltrate nickel positive patch tests. CFSE-labeled skin T cell lines were incubated with autologous monocytes and 20 μg/ml nickel, and the dividing cell population was evaluated as CFSE^{low} cells with flow cytometry (Fig. 3*E*). Nickel dividing cells represented a minor fraction of skin-infiltrating T cells (16.4 and 17.2%, respectively). In aggregate, these findings indicate that despite Th17 being the major IL-17–relasing T cell subpopulation in ACD, only Th1/IL-17 and Th0/IL-17 T cells show a productive response to the metal. Secondly, the great majority of the T cells infiltrating the skin during the acute phase of ACD reactions are not responsive to the causative Ag.

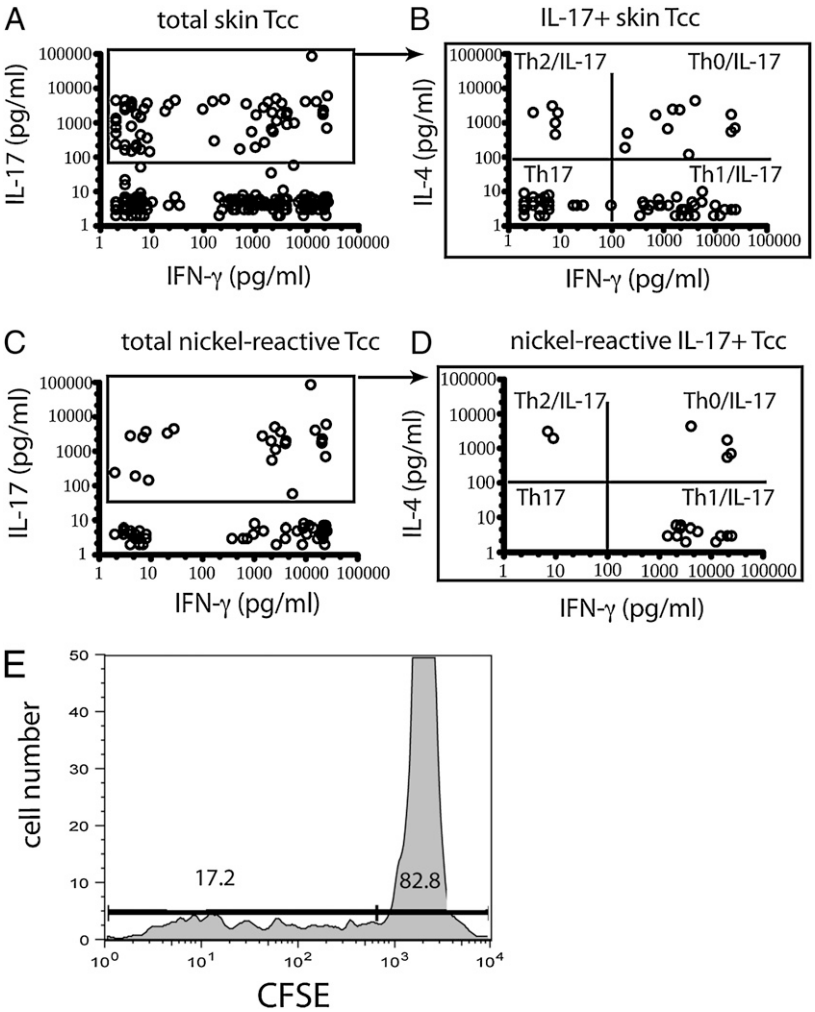

FIGURE 3. In a nickel-induced ACD reaction, only a minority of the infiltrating T cells, comprising Th1/IL-17, Th0/IL-17, and Th2/IL-17, but not Th17 cells, are nickel-specific. Tccs were obtained by limiting dilution from skin T cell lines from two nickel-allergic donors and characterized for cytokine release by ELISA upon PMA/ionomycin stimulation. Each dot represents an individual Tcc. IFN-γ and IL-17 production (*A*) and IFN-γ and IL-4 (*B*) release by IL-17$^+$ skin Tccs pooled from two nickel-allergic individuals. IFN-γ and IL-17 (*C*) and IFN-γ and IL-4 (*D*) release of IL-17$^+$ nickel-specific Tccs. Nickel specificity was measured as cpm in a β counter after overnight incubation with [^{3}H]TdR in the presence and absence of Ag. *E*, Skin T cell lines from skin biopsies of acute ACD reactions of nickel-allergic donors were incubated with CFSE and cocultured 5 d with autologous monocytes in the presence of nickel and examined by flow cytometry. The CFSE^{low} fraction represents the T cell population that undergoes cellular division in the presence of the Ag. Data of one representative T cell line out of two experiments performed is shown.

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IL-17–producing nickel-reactive T cells from peripheral blood of nickel-allergic donors belong to the Th0–Th1/IL-17 subsets

The absence of proliferative in vitro responses to nickel in our skin-derived Th17 Tccs moved us to investigate the cytokine profile of nickel-reactive T cells isolated from peripheral blood of nickel-allergic donors. Purified $CD4^+CD25^-$ T cells were labeled with CFSE and cocultured with adherent monocytes for 5 d in the presence or absence of nickel. T cell lines were restimulated with PMA and ionomycin, and cytokine release in proliferating and nonproliferating T cells was assessed by flow cytometry. Average percentages of IFN-γ– and IL-17–releasing cells were 0.9 ± 0.37% and 0.33 ± 0.11%, respectively (negative control 0.15 ± 0.01% and 0.05 ± 0.03%, respectively) (Fig. 4*A–D*). The dividing, $CFSE^{low}$ fraction of $IL\text{-}17^+$ T cells coreleased IFN-γ or IFN-γ plus IL-4 (Fig. 4*E*), thus belonging to the