Cutting Edge: Inhibition of IL-6 Trans-Signaling Protects from Malaria-Induced Lethality in Mice

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Circulating IL-6 levels correlate with the severity of blood-stage malaria in humans and mouse models, but the impact of IL-6 classic signaling through membrane IL-6Rα, as well as IL-6 trans-signaling through soluble IL-6Rα, on the outcome of malaria has remained unknown. In this study, we created IL-6Rα−deficient mice that exhibit a 50% survival of otherwise lethal blood-stage malaria of the genus *Plasmodium chabaudi*. Inducing IL-6 trans-signaling by injection of mouse recombinant soluble IL-6Rα in IL-6Rα−deficient mice restores the lethal outcome to malaria infection. In contrast, inhibition of IL-6 trans-signaling via injection of recombinant sGP130Fc protein in control mice results in a 40% survival rate. Our data demonstrate that IL-6 trans-signaling, rather than classic IL-6 signaling, contributes to malaria-induced lethality in mice, preceded by an increased inflammatory response. Therefore, inhibition of IL-6 trans-signaling may serve as a novel promising therapeutic basis to combat malaria. The *Journal of Immunology*, 2012, 188: 000–000.

Malaria remains a major health problem (1). All efforts to develop an antimalaria vaccine during the last 30 y have failed (2). This failure is particularly astonishing, because natural immunity against the bloodstages of the infectious agents, protozoan parasites of the genus *Plasmodium*, can be acquired, although only slowly, after repetitive infections. Formation and/or efficacy of protective mechanisms are apparently impared by not-yet-understood parasite-induced host responses, which contribute to malaria morbidity and mortality (3). Presumably included among these host responses are those that are controlled by the pleiotropic cytokine IL-6, with its pro- and anti-inflammatory activities (4). Circulating IL-6 levels are increased in patients suffering from malaria caused by *P. falciparum* and *P. vivax* (5–8), which is often associated with polyclonal B cell activation (9). Conversely, decreasing IL-6 levels were described after antimalarial treatment (10) and are associated with decreasing hyperpyrexia (11) and decreasing parasitemia (12).

The mode of IL-6 action is complex (4, 13, 14). IL-6 signals through the specificity-defining membrane IL-6Rα, which requires the recruitment of two chains of the membrane receptor GP130 for signal transduction to activate the JAK/STAT pathway. This IL-6 classic signaling is restricted to those cells that express IL-6Rα on their surface (i.e., hepatocytes and immune cells). Nevertheless, IL-6 is also able to engage an alternative pathway through the naturally occurring soluble IL-6Rα (sIL-6Rα), which is derived by shedding of the ectodomain of membrane IL-6Rα and by alternative mRNA splicing. The IL-6/sIL-6Rα complex can communicate with all cells by binding to the ubiquitously expressed membrane GP130, thus initiating the so-called “IL-6 trans-signaling process” (13, 14). sIL-6Rα levels may also maintain the half-life of effective IL-6, as demonstrated previously (15). However, IL-6 trans-signaling can be inhibited when soluble GP130 (sGP130) binds and, thereby, inactivates the IL-6/sIL-6Rα complex (16). In this study, we show that IL-6 trans-signaling contributes to malaria-induced lethality.

**Materials and Methods**

**Generation of IL-6Rα−deficient mice**

Generation of IL-6Rαfloxed mice was done, as described previously (17). Briefly, we flanked exons 2 and 3 of the IL-6Rα gene by loxP sites using standard gene-targeting techniques in C57/BL-6–derived embryonic stem cells. To generate IL-6Rα−deficient (knockout; KO) mice (IL-6RαKO mice), IL-6Rαfloxed mice were crossed with deleter-cre mice, and offspring were intercrossed. IL-6Rα deficiency in liver parenchymal cells (IL-6Rα−K/L-KO mice) was generated, as described previously (17). Myeloid lineage-specific IL-6Rα−deficient mice (IL-6Rα−Mice) were created by crossing IL-6Rαfloxed mice with LysM-cre mice and intercrossing offspring.
livers were genotyped using oligonucleotides 5'CCG-GCC-GGC-ATC-GCC-TAG-G-3', 5'-CCA-GAG-GAG-CCC-AAG-TC-3', and 5'-TAG-GGC-CCA-GTT-CCT-TTA-T-3' in a standard PCR reaction.

