

IL-17 in atopic eczema: Linking allergen-specific adaptive and microbial-triggered innate immune response

Kilian Eyerich, MD,^{a*} Davide Pennino, PhD,^{a*} Claudia Scarponi, PhD,^a Stefanie Foerster, PhD,^b Francesca Nasorri, PhD,^a Heidrun Behrendt, MD,^b Johannes Ring, MD, PhD,^c Claudia Traidl-Hoffmann, MD,^b Cristina Albanesi, PhD,^a and Andrea Cavani, MD, PhD^a *Rome, Italy, and Munich, Germany*

Background: Patients with atopic eczema (AE) regularly experience colonization with *Staphylococcus aureus* that is directly correlated with the severity of eczema. Recent studies show that an impaired IL-17 immune response results in diseases associated with chronic skin infections.

Objective: We sought to elucidate the effect of IL-17 on antimicrobial immune responses in AE skin.

Methods: T cells infiltrating atopy patch test (APT) reactions were characterized for IL-17 secretion to varying stimuli.

IL-17-dependent induction of the antimicrobial peptide human β -defensin 2 (HBD-2) in keratinocytes was investigated.

Results: Approximately 10% of APT-infiltrating T cells secreted IL-17 after phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation. Among these, 33% belonged to the newly characterized subtype T_H2/IL-17. Despite the capacity to secrete IL-17, specific T-cell clones released only low amounts of IL-17 on cognate allergen stimulation, whereas IL-4, IFN- γ , or both were efficiently induced. IL-17 secretion was not enhanced by IL-23, IL-1 β , or IL-6 but was enhanced by the *S aureus*-derived superantigen staphylococcal enterotoxin B. Both healthy and AE keratinocytes upregulated HBD-2 in response to IL-17, but coexpressed IL-4/IL-13 partially inhibited this effect. *In vivo*, additional application of staphylococcal enterotoxin B induced IL-17 in APT reactions, whereas IL-4, IFN- γ , and IL-10 were marginally regulated. Induced IL-17 upregulated HBD-2 in human keratinocytes *in vivo*.

Conclusion: IL-17-capable T cells, in particular T_H2/IL-17 cells, infiltrate acute AE reactions. Although IL-17 secretion by specific T cells is tightly regulated, it can be triggered by bacteria-derived superantigens. The ineffective IL-17-dependent upregulation of HBD-2 in patients with AE is due to a partial inhibition by the type 2 microenvironment, which could partially explain why patients with AE do not clear *S aureus*.

Key words: Atopic eczema/dermatitis, T_H17, IL-17, superantigen, defensin

Atopic eczema (AE) is a chronic relapsing-remitting inflammatory skin disorder beginning mostly in early childhood as a first step of the atopic march.¹ It is highly pruritic and severely affects the quality of life of the individual and his or her environment.² The incidence of AE is continuously increasing,^{3,4} which implies a high socioeconomic effect.⁵ The underlying pathogenesis of AE is a complex interaction of genetic predisposition and environmental factors. Recently, a strong association with loss-of-function mutations in the filaggrin gene was reported,^{6,7} which results in epidermal barrier dysfunction. Moreover, AE is often associated with type I (T_H2-dominated) immune hyperreactivities mediated by allergen-specific IgE to common environmental or food allergens.² The atopy patch test (APT) has been widely accepted as a model for allergen-specific induction of acute AE by type I allergy-inducing proteins, such as pollen- or house dust mite-derived allergens.⁸ AE and APT reactions share histologic similarities with delayed-type hypersensitivity responses, with the exception that in acute AE and APT lesions T_H2 cytokines, such as IL-4, IL-5, and IL-13, are abundantly present.^{9,10} Beyond deregulations of the adaptive branch of the immune system, innate immune responses are critical for the outcome of AE. More than 85% of patients with AE are affected by skin colonization with facultative pathogenic microbials, such as *Staphylococcus aureus*,¹¹ without imminent clinical signs of infection. However, colonization of eczematous lesions with *S aureus* is strongly associated with increased disease severity.¹² A suspected underlying mechanism is stimulation of skin-infiltrating T cells by exotoxins that are frequently produced by *S aureus* species colonizing AE skin.¹² These exotoxins stimulate T cells bearing particular T-cell receptor (TCR) V β chains regardless of their specificity and are therefore called superantigens.¹³ Recently, the superantigen staphylococcal enterotoxin B (SEB) was shown to enhance house dust mite-induced patch test reactions in patients with AE.¹⁴ Although interactions between adaptive and innate immunity in AE in terms of a facilitated T_H2 response by superantigens has been suggested,¹⁵ the underlying mechanisms are not well understood.

The recently described T-cell subtype T_H17 is characterized by the production of IL-17 and IL-22 and the expression of CCR6.^{16,17} In addition to a putative role in autoimmune diseases,¹⁸ 2 recent studies show that T_H17 cells are essential for the first-line defense of the human organism. An impaired or absent IL-17 immune response underlies the orphan syndromes autosomal-dominant hyper-IgE syndrome¹⁹ and chronic mucocutaneous candidiasis,²⁰ both of which are associated with recurrent and persistent infection of skin and mucosal membranes. A possible mechanism for host defense against microorganisms by IL-17 and IL-22 in the skin is the upregulation of antimicrobial peptides, the so-called defensins, in

From ^athe Laboratory of Immunology, IDI-IRCCS, Rome; ^bZAUM-Center for Allergy and Environment, Division of Environmental Dermatology and Allergy Helmholtz Zentrum/TUM, Munich; and ^cthe Department of Dermatology and Allergy, Technische Universität München, Munich.

*These authors contributed equally to this work.

K. Eyerich was supported by a grant of the "Bayerische Forschungsförderung" (BFS). This work was supported by a grant of the European Community (UE-LSHB-CT-2005-018681) and by the Italian minister of health.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Abbreviations used

AE: Atopic eczema
APC: Antigen-presenting cell
APT: Atopy patch test
DC: Dendritic cell
HBD-2: Human β -defensin 2
FITC: Fluorescein isothiocyanate
PE: Phycoerythrin
PMA: Phorbol 12-myristate 13-acetate
SEB: Staphylococcal enterotoxin B
TCR: T-cell receptor

human keratinocytes.¹⁷ Defensins are critical for killing *S aureus*,²¹ and they induce migration of CCR6⁺ cells into the skin.²² In patients with AE, however, evidence exists that defensin levels are reduced compared with those seen in other immune-mediated skin diseases, such as psoriasis, and this might be responsible for the high *S aureus* skin colonization.²³

Therefore the aim of this study was to elucidate the effect of IL-17 on antimicrobial immune responses in AE skin.

METHODS**Patients**

Patients with AE according to the criteria of Hanifin and Rajka ($n = 3$) and a positive skin prick test response to *Dermatophagoides pteronyssinus*, RAST class of 3 or greater to Der p 1, and positive APT reaction to *D pteronyssinus* were included in the study. Before blood or skin samples were obtained, each participant provided informed consent. The study was approved by the ethics committee of the Istituto Dermatologico Dell'Immacolata.

