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IL-12Rβ2 Is Essential for the Development of Experimental Cerebral Malaria

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**A** Th1 response is required for the development of *Plasmodium berghei* ANKA (*PbA*)-induced experimental cerebral malaria (ECM). The role of pro-Th1 IL-12 in malaria is complex and controversial. In this study, we addressed the role of IL-12Rβ2 in ECM development. C57BL/6 mice deficient for IL-12Rβ2, IL-12p40, or IL-12p35 were analyzed for ECM development after blood-stage *PbA* infection in terms of ischemia and blood flow by noninvasive magnetic resonance imaging and angiography, T cell recruitment, and gene expression. Without IL-12Rβ2, no neurologic sign of ECM developed upon *PbA* infection. Although wild-type mice developed distinct brain microvascular pathology, ECM-resistant, IL-12Rβ2−deficient mice showed unaltered cerebral microcirculation and the absence of ischemia after *PbA* infection. In contrast, mice deficient for IL-12p40 or IL-12p35 were sensitive to ECM development. The resistance of IL-12Rβ2−deficient mice to ECM correlated with reduced recruitment of activated T cells and impaired overexpression of lymphoxygen-α, TNF-α, and IFN-γ in the brain after *PbA* infection. Therefore, IL-12Rβ2 signaling is essential for ECM development but independent from IL-12p40 and IL-12p35. We document a novel link between IL-12Rβ2 and lymphoxygen-α, TNF-α, and IFN-γ expression, key cytokines for ECM pathogenesis. The Journal of Immunology, 2012, 188: 1905–1914.

**C**erebral malaria is the most severe neurologic complication in children and young adults infected with *Plasmodium falciparum*. The specific role of T cells in the pathogenesis of cerebral malaria has been difficult to address in humans. In mice, mononuclear cell sequestration and T cell activation are crucial steps for the development of experimental cerebral malaria (ECM) after *Plasmodium berghei* ANKA (*PbA*) infection (1–3), a murine model reproducing several pathophysiological features of the human disease (4). Brain-sequestered CD8+ T cells were shown to play a pathogenic, effector role for ECM development (1).

The implication of Th1-promoting IL-12 in malaria and cerebral malaria, in both humans and mice, is still controversial. IL-12 was implicated in the control of early parasite development (5). Low serum levels of IL-12 and IFN-γ have been shown in children with cerebral malaria, supporting a protective role for the Th1 pathway (6). However, a role for Th1 response was reported in the initiation of CNS vascular permeability, a CD8+ T cell perforin-dependent process (7, 8). CD8+ T cells may mediate circulatory shock, vascular permeability changes, and edema in the brain during *PbA* infection (9). Indeed, CD8+ T cells are the primary effector cells of ECM in the *PbA* mouse model, although their precise mechanism of action remains unresolved (3). We reported recently that the absence of protein kinase C-β expression, a critical regulator of TCR signaling and T cell activation, prevents ECM pathogenesis upon *PbA* infection (10).

IL-12 is a heterodimeric cytokine composed of two subunits, p40 and p35. IL-12 is produced primarily by APCs and acts as an immunoregulatory molecule on T and NK cells. IL-12, formed by the association of IL-12Rβ1 and IL-12Rβ2 chains, is expressed primarily on activated T and NK cells. IL-12 induces IFN-γ, which is involved in protection against blood-stage *Plasmodium chabaudi* infection (11). Furthermore, treatment of susceptible mice with rIL-12 decreases parasite burden and increases survival after *P. chabaudi* infection (11). A crucial role for IL-12 in the early phase of blood-stage *P. chabaudi* infection was shown in terms of IFN-γ induction but also of Ab response involved in suppressing and resolving the chronic phase of infection (12). *P. berghei* NK65 infection induces the activation of TLR-MyD88 signaling, leading to IL-12 expression and liver injury, without a decrease in parasite proliferation (13). A protective immunity against *P. berghei* malaria was seen after treatment with exogenous IL-12 (14), and downregulation of IL-12p70 expression was seen in peritoneal exudate cells from *PbA*-infected mice, mediated through IL-10 inhibition of IL-12p40 message (15).

In this study, we investigated a novel aspect of the IL-12 pathway, namely, an IL-12Rβ2 response, which seems to be involved in the development of ECM but different from the known

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Abbreviations used in this article: ECM, experimental cerebral malaria; L:T:A, lymphoxygen-α; MRA, magnetic resonance angiography; MRI, magnetic resonance imaging; *PbA*, *Plasmodium berghei* ANKA; RT, reverse transcription; WT, wild-type.

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IL-12 responses. We report the prevention of ECM in mice deficient for IL-12Rβ2. Magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) were used as noninvasive tools to document the absence of pathological changes in these mice. Furthermore, mice deficient for IL-12p40 or IL-12p35 were sensitive to ECM, suggesting that IL-12Rβ2 is involved in the development of ECM, whereas IL-12p40 and IL-12p35 were dispensable.

