Epidermal expression of I-TAC (Cxcl11) instructs adaptive Th2-type immunity

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ABSTRACT To decipher early promoters of the local microenvironment for Th2-type immunity, we wanted to identify gene patterns that were induced by Leishmania major in the infected skin of susceptible, Th2-prone BALB/c, but not of resistant, Th1-prone C57BL/6 mice. We found a marked up-regulation of the chemokine I-TAC (Cxcl11) during the first 2 d of infection in the epidermis of susceptible but not of resistant mice. Accordingly, local injection of I-TAC (2×1 μg) in resistant mice on the first day of infection resulted in a Th2-driven, sustained deterioration of disease and dramatically enhanced parasite levels. On the cellular level, I-TAC decreased IL-12 production by dendritic cells (DCs) in skin-draining lymph nodes and by DCs in vitro. Thus, we demonstrate for the first time that epidermis-derived I-TAC triggers a sustained Th2-response that determines the outcome of a complex immunological process.—Roebrock, K., Sunderkötter, C., Münck, N-A., Wolf, M., Nippe, N., Barczyk, K., Varga, G., Vogl, T., Roth, J., Ehrchen, J. Epidermal expression of I-TAC (Cxcl11) instructs adaptive Th2-type immunity. FASEB J. 28, 000–000 (2014). www.fasebj.org

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Differentiation of T-helper (Th) cells into different subsets has a major impact on the course of diverse infections or inflammatory diseases. Th1 and Th17 subsets dominate in parasitic or bacterial infections, as well as in chronic inflammatory diseases, such as arthritis or colitis. A prevalence of Th2 cells, on the other hand, occurs in helminth infections or allergic and atopic disorders.

Experimental leishmaniasis presents a renowned model system to investigate the mechanisms underlying Th-cell differentiation in vivo. A Th1 response is crucial for protective immunity in genetically resistant strains, such as C57BL/6 mice, as it leads to interferon-γ (IFN-γ)-mediated activation of macrophages and subsequent killing of intracellular parasites. Susceptible BALB/c mice develop a Th2 response and succumb to progressive disease due to the release of deactivating cytokines such as interleukin (IL)-4 and IL-13 (for review see refs. 1, 2).

The decisive events that determine either Th1 or Th2 differentiation occur early after infection. The potential time frame may be narrowed to approximately the first 2 d, since this is the only time frame within which pharmacological manipulation is able to change Th1/Th2 differentiation (1, 3). The Th1/Th2-directing mechanisms have long been supposed to occur primarily in the lymph node during antigen presentation (1, 4). However, there is accumulating evidence that regulatory processes in the skin; i.e., at the site of the primary infection, precede and influence Th1/Th2 differentiation in the lymph node. We and others showed that already within 2 d after infection with Leishmania major, a higher percentage of granulocytes is found in the infiltrate of BALB/c compared to C57BL/6 mice (5). Antibody-mediated elimination of these cells in susceptible mice results in a Th1 response and resistance (6). These differences in leukocyte infiltration reflect important differences in the microenvironment of the infected tissue within the first hours of infection. This concept of decisive immunological events taking place at the site of infection that subsequently influence the processes in the lymph node is supported by the observation that a change in the route

Abbreviations: CBA, cytometric bead array; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CD, cluster of differentiation; CXCR, chemokine (CX-C motif) receptor; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IF, immunofluorescence; IFN-γ, interferon γ; IL, interleukin; I-TAC, interferon-inducible T-cell α chemoattractant; LDA, limited dilution assay; LMPC, laser microdissection and presелl cataplasm; PCR, polymerase chain reaction; RISH, RNA in situ hybridization; RT-PCR, reverse transcription polymerase chain reaction; SLA, soluble Leishmania antigen; Th, T helper; TSLP, thymic stromal lymphopoietin

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of infection; e.g., via bloodstream or nasal mucosa instead of skin, resulted in a Th2 response and non-healing disease in originally resistant mice (7, 8). Applying microarray analysis, we were able to detect differentially regulated genes between resistant and susceptible mice in the infected skin (9). We found that not only were keratinocytes the major cellular source for differential gene induction after dermal infection, but also the cutaneous gene expression of several Th1/Th2-influencing cytokines was temporally restricted to the crucial time of Th1/2 differentiation. IL-6 and IL-4 belong to those genes that were more strongly regulated in resistant mice. Astonishingly, although IL-4 had already been demonstrated to induce a Th1 response when administered to susceptible mice exclusively in the first hours after infection (10), its cellular source had not been revealed. We showed that keratinocytes produce IL-4 in the respective time span and that its early local neutralization in resistant mice resulted in a Th2 switch (9). Thus, we clearly demonstrated that early tissue signals produced by local resident keratinocytes in the skin can direct a Th1 response in experimental leishmaniasis.