Cytokines and antibodies

The following antibodies were used in flow cytometric analysis: CD4-fluorescein isothiocyanate (FITC; SK3), CD14-FITC (M ϕ P9), CD4-phycoerythrin (PE; clone SK3), CD8-PE (clone SK1), CD56-FITC (NCAM16.2; all BD Biosciences Mississauga, Ontario, Canada), CD83-FITC (HB15e), CD86-FITC (2331 FUN1), CD1a-FITC (HI149), CD8-FITC (RPA-T8), CCR6-PE (clone 11A9), IL-10-PE (JES3-19F1), IL-4-FITC (MP4-25D2), IFN- γ -FITC (B27), IFN- γ -allophycocyanin (B27), TNF- α -FITC (Mab11; all BD Pharmingen, San Jose, Calif), CCR4-PE (Clone 205410), CXCR3-PE (clone 49801.111), IL-22-PE (142928), IL-4-PE (3007.11; all R&D Systems, Minneapolis, Minn), and IL-17A-PE (clone SK3; eBioscience, San Diego, Calif).

For cell culture and stimulation, the following cytokines were used: purified natural Der p 1, recombinant Der p 1 (both Indoor Biotechnologies, Charlottesville, Va), anti-CD3, anti-CD28 (both BD Bioscience), IL-2 (Novartis), IL-1 β , IL-4, IL-6, IL-13, IL-17, IL-23 (all R&D Systems), GM-CSF (Schering-Plough, Deerfield, Ill), IFN- γ (BD Pharmingen), LPS, SEB (both Sigma, St Louis, Mo), anti-HLA-DP, anti-HLA-DQ, anti-HLA-DR, anti-IL-4 receptor, and anti-IL-17 (AF-317; all R&D systems).

Isolation and expansion of T cells, antigen-presenting cells, and keratinocytes

T-cell clones derived from 48-hour positive APT reactions to *D pteronyssinus* (Stallergenes, Antony, France) were isolated as previously described.²⁴ Briefly, biopsy specimens were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, 0.05 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 mg/mL streptomycin (all Invitrogen, Carlsbad, Calif; RPMI complete) and 5% human serum (Sigma) supplemented with 60 U of IL-2/mL. Migrated cells were collected after 2 days and cloned after 6 days by means of limiting dilution (0.6 cells/well in 96-well U-bottom microplates) in RPMI complete 5%

human serum and 10% heat-inactivated FBS on a feeder layer of irradiated PBMCs, 30 U/mL IL-2, and 1% PHA (Sigma). Fresh medium containing IL-2 was added 3 times a week, and clones were restimulated with irradiated feeder PBMCs all 3 weeks. Der p 1 reactivity of both T-cell lines and clones was determined by using the tritiated thymidine assay.

Dendritic cells (DCs) were generated from CD14⁺ monocytes, as described previously.²⁵ Briefly, CD14⁺ monocytes were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 0.5 mmol/L 2-mercaptoethanol, 20 μ g/mL gentamicin (all from Invitrogen), 10% FBS, 50 U/mL human rGM-CSF, and 200 U/mL human rIL-4 (complete DC medium), with a complete change of medium after 3 days. At day 5, a part of the cells was stimulated with 50 μ g/mL LPS (Invitrogen) for 24 hours. Immediately before coculture experiments, cells were harvested and characterized for maturation markers (CD83, CD86, and HLA-DR) and CD1a by using flow cytometry.

Autologous keratinocytes were isolated by using the suction blister method, as described previously.²⁶ Briefly, blisters were induced by generating a vacuum on normal skin of the forearms. Epidermal sheets were obtained from blister roofs, treated with 0.05% trypsin (Invitrogen) to obtain single-cell suspensions, and seeded on a feeder layer of irradiated 3T3/J2 fibroblasts in modified Green's medium. At 70% to 80% confluence, keratinocytes were detached with 0.05% trypsin, placed in aliquots, and cryopreserved in liquid nitrogen. Keratinocytes of second and third passages were used in experiments.

Coculture experiments

T cells (10^5) and between 100 and 10,000 immature or mature DCs were cocultured in flat-bottom 96-well plates (Falcon, BD Biosciences) in RPMI complete 5% human serum for 36 hours with 5 μ g/mL Der p 1, 10 μ g/mL phorbol 12-myristate 13-acetate (PMA)/1 μ g/mL ionomycin, 5 μ g/mL SEB, or full medium as a negative control, respectively. The cell-free supernatant was obtained, and T-cell proliferation was read by using the tritiated thymidine method.

Substantial expression of IL-17 was defined as a cytokine expression of at least 10% of the total releasing capacity measured by means of ELISA after 48 hours of PMA/ionomycin stimulation and a biologic effect of IL-17 in the supernatant. Biologic effect was defined by inducing detectable amounts of human β -defensin 2 (HBD-2) in human keratinocytes measured by means of ELISA.

Flow cytometric analysis

Intracellular cytokine staining was performed with a kit (BD Biosciences), according to the manufacturer's instructions. Briefly, cells were stimulated with PMA and ionomycin for 6 hours in the presence of monensin. After 2 hours, brefeldin A was added. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and incubated with antibodies. Acquisition and analysis was done with a FACSCalibur (BD Biosciences).

ELISA

Concentrations of IFN- γ , IL-4, IL-10, IL-13, IL-17, IL-22, TNF- α (all R&D systems), and HBD-2 (Phoenix Pharmaceuticals, Inc, Burlingame, Calif) in cell-free culture supernatants were measured by using commercially available sandwich ELISA kits from indicated companies.

RNA isolation and real-time PCR

Total cellular RNA was extracted with the TRIzol (Invitrogen) method, according to the manufacturer's instructions. RNA was reverse transcribed with oligo(dT)primers. PCR reactions were performed with the following synthetic oligonucleotides for HBD-2: 5'-TCCTCTCGTTCCTTCATATT-3' and 5'-TTAAGGCAGGTAACAGGATCGC-3'.