Materials and Methods

Mice

Mice deficient for IL-12Rβ2 (16), IL-12p40 (17), or IL-12p35 (18) were bred in our animal facility at the Transgene Institute (Unité Propre de Services 44, Centre National de la Recherche Scientifique, Orléans, France). All of the mice were on a C57BL/6J genetic background, backcrossed 7 times for IL-12Rβ2-deficient mice and at least 10 times for IL-12p40 and IL-12p35-deficient mice. The C57BL/6 genetic background in IL-12Rβ2-deficient mice was verified using a set of 20 microsatellites (D1N1d9, D2mit148, D3mit22, D4mit2, D5mit18, D6mit18, D7mit22, D8mit4, D9mit10, D10mit10, D11mit5, D12mit46, D13mit159, D14mit30, D15mit121, D16mit15, D17mit16, D18mit188, D19mit1, and D20mit40). For the experiments, adult (7- to 10-wk-old) mice were kept in filtered-air cages at the Biosafety Level 2 animal facility. All of the animal experiments complied with the French Government’s ethical and animal experiment regulations (CLE CCO 2009-007).

Experimental malaria infection

A recombinant P. berghei that constitutively expresses GFP at a high level throughout the complete life cycle, from a transgene controlled by a strong promoter from a P. berghei elongation factor-1 gene, was obtained from Dr. A. Waters (University of Glasgow, Glasgow, Scotland) (19). The parasite was frozen and amplified by passage in C57BL/6J mice. Mice were infected by i.p. injection of 10^6 parasitized erythrocytes as described (22). Mice were followed daily for neurologic signs of ECM, and blood-brain barrier integrity was assessed by Evans blue extravasation as described (10).

Parasitemia

Parasitemia was assessed with enhanced GFP-P. berghei as described (21), and fluorescent cells were analyzed with a BD Canto II flow cytometer. Data were acquired by using DIVA software (BD Biosciences, Rungis, France) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Hematology

Blood was drawn into EDTA-containing tubes (Vacutainer; Becton Dickinson, Grenoble, France), and hematological parameters were determined using a five-part differential hematological analyzer (MS9–5; Melet Schloesing Laboratories, Oxnard, France).

Histology and immunohistological staining

Histological analysis and immunohistological staining were performed as described (10). Mice were euthanized and perfused with intracardiac sterile PBS/0.002 M EDTA to remove circulating RBCs and leukocytes from the brain and lungs. The brain and lungs from P. berghei-infected mice were fixed in 3.6% PBS/formaldehyde for 72 h. Longitudinal sections (3 μm) were stained with H&E. Brain microvascular pathology with endothelial damage, accumulation of mononuclear cells, and extravasated erythrocytes in the Virchow-Robin space were assessed semiquantitatively from a whole-brain section typically between ~5.64 and ~6.84 from the bregma. Erythrocyte accumulation in the alveoli and thickening of alveolar septae of the lung were quantified using a semiquantitative score with increasing severity of changes (0–5) by two independent observers, including a trained pathologist (B.R.). Brain microvascular obstruction was evaluated by scoring the number of vessels containing sequestered RBCs in multiple fields corresponding to whole-brain sections (0% microvascular obstruction = 0; 1–19% microvascular obstruction = 1; 20–39% microvascular obstruction = 2; 40–59% microvascular obstruction = 3; 60–79% microvascular obstruction = 4; ≥80% microvascular obstruction = 5). Lung sections were scored on 10 fields per section for erythrocyte accumulation in the alveoli (<1 cell/alveoli = 0, 1 cell/alveoli = 1; 2–5 cells/alveoli = 2, 5–10 cells/alveoli = 3; 10–30 cells/alveoli = 4; >30 cells/alveoli = 5), alveolar size (all normal alveoli = 0, mix of normal and medium-sized alveoli = 1; medium-sized alveoli only = 2; mix of medium and small alveoli = 3; all small alveoli = 4; full obstruction would be 5 but was not observed), and thickening of alveolar septae due to inflammatory cells, and edema was scored with increasing severity from 0 to 5 as an estimate of interstitial inflammation.

For immunohistochimistry staining, euthanized mice were perfused as above, and brains were harvested, embedded in OCT media (Tissue Tek; Sakura), and frozen rapidly. Cryosections (10 μm) were fixed in acetone, washed in PBS/0.05% Tween and PBS, and incubated for 30 min with 2% wild-type (WT) mouse serum in PBS. All of the incubations were carried out in a wet chamber. Brain sections then were incubated overnight at 4°C with biotinylated rat Abs to CD54 (clone 3E2; BD Pharmingen). After being washed, sections were incubated for 30 min with an avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA), followed by the peroxidase substrate (diaminobenzidine; Vector Laboratories). The sections were washed with PBS and colored with hematoxylin. Finally, sections were mounted using Eukitt (O. Kindler, Freiburg, Germany) and examined microscopically.

Brain imaging using MRI

MRI and MRA measurements of vascular cerebral blood flow were performed as described previously (21) using a horizontal 9.4 T/20 Bruker BioSpec magnetic resonance system (Bruker BioSpin, Wissembourg, France).

