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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Disease Control in Cutaneous Leishmaniasis Is Independent of IL-22

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TO THE EDITOR

Cutaneous leishmaniasis is a parasitic disease caused by dermatotropic subspecies of *Leishmania*. The disease is endemic in several parts of the world with approximately 12 million people infected worldwide. In mice and man, healing and lifelong protection is mediated by IFNγ-producing $CD4^+$ Th1 and $CD8^+$ Tc1 cells, whereas Th2- and regulatory T-cell (Treg)–associated immune responses with high levels of IL-4 and IL-10 are associated with a non-healer phenotype (Sacks and Noben-Trauth, 2002; Kautz-Neu *et al.*, 2011). Recently, we and others showed that IL-17A contributes significantly to genetically determined disease susceptibility in BALB/c mice, whereas lower levels of IL-17A are detected in resistant C57BL/6 mice (Lopez Kostka *et al.*, 2009; Gonzalez-Lombana *et al.*, 2013). As a result, IL-17A-deficient BALB/c mice were protected from progressive disease, because, in wild types, IL-17A is responsible for maintaining persisting neutrophil infiltrates in BALB/c lesions associated with impaired wound repair and parasite killing, ultimately leading to parasite visceralization. In humans, IL-17A and nitric oxide release were negatively correlated in self-healing lesions exhibiting high nitric oxide and low IL-17A levels in *L. braziliensis* infections (de Assis Souza *et al.*, 2013). In addition, IL-17A was strongly associated with protection against Kala Azar (Pitta *et al.*, 2009). Overall, these first results demonstrated that, in addition to Th1/Th2 cells and Treg, Th17 cells are also relevant for protection against this important human pathogen.

Among the cytokines produced by Th17 cells, IL-22 is most prominent. Receptors to IL-22 are specifically expressed by epithelial cells. Also, overexpression of IL-22 has been demonstrated to initiate skin inflammation. In the present study, we addressed the role of IL-22 in experimental cutaneous leishmaniasis. First, murine experimental leishmaniasis was induced in resistant C57BL/6 mice and susceptible BALB/c mice using physiological low-

Abbreviations: DC, dendritic cell; LACK, Leishmania homolog of receptors for activated C kinase; LN, lymph node; Treg, regulatory T cell

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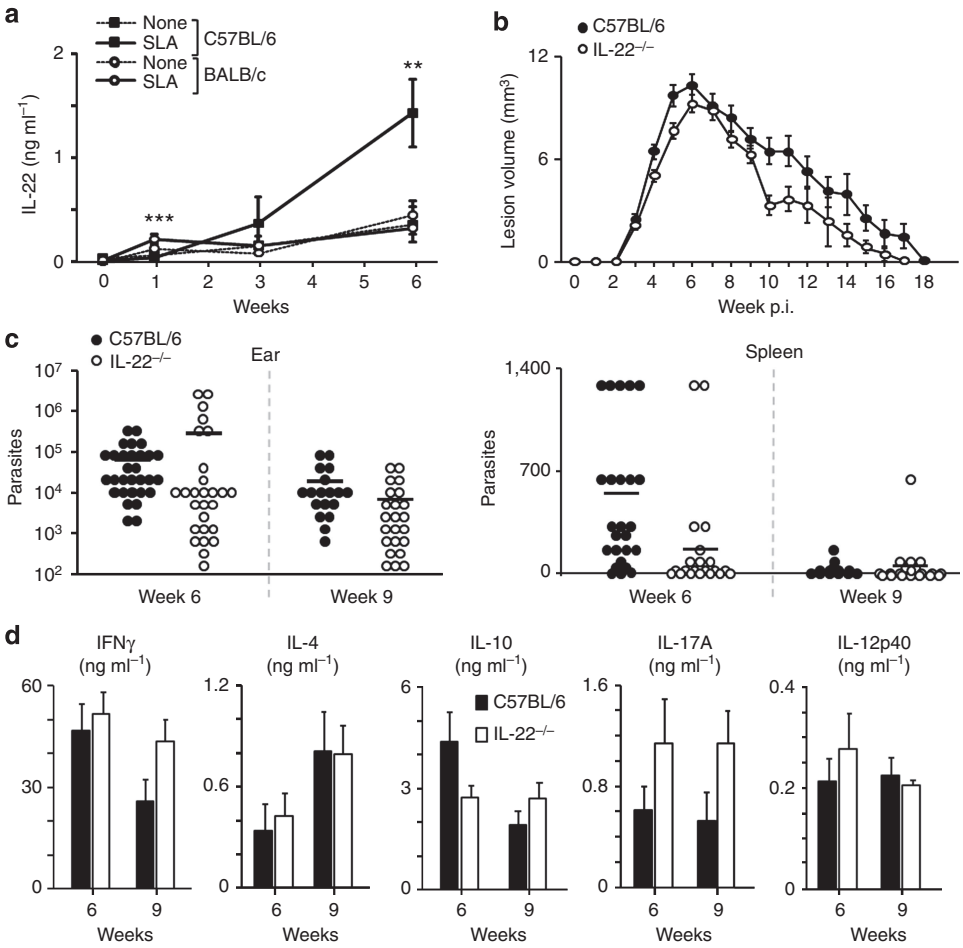

