Targeting IL-27 and/or IL-10 in Experimental Murine Visceral Leishmaniasis

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Abstract. Interleukin-10 (IL-10) and interleukin-27 (IL-27) both exert counterregulatory immunodeactivation in visceral *Leishmania donovani* infection. We studied experimental *L. donovani* infection in the livers of IL-10^{-/-} and IL-27Rα^{-/-} mice and observed that in IL-27Rα^{-/-}, but not IL-10^{-/-} mice, interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) were required for heightened granulomatous inflammation and accelerated control of intracellular parasite replication. This difference in mechanism, along with residual IL-10 activity in IL-27Rα^{-/-} mice, suggested targeting IL-27 in addition to IL-10 in a macrophage-activating, anti-counterregulatory cytokine treatment strategy. In C57BL/6 wild-type mice with established liver infection, a single injection of anti-IL-27 p28 or anti-IL-10R monoclonal antibody enhanced granuloma assembly, enabled macrophage activation, and induced comparable parasite killing (49–56%). However, anti-IL-27 p28 plus anti-IL-10R combination treatment did not increase leishmanicidal effects. These results suggest that IL-27 and IL-10 may operate in a linked deactivating mechanism and that in this intracellular infection, either IL-27 or IL-10 is a suitable immunotherapeutic target.

In a model of visceral leishmaniasis (*Leishmania donovani* infection in the liver), IL-10-deficient mice and IL-10 receptor (IL-10R) blockade in wild-type (WT) mice were used to demonstrate IL-10's counterregulatory, macrophage-deactivating role and the therapeutic antileishmanial effects of targeting IL-10.^{1,2} In WT mice with established infection, injection of anti-IL-10R monoclonal antibody (MAb) enhanced T helper cell type 1 (Th1) cell-associated cytokine and tissue granulomatous responses, induced macrophage activation and intracellular parasite killing, and synergistically augmented the T cell-dependent efficacy of antileishmanial chemotherapy.^{2,3}

This report extends the analysis of anti-cytokine immunotherapy in the *L. donovani* model to IL-27, an additional counterregulatory cytokine.⁴ IL-27 induces IL-10 secretion,^{4–6} including in mononuclear cells from *Leishmania*-infected mice,^{7,8} and is already recognized as an immunodeactivating cytokine in experimental *L. donovani* infection.⁹ Whether this latter role in visceral infection primarily reflects induction of and effects produced by IL-10 or extends to separate mechanisms mediated by IL-27^{4,5,10,11} is unknown. Making observations suggesting that the two cytokines act differently in *L. donovani* infection would provide a rationale for therapeutically targeting IL-27 and IL-10.

C57BL/6 (B6) WT mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Breeding pairs of gene-deficient mice (B6 background) were obtained and bred at Weill Cornell Medical College: 1) (TLR2) Toll-like receptor 2^{-/-},¹² 2) IL-6^{-/-},¹³ 3) IL-10^{-/-} (Jackson), and 4) IL-27Rα^{-/-} (WSX-1^{-/-}) (Amgen, Thousand Oaks, CA).^{7,10} Seven- to 10-week-old female mice were injected via the tail vein with 1.5 × 10⁷ *L. donovani* amastigotes (LV9 strain).¹² Liver parasite burdens, determined microscopically using Giemsa-stained liver imprints, are expressed as Leishman–Donovan units (LDUs)—the number of amastigotes per 500 cell nuclei × organ weight (mg).¹² Differences between mean values were analyzed by a two-tailed Student's *t* test; *P*-value of < 0.05 was considered significant. The histologic response to infection was evaluated in liver sections stained with hematoxylin and

eosin; the granulomatous reaction at infected foci was considered as none, developing, or mature and/or parasite-free.¹² Supernatants of spleen cell cultures (5 × 10⁶ cells/mL stimulated for 48 hours with 30 μg/mL of soluble *L. donovani* antigen) were assayed for IFN-γ and IL-10 activities using ELISA kits from BD Biosciences Pharmingen (San Diego, CA) and BioLegend (San Diego, CA), respectively.¹⁴