In vivo experiments

Commercially available *D pteronyssinus* (Stallergenes) was applied at the 2 forearms of a patient with AE. Thirty-six hours after allergen application, 50 μ g/cm² SEB was added on 1 forearm. Sixty hours after allergen application, epidermal sheets and blister fluid of induced eczematous reactions were

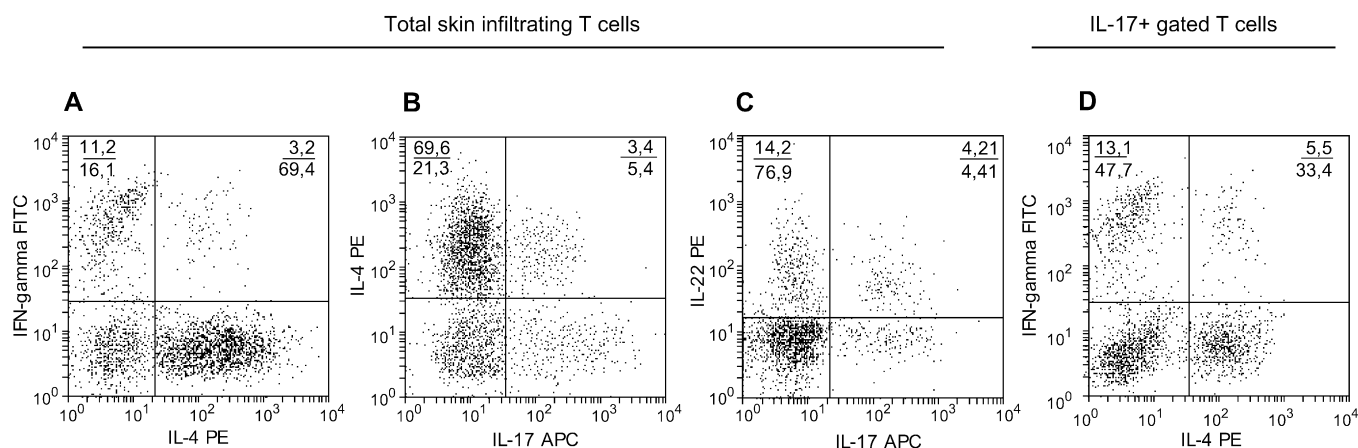


FIG 1. Distinct populations of IL-17-producing T cells in the skin infiltrate of a positive APT reaction. **A-C**, Intracellular cytokine staining of skin-derived lymphocytes with IL-4/IFN- γ (Fig 1, A), IL-4/IL-17 (Fig 1, B), and IL-17/IL-22 (Fig 1, C). **D**, Gating on IL-17⁺ cells after triple staining with IL-4, IFN- γ , and IL-17. Percentage of cells is indicated for each quadrant. Shown is 1 representative experiment. APC, Allophycocyanin.

TABLE I. Functional characterization of APT-derived IL-17⁺ T-cell clones

Clone	Proliferation		Cytokine profile (after PMA/ionomycin stimulation)							Subtype
	SI to Der p 1	SI to SEB	IFN- γ	IL-4	IL-10	IL-13	IL-17	IL-22	TNF- α	
3	75	45	496	4,285	13,484	13,738	12,494	2,931	7,779	T _H 2/IL-17
27	1	20	0	3	1,578	947	13,788	2,595	1,596	T _H 17
60	25	30	5,385	2,922	10,623	13,074	11,000	126	5,348	T _H 0/IL-17
91	20	15	0	3,252	16,260	18,317	3,519	7,000	4,620	T _H 2/IL-17
96	40	45	9,946	3,477	16,159	14,302	2,394	3,568	3,840	T _H 0/IL-17
141	40	1	0	4,710	1,149	20,472	5,500	219	861	T _H 2/IL-17

Cytokine levels are measured by means of ELISA after PMA/ionomycin stimulation.
SI, Stimulation index to Der p 1 and SEB.

TABLE II. Surface markers of APT-derived IL-17⁺ T-cell clones: Flow cytometric analysis of resting T-cell clones

Clone	TCR V β chain	Surface markers (in resting state)				
		CD4	CD8	CCR4	CCR6	CXCR3
3	14	94%	0%	73%	85%	14%
27	12	98%	0%	95%	99%	1%
60	3	96%	2%	85%	90%	67%
91	14	98%	0%	89%	50%	3%
96	ND	90%	0%	1%	54%	45%
141	ND	99%	0%	45%	55%	1%

Shown is the percentage of positive cells compared with the isotype control.

ND, Clone expresses a TCR V β chain that is not detectable by the Iotest Beta Mark Repertoire Kit (Beckman Coulter, Fullerton, Calif).

obtained by using the suction blister method. RNA was prepared from epidermal roofs, as described above; suction blister fluid was analyzed by using the Luminex method (Fluorokine MAP multiplex human cytokine panel A, R&D systems) and by using HBD-2 ELISA, as described above.

Statistical analysis

Statistical analysis was done with the Student *t* test.

RESULTS

IL-17-producing T lymphocytes infiltrate the skin during APT reactions: Newly characterized T_H2/IL-17 subset

Three patients with AE with a documented hypersensitivity to *D pteronyssinus* were challenged with APTs to *D pteronyssinus*.

Biopsy specimens were taken from the resulting eczematous reactions, and infiltrating T cells were isolated and characterized by means of intracellular cytokine staining with flow cytometric techniques. In line with the hypothesis of a type 2 domination in early AE, the majority of skin-derived T cells activated *in vitro* by PMA plus ionomycin expressed high levels of IL-4 (Fig 1, A). Moreover, about 9% (9% \pm 3%) of all infiltrated T cells were capable of producing IL-17 (Fig 1, B). IL-17 and IL-22 were not necessarily coexpressed (Fig 1, C). Interestingly, about one third of IL-17-releasing T cells coexpressed IL-4 (T_H2/IL-17 T cells) or IL-4 plus IFN- γ (T_H0/IL-17 T cells). Fifty percent of IL-17-producing T cells were pure T_H17 T cells, and a minor proportion coproduced IL-17 and IFN- γ (T_H1/IL-17; Fig 1, D).

