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Interleukin-10 modulates susceptibility in experimental cerebral malaria

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SUMMARY

In this study, we examined the effects of interleukin-10 (IL-10) on the outcome of experimental cerebral malaria (CM), a lethal neurological syndrome that occurs in susceptible strains of mice after infection with *Plasmodium berghei* ANKA (PbA). Constitutive IL-10 mRNA levels were significantly higher in the spleen and brain of resistant animals. *In vivo* neutralization of endogenous IL-10 in CM-resistant mice induced the neurological syndrome in 35·7% of these mice, as opposed to 7·7% in controls. IL-10 inhibited PbA antigen-specific interferon-γ (IFN-γ) production *in vitro* but not tumour necrosis factor (TNF) serum levels *in vivo*. Susceptible mice, on the other hand, were significantly protected against CM when injected with recombinant IL-10. Overall, our findings suggest that IL-10 plays a protective role against experimental cerebral malaria.

INTRODUCTION

Malaria can be considered to be the most important parasitic disease, affecting over 300 million people annually worldwide. While infection with *Plasmodium vivax*, *P. malariae* and *P. ovale* cause a debilitating and incapacitating disease, infection with *P. falciparum* is responsible for life-threatening complications such as anaemia and cerebral malaria (CM) which account for 500 000–1·5 million deaths annually, principally among young African children. An experimental model of CM has been developed in susceptible strains of mice after infection with *P. berghei* ANKA (PbA). Several lines of evidence indicate that tumour necrosis factor (TNF) and interferon-γ (IFN-γ) play a central part in the pathogenesis of murine CM.[1–4] Certain observations suggest that TNF is also possibly involved in the pathogenesis of human malaria.[5–7]

Inerleukin-10 (IL-10) is a pleiotropic cytokine known to inhibit the production of IFN-γ by T helper 1 (Th1) cells[8,9] and of TNF secretion by macrophages.[10] Also, IL-10 acts a suppressor of the immune response by decreasing the antigen-presenting function of macrophages.[11] Recent studies have shown markedly elevated levels of circulating IL-10 in patients with cerebral and severe, as compared to mild malaria.[12] Furthermore, IL-10 levels were shown to return to normal after antimalaria chemotherapy.[12,13] The role IL-10 plays in CM remains to be determined: while it can play a beneficial role by reducing the parasite-induced inflammatory response, it may also play a detrimental one by decreasing cellular immune responses. A strong indication that IL-10 plays a protective role in CM was provided by a study in which IL-10 was shown to mediate the murine acquired immune deficiency syndrome (MAIDS)-induced protection against CM.[14] In the present study we evaluated whether resistance to CM correlated with IL-10 expression. We assessed the effects of both passive immunization against endogenous IL-10 and treatment with exogenous IL-10 on the outcome of CM.

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MATERIALS AND METHODS

Mice

Susceptible CBA/J (H-2^k) and resistant BALB/c (H-2^b) mice were originally purchased from IFFA-CREDO (Lyon, France) and housed in our facilities. Mice were injected intraperitoneally (i.p.) with 10^6 PbA-infected erythrocytes, as described previously.[15] Between 90 and 100% of susceptible mice showed neurological complications (mono-, hemi- para- or tetraplegia, ataxia and convulsions) and started dying 7 days after infection. Parasitaemia at the time of death was relatively low (below 14%). Resistant mice did not present neurological lesions and died 3 or 4 weeks after infection of hyperparasitaemia (70–80%) and severe anaemia (less than 10^6 red blood cells/ml).

Reagents

Murine recombinant IL-10 was obtained as culture supernatant from chinese hamster ovary (CHO)-K1 cells stably transfected with the corresponding cDNA, as previously described.[16] Supernatant from mock-transfected cells was used as control. The endotoxin content of IL-10 and mock preparations was below 1 ng/ml, as determined by the limulus

assay. The JES5-2A5 monoclonal rat anti-mouse IL-10 (immunoglobulin G1: IgG1) neutralizing antibody was a kind gift from T. Mosmann (Department of Immunology, University of Alberta, Edmonton, Canada).