Southern blot
Tail DNA was isolated from IL-6RxKO and IL-6RxFL mice and digested with BglI with the probe that was amplified using primers 5'probeB (5'-TTG-TAG-TCT-CAT-CCA-GAG-GCC-GC-3') and 3'probeB (5'-AGC-TGG-GCA-CTT-CCT-GAG-AC-3').

Western blot
Western blot analysis of protein lysates from indicated tissues and macrophages was performed using anti-IL-6Rα (#sc-660; Santa Cruz), anti-pSTAT3 (#9272 and #9145; Cell Signaling), anti-pAKT (#4056; Cell Signaling), anti-pERK (#4377; Cell Signaling), anti-calnexin (#208880; Calbiochem), and anti-β-actin (#A5441) Abs as primary Abs. Secondary anti-rabbit and mouse Abs were coupled to HRP, and detection was performed using ECL solutions (Pierce).

Flow cytometry
Single-cell suspensions were isolated from secondary lymphoid organs of IL-6RxFL control and IL-6RxKO mice by standard techniques and examined for TCRβ+ T cells and CD19+ B cells (BD Biosciences).

Malaria infections
Mice were kept under specific pathogen-free conditions and received water and food ad libitum. The experiments were approved by the state authorities. Only female mice aged 10–14 wk were used in the experiments. Challenge was i.p. with 107 erythrocytes parasitized with virulent P. chabaudi as previously (18, 19).

Injections with sIL-6Rx and sGP130Fc
Commercially available mouse recombinant sIL-6Rα protein and sGP130Fc protein (R&D Systems, Minneapolis, MN) were diluted in PBS. Final concentrations of 1 μg sIL-6Rx were injected i.p. into IL-6RxKO mice and different doses of sGP130Fc were injected into IL-6RxKO mice during infection with P. chabaudi malaria on days 1, 4, and 7 postinfection (p.i.).

ELISA for sIL-6Rx, IL-6, IL-1β, and TNF-α
sIL-6Rx, IL-6, IL-1β, and TNF-α were determined in sera using commercially available ELISA kits (R&D Systems), according to the manufacturer’s manual.

Northern blot
Total RNA (20 μg) was glyoxylated, separated in agarose gels, transferred to positively charged Biodyne/PPLUS nylon membrane (Pall, Pensacola, FL), and subjected to Northern blot analysis, as detailed previously (19).

Ex vivo experiments
Macrophages were isolated from IL-6RxFL, IL-6RxKO, and IL-6RxMyel-KO mice, as described previously (20), and stimulated in vitro with 25 ng/ml IL-6. Hepatocytes were isolated from IL-6RxFL- and IL-6RxKO mice, as detailed previously (21). A total of 105 ex vivo macrophages and hepatocytes was stimulated in vitro with 25 ng/ml IL-6, 25 ng/ml IL-6 plus 200 ng/ml sIL-6Rx, or 10 ng LPS for 4 h. mRNA expression for IL-1β, TNF-α, and IL-6 was quantified by quantitative PCR analysis (20).

