Cytokine profile of murine malaria: stage-related production of inflammatory and anti-inflammatory cytokines

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ABSTRACT
Balance between inflammatory and anti-inflammatory cytokines may be important in malaria presentation and outcome. To clarify cytokine interactions that produce pathology of malaria and control infection, C57BL/6 mice were infected with 10⁴ parasitized RBCs from a non-lethal strain of Plasmodium yoelii. Kinetics was monitored showing the course of parasitemia, and cytokines were determined by RT-PCR from liver and spleen tissues. Inflammatory cytokines such as interferon-γ (IFNγ), interleukin (IL)-12, IL-6, tumor necrosis factor-α (TNFα) and anti-inflammatory cytokines, including IL-4 and IL-10, were investigated as key molecules that interact with immune cells in the activation of the immune responses. The production of IFNγ mRNA was found to be higher on day 7 than on day 21 after infection, and IL-12 and IL-6 showed higher expression in the liver than in the spleen. Though TNFα was highly expressed on day 14 after infection and on day 21 in the liver, such expression was decreased on day 21 in the spleen. Anti-inflammatory cytokines showed high expression in both the liver and spleen. The results suggest that a relative balance between inflammatory and anti-inflammatory cytokines is crucial and that the increase of inflammatory cytokine levels during the acute phase of malaria may reflect an early and effective immune response. The counteraction effect of anti-inflammatory cytokines is thought to play a role in limiting progression from uncomplicated malaria to severe life-threatening complications.

Malaria is a major cause of morbidity and mortality in tropical countries. Despite more than two decades of intense research, there is currently no vaccine that reliably protects against blood stage malaria infection. Vaccine-related research has tended to focus on adaptive rather than innate immune responses. The innate response to malaria has, until recently, received relatively little attention. However, as well as other investigators have reported that protection against malaria is an event of innate immunity mediated by intermediate T (TCRint) cells due to its feature as an intracellular pathogen (3, 4, 17).

Studies in both mice and humans have repeatedly shown that inflammatory cytokines, especially IL-12, IFNγ and TNFα, are essential mediators of protective immunity to erythrocytic malaria; these cytokines can be derived from either the innate or adaptive arm of the immune response (9, 26). Resistance to rodent malaria is absolutely dependent on signals mediated by IFNγ (9). TNFα and IFNγ act synergistically to optimize nitric oxide (NO) production (14) which is involved in parasite killing (25). Similarly, in humans, IFNγ production is correlated with resistance to reinfection with Plasmodium falciparum (6, 16) and protection from clinical attacks of malaria (7).

An increasing body of evidence from both murine models and human studies indicates that cytokines,
along with T cells, natural killer (NK) cells and macrophages, contribute either to resolution of malaria infection or to a persistent increase in concentrations of malaria parasitemia which leads to complicated malaria such as cerebral malaria (27, 32). Specific cytokines reported to be important in human malaria include TNFα (5, 12), IFNγ (30, 31, 33), IL-6 (5, 31), and IL-10 (5, 12, 24, 30–32).

Some studies have suggested that the balance between type 1 and type 2 immune responses and that between pro- and anti-inflammatory responses determine the degree of malaria parasitemia, the level of anemia, the clinical severity and the presentation and/or outcome. Murine models suggest that parasite clearance requires an early IFNγ (type 1) response, followed by an IL-10 (type 2) response which is needed to complete parasite removal (27). Ho et al. showed that parasite clearance was related to levels of both IL-10 and IFNγ but that severe malaria was associated with an intense type 1 response (12).

Results of other studies support the idea that absolute levels of both type 1 and type 2 cytokines determine disease outcome. Specifically, extreme levels of either pro- or anti-inflammatory cytokines may be associated with heightened disease severity and mortality (5). Day et al. found mortality to be associated with high levels of IL-6, IL-10 and TNFα, the last of these being a strong pro-inflammatory, type 1 cytokine (5).

We had an opportunity to explore the role of some Th1 and Th2 cytokines in malaria parasitemia by assessing cellular cytokines extracted from liver and spleen tissues, aiming to clarify the interactions between inflammatory cytokines such as IFNγ, IL-12, IL-6 and TNFα, and anti-inflammatory cytokines such as IL-4 and IL-10 that determine the pathology of malaria and control infection.

MATERIALS AND METHODS

Mice and parasites. C57BL/6(B6) mice at the age of 8–10 weeks were used. The mice were maintained at the animal facility of Niigata University (Niigata, Japan) under specific pathogen-free conditions. P. yoelii 17XNL (non-lethal strain) was used (29). Parasites were maintained by routine passages in mice. Mice were infected by intraperitoneal (ip) injection with 10^4 parasitized erythrocytes per mouse. Parasitemia of erythrocytes in the blood was observed by Giemsa staining every 2 or 3 days.

Hematocrit percentage. Packed cell volume (PCV) can be determined by centrifuging heparinized blood obtained from mice tails in a capillary tube at 10,000 rpm for five min. This separates blood into layers. The volume of packed red blood cells divided by the total volume of blood sample gives PCV. Because a tube is used, this can be calculated by measuring the lengths of the layers.