The molecular mechanisms by which the early microenvironment could instruct the ensuing immune response are currently not clear. It has been suggested that signals generated in the infected tissue are integrated and transferred via dendritic cells (DCs) to T cells in draining lymph nodes (11). Thus, in addition to an antigen-specific signal and a costimulatory signal, the tissue microenvironment induces a so-called signal 3 in DCs that influences the direction of the T-cell response (11, 12). Some of these third signals have been characterized. As such, DC-derived IL-12 is the major signal that shifts the Th-cell response toward Th1, while its absence favors a Th2 response. We and others provided evidence that IL-4 could be a tissue signal that stimulates IL-12 secretion from DCs and subsequently instructs Th1 priming (9, 10). Similarly, it was shown that transforming growth factor β (TGF-β), IL-23, and IL-6 act as Th17-promoting cytokines (13). OX40L and jagged on DCs have recently been described to drive Th2 differentiation (14, 15).

The nature of the tissue signals that influence expression of Th2-promoting signals in DCs, however, is currently not well defined. To clarify the enigmatic early mechanisms of Th2 cell differentiation, we have already analyzed the expression of immunomodulatory mediators at the initial site of infection during experimental leishmaniasis; i.e., the skin, by microarray technology in susceptible as compared to resistant mice (9). However we found only 4 genes up-regulated in BALB/c but not C57BL/6, but none of them a likely candidate for a Th2-inducing tissue signal. Therefore, we now extended our analysis using the more sensitive real-time polymerase chain reaction (PCR) technique, employing probes for cytokines that had previously been directly or indirectly linked to T-cell responses and not yet detected by the less sensitive gene array [such as IL-13, IL-17, IL-18, CC chemokine ligand 17 (Ccl17), thymic stromal lymphopoietin (TSLP), and interferon-inducible T-cell α chemottractant (I-TAC)].

We found a marked up-regulation of the chemokine I-TAC [U.S. National Center for Biotechnology Information (NCBI) ID 56066] in the epidermis of susceptible, Th2-prone BALB/c mice during the first 2 d of infection. Since this induction of I-TAC did not occur in resistant, Th1-prone C57BL/6 mice, we tested the capacity of I-TAC to act as a Th2-inducing tissue signal in infection. Strikingly, treatment of resistant mice with I-TAC during the first hours of infection resulted in decreased IL-12 expression in lymph nodes with an ensuing Th2 shift, which resulted in a sustained deterioration of disease. Coherent with our in vivo findings, we noticed an I-TAC-triggered decrease of L. major-induced IL-12 production by DCs in vitro. Thus, I-TAC is a novel Th2-inducing tissue signal, generated at the site of infection that acts via an impairment of IL-12 production by DCs.

**MATERIALS AND METHODS**

**Reagents**

Recombinant murine CXCL11 (I-TAC) and CXCL10 (IP10) (purity >98%) were purchased from Pepro Tech (London, UK). Anti-mouse CXCL11 Rat IgG2a monoclonal antibody clone 131237 for in vivo neutralization was purchased from R&D Systems (Wiesbaden, Germany). Recombinant murine IFN-γ was purchased from Promokine (Heidelberg, Germany). Functional grade anti-mouse, chemokine (C-X-C motif) receptor 3 (CXCR3) antibody clone CXCR3-173 was purchased from eBioscience (San Diego, CA, USA). CXCR7 agonist VUF11207 was purchased from Merck (Darmstadt, Germany). Polyclonal goat anti-mouse I-TAC and donkey anti-goat IgG-FITC antibodies for immunofluorescence were purchased from Santa Cruz Biotecology (Santa Cruz, CA, USA).

**Animals**

C57BL/6 and BALB/c mice were purchased from Harlan (Borchen, Germany) and kept under sterile conditions in microisolator cages in a specific pathogen-free (SPF) animal facility with unlimited access to food and water. All animal studies were reviewed and approved by the Nordrhein-Westfalen State Office for Nature, Environment, and Consumer Protection.

Experimental leishmaniasis L. major [World Health Organization (WHO) nomenclature MHOM/IL/81/FE/BNI] were cultivated in Schneider’s Drosophila medium supplemented with 10% FCS, 2% human urine, 2% glutamine, and 1% penicillin/streptomycin, as described previously (16). Mice were infected subcutaneously by application of 2 × 10⁷ promastigotes (stationary phase) in 50 μl PBS into the left hind footpad. The right footpad was injected with 50 μl PBS and served as internal control for analysis of gene expression and footpad swelling. At indicated times footpads, lymph nodes, serum, liver, and spleen were harvested for limiting dilution assay (LDA) and determination of cytokine profile (4).