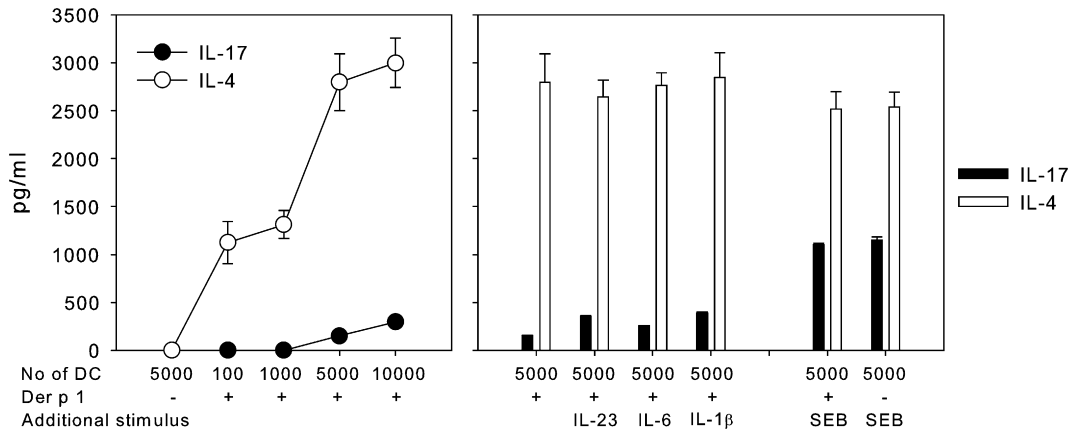
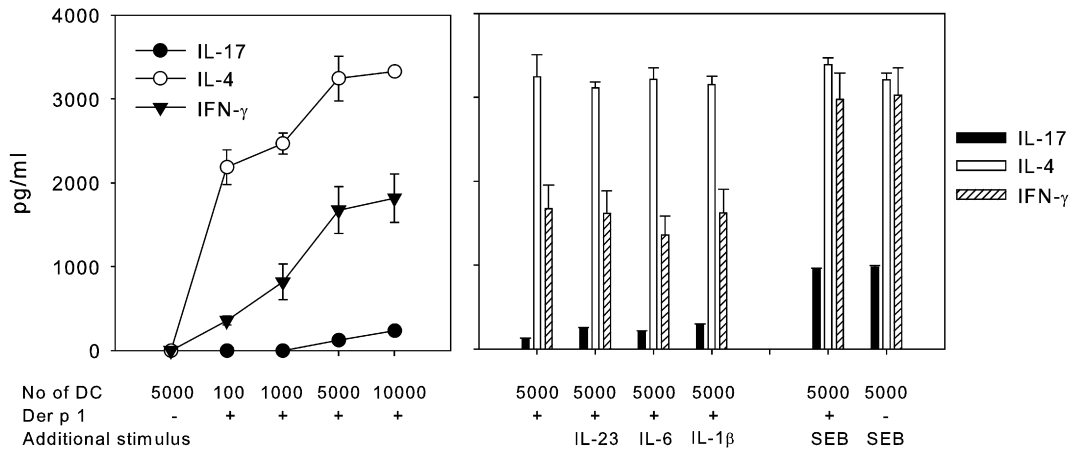
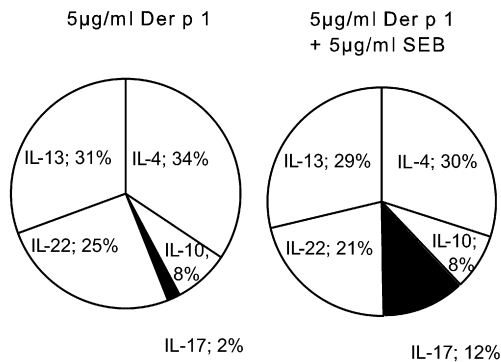
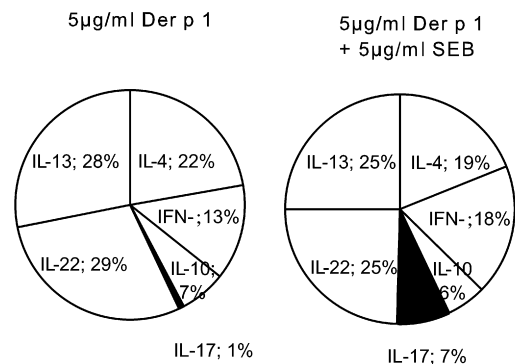
A Clone 3 (Th2/IL-17)**B Clone 96 (Th0/IL-17)****C Clone 3 (Th2/IL-17)****D Clone 96 (Th0/IL-17)**

FIG 2. IL-17⁺ Der p 1-specific T-cell clones secrete IL-17 on stimulation with SEB. **A and B,** Dose-dependent secretion of IL-17/IL-4/IFN-γ (left panel) and addition of cytokines and SEB (right panel). Type of APC: immature DCs. Error bars indicate SDs of 1 representative experiment performed in triplicate. **C and D,** Relative cytokine profile (mean percentage of 3 independent experiments) of clones shown in A and B.

A subpopulation of Der p 1-specific T cells has the capacity to produce IL-17

T-cell lines isolated from biopsy specimens with positive APT reactions to *D pteronyssinus* were cloned by means of limiting dilution to further characterize skin-derived IL-17-producing T lymphocytes. Expanded T-cell clones (in total 142 T-cell clones

obtained from 3 patients with AE) were characterized for Der p 1 specificity, chemokine receptor expression, and the release of IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-22, and TNF-α. Consistent with the observations obtained from the APT-derived T-cell line, a high number of APT-derived T cells (24% ± 1.9%) were capable of producing IL-17 after activation with PMA plus ionomycin. The

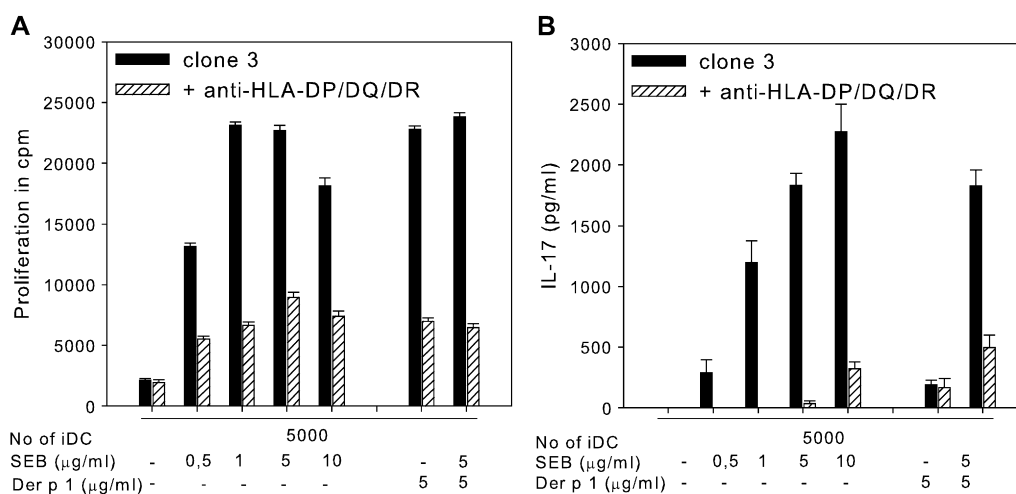


FIG 3. SEB activates T cells through a TCR-dependent mechanism. Clone 3 was stimulated with increasing amounts of SEB in the presence of 5000 immature DCs for 48 hours. Experimental end points were proliferation (A) and IL-17 secretion (B). Addition of anti-HLA-DP/DQ/DR almost completely abrogated SEB stimulation (hatched bars). Error bars indicate SEMs of 2 independent experiments. *iDC*, Immature DCs.

newly identified $T_H2/IL-17$ subset was identified also on the clonal level. The relative distribution of IL-17-producing subpopulations was comparable with the results obtained from T-cell lines, with a more than 40% pure T_H17 phenotype ($41\% \pm 4.1\%$), one third $T_H2/IL-17$ cells ($32\% \pm 3.6\%$), and one fourth $T_H1/IL-17$ cells ($26\% \pm 4.5\%$). Pure T_H17 cells were not specific for Der p 1. A correlation of IL-17 with IL-22 was not obvious on the clonal level. Sixty-nine percent ($\pm 4.8\%$) of skin-infiltrating T-cell clones were capable of producing IL-22. A characterization of IL-17-producing T-cell clones is shown in Tables I and II (see also Fig E1 in this article's Online Repository at www.jacionline.org).