Immunophenotyping of brain-sequestered leukocytes

Mice were euthanized on day 7, a time point when all of the WT mice showed neurologic symptoms of ECM, and perfused with 10 ml PBS/0.02% EDTA intracardiac to remove circulating RBCs and leukocytes as described (21). Brains were removed and homogenized gently (30 s at 4000 rpm) using the sterile disposable homogenization system Dismopix (Medic Tools, Zug, Switzerland) in RPMI 1640 medium containing 2% FCS. Homogenates were passed through a nylon cell strainer (100 μm; Becton Dickinson), and cells were centrifuged at 400 × g for 10 min. The mononuclear cells then were separated over a 35% Percoll gradient (Amersham Biosciences, Uppsala, Sweden).

Brain-sequestered leukocytes were collected, washed, and saturated with mouse serum before being stained with fluorescein previously labeled Abs for 30 min. The cells then were phenotyped by flow cytometry using the following rat Abs specific for mouse CD3ε conjugated to FITC (clone 145-2C11), CD4 conjugated to PerCP (clone L3T4 RM4-5), CD8α conjugated to allophycocyanin-cyanin7 (clone 53-6.7), CD69 (clone H1.2F3) or peroxidin (clone cBioOMAK-D) conjugated to PE and isotype-match control Abs (all from BD Pharmingen, San Diego, CA). For the analysis of perfusor expression, the cells were permeabilized for 20 min with Cytofix/ Cytoperm (BD Biosciences) before Ab staining. For each sample, 5000 cells from the mononuclear population (gated on the CD3+ cell population) were scored. Data were analyzed using a BD Canto II flow cytometer and FlowJo software (10).

mRNA expression of brain tissue by semiquantitative and real-time quantitative PCR

Total RNA was isolated from homogenized mouse brain and lung using TRI reagent (Sigma-Aldrich) and purified by RNasey Mini Kit (Qiagen, Valencia, CA), and quality was determined by NanoDrop (ND-1000). Reverse transcription (RT) was performed in a mix containing RT buffer, MgCl2, dNTPs, random hexamers, RNase inhibitor, and Multi-Scribe SuperScript III reverse transcriptase (Invitrogen). Incubations were performed in a thermocycler (Eppendorf, Westbury, NY) for 10 min at room temperature, 30 min at 50°C, and 5 min at 85°C. After this amplification, RNA H was incubated for 20 min at 37°C. These cDNAs were subjected to semiquantitative PCR or real-time quantitative PCR.

The brain transcript level of IL-12Rβ2 also was assessed by semiquantitative PCR, because IL-12Rβ2-deficient mice have a deletion in exons 2 and 3 (16) and commercially available primers for quantitative PCR targeting exon 6 were inappropriate. We thus designed specific primers in exon 3 (5′-TCTTGGCCATGAGGATTTCCACAGACAT-3′ and 5′-CAGACTTGAAGAATGTCTGGACACTTC-3′; Sigma-Aldrich) with hypoxanthine phosphoribosyltransferase 1 as a housekeeping gene (5′-CTGGTGAAGAGAGCCCTCTGCTGAAA-3′ and 5′-TGAAGTACTCATTTGCATGCAGAA-3′; Sigma-Aldrich) and used Taqman polymerase mix for amplification. An initial denaturation (10 min at 95°C) was followed by 40 cycles of amplification (30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of amplification at 72°C). PCR products were resolved by electrophoresis on an agarose gel and analyzed by densitometry (ImageJ).
Quantitative real-time PCR and RNA identification were performed using primers from Qiagen for IL-12Rβ2, IFN-γ, lymphoxygenin-α (LT-α), LT-β, LT-βR, and TNF-α message expression and Fast SYBR Green Master Mix for amplification and detection. Expression of actin B, GAPDH, and 18S housekeeping genes was used for normalization. Reactions were performed in triplicate using an Mx4000 Stratagene thermocycler (Agilent) and the following program: 95°C for 15 min and 40 cycles of 95°C for 10 s and 56°C for 30 s. Raw data were analyzed using the Relative Expression Software Tool (http://www.rest.de.com/).

Statistical analysis

Statistical significance was determined with GraphPad Prism software (version 4.0; San Diego, CA). Differences between multiple groups were analyzed for statistical significance by means of one-way parametric ANOVA test followed by Bonferroni posttest for the analysis of large groups of samples or by a nonparametric Mann–Whitney U test for the analysis of small groups of samples. The p values < 0.05 were considered statistically significant.

Results

IL-12Rβ2 is essential for ECM development

The functional role of IL-12Rβ2 in the development of ECM was assessed by investigating neurologic signs and survival in mice deficient for IL-12Rβ2 expression (Fig. 1A). After PhA infection, WT mice developed neurologic symptoms of ECM, such as ataxia, loss of grip strength, progressive paralysis, and coma, and most succumbed within 1 wk, whereas 90% of IL-12Rβ2–deficient mice survived >21 d with no sign of ECM (Fig. 1A). Mice deficient for IL-12p35 or IL-12p40 were sensitive to ECM (Fig. 1A); they died between days 6 and 11 with the same phenotype as WT mice. Similarly, mice deficient for IL-23p19 were also sensitive to ECM (Supplemental Fig. 1). We verified the expression of IL-12Rβ2 during ECM, which was overexpressed in the brains of PhA-infected WT mice undergoing ECM (Fig. 1B) and absent in IL-12Rβ2–deficient mice (Supplemental Fig. 2). Parasitemia was analyzed by flow cytometry using GFP-transfected parasites (19). Although the onset of parasitemia was similar in all of the groups on day 7, IL-12Rβ2–deficient mice failed to control parasite growth thereafter, with parasitemia increasing up to 30% at day 14 and reaching 55% at day 21 (Fig. 1C). IL-12Rβ2–deficient mice succumbed after day 21 with severe anemia (Fig. 1D) in the absence of neurologic signs. Therefore, our data point to a critical role for IL-12Rβ2 in ECM development, which cannot be attributed to the known ligands IL-12p40 and IL-12p35 or to a direct effect on parasitemia.