**Laser microdissection and pressure catapulting (LMPC)**

Frozen feet embedded in Tissue-Tec optimal cutting temperature (OCT) compound (Sakura, Staufen, Germany) were...
cut into 12-μm sections, transferred onto PEN-covered glass slides (PALM, Bernried, Germany), and immediately stored at −80°C. Sections were stained with cresyl violet (1% in H2O), followed by rehydration in 99, 96, and 70% ethanol, and dried at 37°C for 0.5 h.

Collection of keratinocytes by LMPC was performed with a laser microbeam microdissection system with laser pressure catapulting (PALM), as described previously (17). Approximately 1000 keratinocytes were excised. Harvested cells were collected in TRIzol reagent (Invitrogen, Darmstadt, Germany) until further processing of RNA.

RNA isolation, reverse transcription, and random-based PCR amplification

For total skin RNA extraction, skin from the footpad was excised and ground in liquid nitrogen. The lyophilizate was Potter homogenized in TRIzol reagent according to manufacturer’s instructions and further processed by RNeasy Minikit (Qiagen, Hilden, Germany) cleanup preparation including DNase digestion. The RNA from 1000 laser microdissected keratinocytes was TRIzol extracted, following the manufacturer’s protocol for small quantities of cells. RNA quality and quantity were determined using the microfluidics system (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA). Whole skin material was transcribed into cDNA utilizing RevertAid H-minus M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany), cDNA synthesis and amplification of laser microdissected samples were performed with the Microarray Target Amplification Kit (Roche Diagnostics, Mannheim, Germany), following the manufacturer’s instructions.

Reverse transcription PCR (RT-PCR) quantification of gene expression

Real-time RT-PCR was performed on a Prism T 7000 RT-PCR system (Applied Biosystems, Foster City, CA, USA) using the absolute qPCR SYBR Green Mix (ABgene; Thermo Fisher Scientific, Waltham, MA, USA). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Oligonucleotides used for amplification were for I-TAC, 5′-GGGCTCAAAACATGTGACATCC-3′ and 5′-TTGCTGATGTTCCAAAGACAG-3′; IL-12p40, 5′-CCTAAGTCTCATGACACCTTTGC-3′ and 5′-CCAAGTGCTAGAATATCTATGC-3′; and IL-12p70, 5′-AGGGCCAGAGAAAACTGAAAC-3′; IL-12p40, 5′-GAGCATCAACTGAGTTGGC-3′ and 5′-GCCAGGCGAGCCCTCTCAG-3′.

Cytokine detection

At 1, 2, and 4 wk after infection with L. major, mice were euthanized, and lymph nodes were aseptically removed. Single-cell suspensions were prepared by gentle teasing of the draining lymph node through a 50-μm mesh filter. Cluster of differentiation 4-positive (CD4+) T cells were generated as described previously (16). Briefly, cells were depleted of contaminating cells using magnetic assorted cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

DCs were differentiated in vitro from C57BL/6 bone marrow as described previously (16). DCs (1×10⁶ cells/ml) were incubated with soluble Leishmania antigen (SLA) equivalent to 5 × 10⁶ L. major for 48 h. SLA was prepared by 5 freeze/thaw cycles in PBS. For assessment of cytokine secretion, DCs and CD4+ T cells were mixed at a ratio of 1:5 and cultured in RPMI1640 plus 2 mM glutamine, 50 μM mercaptoethanol, and 10% FCS for 5 d.

For IL-12p70 detection, DCs were added to 96-well plates, incubated with 100 ng/ml I-TAC, L. major (ratio 1:5 DCs:L. major) in complete RPMI medium for 48 h. For detection of secreted IL-12p70 in lymph nodes, 10 individual lymph nodes were homogenized in 150 μl PBS, cells and debris were collected by centrifugation, and IL-12p70 was measured in the supernatants. IL-4, IL-12p70, and IFN-γ were assayed by cytometric bead array (CBA; BD Bioscience, San Jose, CA, USA) according to the manufacturer’s instructions.

RNA in situ hybridization (RISH), immunofluorescence (IF), and enzyme-linked immunosorbent assay (ELISA)

For RISH, a 1020-bp fragment corresponding to nt 68–1087 of the cDNA encoding murine I-TAC (mCxcl11; AF179872) was amplified by PCR and cloned into a pBluecript KSII+ plasmid (Stratagene, La Jolla, CA, USA). RISH was performed using frozen sections of 4 mm thickness and digoxigenin labeled probes according to a previously described protocol (18).