Th1/IL-17 and Th0/IL-17 subsets. In contrast, the nonproliferating fraction of $IL\text{-}17^+$ T cells consisted mainly of pure Th17 T cells. To confirm the unresponsiveness of pure Th17 to nickel, PBMCs from allergic donors were cocultured 3 d with 20 μg/ml $NiSO_4$, and the purified $CD4^+$ fraction was evaluated for expression of the activation marker CD69 and expression of IL-17, IFN-γ, and IL-4 in a four-color cytofluorimetric analysis (Fig. 4*G–M*). Results showed that ~1.5% of circulating $CD4^+$ lymphocytes express the CD69 activation marker and release IL-17. Interestingly, the great majority of the resulting $IL\text{-}17^+$ gated subpopulation coreleased IFN-γ (Fig. 4*M*), thus confirming that most IL-17–producing nickel-reactive $CD4^+$ T cells belong to the Th1/IL-17 subset. This finding confirms the hypothesis that IL-17 is coreleased with IFN-γ in most of the hapten-reactive T cells isolated both from peripheral blood and inflamed tissues.

Supernatants of Th1/IL-17 clones modulate innate immune function of keratinocytes

The intensity of ACD reactions depends on the cytotoxic potential of infiltrating T cells, which target hapten-loaded keratinocytes, resulting in spongiosis and microvesiculation (27, 28). In addition, keratinocytes activated by T cell-derived mediators release a plethora of cytokines and chemokines, which promote the accumulation of leukocytes and modulate the inflammatory response. To disclose how IL-17–releasing T cells could affect keratinocyte immune function, we compared the effects of supernatant of skin-derived activated Th1/IL-17 cells to those of Th1 lymphocytes on primary human keratinocytes. As a control, cocktails of recombinant cytokines were used. Th1/IL-17 supernatant was 4-fold more efficient than supernatant of Th1 T cells (both releasing comparable amounts of IFN-γ and TNF-α) to induce CXCL8 production in human keratinocytes. Th1/IL-17 supernatant also increased keratinocyte release of IL-6, as compared with Th1 supernatant. Both effects were IL-17–dependent and could be reverted by anti–IL-17–neutralizing Ab. Conversely, natural IL-17 inhibited the IFN-γ–induced CCL5 production by human keratinocytes, confirming previous reports on the effects of recombinant IL-17 on cultured keratinocytes (8). Other chemokines involved in T cell recruitment at the ACD site, such as CXCL10, CCL2, and CCL20, were not affected by IL-17 (Fig. 5*A*). In addition to the modulation of chemokines, Th1/IL-17 strongly induced mRNA of HBD-2 in an IL-17–dependent manner, without affecting HBD-3 or LL-37 (Fig. 5*B*). In line with previous reports indicating a synergistic effect of IL-17 on the IFN-γ–dependent induction of ICAM-1 expression on keratinocytes, we could demonstrate that Th1/IL-17 was much more effective than Th1 supernatant in inducing keratinocyte expression of ICAM-1 but not MHC molecules (Fig. 5*C*).