Results and Discussion
To address the role of IL-6 signaling in malaria, we decided to investigate experimental P. chabaudi malaria in the mouse, which shares several characteristics with P. falciparum, which causes the most dangerous form of human malaria (22). First, we created an IL-6Rx-deficient mouse strain on the C57BL/6 background (Supplemental Fig. 1A–D). These IL-6RxKO mice did not exhibit any apparent phenotype. In particular, the immune system did not appear to be grossly affected, as indicated by similar numbers of CD19+ B cells and TCRβ+ T cells in major immune organs (Supplemental Fig. 1E). Moreover, we also created IL-6Rx deficiency specifically in liver parenchymal cells (IL-6RxL-KO mice) (17) and in the myeloid lineage (IL-6RxMyel-KO mice). Macrophages and neutrophils in IL-6RxMyel-KO mice lack IL-6Rx (Supplemental Fig. 1F), which, upon IL-6 stimulation in vitro, were unable to activate STAT3 (Supplemental Fig. 1G).

Surprisingly, infection with blood-stage P. chabaudi malaria took a lethal course in IL-6RxFL control mice (Fig. 1A), IL-6RxMyel-KO mice (Supplemental Fig. 1H), and IL-6RxL-KO mice (Supplemental Fig. 1I), whereas 50% of IL-6RxKO mice were able to self-heal and, thus, survive the infection (Fig. 1B). The precrisis phase of infection was similar, reaching peak parasitemia on day 8 p.i. Mice succumbed to infection during the subsequent crisis phase characterized by dramatically decreasing parasitemia (Fig. 1B). P. chabaudi-induced lethality is known to result from multiple organ failure that varies in individual mice (18). Control IL-6RxFL mice exhibited a continuous increase in circulating sIL-6Rα during the precrisis phase, from 16 to 190 ng/ml on day 8 p.i., whereas sIL-6Rx was absent in IL-6RxKO mice (Fig. 1C). The infection-induced increase in circulating IL-6 followed a biphasic pattern in both control and IL-6RxKO mice, with the first peak on day 1 p.i. and the second peak on day 8 p.i. (Fig. 1D), and the latter being higher in IL-6RxKO mice.

FIGURE 1. IL-6Rx deficiency protects from P. chabaudi malaria-induced lethality. Outcome and course of blood-stage infections after challenge with 105 P. chabaudi-infected erythrocytes. IL-6RxFL mice (n = 20) succumb to infection (A), whereas 50% of IL-6RxKO mice (n = 14) are able to self-heal the same infections (B). Circulating levels of sIL-6Rx (C), IL-6 (D), IL-1β (E), and TNF-α (F) during infection (n = 5–6). Northern blot analysis of hepatic mRNA expression of serum amyloid A3 (G) and C-reactive protein (H) in IL-6RxFL and IL-6RxKO mice during P. chabaudi infection. *p < 0.01, control versus KO mice, two-way ANOVA with Dunnett and Bonferroni post hoc tests (SPSS version 17.0).
Similarly, the proinflammatory cytokines IL-1β and TNF-α exhibited a biphasic increase in IL-6Rα<sup>FL</sup> control mice during infection (Fig. 1E, 1F), whereas IL-6Rα<sup>KO</sup> mice had lower IL-1β and TNF-α on day 8 p.i. In the liver, IL-1β and TNF-α are known to induce acute-phase and other innate responses (13). Indeed, the hepatic expression of two acute-phase proteins, serum amyloid A3 and C-reactive protein, was lower in IL-6Rα<sup>KO</sup> mice than in IL-6Rα<sup>FL</sup> control mice on day 8 p.i. (Fig. 1G, 1H). Importantly, the inflammatory response in IL-6Rα<sup>Mycel-KO</sup> mice and IL-6Rα<sup>L-KO</sup> mice on day 8 p.i. (Supplemental Fig. 1J, 1K) was intermediate to that in IL-6Rα<sup>FL</sup> mice and IL-6Rα<sup>KO</sup> mice, indicating that only complete IL-6Rα deficiency effectively reduced inflammation.