IF was performed as described previously (5), using polyclonal goat anti-mouse I-TAC (dilution 1:100), and donkey anti-goat IgG-FITC (dilution 1:400) purchased from Santa Cruz Biotechnology, using 5-μm-thick sections.

ELISA was performed using the mouse CXCL11/I-TAC DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions. Serum of infected and control mice was acquired 1 and 2 d after infection.

Statistical analysis

Data are presented as means ± sem, unless indicated otherwise. Statistical analysis was performed using unpaired 2-tailed Student’s t test. Differences were considered to be statistically significant at values of P < 0.05.

RESULTS

Early up-regulation of I-TAC in L. major-infected BALB/c mice

To identify immunomodulatory mediators that could determine early Th2 differentiation, we analyzed L. major-induced cytokine expression patterns 16 h after infection in the skin of susceptible BALB/c compared to resistant C57BL/6 mice using real-time PCR. We aimed to identify cytokines more strongly induced in susceptible, Th2-prone BALB/c mice using probes for cytokines directly or indirectly linked to a T-cell response (such as IL13, IL17, IL18, Ccl17, TSLP, and I-TAC).

The chemokine I-TAC was the only cytokine for which we could demonstrate a higher expression in BALB/c mice. It was originally only reported to attract activated Th1 cells (19). Early after infection, it was rapidly and significantly more strongly induced in the skin of BALB/c compared to C57BL/6 mice (Fig. 1A). IFN-γ is a well-known inducer of I-TAC expression. Thus, we locally treated susceptible and resistant mice with IFN-γ for 16 h. I-TAC mRNA was induced in both
strains, but less so in C57BL/6 (Fig. 1B). To detect the cellular source of I-TAC expression in BALB/c mice, we applied in situ hybridization. We found high levels of I-TAC transcripts in the epidermis of infected BALB/c (Fig. 1C, D), but not of C57BL/6 mice (data not shown). We also detected some I-TAC\textsuperscript{+/−} infiltrating cells; however, cells clearly discernible as keratinocytes were by far the major source of I-TAC mRNA (Fig. 1C, D). To verify epidermal I-TAC expression, we applied laser microdissection (Fig. 1E). Real-time PCR of microdissected keratinocytes clearly confirmed significantly stronger up-regulation of I-TAC in keratinocytes from BALB/c mice (Fig. 1F).

Immunofluorescence confirmed these results on the protein level (Fig. 2). Isotype and uninfected controls did not show any signal (data not shown).

We measured I-TAC in serum of infected and control C57BL/6 and BALB/c mice 1 and 2 d after infection using ELISA, but we were not able to detect I-TAC.

Local administration of I-TAC leads to a Th2 switch in resistant C57BL/6 mice

Since marked amounts of I-TAC were detected only in Th2-prone BALB/c, and not in C57BL/6 mice, it could be a critical component in early Th2 differentiation. This is why we infected BALB/c mice with L. major and treated them locally with various amounts of anti-I-TAC antibody in different treatment regimes. After 1 wk, we analyzed secretion of L. major-specific cytokines by CD4\textsuperscript{+} T cells from lesion-draining lymph nodes after in vitro restimulation with L. major-primed DCs. No observation that anti-I-TAC-antibody treatment had an effect on cytokine secretion of L. major-specific CD4\textsuperscript{+} T cells could be made (data not shown). The fact that neutralizing I-TAC did not change Th differentiation in BALB/c mice most likely reflects the absence of early skin-derived Th1-inducing tissue signals, as shown previously (9).
These results prompted us to investigate whether the opposite treatment, namely the application of I-TAC, would have any effect in a genetic resistant setting. Thus, we treated genetically Th1-prone C57BL/6 mice locally with $\frac{1}{H9262}$ g of recombinant I-TAC at the time of parasite inoculation and again 4 h later. Remarkably, CD4$^+$ T cells obtained from I-TAC-treated C57BL/6 mice showed a strongly increased, L. major-specific secretion of IL-4, while L. major-specific secretion of IFN-γ was reduced (Fig. 3). We also injected mice with 100 or 10 ng of I-TAC at the time of parasite inoculation and 4 h later and found that 200 ng of I-TAC resulted in a similar Th2 shift as seen for 2 μg of I-TAC, while treatment with 20 ng of I-TAC did not result in a clear alteration of Th1/2 differentiation [IFN-γ/IL-4 ratio as indicator for Th1/2 pattern: BALB/c 1.3, control C57BL/6 72, I-TAC-treated C57BL/6: 8.3 (2 μg), 12.2 (200 ng), and 64 (20 ng)]. Thus, despite the presence of Th1-inducing tissue signals in C57BL/6 mice, in I-TAC-treated C57BL/6 mice, the ratio between Th1 and Th2 cytokines was clearly skewed toward a Th2 response, as seen in BALB/c mice (Fig. 3).

