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J Immunol 2011; 186:1495-1502; Prepublished online 20 December 2010;

doi: 10.4049/jimmunol.1001001

http://www.jimmunol.org/content/186/3/1495

Supplementary Material http://www.jimmunol.org/content/suppl/2010/12/22/jimmunol.1001001.DC1

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
IL-23–Mediated Psoriasis-Like Epidermal Hyperplasia Is Dependent on IL-17A

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IL-23 and Th17 cells producing IL-17A and IL-22 are found in excess in skin affected by psoriasis. Previous studies showed that IL-22, but not IL-17A, mediates psoriasis-like epidermal hyperplasia following recombinant murine (rm)IL-23 injections into skin. To further investigate the role of IL-17A, ears of mice were injected with rmIL-23. Investigators blinded to treatment conditions and mouse genotypes measured ear swelling, epidermal thickness, and cytokine expression. In wild-type (WT) mice, rmIL-23 induced ear swelling ($p < 0.001$, all $p$ values versus saline), epidermal hyperplasia by histology ($p < 0.001$) and confocal microscopy ($p < 0.004$), and expression of both IL-17A and IL-22. As expected, rmIL-23 injections into IL-22$^{-/-}$ mice resulted in relatively little ear swelling ($p < 0.09$) and epidermal hyperplasia ($p < 0.51$ by histology and $p < 0.75$ by confocal microscopy). Notably, rmIL-23 injections into IL-17A$^{-/-}$ mice produced little ear swelling ($p < 0.001$, versus IL-23–injected WT mice) and epidermal hyperplasia ($p < 0.001$ by histology and $p < 0.005$ by confocal microscopy), even though IL-22 was readily induced in these mice. Furthermore, systemic delivery of blocking Abs directed against either IL-22 or IL-17A completely inhibited IL-23–induced epidermal hyperplasia in WT mice. These results demonstrate that IL-17A, like IL-22, is a downstream mediator for IL-23–induced changes in murine skin and that both of these Th17 cytokines are necessary to produce IL-23–mediated skin pathology. IL-17A may represent an attractive therapeutic target in individuals with psoriasis by blocking downstream effects of IL-23. The Journal of Immunology, 2011, 186: 1495–1502.

Cytokines produced by T cells are predominant mediators of skin pathology in psoriasis, a chronic inflammatory skin disease. Accumulating evidence suggests that IL-23 and the subsequent Th17 cell response it promotes are central factors in the pathogenesis of this disease (1). In psoriatic lesions, mRNA for both IL-23 subunits (IL-23p19 and IL-12/23p40) are elevated (2–4) and numerous IL-23$^+$ dendritic cells are present (2, 5–7). Messenger RNAs for Th17 cytokines and chemokines (IL-17A, IL-17F, IL-21, IL-22, TNF-α, and CCL20) along with Th17 cells are increased (4, 8–13). Psoriasis also has high circulating levels of Th17 cells and cytokines (14). In addition, specific polymorphisms in IL-23p19, IL-12/23p40, and IL-23R are associated with increased risk of developing psoriasis (15–17).

Although it is not yet known what triggers dendritic cells to produce IL-23 in individuals predisposed to develop psoriasis, it is well established that IL-23 is a central growth factor for Th17 cells as it influences both their differentiation and expansion (18–20).

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Received for publication April 1, 2010. Accepted for publication November 23, 2010.

This work was supported in part by a Veterans Affairs Merit award (to A.B.), National Institutes of Health Grants 1 R21 AR054495-01A1 (to A.B.) and CA069533 (to K.G.P.), the Naito Foundation (to S.E.K.), and the National Institutes of Health under Ruth L. Kirschstein National Research Service Award 5-T32 CA106195-05 (to K.G.P.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: qRT-PCR, quantitative RT-PCR; rCSLM, reflectance mode confocal scanning laser microscopy; rm, recombinant murine; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001001