Stimulation with cognate antigen induces IL-4 release, IFN- γ release, or both but no or very low amounts of IL-17

We then analyzed the physiologic reaction pattern of specific skin-derived $T_H2/IL-17$ and $T_H0/IL-17$ T-cell clones stimulated with native or recombinant Der p 1 in the presence of different antigen-presenting cells (APCs). Surprisingly, IL-17 was not or only marginally secreted by specific $T_H2/IL-17$ and $T_H0/IL-17$ cells when the allergen was presented by varying numbers of immature (Fig 2) or mature (see Fig E2 in this article's Online Repository at www.jacionline.org) DCs or CD14⁺ monocytes (see Fig E3 in this article's Online Repository at www.jacionline.org). Incrementing TCR stimulation intensity by increasing allergen concentration (see Fig E4 in this article's Online Repository at www.jacionline.org) or the number of APCs (up to a DC/clone ratio of 1:10) only marginally upregulated IL-17 secretion (Fig 2, A and B). In contrast, even at low levels of stimulation intensity (DC/T-cell ratio of 1:1000 and 5 μg/mL Der p 1), high amounts of IL-4 and a strong induction of proliferation were detected, without substantial differences between different APC populations. Thus in contrast to the maximal stimulation induced by PMA and ionomycin, physiologic TCR triggering failed to upregulate the release of IL-17, although it was capable of inducing T_H2 cytokines, which predominate in the early phase of AE.

T_H17 -associated cytokines IL-1 β , IL-6, and IL-23 do not increase IL-17 secretion in allergen-specific stimulated effector T-cell clones

To explain the divergence between the capacity of T-cell clones to produce IL-17 and the *de facto* secretion on cognate antigen recognition, we sought to find tissue-derived stimuli that could induce IL-17 secretion in $T_H2/IL-17$ and $T_H0/IL-17$ cells. In a first step, we investigated the effect of presently identified cytokines known to be involved in the differentiation and maintenance of human T_H17 cells. Addition of IL-1 β , IL-6, or IL-23 did not increase IL-17 secretion after stimulation with DCs and Der p 1 (Fig 2, A and B, see also Figs E2 and E3) or TCR stimulation with anti-CD3/anti-CD28 (data not shown).

SEB induces high secretion of IL-17 by Der p 1-specific T cells

Because a defect in IL-17 secretion results in recurrent infections of the skin and mucosal membranes,^{19,20} we investigated whether microbial-derived products could induce substantial production of IL-17. We stimulated skin-derived Der p 1-specific T-cell clones ($n = 5$) with the proinflammatory bacterial substances LPS and SEB, which is commonly present on AE skin. Although 50 μg/mL LPS did not alter the cytokine secretion of T-cell clones (data not shown), addition of 5 μg/mL SEB to DC/T-cell cocultures strongly promoted IL-17 release by T cells expressing SEB-sensitive TCR V β chains (4/5; Fig 2, A and B; see also Figs E2 and E3). Secretion of IL-10 and IFN- γ , but not that of IL-4, was also affected in these clones. However, the increase in IL-17 secretion was by far most prominent, resulting in an increased percentage relative to other T-cell cytokines in all clones examined (Fig 2, C and D). A predominating V β chain was not detected in IL-17-producing T-cell clones (Table II). In line with the literature, blocking TCRs by adding neutralizing antibodies against MHC class II molecules abrogated SEB stimulation almost completely (Fig 3). This finding indicates that secretion of inflammatory IL-17 by T lymphocytes is tightly regulated and requires additional stimulation beyond cognate antigen

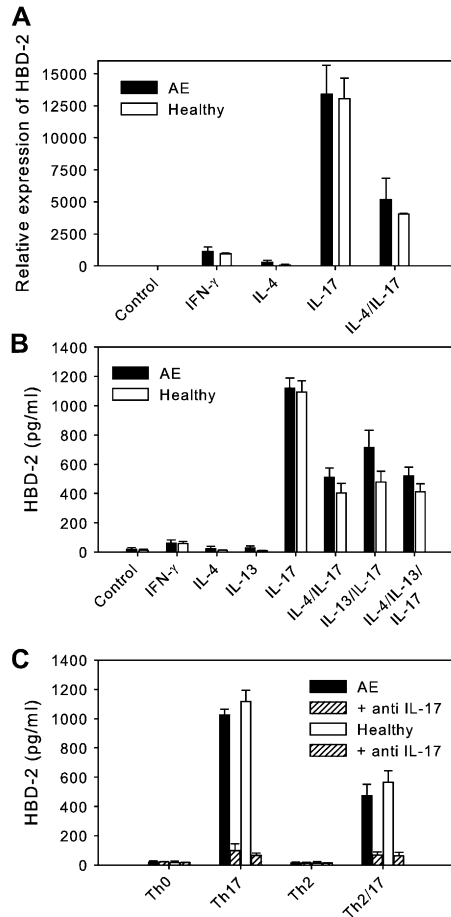


FIG 4. T cell-derived IL-17 induces HBD-2 in primary human keratinocytes, and IL-4 and IL-13 partially block this effect. Induction of HBD-2 mRNA (A) and protein (B) in AE and healthy keratinocytes in response to recombinant cytokines and to cell-free supernatant obtained from stimulated T-cell clones (C) is shown. Error bars indicate SEMs of 3 independent experiments.

presentation by professional APCs. In AE, such hyperstimulation could be provided by microbial-derived superantigens.

T cell-derived IL-17 potently induces HBD-2 production in AE keratinocytes, whereas secreted IL-4 partially inhibits IL-17-dependent HBD-2 upregulation

IL-17 strongly induces HBD-2, but this antimicrobial peptide is diminished in patients with AE. To clarify whether the ineffective upregulation in AE is due to intrinsic defects in AE keratinocytes or due to inhibitory effects of the microenvironment, we stimulated primary keratinocytes from patients with AE ($n = 3$) and healthy donors ($n = 3$) with different T-cell cytokines. We found that IL-17 strongly induced HBD-2 release in both AE and healthy keratinocytes *in vitro* (Fig 4). However, IL-17-induced HBD-2 upregulation was partially inhibited by the T_H2 cytokines IL-4 and IL-13 (Fig 4, A and B). Accordingly, experiments performed with supernatants from APT-derived T-cell clones demonstrated that neither T_H2 nor T_H0 could induce HBD-2 release by AE keratinocytes. The T_H17 -derived supernatant was the most effective in HBD-2 induction, whereas the coexpression of IL-4 in the supernatant of

$T_H2/IL-17$ cells partially, but not completely, blocked the induction of HBD-2 release. Finally, preincubation of T_H17 and $T_H2/IL-17$ supernatant with a neutralizing antibody against IL-17 abrogated HBD-2 induction almost completely (Fig 4, C).