Reduced cerebral ischemia and blood flow perturbation in the absence of IL-12Rβ2

Noninvasive MRI and MRA were used to follow the neuropathology caused by PhA infection, as in human patients to study brain changes during ECM (21–25). In ECM, MRI and MRA allow a semiquantitative analysis of edema/swelling, focal ischemia, morphological changes, and vascular blood flow (21, 26). This could be indicative of microvascular pathology of the brain due to the obstruction of small vessels with parasitized erythrocytes and leukocyte sequestration and endothelial cell damage (21, 26–28). WT and IL-12 pathway-deficient mice were examined at day 7, when sensitive WT mice started developing acute ECM. Typical MRI and MRA images of uninfected and infected mice are shown in Fig. 2A and 2B, respectively.

WT mice presented distinct signs of ischemic brain damage upon PhA infection, with a clearly increased MRI bilateral hyperintense signal at the corpus callosum, whereas IL-12Rβ2–deficient mice displayed normal MRI parameters without any signs of edema upon infection, similar to those of WT uninfected mice (Fig. 2A, 2C). IL-12p35– or IL-12p40–deficient mice exhibited increased

**FIGURE 1.** IL-12Rβ2–deficient mice do not develop PhA-induced ECM. A. WT mice and mice deficient for IL-12Rβ2, IL-12p35, or IL-12p40 were infected with 10⁵ PhA-parasitized RBCs, and their survival was monitored daily. Results are pooled from nine independent experiments, n = 16–88 mice per group. B. Transcription of IL-12Rβ2 7 d after PhA infection of WT mice was evaluated by real-time RT-PCR in the brain and in the lung. mRNA gene expression was normalized relative to that of uninfected WT mice. Results are mean ± SD of n = 4 mice per group and are from one experiment representative of two independent experiments. C. The kinetics of parasitemia are shown up to day 21 after PhA infection of WT mice and IL-12p35– or IL-12p40–deficient mice and day 21 postinfection for ECM-resistant, IL-12Rβ2–deficient mice. Results expressed as the percentage of parasitized RBCs are mean values ± SD from nine independent experiments, n = 26–50 mice per group. D. Peripheral RBC count kinetics are shown up to day 7 for WT mice and day 21 after PhA infection for ECM-resistant, IL-12Rβ2–deficient mice. Results are mean ± SD from five independent experiments, n = 5–33 mice per group. ***p < 0.001.
ischemic parameters, similar to those of WT mice, after PbA infection (Fig. 2A). Brain edema/swelling was documented in infected WT mice during acute ECM by measuring three distances in the brain (Fig. 2D–F), namely, line 1 from the pituitary gland to the Sylvius aqueduct, median line 2 crossing the medial cerebellar nucleus, and medium line 3 stemming from the cerebellar obex (26). Distances 1 and 3 were increased significantly, whereas distance 2 was reduced significantly, in infected WT mice and IL-12p35– or IL-12p40–deficient mice, as compared with those in noninfected WT mice (Fig. 2D–F), in agreement with Penet et al. (26). In contrast, these metric parameters were not altered significantly in infected IL-12Rb2–deficient mice as compared with those in noninfected mice (Fig. 2D–F).

Distinct vascular blood flow perturbations were analyzed by MRA, and a dramatic reduction in blood flow was documented in WT and IL-12p35– or IL-12p40–deficient mice upon PbA infection (Fig. 2A). MRI/MRA analyses showing the absence of ECM-associated brain edema and reduced blood flow in PbA-infected IL-12Rb2–deficient mice. WT and IL-12Rb2–, IL-12p35–, or IL-12p40–deficient mice were infected with 10^7 PbA-parasitized RBCs as in Fig. 1A. MRI of uninfected and infected mice 7 d postinfection. Left column, midsagittal MRI image [cc, corpus callosum (indicated by arrows); cb, cerebellum; pg, pituitary gland]; right column, typical axial MRI image. Distinct anomalies were visible in PbA-infected WT and IL-12p35– or IL-12p40–deficient mice; namely, edema formation was apparent as a bilateral hyperintense signal at the corpus callosum, whereas WT uninfected mice and IL-12Rb2–deficient infected mice did not show such alterations. MRI images are representative of three independent experiments, n = 4–7 mice per group. B, MRA vascular blood flow perturbations are evident in WT and IL-12p35– or IL-12p40–deficient mice, 7 d after PbA infection, whereas infected IL-12Rb2–deficient mice seemed unaffected, similar to naive WT mice. The arrows point to an area showing reduced brain vascularization in WT mice undergoing ECM, vascularization that was not impaired in PbA-infected IL-12Rb2–deficient mice. MRA images are representative of three independent experiments (as in A). C, Corpus callosum mean gray levels of intensity from MRI (data from A) of uninfected and infected mice 7 d postinfection. The data represent the mean ± SD from three independent experiments as in A. ***p < 0.001. D–F, Assessment of brain swelling in uninfected and 7 d PbA-infected mice in terms of the measurement of distances 1–3 from the midsagittal MRI images in A. D, Line 1 going from the pituitary gland to the Sylvius aqueduct. E, Line 2 median line crossing the medial cerebellar nucleus. F, Line 3 medium line stemming from the cerebellar obex. The data represent the mean ± SD from three independent experiments as in A. *p < 0.05, **p < 0.01, ***p < 0.001.
tion (Fig. 2B). In contrast, IL-12Rβ2–deficient mice displayed normal MRA parameters without signs of microvascular obstruction after PbA infection, similar to those in uninfected WT mice (Fig. 2B). Therefore, IL-12Rβ2 signaling is involved critically in the development of ischemic brain damage and microvascular obstruction after PbA infection.