Serum levels of anti-DNA antibody. Measurements of immunoglobulin G (IgG) and IgM antibodies reacting with single-stranded (ss) DNA obtained with the enzyme-linked immunosorbent assay (ELISA) method were modified as previously described (20). Standard sera were obtained from MRL-lpr/lpr mice (after the onset of disease) and arbitrarily determined to contain 100 units of anti-DNA antibody. In each test, the titer was expressed as a percentage in comparison with standard sera.

ELISA assay for detection of serum IL-4 and IFNγ. Blood samples were taken on the indicated days after infection. After centrifugation, sera were immediately aliquoted and stored at −80°C until tested. Pooled sera taken from mice were used to detect the concentrations of IL-4 and IFNγ by ELISA assay using Opt EIA mouse IL-4 and IFNγ sets (BD PharMingen). Optical density was read at 450 nm.

Reverse transcription (RT)-PCR for cytokines mRNA. Mice were sacrificed on the indicated days after infection. Total RNA was extracted from liver and spleen tissues of B6 mice (29). To detect mRNAs of IFNγ, IL-12P40, IL-6, TNFα, IL-4 and IL-10, RNA was subjected to reverse transcription using primers of these genes and such cDNA was further amplified by the PCR method (29). Briefly, total RNA was prepared from (30 mg) samples from liver and spleen tissues of control and infected mice by using Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized using 5 μg RNA with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) and Oligo (dT) 15 Primer (Promega, Madison, WI). PCR amplification of synthesized cDNA was conducted as previously described (7). Briefly, PCR was performed in a mixture containing 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) (BRL), 1.0 mM MgCl2, 0.2 mM dNTP, 0.2 mM sense and antisense primers, 2.5 U Taq polymerase (BRL), and 1 mL cDNA template (80 mM). PCR amplification was performed by Gene Amp PCR System 9700 (Perkin Elmer, Norwalk, CA). Thirty cycles of amplifications were run under conditions of denaturation at 94°C for 1 min,
annealing at 60°C for 1 min and elongation at 72°C for 2 min. PCR products as well as markers were electrophoresed on 2% agarose gel and stained by ethidium bromide to confirm single-band products. Primers of IFNγ, IL-12P40, IL-6, TNFα, IL-4, IL10 and G3PDH were as follows: IFNγ (sense 5’-AAT GAA CGC-TAC ACA CTG CA-3’ and antisense 5’-TGA AGA AGG TAG TAA TCA GG-3’); IL-12P40 (sense 5’-CGT GCT CAT GGC TGG TGC AAA G-3’ and antisense 5’-CTT GAT CTG CAA GTT CTT GGG C-3’); IL-6(sense 5’-GAC TTC CAT CCA GTT GCC TTC TTG GGA-3’ and antisense 5’-AGG AGA GCA TTG GAA ATT GGG GTA G-3’); TNFα (sense 5’-ATG AGC ACA GAA AGC ATG ATC CGC-3’ and antisense 5’-AAA GTA GAC CTG CCC GGA CTC-3’); IL-4(sense 5’-CCA GCT AGT TGT CAT CCT GC-3’ and antisense 5’-CCA GCT ATG ATC CGC-3’ and antisense 5’-GTG ATG TGG ACT TGG ACT CA-3’); IL-10 (sense 5’-GGA CAA CAT ACT GCT GAC TCT GGG AGA GAG GTA CA-3’); G3PDH (sense 5’-ACC ACA GTC CAT GAA ATC AC-3’ and antisense 5’-TCC ACC ACC CTG TTG CTG TA-3’). Primers for G3PDH were used to assess the integrity of the RNA preparation.

RESULTS

Parasitemia and hematocrit
Time kinetic study for parasitemia showed that parasites appeared in the blood on day 3, and that parasitemia gradually increased and reached a peak of around 60% on day 15 and started to recover on day 21 with a complete recovery on day 25 (Fig. 1A). On the other hand, hematocrit percentage showed that mice became severely anemic at the peak and that the percentage returned to normal value during recovery (Fig. 1B).

Serum levels of anti-DNA Ab
The levels of both IgM and IgG-type autoantibodies against denatured DNA were measured (Fig. 2). Both of them gradually increased from day 7 with an obvious high level at day 14 more in IgM than in IgG. Though the level of IgG and IgM decreased on day 21, it was still higher than the normal one (*P < 0.05).

Serum cytokine profile during malaria infection
The serum cytokine profile was examined in terms of IFNγ and IL-4 (Fig. 3). A time kinetic study showed that both IFNγ and IL-4 were detected after infection and that the level of IFNγ was higher than that of IL-4 on day 7. IL-4 was still high while
each other in the liver, although their expressions in the spleen were higher than in the liver.