Figure 1. Antigen-dependent IL-22 production by T cells is not relevant for disease outcome in cutaneous leishmaniasis. Groups of five wild-type C57BL/6, C57BL/6 IL-22$^{-/-}$, or BALB/c mice were infected with 10^3 metacyclic promastigotes of *L. major*. (**a**) At weeks 0, 1, 3, and 6, draining lymph node cells were collected and restimulated at 1×10^6 cells ml^{-1} in the presence of soluble *Leishmania* antigen (SLA, 25 μg ml^{-1}). Data are presented as mean ± SEM ($n = 3$ independent experiments, ⩾10 mice per group, $^{**}P \leqslant 0.05$, $^{***}P \leqslant 0.002$). (**b**) Lesion development was monitored weekly and lesion sizes calculated in three dimensions as ellipsoid (mean ± SEM, $n \geqslant 13$ mice per group). (**c**) Parasite burdens of ear lesions and spleens were determined by the limiting dilution assay. One ear is represented by a dot; means are indicated as bars. (**d**) Draining lymph node cells of IL-22$^{-/-}$ and C57BL/6 control mice were collected at week 6 and week 9 post infection (p.i.) and restimulated as indicated in **a**. Data are presented as mean ± SEM.

dose inocula with metacyclic promastigotes of *L. major* (10^3 parasite *i.d.*) mimicking natural parasite transmission by sand flies (Belkaid *et al.*, 2000). In weeks 1, 3, and 6 post infection, draining lymph node (LN) cells were restimulated with soluble *Leishmania* lysate, and cytokine responses were determined in 48 hour supernatants. As expected, IFNγ levels were high in C57BL/6 supernatants, whereas an early IL-4 release from pre-primed, *Leishmania* homolog of receptors for activated C kinase (LACK)-reactive CD4$^+$ T cells together with high IL-17A production was detectable from BALB/c cells (data not shown and Sacks and Noben-Trauth, 2002; Lopez Kostka *et al.*, 2009). Interestingly, however, IL-22 release was significantly increased in supernatants of C57BL/6 cells restimulated with antigen, reaching highest levels at peak of lesion evolution (Figure 1a). Using C57BL/6 mice deficient for βγ T-cell receptors (TCRs), we identified αβ T cells as the main source for IL-22, whereas in mice lacking only γδ T cells (γTCR$^{-/-}$) IL-22 levels were unaffected (Supplementary Figure S1a online, and data not shown). In addition, isolated C57BL/6 CD4$^+$ T cells, but not CD8$^+$ T cells, produced high levels of IL-22 upon restimulation with *L. major*-infected DCs (Supplementary Figure S1b online). Induction of IL-22 production was not observed in BALB/c draining LN cells. Thus, IL-22 was predominantly detected in *Leishmania*-resistant mice suggesting differences in the Th17 compartment in these as compared with susceptible BALB/c mice.

To further address the physiological relevance of IL-22 in cutaneous leishmaniasis, low-dose infections with *L. major* were initiated in wild-type and IL-22-deficient C57BL/6 mice (Figure 1b–d). Lesion sizes were monitored over the course of 4 months. Interestingly, no obvious alteration of disease outcome was observed in IL-22$^{-/-}$ mice with regard to both lesion sizes and lesion evolution. Similar to wild-type C57BL/6 mice, lesions of

IL-22$^{-/-}$ mice healed within 4 months (Figure 1b); in addition, similar to wild types, IL-22-deficient mice were protected from lesion formation upon reinfection (data not shown), indicating no overt defects in the acute immune response as well as in the development of efficient memory responses against *L. major*.