**IL-12Rβ2 is critical to cerebral but not pulmonary microvascular lesions after PbA infection**

Microvessel obstruction and disruption led to increased protein extravasation from the capillary bed, which can be quantified functionally by Evans blue leakage into the tissue. Although PbA-infected WT mice showed strong vascular leakage, infected IL-12Rβ2–deficient mice had no discoloration of the brain parenchyma (Fig. 3A). The integrity of the blood–brain barrier in IL-12Rβ2–deficient mice was confirmed further by measuring Evans blue extravasation, significantly increased in WT mice but not in IL-12Rβ2–deficient mice upon PbA infection (Fig. 3B).

**FIGURE 3.** IL-12Rβ2 deficiency prevents ECM-associated brain vascular leakage, not lung inflammation, after PbA infection. Mice were infected with 10^7 PbA-parasitized RBCs as in Fig. 1. A, Qualitative assessment of brain vascular leakage by Evans blue extravasation. WT mice with severe ECM on day 7 postinfection (WT D7) showed blue discoloration of the brain, whereas uninfected control (WT D0) and uninfected or day 7 infected IL-12Rβ2–deficient mice showed no discoloration. Images are representative of three independent experiments, n = 4–16 mice per group. B, Quantitative assessment of brain capillary permeability using Evans blue extravasation in formamide was measured by absorbance at 610 nm in brain extracts of naive or PbA-infected WT or IL-12Rβ2–deficient mice as in A. Results are mean ± SEM from three different experiments as in A. C, Microvascular damage with mononuclear cell adhesion and perivascular hemorrhage was visible 7 d after PbA infection in the brains of WT and IL-12p35– or IL-12p40–deficient mice but not in infected IL-12Rβ2–deficient mice. Microscopy sections (magnification ×40) showing H&E staining representative of three independent experiments, n = 4–8 mice per group. D, Semiquantitative scores of brain infiltration, obstruction, and hemorrhages. The bar graphs show the score mean ± SEM from three independent experiments as in C. E, Lung inflammation with alveolar septae thickening, hemorrhage in alveoli, and interstitial edema of PbA-infected WT and IL-12Rβ2–, IL-12p35–, or IL-12p40–deficient mice. Magnifications shown are ×40 of H&E staining representative of three independent experiments as in C. F, Semiquantitative scores of thickening of alveolar septae, cell accumulation in the alveoli, and reduction in size of the alveoli. The bar graphs show the mean ± SEM from three independent experiments as in E. *p < 0.05, **p < 0.01, ***p < 0.001.
and we thus investigated the effects of the IL-12Rβ2 pathway on lung pathology by analyzing pulmonary microvascular beds in PbA-infected mice. The lungs of PbA-infected WT mice displayed congested capillaries of the alveolar septae, hemorrhage in the alveoli, and interstitial edema postinfection, similar to those of PbA-infected IL-12Rβ2−, IL-12p35−, or IL-12p40−deficient mice (Fig. 3E). The lung pathology was investigated semiquantitatively, and a significant difference was found between WT uninfected mice and PbA-infected mice, either WT or deficient for IL-12Rβ2, IL-12p40, or IL-12p35 (Fig. 3F), suggesting that the lung pathology is largely independent of IL-12Rβ2, IL-12p35, and IL-12p40. Therefore, disruption of IL-12Rβ2 prevents brain microvascular pathology, but not lung inflammation, induced by PbA infection.

Reduced LT expression and T cell recruitment and activation upon PbA infection in the absence of IL-12Rβ2

We next focused on the different immunological pathways likely triggered by PbA infection in the absence of IL-12Rβ2. The adhesion molecule ICAM (CD54) expression was correlated with macrophage and parasitized erythrocyte brain sequestration during PbA-induced ECM (29), and we investigated ICAM expression in the brains of PbA-infected IL-12Rβ2−deficient mice by immunostaining (Fig. 4). ICAM expression increased significantly in both WT uninfected mice and PbA-infected mice, indicating that ICAM expression is independent of IL-12Rβ2. ICAM expression was also similar in the brains of PbA-infected IL-12p40− or IL-12p35−deficient mice (Supplemental Fig. 3). This was confirmed at the level of CD54 expression on brain-infiltrating CD4+ or CD8+ T cells from either IL-12Rβ2−, IL-12p35−, or IL-12p40−deficient mice 7 d after PbA infection (data not shown). Thus, ICAM expression seems to be independent of the IL-12Rβ2 and IL-12 pathways.