Comparative reverse transcriptase–polymerase chain reaction (RT–PCR)

RNA was prepared from spleen and brain samples using the single-step method by acid guanidinium thiocyanate–phenol–chloroform extraction, as described.[17] Genomic DNA was removed by incubating RNA for 30 min at 37° in 40 mM Tris–HCl pH 7·5, 10 mM NaCl, 6 mM $MgCl_2$ and 2·5 units of RQ1 DNAse (Promega, Madison, WI). After phenol–chloroform extraction and ethanol precipitation, pellets were resuspended in water. Synthesis of the first strand of cDNA was performed according to the instructions delivered with the cDNA synthesis kit (Boehringer Mannheim AG, Rotkreuz, Switzerland), using random primers and avian myeloid leukaemia virus (AMV) reverse transcriptase (10 units/sample). PCR reactions were performed using the Gene-Amp PCR reagent kit (Perkin-Elmer/Cetus, Rotkreuz, Switzerland), 2·5 μCi of [α-^{32}P]-dCTP (3000 Ci/mmol, Amersham, Zurich, Switzerland) and specific primers. The following primers were used: glyceraldehyde-phosphate dehydrogenase (GAPDH) 1, 5′ primer TGA AGG TCG GTG TGA ACG GAT TTG G, GAPDH 2, 3′ primer ACG ACA TAC TCA GCA CCA GCA TCA C. The murine IL-10 amplimer sets were purchased from Clontech (Clontech, Palo Alto, CA). PCR was performed using the automated DNA Thermal Cycler (Perkin-Elmer/Cetus) and the programme consisted of 1 cycle at 94° for 5 min, 55° for 1 min and 72° for 30 s followed by either 25 (for GAPDH gene) or 35 (for IL-10) cycles consisting of 94° for 30 s, 55° for 1 min and 72° for 30 s. PCR products were visualized under UV light after electrophoresis on 1% agarose gels containing ethidium bromide. The GAPDH primers yield an amplified PCR fragment of 297 bp and the IL-10 primers yield an amplified PCR fragment of 455 bp. Gels were then dried and exposed to XAR-5 film (Kodak, Rochester, NY) at room temperature. For quantification, phosphor-image analysis of the specific bands was performed. Values obtained when IL-10 fragments were quantified were normalized to their corresponding values obtained by quantitating the GAPDH amplification products. Three animals per group were individually analysed and PCR was performed twice, with comparable results. In these conditions of RNA amount and numbers of cycles, there was a linear relationship between numbers of mRNA molecules and signal (data not shown).

In vitro *cytokine production*

Single cell suspensions from infected CBA/J mouse spleens were prepared by homogenizing organs in a tissue grinder (Bellco Glass, Vineland, NJ) in Hanks' balanced salt solution (HBSS) + 5% fetal bovine serum (FBS). Debris were removed after sedimentation and cells were washed in HBSS + 5% FBS. Red blood cells (RBC) were eliminated by incubating cell suspensions with NH_4Cl for 3 min at 37° and extensive washing with HBSS + 5% FBS. Cells were finally resuspended at 4×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) + 0·5% normal mouse serum (NMS) and cultured in flat-bottomed 24-well plates (Falcon, Lincoln Park, NJ; Becton Dickinson, Lincoln Park, NJ) in the presence of phytohaemagglutinin as a positive control (PHA, 1 μg/ml, Wellcome, Dartford, UK), nRBC or PbA antigen at the concentrations shown below (see figures). As a negative control, cells were cultured in the presence of medium alone. Supernatants were collected after 3 days incubation in an atmosphere containing 5% CO_2, and stored frozen (−20°) until used.

