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Disease control in cutaneous leishmaniasis is independent of IL-22

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Cutaneous leishmaniasis is a parasitic disease caused by dermatotropic subspecies of *Leishmania*. The disease is endemic in several parts of the world with approx. 12 mio. people infected worldwide. In mice and man, healing and life-long 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 & Noben-Trauth, 2002; Kautz-Neu *et al*., 2011). Recently, we and others have shown 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 visceralisation. In humans, IL-17A and nitric oxide release were negatively correlated in self-healing lesions exhibiting high NO 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, also Th17 cells are 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 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 (SLA) and cytokine responses were determined in 48 hrs supernatants. As expected, IFNγ levels were high in C57BL/6 supernatants, whereas an early IL-4 release from pre-primed, *Leishmania* homologue 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 (Lopez Kostka *et al.,* 2009; Sacks and Noben-Trauth, 2002)). 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 (Fig. 1A). Using C57BL/6 mice deficient for βγ T cell receptors (TCR), we identified αβ T cells as main source for IL-22, whereas in mice lacking only γδ T cells (γ $TCR^{-/-}$), IL-22 levels were unaffected (Fig. S1a, 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 DC (Fig. S1b). 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 to 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 (Fig. 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 or lesion evolution. Similar to wild type C57BL/6 mice, lesions of IL-22$^{-/-}$ mice healed within 4 months (Fig. 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 (Wolk *et al*., 2010; Rubino *et al.,* 2012; Sonnenberg *et al*., 2010). 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 Fig. 1C and in line with the lesion sizes measured, no alteration of parasite killing was detectable both for the number of lesional parasites in infected skin (left panel) or the degree of parasite dissemination into 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 in week 6 and week 9 post infection using flow cytometry (data not shown). Lesions of IL-22-deficient mice harboured similar numbers of CD4$^+$ and CD8$^+$ T cells, neutrophils, macrophages and antigen-presenting dendritic cells (DC) as wild type control mice.

Finally, antigen-specific cytokine responses in IL-22$^{-/-}$ mice were assessed in weeks 6 and 9 as shown in Fig. 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$^{-/-}$ as well as 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 harbour 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 ($\alpha\beta$ or $\gamma\delta$ 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 down modulated by anti-IL-23 (Ghosh *et al*., 2013). Another study using BALB/c mice revealed that a plasmid-based vaccine comprised of 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 key player in anti-microbial defence, regeneration and protection against damage (Wolk *et al.,* 2010). Our results add to those of Wilson *et al.,* who showed that neutralization of IL-22 in a murine model of *M. tuberculosis* infection did not affect bacterial burdens of lungs (Wilson *et al.,* 2010), 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 with the important human pathogen *Leishmania* need to be performed to fully clarify the contribution of this Th subset for infection control and its potential as vaccine target.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legend

Figure 1: Antigen-dependent IL-22 production by T cells is not relevant for disease outcome in cutaneous leishmaniasis.

Groups of 5 wild type C57BL/6, C57BL/6 IL-22$^{-/-}$ or BALB/c mice were infected with 10^3 metacyclic promastigotes of *L. major*. **A**, In weeks 0, 1, 3, and 6, draining lymph node cells were harvested and restimulated at $1x10^6$ cells/ml in the presence of soluble *Leishmania* antigen (SLA, 25 µg/ml). Data are presented as mean±SEM (n= 3 independent experiments, ≥10 mice/group, **=p≤0.05, ***=p≤0.002). **B**, Lesion development was monitored weekly and lesion sizes calculated in 3 dimensions as ellipsoid (mean±SEM, n≥13 mice/group). **C**, Parasite burdens of ear lesions and spleens were determined by 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 harvested in week 6 and week 9 post infection and restimulated as indicated in (A). Data are presented as mean±SEM.