Approximately 70% of circulating IL-6 was estimated to bind to sIL-6Rα (16), and the IL-6/sIL-6Rα complex is known to induce IL-6 trans-signaling in all cells via membrane-bound GP130. Therefore, it is conceivable that a lethal outcome of malaria is due to the <i>P. chabaudi</i>-induced increase in circulating endogenous sIL-6Rα in control IL-6Rα<sup>FL</sup> mice causing increased IL-6 trans-signaling. To examine this hypothesis, we next aimed at restoring IL-6 trans-signaling by injecting mouse recombinant sIL-6Rα into IL-6Rα<sup>KO</sup> mice. Injections of 1 μg sIL-6Rα on days 1, 4, and 7 p.i. caused an increase in circulating sIL-6Rα from undetectable levels to 70 ng/ml on day 8 p.i. (Fig. 2A) and a concomitant decrease in circulating IL-6 from 600 to 110 pg/ml (Fig. 2B). These data suggest the occurrence of IL-6 trans-signaling that was also evidenced by increased phosphorylation of hepatic STAT3, a downstream target of IL-6 signaling (Fig. 2C). Control IL-6Rα<sup>FL</sup> mice and IL-6Rα<sup>KO</sup> mice did not exhibit any hepatic STAT3 phosphorylation at the steady state. At peak parasitemia, the control mice displayed greater STAT3 phosphorylation than did IL-6Rα<sup>KO</sup> mice (Fig. 2C). STAT3 phosphorylation was activated in response to IL-6 signaling induced by malaria, as well as by other factors (e.g., other GP130-acting cytokines or IFNs and IL-10) (4, 13). Remarkably, however, a much stronger STAT3...
phosphorylation occurred upon injection of sIL-6R into IL-6KO mice (Fig. 2C), thus demonstrating the induction of IL-6 trans-signaling by recombinant sIL-6R. Importantly, all IL-6REKO mice injected with sIL-6R succumbed to malaria during the crisis phase (Fig. 2D). This was associated with an increased inflammatory response to P. chabaudi malaria, as indicated by increasing levels of IL-1β and TNF-α in sIL-6RE–injected IL-6REKO mice that were similar to those found in control IL-6REFL mice on day 8 p.i. (Fig. 2E, 2F). Notably, myeloid lineage cells, rather than nonimmune cells, were affected by IL-6 trans-signaling that induced inflammatory cytokine expression in ex vivo-isolated macrophages but not in primary hepatocytes (Supplemental Fig. 1L, 1M).

IL-6 trans-signaling can be inhibited by sGP130, which is derived by shedding of the ectodomain from membrane GP130 and alternative splicing (13, 16). This sGP130 is able to bind the IL-6/sIL-6R complex, thus preventing IL-6 trans-signaling (16). To further substantiate a critical role for IL-6 trans-signaling in the outcome of the malaria, we also attempted to reduce IL-6 trans-signaling in the IL-6RE–expressing control mice. When control IL-6REFL mice were injected with mouse recombinant sGP130Fc protein (16, 8, and 8 μg) during infection on days 1, 4, and 7 p.i., respectively, endogenous sIL-6R decreased from 200 to 35 ng/ml (Fig. 2G), and concentrations of IL-6 decreased from 350 to 100 pg/ml on day 8 p.i. (Fig. 2H). IL-6 trans-signaling was indeed inhibited, as evidenced by dramatically lowered STAT3 phosphorylation (Fig. 2C), whereas the MAPK/ERK- and IL-6Rα levels also increased with the severity of human malaria. Although malaria.