Cytokine assays

IFN-γ production was assayed by a specific enzyme-linked immunosorbent assay (ELISA) using as a first antibody a rat anti-mouse IFN-γ (5 μg/ml, hybridoma 01.E70.3b218) and as second antibody a biotin-coupled rat anti-mouse IFN-γ (hybridoma ANI-8.03[18]), kind gift of Dr S. Landolfo. The standard curve was performed with purified recombinant mouse IFN-γ (Holland Technology, Amsterdam, the Netherlands). Serum mouse IL-10 levels were measured by ELISA using the JES5-2A5 and the SXC1 monoclonal antibodies (mAb) purchased from Pharmingen (San Diego, CA), as described before.[19] Immuno-reactive TNF serum levels were quantified by ELISA.[20]

Statistical analysis

Significance analysis on survival curves was determined by Fisher's exact test. Analysis of differences in mRNA levels from various groups was performed by using the non-parametric Mann–Whitney *U*-test.

RESULTS

Expression of IL-10 mRNA during PbA infection

Spleen and brain samples were collected from three individual CBA/J mice and BALB/c mice before and 8 days after PbA infection. Spleen samples from non-infected resistant mice contained significantly higher levels (four-fold more, P0·008) of IL-10 mRNA than spleen tissue from non-infected susceptible mice. Eight days after PbA infection, IL-10 mRNA levels increased in susceptible, but not in resistant mice. At this stage, the levels of IL-10 transcripts were not significantly different between both strains of mice. Brain tissue samples from non-infected resistant mice were also found to express higher levels of IL-10 mRNA than susceptible mice. While IL-10 mRNA expression decreased after PbA infection in BALB/c mice, it did not change in CBA/J. No significant difference was found in the expression levels between resistant and susceptible strains at this time (Fig. 1). Circulating IL-10 was not detectable in basal conditions nor 7 days after PbA infection (not shown).

Effect *in vivo* of anti-IL-10 and IL-10

We further investigated the relationship between endogenous IL-10 and protection against CM by passive immunization against this cytokine. Two groups of CM-resistant BALB/c mice were infected with PbA and injected on the same day and after 3, 6 and 9 days with either 1 mg anti-IL-10 antibody or an irrelevant IgG. Survival was decreased among the anti-IL-10-treated animals (Fig. 2), albeit not significantly. Anti-IL-10-treated mice died between days 7 and 10 with neurological symptoms, with a cumulative incidence of CM of 35·7%, as compared to 7·7% of control animals. Overall,

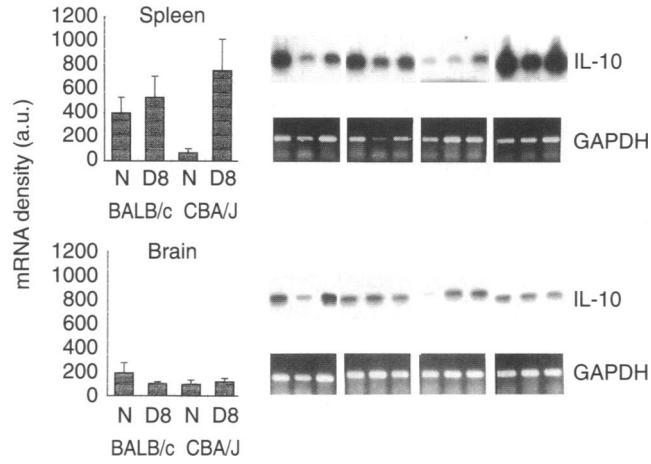

Figure 1. Expression of IL-10 mRNA during *Plasmodium berghei* ANKA (PbA) infection. Total cellular RNA was individually extracted from the spleen (top) and brain (bottom) of three mice per group: resistant BALB/c, before (N) or 8 days after infection (D8), and susceptible CBA/J, before (N) and 8 days after infection (D8). RNA was reverse transcribed and specific messages for IL-10 and GAPDH (housekeeping gene) amplified by PCR, in the presence of ^{32}P-radiolabelled dCTP. Specific bands were visualized after electrophoresis and ethidium bromide staining of the gel (GAPDH PCR fragments) or autoradiography (IL-10 PCR fragments) (shown on the right). Radioactivity of the PCR products was quantifyd with a PhosphorImager System, the values obtained for IL-10 fragments were normalized to the corresponding values obtained by quantitating the GAPDH amplification products (expressed as mean ± SD, 3 mice per group, shown on the left).