Finally, to disclose whether IL-17 could directly affect survival or susceptibility to apoptosis of human keratinocytes, we investigated the effects of IL-17 exposure on keratinocytes treated with IFN-γ plus TNF-α, two potent inducers of apoptosis (Fig. 5*D*). A 48 h treatment with IFN-γ plus TNF-α doubled the percentage of caspase 3/7 keratinocytes (Fig. 5*D*) and strongly reduced the expression of the antiapoptotic molecule Bcl-2 but not that of Bcl-xl. Addition of IL-17 did not affect the proapoptotic

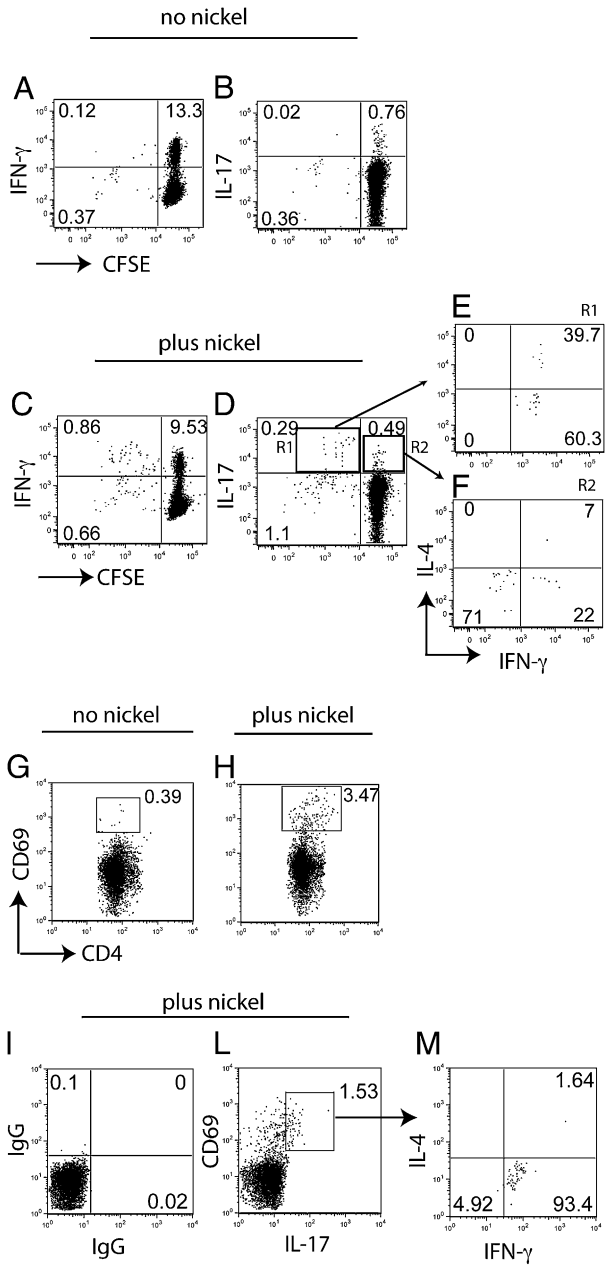

FIGURE 4. Th1/IL-17 and Th0/IL-17 but not pure Th17 cells from peripheral blood of allergic patients are responsive to nickel. Purified $CD4^+CD25^-$ cells from peripheral blood of nickel-allergic patients were stained with CFSE and cocultured with autologous monocytes in the presence or absence of nickel. At day 5, T