Groups of 3–4 infected mice were injected IP with 0.2 mL of saline containing the following: 1) pentavalent antimony (sodium stibogluconate [Sb], Pentostam, Wellcome Foundation Ltd., London, United Kingdom) once 7 days after infection (day +7) using a suboptimal dose (50 mg/kg)¹⁴; 2) 0.25 mg of anti-IFN-γ (clone XMG1.2), anti-TNF (clone XT3.11), anti-IL-10R (clone 1B1.3A) MAb, or rat IgG (all from Bio X Cell, West Lebanon, NH) 2 hours after infection and on days +5 and +10; 3) 0.25 mg of anti-IL-10R MAb or rat IgG or 0.2 mg of polyclonal goat anti-IL-27 p28 Ab or goat IgG (R&D Systems, Minneapolis, MN) on day +14; or 4) 0.25 mg of anti-programmed death 1 (PD-1) (clone RMP1-14) or anti-programmed death-ligand 1 (PDL)-1 (clone 10F.9G2) MAb or rat IgG (all from Bio X Cell) on days +14 and +17. Liver parasite burdens were subsequently determined on the days indicated in the legends to the tables and figures. These studies were approved by the medical college's institutional Animal Care and Use Committee.

IL-27Rα^{-/-} and IL-10^{-/-} mice effectively controlled initial parasite replication at week 2 and rapidly reduced liver burdens by week 4 (Figure 1A); these effects, including the onset and extent of intracellular parasite killing, were more prominent in IL-10^{-/-} mice (Figure 1B). Compared to WT mice, IL-27Rα^{-/-} and IL-10^{-/-} mice also similarly displayed other enhanced responses to *L. donovani* infection: 1) rapid mononuclear inflammatory cell recruitment with widespread granuloma assembly at infected liver foci, clearly evident at week 2 and fully developed by week 4 (Figure 2, Supplemental Figure S1); 2) ~ 5-fold increases in IFN-γ secretion by antigen-restimulated spleen cells (Supplemental Table S1); and 3) > 10-fold increases in leishmanicidal activity following low-dose Sb chemotherapy (Supplemental Table S2). Compared to IL-10^{-/-} mice, livers of IL-27Rα^{-/-} mice showed appreciably greater, more diffuse inflammation early on (week 2) with scattered foci of tissue necrosis, as reported.⁹ However, necrotic lesions, not present in either IL-10^{-/-} or WT livers, resolved by week 4 at which time versus WT mice, IL-27Rα^{-/-} and IL-10^{-/-} mice similarly expressed more numerous and larger

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FIGURE 1. Course of *Leishmania donovani* infection in livers of wild-type (WT) and gene-deficient mice. For clarity, data for IL-10R^{-/-} and IL-27R^{-/-} mice in (A) are duplicated in (B) using an expanded vertical scale, and data for TLR2^{-/-} mice (not shown in [A]) are included. In one experiment carried out to week 8, Leishman-Donovan units (LDUs) were 133 ± 19, 2 ± 1, and 0 in WT, IL-10^{-/-}, and IL-27R^{-/-} mice, respectively (*n* = 4 mice per group). Results in (A) and (B), from 2 to 3 experiments, indicate mean ± standard error of the mean (SEM) values for 8–13 mice at each time point. *P* < 0.05 in (A) for all gene-deficient mice at all time points vs. WT LDU values and in (B) for IL-10^{-/-} vs. IL-27R^{-/-} mice at weeks 2 and 3.

mature parasite-free granulomas (Figure 2). By week 8 in WT and gene-deficient mice, most granulomas had involuted and inflammation had largely receded (not shown); thus, neither IL-27R α nor IL-10 was required by themselves to terminate initially exaggerated tissue inflammation.