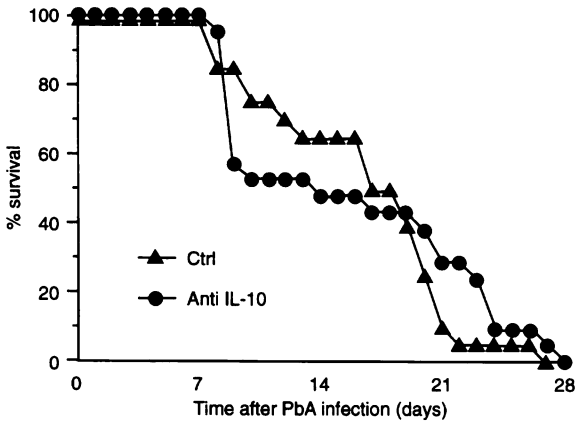

Figure 2. Anti-IL-10 treatment *in vivo* does not render resistant mice susceptible to cerebral malaria. Resistant BALB/c mice ($n=14$) were injected i.p. with 1 mg polyclonal anti-IL-10 antibodies on days 0, 3, 6 and 9 after PbA infection. Control mice ($n=13$) received irrelevant immunoglobulins on the same days after infection. The figure represents the pool of two separate and comparable experiments.

the cumulative incidence of CM in BALB/c mice treated with anti-IL-10 antibody was increased as compared to untreated animals, although not significantly (Fisher exact test on d14: $P=0{\cdot}09$). Parasitaemia levels were not modified by the anti-IL-10 treatment (on days 7 and 14, respectively: $10{\cdot}6\pm2{\cdot}1\%$ and $44{\cdot}3\pm6{\cdot}2\%$ in BALB/c versus $11{\cdot}2\pm2{\cdot}8\%$ and $36{\cdot}2\pm3{\cdot}9\%$ in BALB/c injected with anti-IL-10 antibody, mean ± standard deviation).

Because the basal expression of IL-10 was lower in susceptible mice, we hypothesized that administration of exogenous IL-10 might protect them against CM. Two groups of CM-susceptible mice (CBA/J) were infected with PbA. Three days later, and until day 10, animals received intraperitoneal injections (three times daily) of either murine recombinant IL-10 (1000 U), or supernatant from mock-transfected CHO cells. The cumulative incidence of CM was significantly reduced in mice having received the IL-10 therapy (38·6% versus 100%, Fisher's exact test on d15 $P=0{\cdot}0003$, Kaplan Meier's $P<10^{-4}$) The survival of IL-10-injected mice was prolonged (Fig. 3), these mice died between weeks 3 and 4 of severe anaemia and overwhelming parasitaemia, but without neurological lesions. IL-10 therapy did not modify the levels of parasitaemia (at day 7 after infection: $14{\cdot}0\pm2{\cdot}3\%$ in CBA/J mice versus. $10{\cdot}9\pm1{\cdot}3\%$ in CBA/J mice receiving the