cells were activated with PMA/ionomycin and evaluated for cytokine release by four-color FACS analysis. IFN-γ intracellular staining in the absence (*A*) and presence (*C*) of nickel and IL-17 staining in the absence (*B*) and presence (*D*) of nickel. IFN-γ and IL-4 release by $CFSE^{low}$ nickel-reactive (*E*) and $CFSE^{high}$ nondividing (*F*) $IL\text{-}17^+$ T cells. In parallel experiments, $CD4^+$ T lymphocytes purified from unstimulated (*G*) or nickel-stimulated (*H–M*) PBMCs showed a subpopulation of $CD69^+IL\text{-}17^+$ cells (*L*). The great majority of gated $CD69^+$ $IL\text{-}17^+$ cells corelease IFN-γ (*M*). The figure shows one representative experiment performed with the peripheral blood of a nickel-allergic donor out of two donors investigated.

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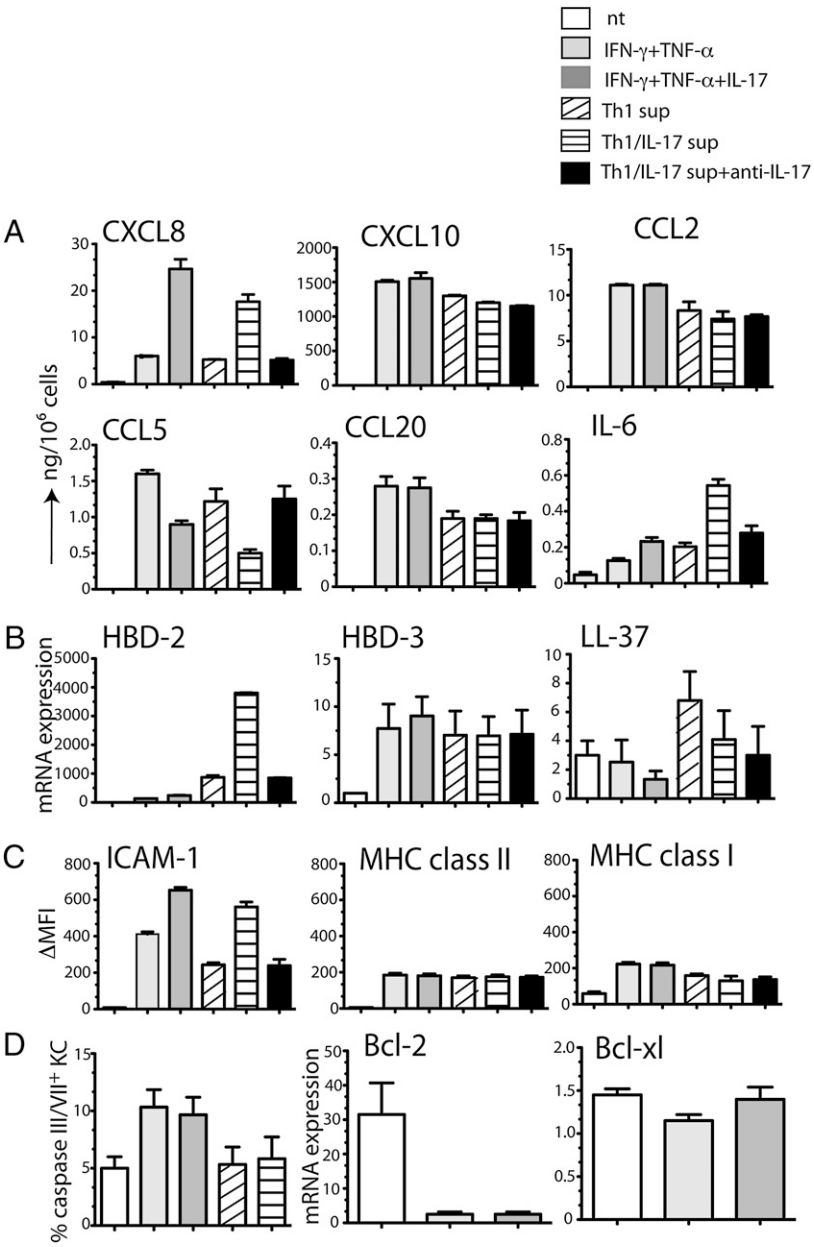