Materials and Methods

Animals

The Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee reviewed and approved all breeding and experiments. IL-22$^{-/-}$ mice were a kind gift from Dr. Ouyang Wenjun, Genentech (25), and IL-17A$^{-/-}$ mice were a kind gift from Dr. Yoichiro Iwakura, Center for Inflamma...
for Experimental Medicine, Institute of Medical Science, University of Tokyo (29). Prior to using the mice, IL-22 \(/\) mice and IL-17A \(/\) mice were backcrossed to C57BL/6 for six and eight generations, respectively. C57BL/6 wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mouse strains were evaluated to assure proper genotype by analyzing DNA isolated from tail snips at 4 wk of age and purified using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). DNAs were genotyped by PCR amplification using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ) and locus specific primers. Primer sequences and product sizes (in bp) are as follows: IL-22 common forward 5’-CTCAGACCTCTACAGAATCTC-3’ with WT specific reverse 5’-CAGCTGGGCGGCAATGGC-3’ (196 bp) and mutant specific reverse 5’-GATACAGTGACGCTTAAAGCC-3’ (374 bp); IL-17A common forward 5’-ACTCTTCTCCACTCTGAGAAG-3’ with WT specific reverse 5’-GCCATGATATAGACGTTGTGC-3’ (500 bp). The annealing temperature was 56˚C for IL-22 primers and 58˚C for IL-17A primers.

Intradermal cytokine injections

Mouse ears were injected intradermally with 20 \(\mu\)l PBS/0.1% BSA into one ear and 1 \(\mu\)g rmIL-23 (eBioscience, San Diego, CA) in PBS/0.1% BSA into the contralateral ear. Injections were continued in this manner daily for 4 d (days 0, 1, 2, and 3). For the Ab blocking experiments, 100 \(\mu\)g anti–IL-17A Abs (eBioscience), 50 \(\mu\)g anti–IL-22 Abs (eBioscience), 100 \(\mu\)g mouse IgG1 (eBioscience), or 50 \(\mu\)g rat IgG2a (eBioscience) were injected i.p. 1 h before injections into ear skin on days 0 and 2. Ear thickness was measured on day 4 using a G-1A dial thickness gauge (Peacock, Japan) by an observer blinded to treatment conditions and mouse genotype. Mice were then used for noninvasive confocal imaging, immunohistochemistry, or extraction of RNA.

Confocal imaging

Mice were placed in the supine position on a heated optical stage so that the ventral side of their ears could be imaged through a metal plate with a 3-mm diameter hole. The reflectance profile from the center of each ear was resolved using confocal detection through the 3-mm hole. During imaging, mice were anesthetized using vaporized isoflurane. Their breathing rates were regulated by providing 0.2 l/min oxygen and 0.8 l/min air mixed with the isoflurane vapor (1.5% v/v at 1 ATM). To restrict movement of the tissue...
volume under investigation, the ear of the mouse was secured by placing a coverslip on the dorsal side of the ear.

Mouse imaging was performed using reflectance mode confocal scanning laser microscopy (rCSLM). The rCSLM system consists of an argon ion laser, \( \lambda = 488 \text{ nm} \), with an average output power of 10 mW, providing illumination through a water-coupled objective lens with numerical aperture 0.90 and magnification \( \times 60 \) (Olympus America, Melville, NY). The objective lens was set up in an inverted orientation. \( (x, y) \) translation of the focus was achieved using a scanning assembly consisting of two galvano-meter mirrors (Nurfitel Technology, Windham, NH) and a pair of relay lenses that directed the laser beam into the rear of the objective lens at varying angles. Confocal detection of light originating from the focus in the mouse tissue was achieved by recollimating the reflected signal and then returning the signal through the optical train until the beam was redirected by a beam-splitter toward a lens/pinhole/photo multiplier tube assembly. The \( z \) position of the sequential \( (x, y) \) scans was incremented in 1- \( \mu \text{m} \) steps by computer control of the translation stage. The spatial resolution along the \( x \) and \( y \) directions is 0.4 \( \mu \text{m} \); \( z \) resolution is 1 \( \mu \text{m} \). Data were acquired using an A/D converter controlled by Labview software (National Instruments, Austin, TX), and image reconstruction was conducted using Matlab software (The Mathworks, Natick, MA).

The confocal microscope records a three-dimensional image volume. These are a collection of images stacked along the \( z \) direction. \( z \) measures depth into the tissue. This image volume is divided into rectangular cubes (regions of interest) of size 10 pixels \( \times \) 10 pixels \( \times \) 100 pixels. In each region of interest, we average over all of the \( x \), \( y \) locations to yield an average reflectance profile, or brightness of the reflected light as a function of depth. This reflected brightness has two local maxima: one at the tissue–light interface (stratum corneum) and one in the upper dermis. The peaks are a result of the tissue–light interaction. The dermal–epidermal junction corresponds to the local minima between the two peaks (it appears as a dark fringe in the sagittal confocal images). The thickness of the epidermis was determined by taking the average distance between the first reflective surface (the beginning of the stratum corneum) in the confocal images to the dark fringe before the second reflective surface (the upper dermis) in 2500 regions of interest (30).