Prior studies in other infectious settings observed a role for IL-22 in antimicrobial peptide induction in barrier organs (Sonnenberg *et al.*, 2010; Wolk *et al.*, 2010; Rubino *et al.*, 2012). We assessed parasite clearance at week 6 (peak disease) and week 9 (lesion resolution) post infection by measuring parasite burdens using limiting dilution assays. As shown in Figure 1c and in line with the lesion sizes measured, no alteration in parasite killing was detectable both for the number of lesional parasites in infected skin (left panel) and for the degree of parasitic dissemination into the spleen, which is a prominent feature of visceral leishmaniasis (right panel). Even though IL-22 does not directly signal to immune cells, it can initiate skin inflammation (Wolk *et al.*, 2011). We thus studied inflammatory cell infiltrates into lesions at week 6 and week 9 post infection using flow cytometry (data not shown). Lesions of IL-22-deficient mice harbored similar numbers of CD4$^+$ and CD8$^+$ T cells, neutrophils, macrophages, and antigen-presenting dendritic cells (DCs) as wild-type control mice.

Finally, antigen-specific cytokine responses in IL-22$^{-/-}$ mice were assessed at weeks 6 and 9 as shown in Figure 1d. As expected from lesion sizes and parasite burdens, high levels of IFNγ and low levels of IL-4 and IL-10 were found in supernatants from IL-22$^{-/-}$ and wild-type mice, indicating efficient priming of Th1/Tc1 cells capable of mediating protection. This was further substantiated by equivalent amounts of DC-derived IL-12p40 responsible for Th1/Tc1 priming (Wölbing *et al.*, 2006). Interestingly, however, elevated levels of IL-17A were found in IL-22$^{-/-}$ LN cultures, suggesting that, in the absence of IL-22, IL-17A is upregulated.

In summary, we observed that, in contrast to BALB/c mice, in which IL-17A is, at least to a substantial degree, responsible for susceptibility, resistant C57BL/6 mice harbor CD4$^+$ T cells capable of releasing IL-22, instead of IL-17A, upon antigen-specific restimulation. Our data suggest that cells of the adaptive immune system (αβ or γδ T cells) capable of responding to antigen-specific restimulation, instead of NK cells, innate lymphoid cells, or even other cells, are the primary producers of IL-22 in leishmaniasis (Zenewicz and Flavell, 2011). In line, in BALB/c mice, IL-17A and IL-22 production was downmodulated by anti-IL-23 (Ghosh *et al.*, 2013). Another study using BALB/c mice revealed that a plasmid-based vaccine comprising LACK and IL-22 was superior to plasmid alone by preferential induction of IFNγ (Hezarjaribi *et al.*, 2013). Thus, in BALB/c mice, the lack of relevant amounts of IL-22 may contribute to disease susceptibility via cytokine modulation. However, on a genetically resistant background best mimicking the situation in humans (Sacks and Noben-Trauth, 2002) using physiologically relevant experimental infections, IL-22 production does not appear to contribute to immunological parasite growth control or disease resistance against *L. major* despite its known function as a key player in antimicrobial defense, regeneration, and protection against damage (Wolk *et al.*, 2010). Our results add to those of Wilson *et al.* (2010), who showed that neutralization of IL-22 in a murine model of *M. tuberculosis* infection did not affect bacterial burdens of lungs, suggesting that control of intracellular pathogens is independent from IL-22.