FIGURE 5. Th1/IL-17 T cells modulate the immune function of human keratinocytes by affecting their chemokine, cytokine, and defensin release and by increasing the expression of ICAM-1. Primary cultures of human keratinocytes were treated 48 h with recombinant cytokines or with supernatants of activated Th1 and Th1/IL-17 T cell clones, releasing comparable amounts of IFN-γ and TNF-α. Chemokine and cytokines released by keratinocytes were detected by ELISA in the culture supernatants (*A*), mRNA of HBD-2, HBD-3, and LL-37 was measured by RT-PCR (*B*), and expression of surface molecules was determined by flow cytometry and shown as mean fluorescence intensity (*C*). Keratinocyte spontaneous apoptosis was measured as the percentage of caspase III/VII$^+$ cells by flow cytometry, whereas mRNA expression of Bcl-2 and Bcl-xl were determined by RT-PCR (*D*).

effect of IFN-γ and TNF-α or the mRNA levels of Bcl-2 and Bcl-xl, indicating that IL-17 does not prevent apoptosis in a proinflammatory environment. In contrast to the results obtained with recombinant cytokines, keratinocyte exposure to Th1- or Th1/IL-17–derived supernatants did not significantly affect keratinocyte apoptosis, as measured by the percentage of caspase III/VII$^+$ cells. We may speculate that additional soluble factors released by T lymphocytes could exert a protective role in keratinocyte survival, thus preventing or counteracting the apoptosis induced by IFN-γ and TNF-α.

Overall, these results suggest that IL-17 strongly affects the innate immune function of human keratinocytes, by inducing HBD-2, CXCL8, and IL-6, and has a prominent role in increasing ICAM-1, an adhesion molecule involved in keratinocyte–T cell adhesiveness.

Th1/IL-17 T cells are cytotoxic toward Ag-loaded target cells

The observation of a high number of IL-17$^+$ cells in close proximity to spongiotic areas in ACD skin prompted us to investigate the cytotoxic properties of Th1/IL-17 cells compared with those of Th1 cells. For that purpose, nickel-specific Th1 and Th1/IL-17 T cell clones were incubated for 6 h with nickel-loaded EBV-transformed autologous B cell lines, and cytotoxicity was measured as DNA fragmentation by the [^{3}H]TdR release assay. Although Th1 cells were generally more efficient in killing target cells, all Th1/IL-17 T cells showed a significant cytotoxic activity, thus suggesting that they could directly contribute to the expression of ACD (Fig. 6).

T cell-derived IL-17 augments the ICAM-1–dependent T cell–keratinocyte adhesiveness and induces T cell-mediated keratinocyte killing in an Ag-independent mechanism: the inducer–amplifier model of ACD

To investigate the functional consequences of increased ICAM-1 expression on keratinocytes upon exposure to IL-17, we investigated T cell–keratinocyte adhesiveness in an in vitro cell-contact model. A monolayer of human keratinocytes was treated for 48 h with IFN-γ alone or IFN-γ plus IL-17, labeled with nickel, and cocultured for 5 h with autologous CFSE-labeled nickel-specific T cell clones (Fig. 7*A–C*). After extensive washing,

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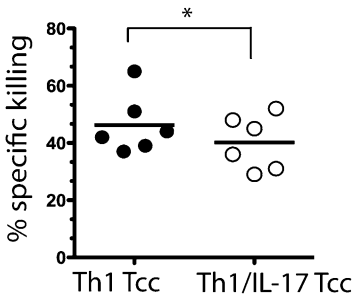