I-TAC treatment results in increased susceptibility of C57BL/6 mice to L. major infection

Next, we analyzed whether the I-TAC-induced skewing toward a Th2 response would correlate with increased susceptibility to L. major infection. We treated C57BL/6 mice locally with I-TAC at the time of infection and 4 h later, reflecting the course of expression observed in susceptible BALB/c mice. Subsequently, we observed a marked increase in footpad swelling (Fig. 4A) and significantly higher parasite numbers compared to non-treated C57BL/6 mice in both draining lymph nodes (Fig. 4B) and footpads (Fig. 4C). These effects remained evident for up to 6 wk after infection. Spleens were devoid of parasites in both groups, indicating that no marked visceralization had occurred at that time (data not shown). When I-TAC-treated C57BL/6 mice were observed for prolonged periods of time, they finally resolved their lesions (data not shown). Thus, when applied exclusively on the first day of infection, I-TAC induces a temporary Th2 shift in genetically resistant C57BL/6 mice, which results in a deterioration of disease evident for up to 6 wk after parasite inoculation.

Involvement of receptors in vivo

The chemotactic effect of I-TAC on Th1 cells is mediated via CXCR3. However, it seems unlikely that this receptor is responsible for the effect of I-TAC in experimental leishmaniasis. In contrast to I-TAC, IP-10 and MIG, two other ligands of CXCR3, were more strongly induced in keratinocytes from resistant compared to susceptible mice during early experimental leishmaniasis, as we published previously (9). The high induction of CXCR3 agonists in both susceptible and resistant mice argues against a Th2-inducing effect of I-TAC via activation of CXCR3. Indeed, early local treatment of resistant mice with 1 μg IP10 at the time of infection and 4 h later did not influence the course of disease (Fig. 5) or Th1/Th2 differentiation (data not shown). Accordingly, local treatment of C57BL/6 mice with 1 μg of anti-CXCR3 antibody at the time of infection and 4 h later did not abolish deterioration of disease induced by treatment with 1 μg I-TAC at 0 and 4 h postinfection (Fig. 5) or the I-TAC-induced Th2 shift (data not shown). In summary, the effects of I-TAC during early experimental leishmaniasis are most likely not mediated via activation of CXCR3.

CXCR7 is another receptor that can be activated by I-TAC. To test whether activation of CXCR7 could be involved in mediating I-TAC effects during early exper-
imental leishmaniasis, we treated mice locally at the time of infection and 4 h later with 1 \mu g CXCR7 agonist VUF11207 and monitored the course of disease. However, local activation of CXCR7 during early experimental leishmaniasis did not result in a significant deterioration of disease.

In summary, we did not find evidence for a role of CXCR3 or CXCR7 in mediating the effect of I-TAC, indicating that other receptors could be involved.

I-TAC treatment inhibits Th1 development by suppressing IL-12 production in DCs

Since the effect of tissue signals, such as I-TAC, could be integrated and transferred to T cells via DCs, we analyzed the effects of I-TAC on DCs.

Bone marrow-derived DCs were incubated for 48 h with I-TAC, L. major, or I-TAC plus L. major, or were left unstimulated. No differences in cell-surface expression of MHC-II or costimulatory CD80 and CD86 in response to I-TAC stimulation were observed (data not shown). However, we found a significant suppressive effect of I-TAC on secretion of IL-12, the most important DC-derived signal for Th1 differentiation. Both basal and, more notably, L. major-induced IL-12p70 secretion were markedly reduced by I-TAC (Fig. 6A).

We then assessed whether IL-12 would also be suppressed in vivo in draining lymph nodes during the critical time for priming of T cells (20).

Using real-time PCR, we detected infection-dependent induction of IL-12p40 and IL-12p35 (Fig. 6B, C) transcripts in regional lymph nodes 7 h after infection.