### References


### Disclosures

The authors have no financial conflicts of interest.
SUPPLEMENTAL FIGURE 1. Generation of IL-6Rα-deficient mice. (A) The exons 2 and 3 of the IL-6Rα gene were flanked by loxP-sites to create the IL-6Rα^{FL} allele, as detailed recently (19). Mice carrying the IL-6Rα^{FL} allele were crossed with deleter-Cre mice and further F1 intercrossed to yield knock out (KO) mice termed IL-6Rα^{KO} on a C57BL/6 background. (B) KO was verified by Southern blot analysis of BglI-digested DNA (us = unspecific band). This analysis revealed the 6.3 kb FL allele using the radioactively labeled probe, while the Cre-mediated recombination of the loxP-flanked exons 2 and 3 resulted in the deleted 4.3 kb allele (19). (C) PCR and (D) Western blot analysis using antibodies against IL-6Rα and AKT. (E) Flow cytometric analysis of B- and T-cells in different lymphoid organs of IL-6Rα^{FL} mice and IL-6Rα^{KO} mice. Single cell suspensions of spleens, mesenterial lymph nodes (LN) and axial lymph nodes (LN) were labeled with anti-CD19 and anti-TCR-β monoclonal antibodies coupled to PE and APC, respectively, and measured in a FACS calibur. FACS dotblots are representative for three different mice and numbers indicate percentage of total cells. (F) Western blot analysis of macrophages (upper) and liver lysates (lower) obtained from IL-6Rα^{FL} mice and IL-6Rα^{Myel-KO} mice using antibodies against IL-6Rα and β-Actin. (G) Western blot of macrophages stimulated in vitro with IL-6 for different periods using p-STAT3 and β–Actin antibodies. Outcome of *P. chabaudi* malaria in (H) IL-6Rα^{Myel-KO} mice (n=5) and (I) IL-6Rα^{L-KO} mice (n=5). Levels of (J) IL-1β and (K) TNFα in IL-6Rα^{FL}, IL-6Rα^{Myel-KO}, and IL-6Rα^{L-KO} mice on days 0 and 8 p.i. (n=5). * indicates significant difference (p < 0.01) between IL-6Rα^{FL} vs. IL-6Rα^{Myel-KO} or IL-6Rα^{L-KO} mice. (L) Ex vivo macrophages
and (M) hepatocytes were isolated from IL-6Rα^FL and IL-6Rα^KO mice and plated at a density of 10^6/well. Subsequently, cells were stimulated \textit{in vitro} with 10 ng/ml IL-6, 10 ng/ml IL-6 plus 200 ng/ml sIL-6Rα or 10 ng/ml LPS and mRNA expressions of IL-1β, TNFα, and IL-6 were determined by qPCR analysis. * indicates significant difference (p < 0.01) between control vs. treated macrophages and hepatocytes, respectively. § indicates significant difference (p < 0.01) between IL-6Rα^FL vs. IL-6Rα^KO.

**SUPPLEMENTAL FIGURE 2.** Effects of recombinant sGP130Fc injected into IL-6Rα^FL mice. Western blot analyses of liver lysates from IL-6Rα^FL and IL-6Rα^KO mice infected with \textit{P. chabaudi} and injected with sIL-6Rα and sGP130Fc protein, respectively using (A) p-MAPK/ERK and (B) p-AKT antibodies. Calnexin; loading control. Outcome of \textit{P. chabaudi} malaria in IL-6Rα^FL mice injected (C) with 1 μg (on day 1 p.i.), 1 μg (on day 4 p.i.), and 1 μg (on day 7 p.i.) sGP130Fc, (D) with 4 μg (on day 1 p.i.), 2 μg (on day 4 p.i.), and 2 μg (on day 7 p.i.) sGP130Fc, (E) with 8 μg (on day 1 p.i.), 4 μg (on day 4 p.i.), and 4 μg (on day 7 p.i.) sGP130Fc, and (F) with 32 μg (on day 1 p.i.), 16 μg (on day 4 p.i.), and 16 μg (on day 7 p.i.) sGP130Fc (n=5-8). (G) Plasma levels of sIL-6Rα were determined on day 8 p.i. in IL-6Rα^FL mice injected with indicated concentrations on days 1, 4, and 7 p.i. (n=3). † indicates significant difference (p < 0.01) between non-injected IL-6Rα^FL vs. IL-6Rα^FL injected with 8/4/4 μg sGP130Fc. § indicates significant difference (p < 0.01) between IL-6Rα^FL injected with 32/16/16 μg sGP130Fc vs. IL-6Rα^FL mice injected with 8/4/4 μg sGP130Fc.