FIGURE 6. Th1/IL-17 T cells are cytotoxic toward Ag-loaded target cells. Nickel-specific Th1 (n = 6) and Th1/IL-17 (n = 6) Tccs were cocultured with autologous [^{3}H]thymidine-pulsed EBV-transformed B cell lines in the presence or absence of nickel for 6 h. Specific cytotoxicity was measured in triplicate as the difference between the [^{3}H]TdR release in the presence of the AG and that in the absence of the Ag. Statistical analysis was determined using unpaired Student t test. $*p > 0.3$

the number of adherent T cells was determined by counting CFSE$^+$ cells with a fluorescence microscope. T cells barely adhered to resting keratinocytes, independently of the presence of the relevant Ag (average numbers of T cells per square millimeter = 36 ± 13 and 16 ± 7 in the presence or absence of nickel, respectively). In contrast, numerous T lymphocytes adhere to IFN-γ–treated keratinocytes (average number of T cells per square millimeter = 176 ± 20), and their number doubled in the presence of the cognate Ag (average number of T cells per square millimeter = 374 ± 14). Interestingly, exposure of keratinocytes to IFN-γ plus IL-17 strongly increased adhesiveness of T cells in the absence of nickel (average number of T cells per square millimeter = 387 ± 47) compared with that of IFN-γ–treated keratinocytes and matched that observed in the presence of nickel (average number of T cells per square millimeter = 379 ± 17). Moreover, T cell–keratinocyte adhesiveness was strongly decreased by blocking anti–ICAM-1 Ab in both IFN-γ (average number of T cells per square millimeter in the presence of nickel = 35 ± 20; in the absence of nickel = 42 ± 19) and IFN-γ/IL-17$^+$ (average number of T cells per square millimeter in the presence of nickel = 33 ± 10; in the absence of nickel = 40 ± 13) treated keratinocytes (Fig. 7*B*, 7*C*). This finding confirms that the increased expression of ICAM-1 induced by IFN-γ/IL-17 cotreatment is relevant for T cell–keratinocyte adhesiveness, in particular in the absence of the relevant Ag.

Because ICAM-1 is a critical mediator of CD4$^+$ T cell killing, we further investigated the susceptibility of autologous keratinocytes activated via IFN-γ or IFN-γ plus IL-17 to Th1-mediated killing. As expected, IFN-γ–prestimulated keratinocytes were only efficiently killed by T cells when optimal concentrations of cognate Ag were added (Fig. 7*D*). Surprisingly, upon IL-17 pretreatment, keratinocytes became susceptible to T cell-mediated killing independently of Ag recognition. The role of ICAM-1 in the T cell-mediated keratinocyte killing was confirmed by the strongly reduced keratinocyte apoptosis in the presence of anti–ICAM-1 blocking Abs. Thus, in the IFN-γ–dominated ACD