Figure 3. I-TAC treatment on the first day of infection is sufficient to induce Th2 cytokine patterns. CD4+ cells were isolated from 3 popliteal lymph nodes of BALB/c, C57BL/6, and C57BL/6 mice treated with 1 \mu g I-TAC (0 and 4 h after infection) 1 wk after infection. CD4+ cells were incubated for 5 d with syngeneic dendritic cells stimulated for 48 h with SLA. Supernatants were harvested and measured for IL-4 (A) and IFN-\gamma (B) content by CBA technology. Data are means \pm sem of 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. I-TAC treatment on first day of infection causes aggravation of disease in Th1-prone C57BL/6 mice. A) Five mice per group were injected with 1 \mu g I-TAC (shaded circles) or PBS control (solid circles) 0 and 4 h after infection and footpad swelling compared to the contralateral footpad was monitored. Data from one of >5 similar experiments are shown. B, C) Effect of I-TAC on parasite containment. LDA from regional lymph nodes (B) and footpad lesions (C) 40 d after infection. Numbers of living parasites for single mice (5–6 mice/group; circles) and means (horizontal bars) are indicated. Results are shown from one of >5 similar experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
detected in the lymph nodes during the early phase of experimental leishmaniasis. Using sensitive assays, we succeeded in demonstrating IL-12p70 protein in regional lymph nodes early after infection, with a markedly reduced expression in I-TAC-treated compared to nontreated C57BL/6 mice (Fig. 6D). These data indicate that I-TAC actively suppresses Th1 cell development by down-regulating the most dominant Th1-modulating cytokine, IL-12.

**DISCUSSION**

A Th2-mediated immune response protects against helminths and other extracellular microbes, while it is also responsible for allergic and atopic disorders. While we are beginning to understand the mechanisms responsible for induction of Th1 cells in more detail, the signals directing early Th2 development are still enigmatic. There is increasing evidence that Th differentiation is decisively influenced by early signals generated at the site of primary infection. There are only a few reports that analyzed early expression of chemokines and cytokines in the skin during experimental leishmaniasis (22–26). We recently were able to identify early Th1-inducing tissue signals expressed by keratinocytes in L. major-infected skin of C57BL/6 mice (9).

When we analyzed differential L. major-induced cytokine expression in the skin 6 h after infection by virtue

**Figure 5.** No involvement of the activation of I-TAC receptors CXCR7 and CXCR3. Five C57BL/6 mice per group were injected locally with 1 μg CXCR7 agonist VUF11207, 1 μg CXCR3 ligand IP10, 1 μg I-TAC, and/or 1 μg CXCR3 blocking antibody 0 and 4 h after infection, and footpad swelling compared to the contralateral footpad was monitored. Data from one of 3 similar experiments are shown. *P < 0.05, **P < 0.01 vs. control group.

Transcription rates of IL-12p40 and IL-12p35 were significantly lower in I-TAC-treated compared to nontreated mice (Fig. 6B, C). However, these RNA data had to be confirmed by detection of IL-12 protein, since biological effects of the IL-12p70 heterodimer can be counterbalanced by high concentrations of the (IL-12p70)2 homodimer (21). Despite its well-established role in Th1 induction, IL-12 protein has never been

**Figure 6.** I-TAC-induced inhibition of Th1 development through suppressed IL-12 production in DCs. A) I-TAC suppresses IL-12 secretion in vitro. Bone marrow-derived DCs (1 \times 10^6) were incubated for 4 h in the presence of 100 ng I-TAC, 5 \times 10^6 L. major, or I-TAC + L. major, and supernatants were analyzed for IL-12p70 content by CBA. Data are means ± SEM of 3 independent experiments. *P < 0.05. B, C) I-TAC suppresses IL-12 secretion in vivo. IL-12p40 (B) and IL-12p35 (C) mRNA expression in draining lymph nodes (5 lymph nodes/experiment) was detected by real-time PCR analysis 7 h after s.c. infection with L. major. PCR data were normalized to GAPDH expression and mean n-fold regulation, and SEM of I-TAC-treated (1 μg 0 and 4 h after infection) in comparison to PBS injected controls was calculated. Data are means ± SEM of 3 independent experiments. D) Soluble IL-12p70 protein was measured in draining lymph nodes 8 h after infection with L. major. Lymph nodes (10/ experiment) were pooled and homogenized in 150 μl PBS. Supernatants (50 μl) were analyzed by CBA for detection of IL-12p70. Results are representative of one of 3 independent experiments. No detectable amounts of IL-12p70 were found in regional lymph nodes of mice injected with PBS instead of L. major (not shown). *P < 0.05, **P < 0.01.

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of microarray technology, we found only 4 genes more highly expressed in BALB/c than C57BL/6 mice, but none of them was an established cytokine (9). Therefore, we extended our analysis, using the more sensitive real-time PCR technique. We used probes for cytokines that were directly or indirectly linked to a T-cell response, such as IL-13, IL-17, IL-18, Ccl17, TSLP, and I-TAC.