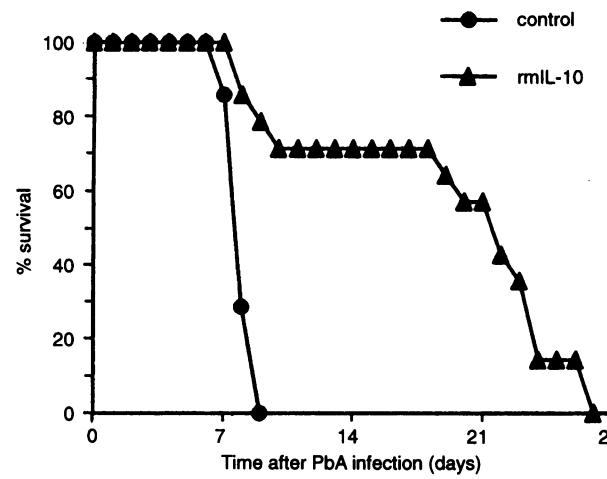

Figure 3. Prolonged survival in PbA-infected CBA/J mice after IL-10 treatment *in vivo*. Susceptible CBA/J mice ($n=14$) were injected three times/day i.p. with 1000 U recombinant IL-10 from days 3–10 after infection with PbA. Control CBA/J mice ($n=14$) were injected following the same schedule but with supernatant from mock-transfected CHO cells. The figure represents the pool of two separate comparable experiments. Kaplan Meier: $P=0{\cdot}0000$; χ^2 (Yate's continuity correction): $P=0{\cdot}00039$.

IL-10 therapy, mean ± standard deviation). All the control mice died before day 10 after PbA infection after showing clinical signs including hemiparesis, paraparesis, ataxia and convulsions. Histopathological lesions were focal intravascular accumulations of mononuclear cells and parasitized erythrocytes (not shown). IL-10 serum levels remained under detection level of the assay (4 U/ml).

Serum TNF levels and *in vitro* IFN-γ production

Because IL-10 is known to inhibit TNF production, we assessed circulating TNF levels in IL-10 and anti-IL-10 treated mice. While baseline serum TNF levels were low in both strains of mice, they increased after infection with PbA, significantly more so in susceptible CBA/J than in resistant BALB/c mice, as measured on day 8 after infection (Fig. 4). *In vivo* treatment with IL-10 reduced the levels of immunoreactive TNF, but not significantly, while anti-IL-10 injections significantly increased the levels of TNF in BALB/c mice (Fig. 4).

We also confirmed the known inhibitory role of IL-10 on antigen-specific IFN-γ production. Spleens were individually harvested from eight CM-susceptible mice, 7 days after PbA infection. Cells were restimulated *in vitro* with PbA antigen (1 μg/ml) either in the presence or absence of recombinant IL-10 (200 U/ml). As control, cells were cultured in medium alone, in the presence or absence of Il-10. IL-10 significantly reduced the PbA antigen-induced IFN-γ production (42·6 ± 7·7 versus 130·7 ± 22·6 U/ml, mean ± standard deviation, $P = 0{\cdot}0009$). IL-10 also significantly reduced the low level IFN-γ production in medium alone ($P = 0{\cdot}03$). IFN-γ was not detectable in serum, in either treated or untreated infected mice.