Histology and immunohistochemistry

Sections from formalin-fixed, paraffin-embedded ears were stained with H&E, and epidermal thickness was measured using ImagePro Plus software (Leeds Precision Instruments, Minneapolis, MN). With this software, the epidermis was outlined for each tissue section using a series of rectangles, and the total epidermal area was determined. Thickness was then calculated as a measure of total area divided by total length of the epidermis. For immunohistochemistry, sections were deparaffinized and hydrated by washing sections in xylene followed by a graded alcohol series. To unmask Ags, sections were incubated in 10 mM citric acid (pH 6) at 95°C for 30 min, and endogenous peroxidase activity was quenched by treating sections with 3% hydrogen peroxide for 5 min at room temperature. Sections were blocked for 60 min at room temperature followed by incubation with primary Abs overnight at 4°C. Samples were washed and incubated for 60 min with secondary Abs and developed using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and DAB substrate kit for peroxidase (Vector Laboratories). All stained sections were counterstained with hematoxylin (Vector Laboratories). Blocking solutions, primary Abs, and secondary Abs used are shown in Supplemental Table I. Sections from four animals per group were stained, and photomicrographs were taken of representative \( \times 40 \) magnification fields. Average numbers of positive cells per photomicrograph were counted.

RNA isolation and quantitative real-time RT-PCR

Injected ears were collected, placed in TRIzol (Invitrogen), and homogenized with a mechanical rotor for 1 min. Total RNA was isolated and purified using TRIzol and the PureLink RNA Mini Kit (Invitrogen) as recommended by the manufacturer. \( \Delta C_{\text{Tm}} \) was calculated from \( 1 \mu \text{g} \) total RNA by reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reactions were diluted three times with RNase-free water for all quantitative RT-PCR (qRT-PCR) experiments. qRT-PCR was performed using TaqMan Gene Expression Master Mix with TaqMan primers and fluorescent probes from Applied Biosystems for Gapdh (catalog no. Mm9999915_g1), Il17a (catalog no. Mm01268754_m1), Il22 (catalog no. Mm00434676_m1), Il17f (catalog no. Mm00434676_m1), Il17f (catalog no. Mm00434676_m1), Il17f (catalog no. Mm00434676_m1), Il12b (catalog no. Mm00436808_m1), and Tofa (catalog no. Mm00436808_m1), on the MyiQ system (Bio-Rad). Expression of each transcript was calculated relative to Gapdh. Fold change relative to the PBS-injected ear was calculated using the 2\( ^{\Delta \text{Ct}} \) method, where \( \Delta \text{Ct} = \text{Ct}_{\text{PBS-injected}} - \text{Ct}_{\text{rmIL-23-injected}} \).

Statistical analyses

The \( \chi^{2} \) goodness-of-fit test was used to evaluate normality for all parameters. The \( F \) test and Bartlett test were used to evaluate equality of variance between two and three groups, respectively. Welch’s \( t \) test was used for analysis of mRNA expression between different strains of mice. Statistical analyses for comparison of PBS-injected ears and rmIL-23-injected ears were performed using the paired \( t \) test or Wilcoxon signed-rank test. For testing equality of population medians among rmIL-23–injected WT, IL-17A–/–, and IL-22–/– mice, Kruskal-Wallis test and Scheffe’s F test were used. \( p \) values of 0.05 or less were considered significant.
IL-23–induced epidermal hyperplasia is dependent on IL-22 and IL-17A

H&E staining of ear sections showed that rmIL-23–injected WT mice had thicker ears and increased infiltration of inflammatory cells within the dermis when compared with saline-injected skin (Fig. 1). Gross ear thickness, as measured in live mice with a caliper by an investigator blinded to the genotypes and treatment conditions of the mice, was also greater in rmIL-23–injected WT mice when compared with saline-injected WT mice (p < 0.001) (Fig. 2A). In addition, epidermal hyperplasia (i.e., acanthosis) as mea-