I-TAC is the only cytokine for which we now demonstrate a significantly higher expression in BALB/c mice, making it strongly suggested as being a decisive factor in the fatal Th2-cell response.

I-TAC is a small cytokine belonging to the CXC chemokine family (CXCL11) that binds to CXCR3 and CXCR7 and is also a natural antagonist for CC chemokine receptor 3 (CCR3) and CCR5 (27–30). I-TAC was originally shown to attract activated Th1 cells (19). While the importance of I-TAC for T-cell migration is well-established (19, 31–34), an effect on Th-cell differentiation has not been described so far.

The distinctly higher induction of a cytokine associated with migration of Th1 cells was in the first instance astonishing. However, the effect of a given cytokine on Th1 vs. Th2 immunity crucially depends on timing and location of action. As such, the classical Th2 cytokine IL-4 has recently been identified as a tissue-derived signal that also induces Th1 differentiation via increasing secretion of IL-12 from DCs when expressed during a restricted crucial time span early in the immune response and in local proximity to DCs in which it increases secretion of IL-12 (9, 10).

We now demonstrate that I-TAC induces a shift toward a Th2 immune response. This is relevant, since it is associated with a long-lasting deterioration of disease. We thus identified the first Th2-promoting cytokine in experimental leishmaniasis.

It is generally accepted that DCs are the most potent generators of an antigen-specific Th-cell response. DCs provide naive Th cells with an Ag-specific and a co-stimulatory signal. In addition, DCs provide a so-called third signal that influences the initial polarization of naive Th cells to a preferential differentiation of specific Th-cell subsets (35, 36). IL-12 is the best characterized DC-derived signal that instructs Th1 differentiation. We previously demonstrated that IL-1 is another DC-derived signal that instructs Th1 differentiation in experimental leishmaniasis (3, 37). In contrast, DC-derived signals that induce Th2 differentiation are less well-established. It has been proposed that Th2 differentiation represents a default pathway implemented if costimulatory and Th1-inducing signals from DCs are weak or impaired (38). However, the Notch ligand jagged and OX40L on DCs have recently been identified as active promoters of Th2 differentiation in helminth infection and type 1 allergies (14, 15).

While we are beginning to understand how DCs can instruct Th1/Th2 differentiation, it is intriguing to see which upstream signals educate DCs to stimulate defined types of Th-cell responses (39). Besides pathogen-derived signals (40), it has been proposed that inflammation-associated tissue factors also affect DC maturation.

Recently, more experimental evidence has been given to support the hypothesis that tissue-derived signals can amplify and fine-tune the pathogen-specific adaptive immune response. While we have identified keratinocyte-derived Th1-inducing tissue signals (e.g., IL-4 and IL-6) expressed within a crucial time span in the early phase of experimental leishmaniasis (9, 10), histamine, TSLP, and prostaglandin E2 were reported to instruct a Th2-promoting DC subset (41–43). However, the kinetics of the expression of these Th2-associated signals in vivo had not been specifically addressed. Thus, it is not yet clear whether they represent the initial tissue signals to educate DCs for priming of Th2 cells.

In this study, we observed that I-TAC indeed represents an essential initial tissue signal, generated early by the epidermis, which instructs DCs for the priming of Th2 cells.

We hypothesize that this Th2-promoting effect of I-TAC is mediated by manipulating DC function, similarly as shown for IL-4. As such, I-TAC repressed L. major-induced IL-12 production from DCs in vitro. Similarly, inhibition of IL-12 secretion from DCs in vivo has been described for ligands of the chemokine receptor CCR2 (44). CCL2 has also been demonstrated to be involved in the generation of a Th2 response pattern in vivo. BALB/c mice that lack CCL2 show diminished Th2-cell response in vaccination experiments and become marginally resistant to Leishmania infection (45). However, the origin of CCL2 expression in vivo remains unclear, and the mechanisms responsible for increased resistance in the absence of CCL2 during L. major infection were not analyzed. For I-TAC, we could correlate reduced IL-12 levels in I-TAC-treated mice during the critical time frame of DC–T-cell interaction, as well as inhibition of IL-12 secretion in I-TAC-treated DCs in vitro, with promotion of Th2 development in I-TAC-treated C57BL/6 mice. A direct effect of locally administered I-TAC on C57BL/6 T cells cannot be ruled out but seems unlikely because of the time point of treatment preceding the development of a T-cell response and due to the fact that CXCR3 is only expressed on activated T cells (19). Also, treatment with CXCR3 antibody could not prevent I-TAC-induced disease deterioration, and CXCR3 agonist IP10 did not cause a similar effect. In BALB/c mice, this notion is further supported by the observation that Th1 cells of L. major infected BALB/c mice have a defect in up-regulating CXCR3 (46). Thus, we conclude that I-TAC acts as an early Th2-promoting tissue signal via the induction of a Th2-promoting DC subset.

