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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: 4141–4144.

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 impaired 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-6Rα floxed 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-6Rα KO mice), IL-6Rα floxed mice were crossed with deleter-cre mice, and offspring were intercrossed. IL-6Rα deficiency in liver parenchymal cells (IL-6Rα LKO mice) was generated, as described previously (17). Myeloid lineage-specific IL-6Rα-deficient mice (IL-6Rα M−/−KO mice) were created by crossing IL-6Rα−/− mice with LysM-cre mice and intercrossing offspring.

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Abbreviations used in this article: KO, knockout; p.i., postinfection; sGP130, soluble GP130; sIL-6Rα, soluble IL-6Rα.

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**PCR**

Litters were genotyped using oligonucleotides 5'-CCG-CCG-GGC-ATC-GCC-TAG-G-3', 5'-CCA-GAG-GAC-CTC-TC-3', and 5'-TAG-GGC-GCA-GTT-CCT-TTA-T-3' in a standard PCR reaction.

**Southern blot**

Tail DNA was isolated from IL-6R KO and IL-6Ra FL mice and digested with BglI with the probe that was amplified using primers 3probeB (5'-TTG-TAG-TCT-CCA-CCC-ACA-AGC-3') and 3probeB (5'-AGC-GAG-CAA-CCT-CAG-ACG-3').

**Western blot**

Western blot analysis of protein lysates isolated from indicated tissues and macrophages was performed using anti–IL-6Ra (5660, Santa Cruz), anti-pSTAT3 (#9272 and #9145; Cell Signaling), anti-pAKT (#4066; Cell Signaling), anti-pERK (#4377; Cell Signaling), anti-calnexin (#20888; 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-6Ra FL control and IL-6Ra KO mice by standard techniques and examined for TCRC- 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 106 erythrocytes parasitized with virulent P. chabaudi, as detailed previously (18, 19).

**Injections with sIL-6Ra and sGP130Fc**

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

**ELISA for sIL-6Ra, IL-6, IL-1B, and TNF-α**

sIL-6Ra, IL-6, IL-1B, 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 Biodyne7PLUS nylon membrane (Pall, Pensacola, FL), and subjected to Northern blot analysis, as detailed previously.

**Ex vivo experiments**

Macrophages were isolated from IL-6Ra FL, IL-6Ra KO, and IL-6Ra Myel-KO mice, as described previously (20), and stimulated in vitro with 25 ng/ml IL-6. Hepatocytes were isolated from IL-6Ra FL- and IL-6Ra KO mice, as detailed previously (21). A total of 106 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-6Ra, or 10 ng LPS for 4 h. mRNA expression for IL-1B, 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-6Ra-deficient mouse strain on the C57BL/6 background (Supplemental Fig. 1A–D). These IL-6Ra KO 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 TCRC- T cells in major immune organs (Supplemental Fig. 1E). Moreover, we also created IL-6Ra deficiency specifically in liver parenchymal cells (IL-6Ra LKO mice) (17) and in the myeloid lineage (IL-6Ra Myel-KO mice). Macrophages and neutrophils in IL-6Ra Myel-KO mice lack IL-6Ra (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-6Ra FL control mice (Fig. 1A), IL-6Ra Myel-KO mice (Supplemental Fig. 1H), and IL-6Ra LKO mice (Supplemental Fig. 1I), whereas 50% of IL-6Ra KO 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-6Ra FL mice exhibited a continuous increase in circulating sIL-6Ra during the precrisis phase, from 16 to 190 ng/ml on day 8 p.i., whereas sIL-6Ra was absent in IL-6Ra KO mice (Fig. 1C).

The infection-induced increase in circulating IL-6 followed a biphasic pattern in both control and IL-6Ra KO 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-6Ra KO mice.

**FIGURE 1.** IL-6Ra deficiency protects from P. chabaudi malaria-induced lethality. Outcome and course of blood-stage infections after challenge with 106 P. chabaudi-infected erythrocytes. IL-6Ra FL mice (n = 20) succumb to infection (A), whereas 50% of IL-6Ra KO mice (n = 14) are able to self-heal the same infections (B). Circulating levels of sIL-6Ra (C), IL-6 (D), IL-1B (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-6Ra FL and IL-6Ra KO 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-6RαKO control mice during infection (Fig. 1E, 1F), whereas IL-6RαKO 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-6RαKO mice than in IL-6RαFL control mice on day 8 p.i. (Fig. 1G, 1H). Importantly, the inflammatory response in IL-6RαKO mice and IL-6RαL-KO mice on day 8 p.i. (Supplemental Fig. 1J, 1K) was intermediate to that in IL-6RαFL mice and IL-6RαKO 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 P. chabaudi-induced increase in circulating endogenous sIL-6Rα in control IL-6RαFL 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-6RαKO 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-6RαFL mice and IL-6RαKO 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-6RαKO 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