In the experiments shown in Figure 1, IL-6^{-/-} and TLR2^{-/-} mice were challenged in parallel to compare kinetics of infection for two reasons: 1) both IL-6 and TLR2 also mediate counterregulatory, macrophage-deactivating effects in this *L. donovani* model,^{12,13} linked for TLR2 to IL-10,¹² and 2) both

FIGURE 2. Histologic responses to *Leishmania donovani* infection in livers of wild-type (WT), IL-10^{-/-}, and IL-27R^{-/-} mice. In WT mice, infected foci show intracellular parasites and inflammatory responses ranging from little or none (arrows) to developing granulomas at week 2 (A); at week 4 (B), WT granulomas are well-developed (mature) and some are coalesced, but few are parasite-free (arrows). In direct contrast at both weeks 2 and 4, IL-10^{-/-} (C and D) and IL-27R^{-/-} mice (E and F) show accentuated mononuclear cell inflammation (initially more diffuse in IL-27R^{-/-} mice [also see Supplemental Figure S1E]), accelerated granuloma assembly and maturation, and few discernible amastigotes. Original magnification, ×400. See Supplemental Figure S1 for corresponding low-power photomicrographs.

IL-6 and TLR2 signaling can also induce IL-27 and IL-10.^{5,12,15–18} Compared to IL-27R $\alpha^{-/-}$ and IL-10 $^{-/-}$ mice, enhanced inhibition of replication and extent of parasite killing were less well-expressed in IL-6 $^{-/-}$ mice (Figure 1A); however, in TLR2 $^{-/-}$ mice, the kinetics and outcome of infection were nearly superimposable (Figure 1B).

IL-10 was not detected in sera from 2- to 4-week infected WT or IL-27R $\alpha^{-/-}$ mice; therefore, antigen-restimulated spleen cells from infected mice were used to gauge IL-27's role in inducing IL-10 (Supplemental Table S1). IL-27R $\alpha^{-/-}$ cells generated less IL-10 in vitro at $\sim 40\%$ of the WT level; decreases in IL-10 production by cells from IL-6 $^{-/-}$ and TLR2 $^{-/-}$ mice were not significant ($P > 0.05$). Different results, however, were evident in an indirect in vivo bioassay based on anti-IL-10R mAb injection, in which further reduction in liver parasite burden was considered to reflect endogenous IL-10 activity.^{7,12} IL-10R blockade produced this result in IL-27R $\alpha^{-/-}$ (and IL-6 $^{-/-}$) mice proportionately similar to the result in WT mice, pointing to residual IL-10 effects (Supplemental Figure S2, see legend); by contrast, IL-10R blockade had no effect in TLR2 $^{-/-}$ mice, as reported.¹² Although not correlating with the in vitro results (Supplemental Table S1), the in vivo observations pointed to factors other than or in addition to IL-27R α (e.g., TLR2) in IL-10 secretion.

In *L. donovani*-infected IL-10 $^{-/-}$ and IL-27R $\alpha^{-/-}$ mice, Th1 cell-type effector cytokines appear to be responsible for both the exaggerated tissue inflammatory reaction and enhanced intracellular antileishmanial activity—IL-12 and, to a noted lesser extent, IFN- γ in IL-10 $^{-/-}$ mice¹ and IFN- γ and TNF in IL-27R $\alpha^{-/-}$ mice.⁹ Our results, using injections of anti-IFN- γ or anti-TNF, demonstrated clear-cut differences in the roles of these two endogenous cytokines in gene-deficient mice (Figure 3). In IL-27R $\alpha^{-/-}$ mice and with comparable effects, both MAbs abolished control over parasite replication and

markedly reduced liver inflammation (Supplemental Figure S3). This result suggested IFN- γ and TNF acting linearly in the same inflammatory mechanism released by an absent IL-27 effect. By contrast, in IL-10 $^{-/-}$ mice, anti-IFN- γ and anti-TNF had comparatively little effect on either host response (Figure 3 and not shown). This observation in IL-10 $^{-/-}$ mice may, in part, reflect an auxiliary IFN- γ -independent mechanism regulated by IL-10 in this model which may involve IL-12^{1,3}; IL-12 also appears to be active in inflammatory effects in IL-27R $\alpha^{-/-}$ mice in other settings.¹⁹ In addition, it is possible that enhanced sensitivity or activation of innate pattern recognition responses in IL-10 $^{-/-}$ mice²⁰ is also involved.