FIGURE 4. IL-17A or IL-22 alone is not sufficient to cause IL-23–mediated epidermal hyperplasia. Bar graphs show relative mRNA expression of IL-17A, IL-17F, IL-21, IL-22, IFN-γ, and TNF-α in rmIL-23–injected and PBS-injected WT mice, IL-22−/− mice, and IL-17A−/− mice. Scatter graphs show fold change in mRNA expression of these cytokines in rmIL-23–injected ears relative to PBS-injected ears in WT mice, IL-22−/− mice, and IL-17A−/− mice. Data are expressed as mean ± SD in bar graphs. Each dot indicates single specimen, and horizontal bars denote the mean of scatter plots. At least six mice from each strain and condition were studied in at least three separate experiments. Bar graphs were analyzed using the Wilcoxon signed-rank test (when not normally distributed) or paired t test (when normally distributed). Scatter graphs were analyzed using the Kruskal-Wallis test and Scheffe’s F test. *p < 0.05; **p < 0.01. AU, arbitrary units.
sion or in fold induction of these genes between the different strains of mice used in this study (Fig 4). Thus, these data suggest that rmIL-23–induced levels of IL-17F, IL-21, and IFN-γ were not sufficient to cause epidermal hyperplasia in our experiments.

Confirmation of IL-17A and IL-22 mRNA results was performed via protein analyses using immunohistochemistry. Both IL-17A−/− and IL-22+ cells were readily observed in the dermis of rmIL-23–injected WT skin (Fig 5). IL-17A−/− cells, but not IL-22+ cells, were also readily apparent in the dermis of IL-22−/− mice injected with rmIL-23, whereas IL-22+ cells, but not IL-17A−/− cells, were readily visualized in the dermis of IL-17A−/− mice injected with rmIL-23 (Figs 5, 6A). Thus, the combined mRNA and protein analyses revealed that IL-22 was present, if not elevated, in IL-22−/− mice and that IL-17A levels were normal to elevated in IL-22−/− mice. These data suggest that neither cytokine alone is sufficient to mediate IL-23–induced epidermal changes. In other words, both of these cytokines are critical for this process to occur.

Characterization of cutaneous leukocyte populations following injections with rmIL-23

To localize and quantify leukocyte populations in skin following injections of rmIL-23, we performed immunohistochemistry for CD3 (T cells), CD11c (dendritic cells), F4/80 (macrophages), and Gr-1 (neutrophils). IL-17A−/− mice injected with rmIL-23 had more F4/80+ cells and Gr-1+ cells when compared with IL-22−/− mice injected with rmIL-23 (p < 0.01 and p < 0.05, respectively) (Figs 5, 6A). There were no differences, however, in F4/80+ or Gr-1+ cells between WT mice and IL-17A−/− or IL-22−/− mice. CD3+ T cells were decreased in both IL-17A−/− and IL-22−/−...
mice compared with WT mice, although differences were only statistically significant for IL-22−/− mice (Figs. 5, 6A). Next, we stained serial sections to detect double-positive cells. IL-17A was mainly produced by CD3+ T cells (Fig. 6B), whereas some Gr-1+ cells also produced IL-17A (Fig. 6C). IL-22 was also primarily produced by CD3+ T cells (Fig. 6B). Some, but not all, IL-17A+ cells were also IL-22+ (Fig. 6B).

Pretreatment with blocking Abs directed against either IL-22 or IL-17A inhibit epidermal hyperplasia induced by rmIL-23 injections into skin

To confirm and extend our studies in genetic knockout mice, we injected WT mice with anti–IL-22 Abs, anti–IL-17A Abs, or isotype control Abs 1 h before rmIL-23 injections on days 0 and 2. Pretreatment with either anti–IL-22 Abs or anti–IL-17A Abs completely blocked induction of epidermal hyperplasia by rmIL-23 (Fig. 7). This result is consonant with findings in our genetic knockout mice that support a critical role for IL-17A, like IL-22, as a downstream mediator of IL-23–induced skin pathology.