**FIGURE 2.** IL-6 trans-signaling causes lethal outcome to *P. chabaudi* malaria. Infected mice were injected i.p. with 1 μg recombinant sIL-6Rα on days 1, 4, and 7 p.i. Levels of sIL-6Rα (A) and IL-6 (B) in serum were determined on day 8 p.i. (n = 5–6). (C) Western blot of liver lysates using pSTAT3 Ab of uninfected and *P. chabaudi*-infected IL-6RαFL and IL-6RαKO mice on day 8 p.i. IL-6RαKO mice were injected with 1 μg recombinant sIL-6Rα and IL-6RαFL mice were injected with 16/8/8 μg sGP130Fc on days 1, 4, and 7 p.i., respectively. Calnexin: loading control. (D) *P. chabaudi* malaria in IL-6RαKO mice (n = 14) injected with 1 μg recombinant sIL-6Rα on days 1, 4 and 7 p.i. Levels of IL-1β (E) and TNF-α (F) in IL-6RαFL and IL-6RαKO mice on days 0 and 8 p.i. Significant differences (p < 0.01) were determined as in Fig. 1. IL-6RαKO mice, day 0 versus day 8 p.i., *IL-6RαFL versus IL-6RαKO on day 8 p.i., #IL-6RαKO injected with recombinant sIL-6Rα, **IL-6RαFL versus IL-6RαKO injected with recombinant sIL-6Rα, $IL-6RαFL versus IL-6RαKO injected with recombinant sGP130Fc. Serum levels of sIL-6Rα (G) and IL-6 (H) in *P. chabaudi*-infected IL-6RαFL mice on day 8 p.i. (n = 6), with or without injections of sGP130Fc during the infection. *p < 0.01, Student t-test. (I) Outcome of *P. chabaudi* malaria in IL-6RαFL mice (n = 14) injected with recombinant sGP130Fc on days 1 (16 μg), 4 (8 μg), and 7 (8 μg) p.i.
phosphorylation occurred upon injection of sIL-6R into IL-6RKO mice (Fig. 2C), thus demonstrating the induction of IL-6 trans-signaling by recombinant sIL-6R. Importantly, all IL-6RKO 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 IL-6RKO–injected IL-6RKO mice that were similar to those found in control IL-6RFl 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 malaria, we also attempted to reduce IL-6 trans-signaling in the IL-6R–expressing control mice. When control IL-6RFl 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 levels 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 JNK- inactivation pathways were not affected (Supplemental Fig. 2A, 2B). Moreover, the infection-induced inflammatory response was decreased, as evidenced by lowered levels of IL-1β and TNF-α (Fig. 2E, 2F). Importantly, under these inhibitory conditions of IL-6 trans-signaling, ~40% of the P. chabaudi–challenged control mice survived the infection (Fig. 2I). Remarkably, survival was not increased further when sGP130Fc doses were increased to 32/16/16 μg, whereas doses <16/8/8 μg on days 1, 4, and 7 p.i. did not prevent P. chabaudi–induced lethality (Supplemental Fig. 2C–G), revealing that only a dosing regimen that efficiently reduced sIL-6R in serum conferred protection from malaria-induced lethal outcomes (Supplemental Fig. 2C–G).

Collectively, this report provides unequivocal evidence that IL-6 trans-signaling, rather than IL-6 classic signaling, contributes to a lethal outcome of P. chabaudi malaria. Although the infection-induced increase in sIL-6R may be considered a beneficial response of the host that inactivates overshooting of IL-6 and its effects through membrane IL-6R, this beneficial response is increasingly superposed by the concomitant increase in IL-6 trans-signaling, which promotes harmful responses, ultimately impairing the development and efficacy of protective immunity. The cell types mediating such harmful responses remain to be identified. Remarkably, some data indicate that sIL-6R levels also increase with the severity of human malaria caused by P. falciparum and P. vivax (12, 23). Therefore, inhibition of IL-6 trans-signaling represents a novel promising therapeutic approach to combat human malaria; it is currently under investigation to treat human rheumatoid arthritis (24), human chronic liver disease (25), and human cachexia (26).

Disclosures

The authors have no financial conflicts of interest.

References


<ref>http://www.jimmunol.org/</ref>
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-6RαFL allele, as detailed recently (19). Mice carrying the IL-6RαFL allele were crossed with deleter-Cre mice and further F1 intercrossed to yield knock out (KO) mice termed IL-6RαKO 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-6RαFL mice and IL-6RαKO 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-6RαFL mice and IL-6RαMyel-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-6RαMyel-KO mice (n=5) and (I) IL-6RαL-KO mice (n=5). Levels of (J) IL-1β and (K) TNFα in IL-6RαFL, IL-6RαMyel-KO, and IL-6RαL-KO mice on days 0 and 8 p.i. (n=5). * indicates significant difference (p < 0.01) between IL-6RαFL vs. IL-6RαMyel-KO or IL-6RαL-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 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 *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 *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.
A. Livers of mice infected with *P. chabaudi*
   - day 0
   - day 8 p.i.
   - IL-6Rα^FL^, IL-6Rα, IL-6Rα, IL-6Rα + sIL-6Rα, IL-6Rα + sGP130
   - p-ERK
   - Calnexin

B. Livers of mice infected with *P. chabaudi*
   - day 0
   - day 8 p.i.
   - IL-6Rα^FL^, IL-6Rα, IL-6Rα, IL-6Rα + sIL-6Rα, IL-6Rα + sGP130
   - p-AKT
   - Calnexin

C. IL-6Rα^FL^ + 1/1 μg sGP130Fc
   - Survival [%]
   - [%] Survival
   - Days p.i.

D. IL-6Rα^FL^ + 4/2 μg sGP130Fc
   - Survival [%]
   - [%] Survival
   - Days p.i.

E. IL-6Rα^FL^ + 8/4/4 μg sGP130Fc
   - Survival [%]
   - [%] Survival
   - Days p.i.

F. IL-6Rα + 32/16/16 μg sGP130Fc
   - Survival [%]
   - [%] Panastemia
   - Days p.i.

G. Injection of sGP130 [μg] on days 1, 4 and 7 p.i.