SEB strongly upregulates HBD-2 mRNA and protein release in *D pteronyssinus*-induced AE

To confirm the role of superantigens in triggering the IL-17/HBD-2 axis *in vivo*, we applied *D pteronyssinus* on both forearms of a patient with AE. Thirty-six hours after allergen application, we added $50 \mu\text{g}/\text{cm}^2$ SEB on 1 forearm. In concordance with previous reports,¹⁴ the clinical reaction was severely aggravated (classified as “++++” vs “+++” according to the European Task Force on Atopic Dermatitis 2000 reading key²⁷) and maintained for substantially longer (10 days vs 4 days) in the SEB-exposed lesion. Sixty hours after allergen application, epidermal sheets of induced eczematous reactions were obtained by using the suction blister method, and suction blister fluid was investigated for cytokine content. In line with our *in vitro* results, IL-17 was induced more than 2-fold in the SEB-challenged patch test site, whereas IFN- γ induction was unchanged. IL-4 levels were less than the detection level, and IL-10 levels were marginally increased (Fig 5, A-D). Consequently, HBD-2 mRNA was 2-fold increased in keratinocytes from SEB-exposed epidermal sheets (Fig 5, E). An increased HBD-2 concentration in the SEB-treated APT reactions was confirmed at the protein level by using ELISA assays performed on blister fluids (Fig 5, F).

DISCUSSION

Increasing evidence suggests a central role of IL-17 in encompassing host defense against microorganisms at surface barriers. In patients with AE, disease severity positively correlates with skin colonization of *S aureus*. This observation led us to investigate IL-17 in the pathogenesis of AE.

In this study we demonstrate that distinct subpopulations of IL-17-secreting T cells infiltrate acute skin lesions, where they trigger keratinocytes to produce the antimicrobial peptide HBD-2. However, this induction is substantially impaired in the presence of the type 2 cytokines abundantly present in the AE skin microenvironment.

By isolating and characterizing the lymphocytic infiltrate in APT reactions, we directly demonstrated IL-17-releasing T cells in acute AE lesions. Hereby we confirm and extend a previous report describing the detection of IL-17 mRNA by means of PCR in AE skin.²⁸ When we further characterized skin-infiltrating T cells, we identified distinct subpopulations of IL-17-producing T-cell clones: in addition to the previously described pure T_H17 and $T_H1/IL-17$ cells that coexpress IFN- γ ,^{26,29} a newly described population coproducing IL-17 and type 2 cytokines was classified as $T_H2/IL-17$ cells. Although in the APT lesion no Der p 1-specific T_H17 cells were found on the clonal level, we were surprised to observe that stimulation of Der p 1-specific $T_H2/IL-17$ and $T_H0/IL-17$ cells with their cognate antigen resulted in a strong induction of proliferation and IL-4 secretion, but IL-17 was poorly secreted. Neither the T_H17 -differentiating cytokines IL-1 β and IL-6³⁰ nor IL-23, described to maintain survival and cytokine secretion of the mouse³¹ and, to a lesser degree, also of human T_H17 cells,³⁰ strongly increased secretion of

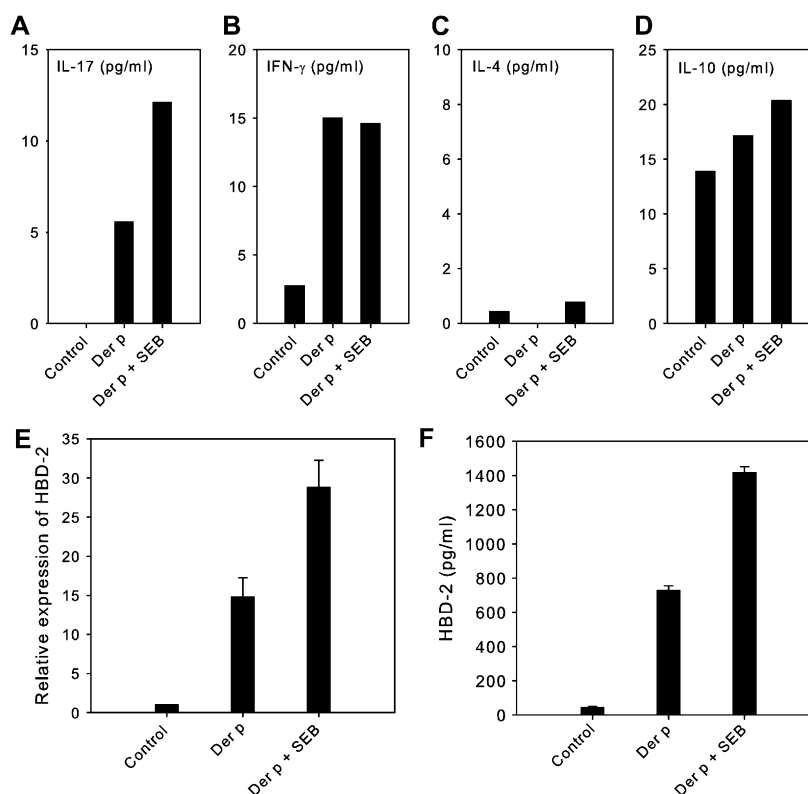


FIG 5. SEB induces expression of IL-17 and HBD-2 *in vivo*. Levels of IL-17 (A), IFN- γ (B), IL-4 (C), and IL-10 (D) in suction blister fluids as detected with Luminex analysis. E and F, Relative induction of HBD-2 mRNA (E) and protein content (F). Error bars indicate SDs of 1 experiment performed in triplicate.

IL-17 in our differentiated effector T cells stimulated with DCs and their cognate antigen. To find an *in vivo* stimulus for substantial IL-17 secretion, we investigated whether microbial-derived products could be adequate stimuli as evidence increases that IL-17 secretion is critical for clearing infections selectively at skin and mucosal membranes.^{19,20} Consensus exists that *S aureus* colonization of AE skin significantly aggravates the intensity and accounts for the persistence of eczematous reactions^{11,12}; however, the mechanisms remain unclear. Under natural exposure conditions, *S aureus*-derived superantigens, such as SEB, could contribute to the amplification of the inflammatory reaction by stimulating infiltrating T cells bearing particular TCR V β chains.^{12,13} Indeed, when we stimulated SEB-sensitive T_H2/IL-17 and T_H0/IL-17 clones with SEB, secretion of proinflammatory IFN- γ , but especially of IL-17, was strongly enhanced compared with that seen with cognate TCR triggering alone both *in vitro* and *in vivo*. Thus our data underline a role of the environment in triggering full effector functions of tissue-infiltrating T cells.