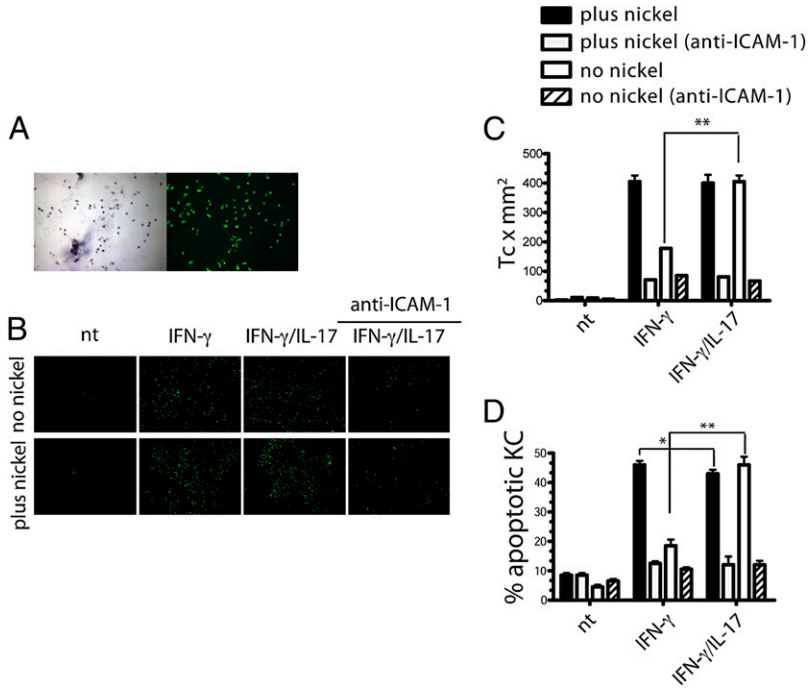

FIGURE 7. IL-17, acting synergistically with IFN-γ, increases keratinocyte–T cell adhesiveness and licenses Th1 lymphocytes to kill autologous keratinocytes in an Ag-independent manner. Nickel-specific Th1 Tccs were stained with CFSE and then cocultured 6 h with untreated, IFN-γ–, or IFN-γ plus IL-17–treated adherent keratinocytes in the presence or absence of nickel. After extensive washing, the number of FITC$^+$ adherent T cells were counted as described in *Materials and Methods*. *A*, Light and fluorescent microscopy of a representative picture of the monolayer of adherent IFN-γ–treated keratinocytes cocultured with CFSE-labeled Tccs. Original magnification ×20. Keratinocytes were counterstained with hematoxylin. *B*, Representative adherence experiment, showing CFSE$^+$ T cells that adhere to untreated, IFN-γ–, or IFN-γ plus IL-17–treated keratinocytes in the presence or absence of nickel. In blocking experiments, keratinocytes were incubated 1 h with anti–ICAM-1 blocking Ab prior to cocultures. *C*, Quantification of adherent T cells per square millimeter ± SD as measured as fluorescent dots in 20 casual fields for each experimental condition. Difference in T cell adherence to IFN-γ– or IFN-γ plus IL-17–treated keratinocytes was analyzed using an unpaired Student t test. $*p < 0.0001$. Representative data of one experiment out of three performed is shown. *D*, IFN-γ– or IL-17/IFN-γ–treated autologous keratinocytes obtained from suction blisters were used as target cells in cytotoxicity assays using nickel-specific Tccs as effector cells. Percentage of apoptotic keratinocytes was measured in triplicate in [^{3}H]TdR release assays. Anti–ICAM-1 Ab was added to selected triplicates at a concentration of 10 μg/ml. The figure shows the result obtained with one representative Tcc out of five Tccs investigated. Statistical analysis was determined using an unpaired Student t test. $*p > 0.05$; $**p < 0.005$.

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environment, the additional release of IL-17 enables non-Ag-specific T lymphocytes, which represent the major part of the infiltrating T cells, to directly attack ICAM-1$^+$ keratinocytes, thus serving as an extremely efficient amplification mechanism of the inflammatory response.

Discussion

In this study, we characterized distinct IL-17–producing T cell populations infiltrating the skin during acute ACD, showing their multiple roles in the amplification of the inflammatory response. We demonstrate that IL-17 secreted by skin-derived T cells modulates innate immunity by keratinocytes, increases T cell–keratinocyte adhesiveness, and thereby promotes ICAM-1–dependent non-Ag-specific keratinocyte killing by T lymphocytes.

Previous reports support a strong impact of IL-17 in murine CHS. IL-17$^{-/-}$ mice show reduced hapten-specific CD4$^+$ T cell responses, a decreased secretion of chemokines and cytokines, and lower expression of ICAM-1 on keratinocytes at the site of hapten challenge (29). Thus, IL-17 may influence multiple steps of the immune response to haptens. In line with these findings, it has been shown that neutralization of IL-17 suppresses the elicitation of murine CHS and that IL-17–producing CD8$^+$ T lymphocytes may be relevant in the efferent phase of murine CHS (30, 31).

In humans, nickel-specific IL-17$^+$CD4$^+$ T lymphocytes isolated from sensitized donors have already been described by our and other groups (8, 32); however, their role in ACD expression remained obscure. Interestingly, a low number of IL-17$^+$ cells is already detectable in normal uninvolved skin, and their number gradually increases at 48–96 h, thus paralleling clinical symptoms of the allergic reaction. Interestingly, in fully expressed eczematous reactions, IL-17$^+$ lymphocytes are greatly enriched at the site of heavy spongiosis and vesiculation. Reduced susceptibility of IL-17–producing T cells to activation-induced cell death, as recently observed, may explain this finding (33).