**DISCUSSION**

Cytokines may be key determinants of malaria severity and outcome (5, 12, 24, 27, 30–33) and thus are potential targets for therapeutic interventions if their effects can be better understood. The main aim of immunological research on malaria over the past 30 years has been vaccine development and has thus, by necessity, focused on adaptive immune responses. However, growing awareness of the protective role of innate immune mechanisms in their own right and their role in the induction of adaptive immunity suggest that studies of innate immunity against malaria are warranted (2).

Rapid induction of cytokines (such as IL-12, IFNγ and TNFα) may enable an infected host to effectively control exponential replication of blood stage parasites until the adaptive immune response can take over. This might be beneficial during primary infection, but given the extent of antigenic polymorphism in malaria, innate responses may also be required to control reinfections of variant genotype until novel adaptive responses can be generated (2).

The present study demonstrated the course of malaria in mice infected with a non-lethal strain of *P. yoelii*, which coincides with the degree of anemia throughout stages of infection. Severe anemia was observed at the peak of infection and hematocrit percentage returned to the normal value during recovery, in good agreement with the findings of Weerasinghe *et al* (29). We and other investigators

**Fig. 3** Assays for serum levels of cytokines in B6 mice after malarial infection. Titers of IFNγ and IL-4 in sera were determined by ELISA method. Mean values with SD were calculated for four mice at each time-point. *P < 0.05.

**Fig. 4** Detection of cytokines mRNA from liver and spleen tissues of B6 mice during malarial infection. Expression of inflammatory and anti-inflammatory cytokine at the indicated points of time. The integrity of RNA was confirmed by detection of G3PDH.
previously reported that resistance to malarial infection was achieved by innate immunity. Kinetics of primary malaria infections in mice suggests that innate responses are essential to limit initial phase of parasite replication (3, 4, 9, 10). Also, there is evidence from experimental human infection that parasite growth can be modulated very early during primary infection (19).

We were not surprised to find that the serum level of autoantibody titers (both IgG and IgM types) increased prominently after malarial infection and that these immunologic responses resembled those seen in autoimmune diseases (13, 18, 21). This phenomenon, seen during malarial infection, seems to be a constituent of innate immunity, which might be due to the nature of Plasmodium as an intracellular pathogen in erythrocytes (the blood stage) and hepatocytes (the liver stage).

The role of cytokines in infection or various physiologic states has usually been inferred from measurements of serum or plasma cytokine levels. This approach has a number of positive aspects, not the least of which is the feasibility of assaying many samples quickly. In this study, we examined cytokine profiles of IL-4 and IFNγ in sera after malarial infection. In the early phase, both IL-4 and IFNγ were produced, namely, a Th0-type cytokine profile; these observations are consistent with those of Paul et al. (23). However, this pattern changed in the late phase, only IL-4 being detected in sera, namely, a Th2-type cytokine profile (8, 34). Despite many research studies involving serum and plasma cytokine assessments, this approach does have a number of limitations. Serum and plasma cytokine levels can be affected by receptor binding, temperature degradation, urinary excretion, and cytokine breakdown within reacting cells (15). To be more precise, the present study demonstrated the role of some inflammatory (i.e., IFNγ, IL-12p40, IL-6 and TNFα) and anti-inflammatory (i.e., IL-4 and IL-10) cytokines mRNA extracted from liver and spleen tissues, as they are considered to be the most affected organs. It was obvious that there is coordination between Th1 and Th2 cytokines and co-operation between affected organs, namely, the liver and spleen throughout stages of infection. Inflammatory cytokines were highly expressed in the acute stage, which signaled the immune system to take action, while some anti-inflammatory cytokines were expressed in early stage to counteract the high level of inflammatory cytokine and continued their effect till recovery to control the inflammatory process and minimize pathology. These observations well agree with those of previous studies and suggest that the balance between pro-inflammatory (TNFα, IFNγ, IL-6 and IL-8) and anti-inflammatory (IL-4 and IL-10) cytokines determines the degree of malaria parasitemia, level of anemia, clinical severity, presentation, and/or outcome (11, 22, 28, 32), while other studies have suggested that absolute levels of cytokines, not a balance between them, determine the outcome of malaria. Specifically, extreme levels of anti-inflammatory as well as pro-inflammatory cytokines may be associated with an increase in disease severity and mortality (5). Our observations demonstrate the correlation between serum and cellular levels of IL-4 and IFNγ in the acute stage. During recovery, the serum level of IL-4 remains high, suggesting that anti-inflammatory cytokines continue their effect till complete recovery.

Taken together, these observations lead to the conclusion that control of the early peak of parasitemia in murine malaria infections is dependent on innate rather than adaptive immune mechanisms, raising important questions about the role of innate immunity in the control of human malaria. It is also suggested that there is a critical balance in the inflammatory response to malaria infection and that the ability to regulate cytokine levels, within quite precise limits, may be a component of clinical immunity. However, further studies are needed to better understand the reciprocal and balancing effects of inflammatory cytokines and anti-inflammatory cytokines in determining resolution or persistence and worsening of malaria infection.

REFERENCES