We identified keratinocytes as the major source of I-TAC mRNA and thus verified its local production by resident tissue cells by applying microdissection, in situ-hybridization and immunofluorescence. This introduces the epidermis as a previously unrecognized early instructor of Th2-immunity in experimental leishmaniasis. Epithelial cells have recently gained attention as
an interface between innate and adaptive immunity especially in atopic disorders (47–49) where they are a source for TSLP in patients with atopic dermatitis (42). TSLP is also induced in epithelial cells by trauma and inflammation (50). It has been considered as one master switch in allergic inflammation (51) and may also be involved in inducing protective Th2-responses during intestinal helminth infections (52), similarly to epithelium-derived IL-33 and IL-25. However, an action of the latter on DCs has not been reported (53, 54), and these cytokines are probably not relevant in early experimental leishmaniasis, as we did not detect a differential induction of these cytokines in susceptible and resistant mouse strains.

The early epidermal expression of I-TAC correlates with Th2 polarization in BALB/c mice, thus adding strong evidence to the hypothesis that factors produced locally within the initially infected tissue are critical for the polarization of the emerging adaptive immune response. This hypothesis is confirmed by our finding that the application of I-TAC at d 1 of infection in resistant C57BL/6 mice instructs a shift toward a Th2 response pattern that leads to a sustained deterioration of disease.

Epithelial barriers represent the first line of defense against invading pathogens. As such, they can deliver the first signals in response to pathogens that are subsequently integrated by DCs. The following interaction of DCs with T cells results in a suitable adaptive immune response that ensures effective clearance of the infectious agent while maintaining or restoring tissue integrity. We demonstrated that the epidermal signal I-TAC can be induced by pathogens, such as L. major, which have no direct contact to the epithelial barrier. This indicates that the epithelium not only reacts directly to pathogens, but could be a critical, hitherto underestimated amplifier of local signaling networks evoked in response to the pathogen.

The question whether the response of keratinocytes to Leishmania infection depends on bystander functions of, for example, resident tissue macrophages, is currently under investigation.

While I-TAC administration resulted in a highly significant deterioration of disease, we did not find visceralization of pathogens to internal organs or progressive ulcerative footpad swelling as seen in genetically susceptible BALB/c mice, and I-TAC-treated C57BL/6 mice finally resolved their lesions. This is most likely due to the fact that I-TAC administration was not able to abolish the Th1-inducing action of cytokines like IL-12, IL-1β, or IL-4, which are strongly produced by keratinocytes in the early infected skin of C57BL/6 mice, as we previously demonstrated (9). Also, neutralization of I-TAC in the absence of strongly produced Th1-inducing tissue signals in BALB/c mice was not sufficient to induce resistance. This is in agreement with an integrative function of DCs as transducers of the local microenvironment into a tissue and pathogen-adapted specific Th-cell response. The relative hierarchy of Th1- and Th2-inducing tissue signals in experimental leishmaniasis has to be determined in future studies.

We also addressed the question of the molecular receptors that mediate the effect of I-TAC during early experimental leishmaniasis. We did not find any evidence that the activation of CXCR3 or CXCR7 is involved in I-TAC-induced deterioration of L. major infection. However, besides CXCR3 and CXCR7, I-TAC also has effects on CC receptors. Also, proteolytic fragments of I-TAC with antagonistic effects have been described (55). However, especially the last mechanism is difficult to address in vivo. The receptor mediating the effect of I-TAC in experimental leishmaniasis has to be addressed in further studies.

In summary, this is the first demonstration of I-TAC as a cytokine that promotes a Th2 response in experimental leishmaniasis and the finding that the epidermis of BALB/c mice generates an early, tissue-derived Th2-inducing signal after subcutaneous infection with L. major. I-TAC is produced exactly in the critical early time frame of DC–T-cell interaction and acts via suppression of IL-12. Hence, we established a connection between epidermal gene expression and Th2 differentiation in experimental leishmaniasis. If the Th2-inducing capacity of I-TAC is not restricted to L. major infection, but can also be established for other conditions, our findings could be of crucial clinical relevance for the treatment of other, noninfectious skin disorders with a predominant Th2 cytokine pattern, such as atopic dermatitis.

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