However, despite the availability of SEB-triggered IL-17, which is a very efficient stimulus for HBD-2,¹⁷ expression of HBD-2 in patients with AE was reported to be diminished in comparison with that seen in T_H1-mediated skin immune diseases, such as psoriasis.²³ We therefore investigated whether AE keratinocytes show an intrinsic defect in responding to IL-17 or whether coexpressed type 2 cytokines could account for the diminished HBD-2 induction, as reported for the IFN- γ - and TNF- α -induced expression of antimicrobial peptides.^{32,33} We found that AE keratinocytes are not hyporesponsive to IL-17

in vitro, but rather the AE skin microenvironment containing abundant IL-4 and IL-13 partially inhibits the IL-17/HBD-2 axis. This could, at least in part, explain the persistent colonization of AE skin with *S aureus* that represents a continuous trigger of cutaneous inflammation.

Key messages

- IL-17-producing T cells, in particular T_H2/IL-17 cells, infiltrate lesional skin in patients with acute AE.
- Secretion of IL-17 by Der p 1-specific T cells is tightly regulated and requires microenvironmental stimuli, such as SEB.
- IL-17 is involved in linking adaptive and innate immunity through the IL-17/HBD-2 axis, which is partially impaired by T_H2 cytokines in patients with AE.

REFERENCES

1. Leung DY, Bonguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. *J Clin Invest* 2004;113:651-7.
2. Leung AK, Hon KL, Robson WL. Atopic dermatitis. *Adv Pediatr* 2007;54:241-73.
3. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911-20.
4. Braback L, Hjern A, Rasmussen F. Trends in asthma, allergic rhinitis and eczema among Swedish conscripts from farming and non-farming environments. A nationwide study over three decades. *Clin Exp Allergy* 2004;34:38-43.
5. Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003;361:151-60.

6. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006;38:441-6.
7. Weidinger S, O'Sullivan M, Illig T, Baurecht H, Depner M, Rodriguez E, et al. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. *J Allergy Clin Immunol* 2008;121:1203-9.
8. Ring J, Darsow U, Gfesser M, Vieluf D. The "atopy patch test" in evaluating the role of aeroallergens in atopic eczema. *Int Arch Allergy Immunol* 1997;113:379-83.
9. Grewe M, Walther S, Gyufko K, Czech W, Schopf E, Krutmann J. Analysis of the cytokine pattern expressed in situ in inhalant allergen patch test reactions of atopic dermatitis patients. *J Invest Dermatol* 1995;105:407-10.
10. Eyerich K, Huss-Marp J, Darsow U, Wollenberg A, Foerster S, Ring J, et al. Pollen grains induce a rapid and biphasic eczematous immune response in atopic eczema patients. *Int Arch Allergy Immunol* 2008;145:213-22.
11. Mempel M, Lina G, Hojka M, Schnopp C, Seidl HP, Schafer T, et al. High prevalence of superantigens associated with the *ecg* locus in *Staphylococcus aureus* isolates from patients with atopic eczema. *Eur J Clin Microbiol Infect Dis* 2003;22:306-9.
12. Bunikowski R, Mielke ME, Skarabis H, Worm M, Anagnostopoulos I, Kolde G, et al. Evidence for a disease-promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis. *J Allergy Clin Immunol* 2000;105:814-9.
13. Choi Y, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc Natl Acad Sci U S A* 1989;86:8941-5.
14. Langer K, Breuer K, Kapp A, Werfel T. *Staphylococcus aureus*-derived enterotoxins enhance house dust mite-induced patch test reactions in atopic dermatitis. *Exp Dermatol* 2007;16:124-9.
15. Ardern-Jones MR, Black AP, Bateman EA, Ogg GS. Bacterial superinfection facilitates epithelial presentation of allergen to T helper 2 cells. *Proc Natl Acad Sci U S A* 2007;104:5556-62.
16. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007;8:639-46.
17. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006;203:2271-9.
18. Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 2007;8:345-50.
19. Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, et al. Impaired Th17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 2008;452:733-6.
20. Eyerich K, Foerster S, Rombold S, Behrendt H, Hofmann H, Ring J, et al. Chronic mucocutaneous candidiasis patients exhibit reduced production of the Th17 associated cytokines IL-17 and IL-22. *J Invest Dermatol* 2008;128:2640-5.
21. Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, et al. *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β -defensins and CAP18, expressed by human keratinocytes. *Infect Immunity* 2003;71:3730-9.
22. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, et al. β -defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 1999;286:525-8.
23. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 2002;347:1151-60.
24. Cavani A, Nasorri F, Prezzi C, Sebastiani S, Albanesi C, Girolomoni G. Human CD4+ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *J Invest Dermatol* 2000;114:295-302.
25. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α . *J Exp Med* 1994;179:1109-18.
26. Albanesi C, Scarponi C, Cavani A, Federici M, Nasorri F, Girolomoni G. Interleukin-17 is produced by both Th1 and Th2 lymphocytes, and modulates interferon- γ - and interleukin-4-induced activation of human keratinocytes. *J Invest Dermatol* 2000;115:81-7.
27. Darsow U, Ring J. Airborne and dietary allergens in atopic eczema: a comprehensive review of diagnostic tests. *Clin Exp Dermatol* 2000;25:544-51.
28. Toda M, Leung DY, Molet S, Boguniewicz M, Taha R, Christodoulopoulos P. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J Allergy Clin Immunol* 2003;111:875-81.
29. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007;204:1849-61.
30. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human helper T cells. *Nat Immunol* 2007;8:903-5.
31. Chen Y, Langrish CL, McKenzie B, Joyce-Shaikh B, Stumhofer JS, McClanahan T, et al. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J Clin Invest* 2006;116:1317-26.
32. Howell MD, Boguniewicz M, Pastore S, Novak N, Bieber T, Girolomoni G, et al. Mechanism of HBD-3 deficiency in atopic dermatitis. *Clin Immunol* 2006;121:332-8.
33. Albanesi C, Fairchild HR, Madonna S, Scarponi C, De Pità O, Leung DY, et al. IL-4 and IL-13 negatively regulate TNF- α - and IFN- γ -induced β -defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. *J Immunol* 2007;179:984-92.