Because IL-12 is a main switch for IFN-γ expression, we next investigated the regulation of IFN-γ expression after PbA infection in the absence of IL-12Rβ2. The increase in IFN-γ expression observed in WT mice after PbA infection was reduced in IL-12Rβ2−deficient mice (Fig. 5A). Similarly, both TNF-α and LT-α messages were increased less upon PbA infection in IL-12Rβ2−deficient mice than in WT mice (Fig. 5B, 5C).

We then characterized brain T cell sequestration in response to PbA (Fig. 6), a parameter necessary for the development of ECM pathology (1, 7). WT mice recruited and sequestered a high number of CD8+ or CD4+ T cells into the brain after PbA infection. However, in the absence of IL-12Rβ2, brain T cell recruitment was reduced strongly (Fig. 6). Furthermore, activated T cells expressing CD69 and perforin after PbA infection also were diminished strongly as compared with those in WT infected mice (Fig. 6C, 6D, 6F, 6G). No such drastic reduction in CD8+ or CD4+ T cell sequestration was seen in mice deficient for IL-12 expression (Supplemental Fig. 4). Thus, the absence of IL-12Rβ2 prevents T cell sequestration in the brain and impairs the expression of IFN-γ and TNF-α/LT-α family cytokines in response to PbA.

Hematological alterations in IL-12Rβ2−deficient mice

Thrombocytopenia and anemia are hallmarks of the course of PbA infection in mice (21, 30). We therefore investigated the hematological parameters in naive and PbA-infected WT and

**FIGURE 4.** ICAM expression in the brains of PbA-infected IL-12Rβ2−deficient mice. Mice were infected with 10^7 PbA-parasitized RBCs as in Fig. 1. A. Immunohistological staining of CD54+ cells in brain vessels of naive and PbA-infected WT mice developing ECM or ECM-resistant, IL-12Rβ2−deficient mice 7 d postinfection (magnification ×100). Results are representative of three to four mice per group and are from one representative of three independent experiments. B. Bar graphs of the score of ICAM staining. Results represent the mean ± SD of n = 3–4 mice per group and are from one representative of three independent experiments. *p < 0.05, **p < 0.01.

**FIGURE 5.** Differential cytokine gene expression in the brains of PbA-infected IL-12Rβ2−deficient mice. Transcription of IFN-γ (A), LT-α, LT-β, and LT-βR (B), and TNF-α (C) 7 d after PbA infection of WT or IL-12Rβ2−deficient mice was analyzed by real-time RT-PCR. mRNA gene expression was normalized relative to that of uninfected controls for each mouse strain, and control baseline mRNA levels did not differ significantly between naive strains. Results are mean ± SEM of n = 3–4 mice per group and are from one representative of three independent experiments. *p < 0.05.
FIGURE 6. Reduced recruitment and activation of CD8+ lymphocytes in the brains of PbA-infected IL-12Rβ2–deficient mice. Mice were infected with 10^5 PbA-parasitized RBCs as in Fig. 1. Flow cytometry analyses of brain-sequestered leukocytes from uninfected (D0) or 7 d PbA-infected (D7) WT and IL-12Rβ2–deficient mice. A, Representative dot plots of CD4+ and CD8+ cells within the gated CD3+ cell populations, from one pool of three mouse brains per group, representative of three independent experiments. B–D, Bar graphs of the absolute numbers of CD8+, CD8+CD69+, and CD8+Perf+ T cells respectively. E–G, Bar graphs of the absolute numbers of CD4+, CD4+CD69+, and CD4+Perf+ T cells, respectively. Results represent the mean ± SEM of n = 3–4 pools of three mice per group, from one representative of three independent experiments.

IL-12Rβ2–, IL-12p35–, or IL-12p40–deficient mice. The hematological analyses revealed similar reductions of platelet counts in WT and IL-12Rβ2–, IL-12p35–, or IL-12p40–deficient mice 7 d after PbA infection (Fig. 7A). Thrombocytopenia was accentuated further on day 14 postinfection in ECM-resistant, IL-12Rβ2–deficient mice (Fig. 7B).

After 7 d of PbA infection, WT mice show a reduction in circulating WBCs (Fig. 7C), largely attributed to a decrease in lymphocyte counts (Fig. 7E). The decrease in leukocyte and lymphocyte counts was seen in all of the groups of mice after 7 d of PbA infection but was however less prominent in IL-12Rβ2–deficient mice than those in WT and IL-12p35– or IL-12p40–deficient mice (Fig. 7C, 7E). Furthermore, leukocytes and lymphocytes increased dramatically on days 14–21 postinfection in IL-12Rβ2–deficient mice (Fig. 7C, 7E). Finally, although no significant anemia developed after 7 d of infection, IL-12Rβ2–deficient mice developed dramatic anemia after 2 and 3 wk of PbA infection (Fig. 1D). Therefore, PbA-infected IL-12Rβ2–deficient mice that were resistant to ECM and developed high parasitemia died at 3 wk with thrombocytopenia, severe anemia, and drastic leukocytosis.