In the future, additional studies on the role of other Th17 cell–derived IL-17 family members (e.g., IL-17F) for disease outcome in infections caused by the important human pathogen *Leishmania* need to be performed to fully clarify the contribution of this Th subset in infection control and its potential as a vaccine target.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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IL-17A Production in Human Psoriatic Blood and Lesions by CD146+ T Cells

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TO THE EDITOR

CD146, also called melanoma cell adhesion molecule, is a cell surface adhesion molecule on endothelial cells involved in homotypic and heterotypic cell interactions (Bardin *et al.*, 2001). CD146 binding in endothelial cells leads to a change in cellular permeability, actin distribution, and redistribution of NF-κB p50 to the nucleus. CD146 has been shown to be present on 1–3% of circulating peripheral blood T cells in healthy humans (Elshal *et al.*, 2005). CD146+ T cells have an effector memory phenotype, demonstrate upregulation of a cluster of genes involved with adhesion, migration, homing, and inflammation, and have enhanced binding to endothelial monolayers *in vitro* (Elshal *et al.*, 2007). These features of the CD146+ T cells in the peripheral circulation have led to the speculation that they represent a small pool of cells primed for extravasation and/or homing of activated T cells (Elshal *et al.*, 2007; Guezguez *et al.*, 2007) in response to inflammatory stimuli. Circulating CD146+ T cells are elevated in several inflammatory autoimmune diseases, such as sarcoidosis, inflammatory bowel disease, multiple sclerosis, connective tissue disease, and Behcet's disease, and produce IL-17 (Dagur *et al.*, 2010, 2011; Larochelle *et al.*, 2012). Whether these cells have a role at the site of active inflammation in these diseases remains unknown. Psoriasis, which is associated with increased vascular inflammation (Mehta *et al.*, 2011) and access to both peripheral blood and the disease target tissue (e.g., skin), is ideal to study CD146+ T cell phenotype and function in an inflammatory condition. Here we present findings from a well-characterized patient population with psoriasis using peripheral blood samples and skin biopsies from psoriatic lesions and uninvolved skin.

Forty-seven patients with psoriasis and sixty-seven healthy controls were included in this study. Diagnosis of psoriasis was confirmed by a dermatologist, and severity was measured by the percentage of body surface area involved and the validated Psoriasis Area and Severity Index. Donor demographics and characteristics are presented in Supplementary Table S1 online. Skin biopsies were isolated from a representative psoriatic target lesion (6 mm) and are identified as lesional psoriatic skin. Nonlesional skin biopsies were obtained from a similar body area at least 10 cm away from the nearest psoriasis skin lesion. Frozen sections were obtained from skin lesions for immunofluorescence studies, and all patients provided written consent as part of an IRB-approved study (NCT01778569).

Venous blood was collected in sodium heparin vacutainers (Becton Dickinson (BD), San Jose, CA). Cells were stained, and flow cytometric analysis was performed as previously described (Guezguez *et al.*, 2007). Skin biopsies were digested in Collagenase IV (GIBCO BRL no. 17104-019, Grand Island, NY) at 5 mg ml^{-1} in RPMI 1640 for 45 minutes, stained, and then sorted in the same manner as peripheral blood. The following antibodies used for staining were obtained from BD: CD3, CD4, CD8, CD33, CD14, CD19, CD45, CD45-RO, and CD146 (Clone P1H12). Anti-IL-17A (clone ebio64DEC17) was purchased from eBiosciences (Grand Island, NY). Immunophenotyping results are expressed as means and standard errors of the mean. RNA was isolated from sorted CD146+ or CD146− T cell subpopulations using RNAquos Micro kits (Ambion, Grand Island, NY), and quantitative real-time reverse-transcriptase–PCR was performed using a 7900-sequence detector (PE-Applied Biosystems, Norwalk, CT).

Data from a single specimen were considered for one experiment (*n*). A *P*-value <0.05 was considered statistically significant. Statistical analysis was performed using STATA version 12.0 (Stata, College Station, TX).

To determine whether CD146+ T cells are prevalent in patients with a Th17 disorder, immunophenotyping was performed on fresh peripheral blood from patients with psoriasis. Psoriasis patients showed a significant elevation of circulating CD3+CD146+ T cells compared with healthy adults (3.91 ± 0.37% vs. 2.96 ± 0.19%, respectively, $P=0.03$; Figure 1a). Increased CD146 expression reached statistical significance with the circulating CD4+ T cells (5.50 ± 0.413% in psoriasis vs. 3.55 ± 0.213%, respectively, $P<0.0001$; Figure 1b) but not the CD3+CD8+ CD146+ T cells (2.75 ± 0.373% in psoriasis vs. 2.30 ± 0.216%, respectively; Figure 1c). CD146+ T cells were abundant within lesional skin biopsies,

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