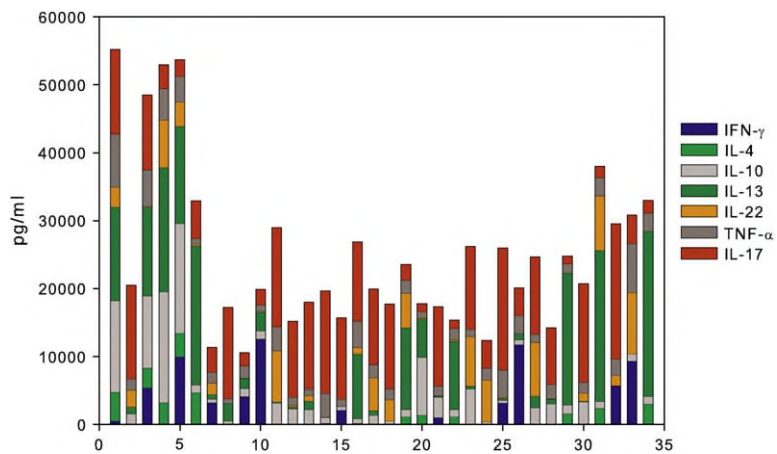


FIG E1. Cytokine pattern of IL-17-secreting T-cell clones confirms the existence of T_H17 , $T_H1/IL-17$, and $T_H2/IL-17$ subpopulations in APT reactions. Cytokine secretion after 48-hour PMA/ionomycin stimulation of all 34 identified IL-17-producing T-cell clones is shown.

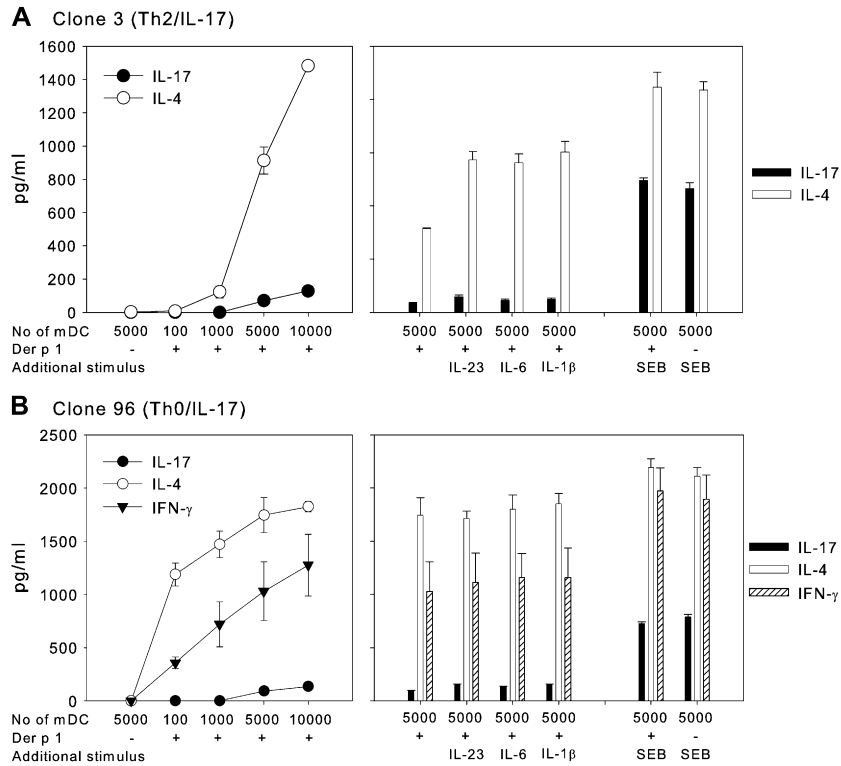
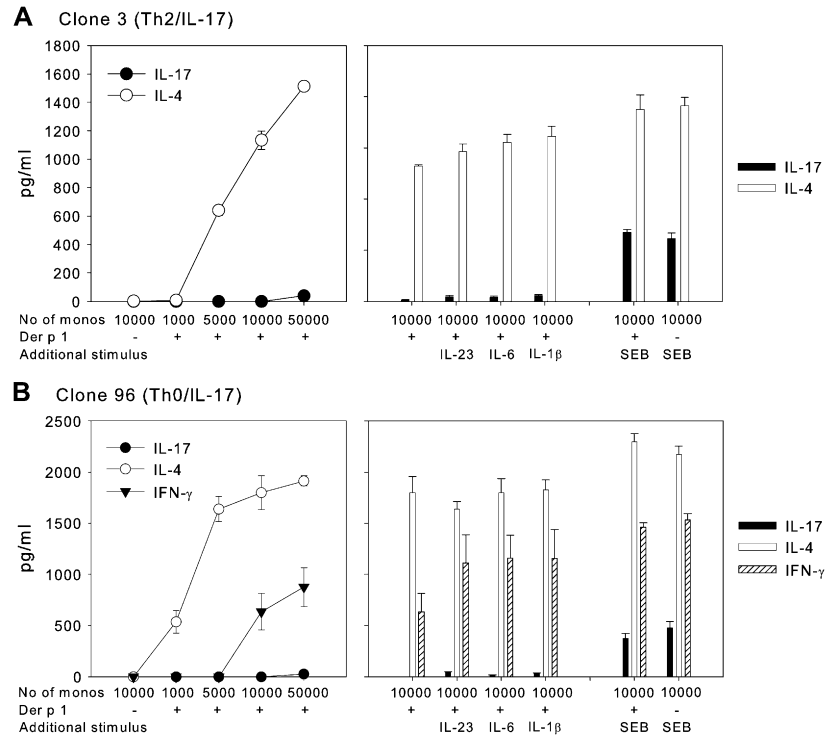


FIG E2. Stimulation of Der p 1-specific T-cell clones with mature DCs results in high IL-17 secretion on SEB but not on allergen stimulation. **A and B,** Dose-dependent secretion of IL-17 and IL-4 (*left panel*) and addition of cytokines and SEB (*right panel*) are shown. Type of APC: mature DCs (*mDC*). *Error bars* indicate SDs of 1 representative experiment performed in triplicate.



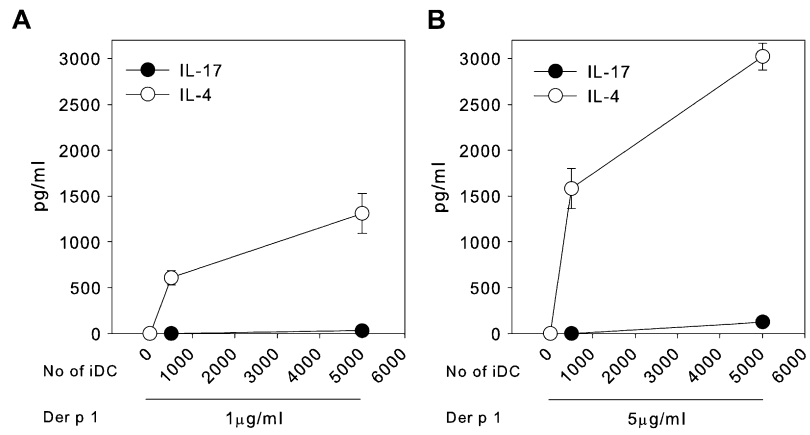


FIG E4. Varying concentrations of Der p 1 have little effect on IL-17 secretion by T_H2 /IL-17 clones: **A**, 1 µg/mL Der p 1; **B**, 5 µg/mL Der p 1. *iDC*, Immature DCs. *Error bars* indicate SEMs from 3 independent experiments with clone 3.