Discussion

The effect of IL-12, required for Th1 cell differentiation, in severe malaria is unclear. In humans, depending on the populations studied, polymorphisms in promoters or genes coding for IL-12p35 and IL-12p40 have been associated with P. falciparum parasitemia (31), linked with mortality from severe malaria in children (32), associated with protection from severe malaria anemia (33), or associated with susceptibility to cerebral malaria (6).

In this study, we show that the disruption of the IL-12Rβ2 pathway prevents microvascular disease leading to fatal ECM in PbA infection. Signaling through IL-12Rβ2 activates the expression of IFN-γ, a critical cytokine for innate and adaptive immune responses (34). Studies in malaria patients or in the P. chabaudi model showed a protective role for IFN-γ in parasite clearance and disease severity (35, 36). In PbA infection, IFN-γ and its receptor are crucial in the development of ECM, genetically deficient mice being protected against ECM (37). The disruption of IL-12Rβ2 prevented cerebral pathology, and we revisited the roles of the different molecules involved in the IL-12 pathway.

We investigated several pathophysiological aspects of ECM in IL-12Rβ2–deficient mice and show that the absence of neurologic signs of ECM is associated with normal cerebral perfusion as assessed by MRI and MRA. In vivo analyses showed no brain sequestration of mononuclear cells, no vascular leak, no hemorrhage, and no inflammation in the absence of IL-12Rβ2, which were evident in PbA-infected WT mice. In contrast, the absence of IL-12Rβ2 did not affect PbA-induced pulmonary microvascular damage, thus confirming the different pathophysiology of PbA-induced ECM and lung pathology (10, 38, 39). Thus, we demonstrated that PbA-induced brain microvascular damage and inflammation are mainly IL-12Rβ2 dependent, whereas PbA-induced lung microvascular damage, edema, and alveolitis are IL-12Rβ2 independent.

Although exogenous IL-12 may have adjuvant, protective effects against experimental malaria (5, 11, 36), IL-12 also may be involved in severe malaria complications, because it contributes to the induction of inflammatory mediators, including IFN-γ (40). We next investigated whether ECM resistance in the absence of IL-12Rβ2 might be attributed to one of the known IL-12 ligands. We document here that IL-12Rβ2– or IL-12p40–deficient mice were susceptible to PbA-induced ECM development. Comparable to WT mice, they presented characteristic signs of ECM, such as
ischemic brain damage, vascular blood flow perturbation, and cerebral sequestration of blood cells. Our results show that PbA-induced ECM development is independent of IL-12p35 and IL-12p40. IL-12p40–deficient mice were shown to develop ECM after P.hKnK65 sporozoite infection (41). Furthermore, mice deficient for IL-23p19 or for IL-12Rβ1 were also sensitive to ECM (Supplemental Fig. 1 and data not shown). These data suggest that IL-12Rβ2 might be involved in another pathway that is unknown currently.

IL-12Rβ2–deficient mice, similar to WT mice, showed thrombocytopenia at day 7, a time when WT mice developed ECM, together with a slight anemia that further increased thereafter. Therefore, disruption of IL-12Rβ2, while protecting from ECM development, had no strong effect on the typical hematological responses to PbA infection in mice, with the notable exception of the lymphocyte counts, which were decreased less than those in WT mice on day 7 postinfection. Furthermore, strong lymphocytosis was evident 2–3 wk postinfection in IL-12Rβ2–deficient mice, as observed previously in ECM-resistant, protein kinase C-θ–deficient mice (10). The propagation of PbA infection in erythrocytes was totally IL-12Rβ2 independent, because the mice succumbed within 3 wk of severe parasitemia and anemia. In agreement with previous studies, there is dissociation among cerebral pathology, modification of the hematological parameters, and parasite growth (21, 28, 37).

Different studies showed that resistance to ECM seems to be linked to reduced parasite sequestration. Whole-body parasite sequestration was reduced in LT-α– or IFN-γ–deficient mice after PbA infection. However, the brain represented only a small proportion of parasite tissue accumulation in ECM-susceptible, WT mice, and sequestration in the brain was not reported for ECM-resistant, LT-α– or IFN-γ–deficient mice (42). Claser et al. (43) reported similar or slightly higher parasitemia in IFN-γ–deficient mice, with a lower total or head parasite biomass than that in WT mice during ECM development, whereas PbA-infected TNF-α– or IL-12p40–deficient mice developed ECM and showed the same level of parasitemia as WT mice.