To complete this analysis which was limited to the liver and did not include other parasitized tissues (e.g., spleen and bone marrow), WT mice were injected with anti-IL-27 and/or anti-IL-10R MAb to test two hypotheses generated from the preceding results. First, anti-IL-27 treatment would by itself enable macrophage activation, produce parasite killing, and promote the tissue inflammatory response. And second, in view of residual IL-10 activity in IL-27R $\alpha^{-/-}$ mice (Supplemental Figure S2) and certain differences between IL-27R $\alpha^{-/-}$ and IL-10 $^{-/-}$ mice (Figures 1B and 3), injecting both anti-IL-27 and anti-IL-10R together would produce enhanced effects. The first hypothesis proved correct (Table 1, Supplemental Figure S4). However, combination MAb treatment produced no additional leishmanicidal effect (Table 1), suggesting that IL-10 and IL-27 may well be components of the same or a related counterregulatory mechanism in this model. Nevertheless, such a mechanism is clearly complex, given the differing roles of two key macrophage activators, IFN- γ and TNF, in the effects regulated by IL-27 and IL-10 (Figure 3). One such effect shared by IL-27 and IL-10 is induction of the inhibitory regulator, PDL-1.^{4,21,22} Expression of PD1/PDL-1 is also provoked in *L. donovani* infection (data not shown),^{23,24} and anti-PDL-1

FIGURE 3. Effect of IFN- γ or TNF neutralization in IL-27R $\alpha^{-/-}$ and IL-10 $^{-/-}$ mice. Mice were injected with control IgG, anti-IFN- γ , or anti-TNF 2 hours after infection and on days +5 and +10, and liver parasite burdens (LDUs) were determined on day +14. Results (two experiments) indicate mean $\pm$ SEM values for 7–8 mice per group. In one experiment (not shown), treatment of IL-10 $^{-/-}$ mice ($n = 4$) 2 hours after infection and on days +5 and +10 with both anti-IFN- γ and anti-TNF (given 1 hour apart) produced no additional effect. * $P < 0.05$ vs. rat IgG-treated controls. See Supplemental Figure S3 for corresponding histologic results in IL-27R $\alpha^{-/-}$ mice.

TABLE 1
Effect of anti-IL-10R and anti-IL-27 p28 treatment*

Treatment	Liver parasite burden (LDU) %		Killing
	Day 14	Day 21	
None	2,487 ± 282	2,884 ± 240	0
A. Control IgG	–	2,955 ± 325	0
Anti-IL-10R	–	1,101 ± 99†	56
B. Control IgG	–	2,803 ± 333	0
Anti-IL-27 p28	–	1,275 ± 148†	49
C. Control IgGs	–	2,756 ± 301	0
Anti-IL-10R + anti-IL-27p28	–	1,003 ± 121†	60

* Two weeks after infection (day +14), wild-type mice received a single IP injection of rat IgG or anti-IL-10R (A), goat IgG, or anti-IL-27 p28 (B), or, spaced 1 hour apart, IP injections of rat and then goat IgG or anti-IL-10R and then anti-IL-27 p28 (C). Day +21 LDUs were compared with day +14 LDUs in untreated mice to determine parasite killing (% reduction in LDUs on day +21). Results (mean ± SEM values) are from two experiments (7–8 mice per group per time point). See corresponding histologic responses in Supplemental Figure S4.

† $P < 0.05$ vs. day 14 result.

treatment appears to induce leishmanistatic effects in both the spleen and liver.²³ In our hands, however, injections of anti-PDL-1 and/or anti-PD-1 MAb in WT mice had little effect on liver parasite burden (Supplemental Table S3), suggesting no meaningful role for either regulator in established infection in the liver. This result contrasted directly with the leishmanicidal effect in the liver of anti-IL-27 p28 or anti-IL-10R treatment (Table 1).

Together, then, these observations suggest that IL-10 and IL-27 are both suitable antileishmanial targets for blockade or neutralization in the *L. donovani*-infected liver. More work in characterizing any potential immunopathology associated with therapeutically targeting IL-27 p28 should also be performed. The prompt induction of appreciable intracellular parasite killing by a single injection of anti-IL-10R or anti-IL-27 p28 MAb supports the still-attractive notion of an anti-cytokine/macrophage-activating treatment strategy,²⁵ logically used in conjunction with chemotherapy.

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