The major sources of IL-17 in ACD skin are infiltrating CD4$^+$ T lymphocytes, whereas IL-17$^+$CD8$^+$ T cells represent $<1\%$ of the total number of T cells obtained from ACD lesions. Whether this limited number of IL-17$^+$CD8$^+$ T cells can affect the magnitude of ACD remains to be determined. Interestingly, skin-infiltrating CD4$^+$ T lymphocytes that release IL-17 upon stimulation are highly heterogeneous. Although in the mouse system the differentiation of Th17 and Th1 appears to be mutually exclusive, a striking T cell plasticity is observed in the human system. Besides Th17, T lymphocytes coreleasing IFN-γ and IL-17 (Th1/IL-17) have been described in many immune-mediated human diseases, including Crohn's disease, CHS, and in healthy subjects (8, 22–24). Additionally, we have recently demonstrated that atopic eczema is enriched in IL-4$^+$IL-17$^+$ (Th2/IL-17) T cells (19). Overall, these findings clearly demonstrate that besides the well-defined Th17 population, IL-17 can be coreleased by a variety of Th1- as well as Th2-polarized T lymphocytes.

Interestingly, although abundantly present in ACD skin, Th17 Tccs are not responsive to nickel in vitro. This finding confirms and extends our previous observation in *Dermatophagoides*-induced atopy patch tests, where we demonstrated *Dermatophagoides* specificity in Th2/IL-17 and Th1/IL-17 but not in Th17 Tccs. Accordingly, all nickel-reactive IL-17$^+$ T cells obtained from skin and peripheral blood of nickel-allergic individuals corelease IFN-γ or IL-4, or both. Experiments are needed to disclose whether unresponsiveness of Th17 is the consequence of an anergic state or a consequence of the plasticity of IL-17$^+$ T cells, which may convert to a Th1/IL-17 phenotype upon terminal differentiation.

In line with the suspected primary function of Th17 cells, T cell-derived IL-17 modulates innate immune responses in the skin. IL-17 directly induces the release of CXCL8, IL-6, and HBD-2 by keratinocytes, while inhibiting CCL5. In contrast to previous reports, we were not able to confirm the effects of IL-17 on CCL20 release by keratinocytes (34). Thus, IL-17 contributes to the recruitment of neutrophils and establishes a protective immune response against extracellular pathogens.

Beyond modulation of innate immunity, this study reveals a second central function of IL-17 in inflammatory skin reactions. Induction of keratinocyte apoptosis by T cells is a key element in the pathogenesis of eczematous disorders. Previous studies demonstrated two pathways eliciting apoptosis in keratinocytes: one being Ag-dependent and mediated via both Fas/Fas ligand and perforin/granzyme B and a second pathway that is Ag-independent and involves IFN-γ and Fas/Fas ligand (27, 35, 36). Our study clearly demonstrates that IL-17 links these two processes in a kind of inducer–amplifier-model. IL-17 is coreleased with IFN-γ by Ag-specific Th1/IL-17 cells and synergistically enhances the nonspecific pathway by increasing ICAM-1 expression on keratinocytes, which in turn strongly enhances T cell–keratinocyte adhesiveness and consequently renders keratinocytes susceptible to non-Ag-specific T cell attack. Given the fact that Ag-specific T cells represent the minor fraction of skin-infiltrating T cells, nonspecific T cell killing represents an extremely efficient amplification mechanism of the initially Ag-specific allergic reaction, rendering virtually all T lymphocytes recruited at the site of skin inflammation capable to directly contribute to tissue damage.

In conclusion, our data demonstrate that IL-17 is a central proinflammatory mediator in the skin microenvironment through modulating keratinocyte immune responses and amplifying a nonspecific cytotoxic cascade that results in a severe and sustained cutaneous inflammatory reaction.

Disclosures

The authors have no financial conflicts of interest.

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