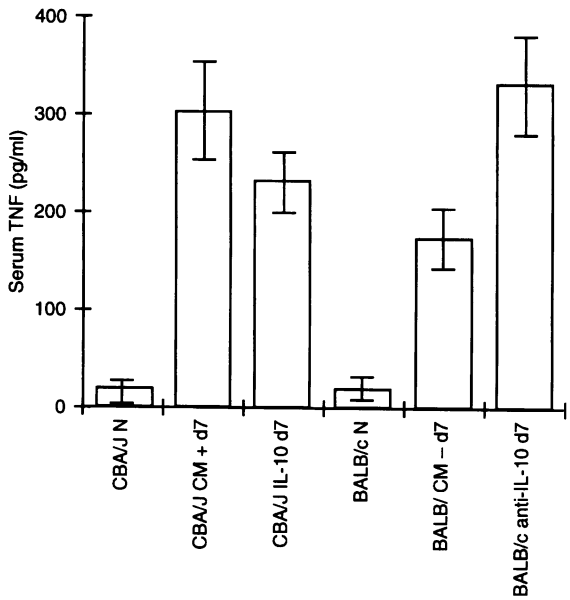

Figure 4. Effect of IL-10 on *in vivo* TNF serum levels. CBA/J and BALB/c mice were bled by retro-orbital puncture before (CBA/J N, $n = 14$ and BALB/c N, $n = 13$)), or 7 days after PbA infection (CBA/J CM + d7 and BALB/c CM- d7). A group of infected CBA/J were injected with anti-IL-10 as described above and bled 7 days later (CBA/J IL-10 d7, $n = 14$) and a group of infected BALB/c mice were injected with anti-IL-10 as described above and bled 7 days later (BALB/c anti-IL-10 d7). Immunoreactive TNF levels were assessed in the individual sera by ELISA. Mann–Whitney: $P0{\cdot}005$ between CBA/J N and CBA CM + d7, BALB/c N and BALB/c CM-d7, CBA CM + d7 and BALB/c CM – d7, BALB/c CM – d7 and BALB/c anti-IL-10 d7.

DISCUSSION

In this report we show that susceptibility to experimental cerebral malaria is accompanied by a decreased constitutive expression of IL-10 mRNA in spleen and brain. The fact that circulating IL-10 in either normal or *Plasmodium berghei* ANKA-infected mice could not be detected is not altogether surprising; other studies on the role of IL-10 in experimental CM[14] do not mention serum IL-10 levels. Also, it has been reported that constitutive IL-10 levels in the serum of mice are undetectable. After lipopolysaccharide (LPS) challenge *in vivo*, IL-10 serum levels peak at 2 hr, returning to basal levels 9 hr later.[21] Resistance to experimental CM could be achieved by treatment with exogenous IL-10 while it was partially reversed by neutralizing endogenous Il-10. The development of cerebral malaria in anti-IL-10 treated CM-resistant mice is the first example where CM can be induced by the neutralization of an endogenous cytokine. For example, administration of anti-IL4 (11B11; a kind gift of Dr W. E. Paul, Laboratory of Immunology, NIAID, NIH, Bethesda, MD) using a similar schedule and even higher doses, did not lead to CM in resistant animals (not shown). IL-10 also plays a decisive role in controlling parasite infection with *Toxoplasma gondii.* Indeed, IL-10 mRNA and protein have been shown to increase during infection of mice with *T. gondii.*[22,23] In the absence of endogenous IL-10, these mice die in response to a lethal immune response accompanied by overproduction of IFN-γ and TNF.[24] What is most interesting in this model is that TNF and IFN-γ are both necessary to achieve protection.[25,26] Likewise, TNF can play a protective role in experimental CM,[2] as can TNF or IFN-γ in murine malaria induced by infection with *Plasmodium chabaudi.*[27] It is the imbalance in time and site of TNF and IFN-γ production which contribute to pathology, both in the *T. gondii* and murine malaria models. IL-10 can also be associated with susceptibility, as shown in a different model, where BALB.Xid mice, which are naturally resistant to experimental Chagas' disease, express lower levels of IL-10 before and during infection, as compared to their susceptible BALB/c counterpart.[28] These reports highlight the paradoxical actions of T helper cell-associated cytokines in infectious diseases, where they may play either a protective or deleterious role (for a review see 29).

The fact that no significant decrease in serum TNF levels was observed in IL-10 treated mice suggest that IL-10 directly inhibited TNF-dependent or independent mechanisms which are important in CM pathogenesis. For example, IL-10 can interfere with the co-stimulatory properties of antigen-presenting cells such as antigen-presenting capacity of endothelial cells.[30] Also, since IL-10 can inhibit intracellular adhesion molecule-1 (ICAM-1) expression on monocytes in response to TNF and LPS,[21] one may hypothesize that IL-10 directly inhibits the up-regulation of ICAM-1 on endothelial cells in response to TNF, therefore decreasing the adherence of leucocyte function-associated antigen-1 (LFA-1)-bearing platelets, mechanisms which has been shown to be crucial in CM pathogenesis.[31] In this context, IL-10 may help control

infection not only by inhibiting excessive production of deleterious cytokines but also by interfering with endothelial cell functions involved in pathology.

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