Discussion

IL-23 and Th17 cells play a central role in psoriasis pathogenesis (1). IL-23 is predominantly produced by inflammatory dendritic cells in the dermis of skin affected by psoriasis (2, 5–7). Experiments in which rmIL-23 is injected into skin of mice have provided useful information regarding the mechanisms involved in IL-23–induced psoriasiform inflammation in skin (3, 25, 28). In this study, a central role for IL-17A in mediating downstream effects of IL-23 in murine skin is demonstrated, to our knowledge, for the first time. Specifically, mice deficient in IL-17A demonstrated little epidermal hyperplasia following repeated injections of rmIL-23 in skin (Figs. 1–3), and WT mice treated with anti–IL-17A Abs showed no IL-23–induced epidermal hyperplasia (Fig. 7). This IL-17A finding is analogous to a prior report (and confirmatory findings shown in this study) that IL-22 is a critical downstream mediator of IL-23–induced epidermal hyperplasia (25). Because IL-22 has a direct proliferative effect on keratinocytes (12, 21, 24–26), whereas IL-17A does not (8, 21, 23), the in vivo role for IL-17A in promoting epidermal hyperplasia is
likely due to indirect effects on keratinocytes. For example, we showed that TNF-α mRNA was not induced in IL-17A knockout mice treated with rmIL-23 (Fig. 5), which could be playing a role in the failure to induce epidermal thickening. Additionally, IL-17A can induce CCL20 expression by keratinocytes and promote chemotaxis of CCR6+ cells into skin (8); thus, IL-17A–deficient mice may demonstrate impaired movement of CCR6+ Th17 cells, which is a critical component of IL-23–mediated psoriasiform inflammation (28). Of note, our Ab results differ from Chan et al. (3), who showed that anti–IL-17A Abs did not block psoriasiform changes in murine skin injected with rmIL-23. This discrepancy can possibly be explained by methodologic differences between these investigators and our laboratory regarding: 1) genetic background of the mice; 2) sources of anti–IL-17A Ab; and/or 3) treatment protocols.

A novel aspect of this report is the use of noninvasive confocal microscopy, or rCSLM, to measure epidermal thickening in live animals (Fig. 3). Findings from this imaging modality corroborated nicely with epidermal thickness measurements on histologic specimens obtained immediately after mice were sacrificed (Fig. 2B). rCSLM is a promising technique for use in live small animal imaging, as it provides a quantitative means of studying morphology associated with cutaneous disease in vivo (30). Applications to quantifying epidermal thickness in humans with skin disease are underway (31).

It is likely that other Th17 cytokines other than IL-17A and IL-22 are also critically involved in IL-23–mediated cutaneous pathology. On a technical note, we used an IL-23 injection model that assessed short-term effects of IL-23 on skin, like some others (3, 28), whereas other investigators have assessed IL-23–induced skin changes over longer periods of time (25). Caruso and colleagues (10) recently highlighted a key role for IL-21 in psoriasis-like keratinocyte hyperproliferation, although we did not observe differences in IL-21 mRNA levels between WT and knockout mice in our experiments (Fig. 4). They found that blocking IL-22 did not prevent IL-21–induced epidermal thickening (10). In addition, members of the IL-10 family of cytokines other than IL-22, such as IL-19, IL-20, and IL-24, were reported to induce epidermal hyperplasia directly (26). rmIL-23 injections induced IL-19 mRNA and IL-24 mRNA, but not IL-20 mRNA, and IL-23–induced epidermal hyperplasia was decreased in IL-20R2−/− mice, but not in IL-19−/− and IL-24−/− mice (3). Moreover, these investigators showed that TNF-α was induced by intradermal injection of rmIL-23.
and that mAbs directed against TNF-α partially inhibited IL-23–dependent epidermal hyperplasia (3). This latter finding may partly explain the clinical efficacy of TNF-α blockade in individuals with psoriasis.

Clinical findings in psoriasis patients support the concept that IL-23 is critical in psoriasis pathogenesis. Thus far, the mAbs ustekinumab and briakinumab, directed against IL-12/23p40, have shown remarkable clinical efficacy in individuals with moderate-to-severe psoriasis (32–35). Several drugs that block only IL-23 (and not IL-12) target IL-23p19 and are currently in phase I and phase II development for psoriasis. In addition, mAbs and receptor antagonists that block either IL-17A or IL-22 function are also under development (36). A critical point shown in this study is that the presence of normal-to-increased levels of IL-22 in IL-17A−/− mice and the presence of normal-to-increased levels of IL-17A in IL-22−/− mice following mAbs injections suggests that neither Th17 cytokine alone is sufficient to promote IL-23–induced psoriasis-like changes in murine skin (Figs. 4, 5). Because genetic and Ab targeting of either IL-17A or IL-22 leads to impaired IL-23–mediated immune responses in skin, these data support the use of drugs for psoriasis that block function of either IL-17A (36) or IL-22.

Disclosures
The authors have no financial conflicts of interest.

References