We next assessed the downstream effects of IL-12Rβ2 disruption on cytokine expression in the brain after PbA infection. In WT mice, IFN-γ expression was increased strongly after PbA infection, whereas this overexpression was attenuated in IL-12Rβ2–deficient mice. A strong reduction of IFN-γ expression also was noted in IL-12p40–deficient mice infected with P. chabaudi (12). Indeed, the LT-α and TNF-α overexpression seen in the brains of WT mice after PbA infection was reduced strongly in infected IL-12Rβ2–deficient mice. Increased expression of LT-α in the CNS was reported in multiple sclerosis and experimental autoimmune encephalitis (44–46). TNF-α was implicated originally in ECM pathogenesis (47). Administration of TNF-α–neutralizing Abs prevented ECM (47), and mice with a combined deletion of TNF-α and LT-α were resistant to ECM development (20). However, LT-α rather than TNF-α was shown to be essential for the development of ECM (28) with the implication of both LT-α homotrimer/TNFFR2 and LT-β heterotrimer/LT-βR (21, 27). The cross talk between IL-12 and TNF-α/LT-α pathways seems to be multifold. Reduced TNF-α and LT-α levels were reported in the absence of IL-12p35 in experimental autoimmune encephalitis (48). CD8+ T cells deficient for LT-α have diminished IL-12Rβ2 expression, and they fail to differentiate into normal effectors (49). LT-β expression was independent of IL-12 and IFN-γ (50). However, IL-12p40 homodimers, which were thought to be inactive or inhibitory, were shown recently to strongly induce LT-α expression in microglial cells (51). This effect of 12p40 homodimers was attributed to IL-12Rβ1 rather than IL-12Rβ2 (51),
a novel activity mediated by IL-12Rβ1, although the precise molecular interactions remain to be defined. The question of how IL-12Rβ2 may be triggered to affect LT-α transcription in response to PbA infection remains and deserves further biochemical investigations.

In conclusion, IL-12Rβ2 clearly has an important role in the development of ECM. The absence of this receptor subunit abrogates the microvascular disease leading to fatal ECM. Notably, ECM pathology mediated by IL-12Rβ2 appears to be independent of the IL-12 ligands, because they are not essential for the pathogenic response. The ECM protection of IL-12Rβ2-deficient mice is likely linked to the diminution of the IFN-γ and TNF-α/LT-α family responses; however, a transcriptomic approach has been initiated to identify further gene expression downstream of IL-12Rβ2 critical for the response to PbA infection.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


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Supplemental data

Supplemental Figure 1: IL-23p19 deficient mice are sensitive to PbA induced ECM.

A. WT and mice deficient for IL-23p19 were infected with \(10^5\) PbA parasitized red blood cells and their survival monitored daily. Results are pooled from seven independent experiments n=54 mice per group.

B. The parasitemia on day 7 post PbA-infection is shown for WT mice and IL-23p19 deficient mice. Results expressed as percent of parasitized RBC are mean values ± SD from seven independent experiments n=27 mice per group.

C. Quantitative assessment of brain capillary permeability using Evans blue extravasation in formamide was measured by Absorbance at 610 nm in brain extracts of naive or PbA-infected WT or IL-23p19 deficient mice as in A. Results are means + SEM from 2 different experiments.

Supplemental Figure 2: IL-12Rβ2 expression in the brain.

Semi-quantitative PCR of IL-12Rβ2 message and HPRT-1 as a reference gene present in the brain from individual WT and IL-12Rβ2 deficient mice either uninfected (D0) or 7 days post PbA-infection (D7)

Supplemental Figure 3: ICAM expression in the brain of PbA-infected mice.

Mice were infected with \(10^5\) PbA parasitized red blood cells as in Figure 1.

A. Immunohistological staining of CD54+ cells in brain vessels of WT naive (D0) and PbA-infected (D07) WT, IL-12Rβ2, IL-12p40 and IL-12p35 deficient mice, 7 days post infection (micrograph 100x). Results are representative of 3-4 mice per group.
B. Bar graphs of the score of ICAM staining. Results represent the mean + SEM of n =3-4 mice per group (*, p < 0.05; ***, p < 0.001; n.s., not significant).

**Supplementary Figure 4 legend:** T cell recruitment in IL-12p35 deficient mice after *Pb*A infection.

WT or mice deficient for IL-12Rβ2 or IL-12p35 were infected with 10⁵ *Pb*A parasitized red blood cells and flow cytometry analyses performed on brain-sequestered leukocytes from uninfected (D0) or 7 days post-infection (D7). Bar graphs of the absolute numbers of CD8⁺, CD8⁺CD69⁺, CD4⁺ and CD4⁺CD69⁺ gated on CD3⁺ cell populations are shown. Results represent the mean + SEM of n =3 pools of 3 mice per group.
Supplemental Figure 1

A

Survival [%]

Days post infection

B

Parasitaemia [%]

WT IL-23p19

C

Parasite burden [pg/μg]

WT IL-23p19

n.s.

**

n.s.
Supplemental Figure 3

A

WT D0  WT D7
IL-12Rβ2 D7
IL-12p40 D7
IL-12p35 D7

B

KAM score [UL]

WT D0  WT  IL-12Rβ2  IL-12p40  IL-12p35

D7

ns

*
Supplemental Figure 4

**A**

- **CD8+**

  - WT D0
  - WT D7
  - IL12Rβ2 D7
  - L12p35 D7
  - IL23p19 D7

**B**

- **CD8+ CD69+**

  - WT D0
  - WT D7
  - IL12Rβ2 D7
  - L12p35 D7
  - IL23p19 D7

**C**

- **CD4+**

  - WT D0
  - WT D7
  - IL12Rβ2 D7
  - L12p35 D7
  - IL23p19 D7

**D**

- **CD4+ CD69+**

  - WT D0
  - WT D7
  - IL12Rβ2 D7
  - L12p35 D7
  - IL23p19 D7