Neutrophil Depletion during *Toxoplasma gondii* Infection Leads to Impaired Immunity and Lethal Systemic Pathology

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The immunomodulatory role of neutrophils during infection with *Toxoplasma gondii* was investigated. Monoclonal antibody-mediated depletion revealed that neutrophils are essential for survival during the first few days of infection. Moreover, neutrophil depletion was associated with a weaker type 1 immune response as measured by decreased levels of gamma interferon, interleukin-12 (IL-12) and tumor necrosis factor alpha. IL-10 was also decreased in depleted animals. Additionally, splenic populations of CD4+ T cells, CD8+ T cells, and NK1.1+ cells were decreased in depleted mice. Neutrophil-depleted mice exhibited lesions of greater severity in tissues examined and a greater parasite burden as determined by histopathology and reverse transcription-PCR. We conclude that neutrophils are critical near the time of infection because they influence the character of the immune response and control tachyzoite replication.

The immune system is a complex nonlinear system, involving the coordination of multiple cell types. Disease often results from an insufficient immune response. Lack of protection can occur when any one or more of the components of the immune system are impaired. Neutropenia is a risk factor associated with Aspergillus fumigatus, Candida albicans, Strongyloides ratti, Yersinia enterocolitica, Chlamydia trachomatis, Mycobacterium tuberculosis, Listeria monocytogenes, and *Toxoplasma gondii* infections (2, 3, 5, 7, 8, 22, 27, 31, 41). In some cases, neutropenia has been correlated with impaired protective acquired immunity, suggesting that neutrophils function as immunomodulators of acquired immunity (27, 31). The list of microbes affected by the actions of neutrophils is ever-growing, and it is evident that these cells are involved in the immune response to a highly diverse array of both intracellular and extracellular pathogens.

We examined the development of immunity during *T. gondii* infection in mice depleted of neutrophils by monoclonal antibody (MAb) administration. *T. gondii* is an obligate intracellular protozoan parasite that poses an important public health risk. Congenital infections may result in severe birth defects, and reactivation of chronic infection can lead to development of encephalitis, a particular problem for persons who are immunosuppressed (25, 28). We found that neutrophil depletion at the time of infection led to development of lesions in multiple organs, including the spleen, lung, liver, and brain, and was associated with an impaired ability to produce early gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12). Splenic populations of T cells and NK cells were decreased in neutrophil-depleted infected mice. Moreover, neutrophil-depleted mice harbored an increased parasite burden. We conclude that neutrophils are important immunomodulators early in the course of *T. gondii* infection and play a critical role in protecting the host from uncontrolled tachyzoite replication.

**MATERIALS AND METHODS**

**Mice.** C57BL/6 female mice (6 to 12 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, Maine). The animals were housed under specific-pathogen-free conditions at the College of Veterinary Medicine animal facility at Cornell University. The college maintains an animal facility that is accredited by the American Association for Accreditation of Laboratory Animal Care.

**Parasites and infections.** ME49 bradyzoite cysts were maintained in Swiss Webster mice as described previously (5). Mice were rendered neutropenic with an anti-Gr-1 MAb (RB6C6.3C5 hybridoma originally provided by R. L. Coffman, DNAX Research Institute, Palo Alto, Calif.) or a control rat immunoglobulin G (IgG) (Accurate, Westbury, N.Y.) and infected intraperitoneally (i.p.) with 100 ME49 cysts as described previously (3).

Soluble tachyzoite antigen (STAg) was prepared as previously described (5). Briefly, tachyzoites were sonicated in the presence of protease inhibitors (0.2 mM phenylmethylsulfonlfuoride, 0.2 mM aprotinin, 1 mM leupeptin, and 1 mM EDTA), dialyzed into phosphate-buffered saline, filter sterilized through a 0.2-μm-pore-size membrane. (Corning Costar Corp., Cambridge, Mass.), assayed for protein concentration, and stored at −70°C. Parasite extracts were found to be free of endotoxin as measured by the Limulus amebocyte assay.

**Splenocyte cultures.** Mice were depleted of neutrophils by MAb administration (depleted infected mice) or given a control rat IgG (control infected mice) on days −2, 0, +2, and +4 and were infected i.p. with 100 ME49 cysts on day 0. Mice were euthanatized on days +2,+4,+6, and +8, and spleens were harvested. Plasma was also obtained at the time of euthanasia. Splenocytes from uninfected, control infected, and depleted infected mice were cultured at 5 × 10⁶ cells/ml at 37°C and 5% CO₂ or stained for flow cytometry (see below). Cells were stimulated with medium or STAg at 2, 20, or 200 μg/ml. After 3 days, cell-free supernatants were collected and stored at −20°C until cytokine analysis.

**Cytokine measurement.** IL-12 p40 and IL-10 were measured in cell-free supernatants by enzyme-linked immunosorbent assay (ELISA) as described in detail previously (5, 39). To measure IFN-γ, the protocol for determining p40 levels was followed, except that clone HB170 (American Type Culture Collection, Manassas, Va.) was used as the coating antibody (Ab) at 10 μg/ml and clone XMG-biotin (PharMingen, San Diego, Calif.) was used as the secondary Ab at 1:4,000 dilution. TNF-α levels were determined using a murine-specific kit according to the manufacturer's instructions (R & D Systems, Minneapolis, Minn.). Detection sensitivities were 10 pg/ml for IL-12 p40, 20 pg/ml for TNF-α, 30 pg/ml for IL-10, and 75 pg/ml for IFN-γ.

**Flow cytometric analysis.** Splenocytes at 5 × 10⁶/ml were immediately stimulated ex vivo for 4 hours with phorbol myristate acetate and ionomycin (5 and 500 ng/ml, respectively; Sigma) according to the PharMingen protocol for intracellular murine IFN-γ detection. Brefeldin A (GolgiPlug) was added for the last 2
FIG. 1. Granulocytes are necessary for survival of T. gondii infection only during the first few days of infection. C57BL/6 mice were injected i.p. either with 200 μg of the granulocyte-depleting Ab RB6C6.8C5 on days −2, 0, +2, +4, and +6, or days +2, +4, +6, and +10 or with a control rat IgG on days −2, 0, and +2. Mice (four per group) were infected i.p. with 100 ME49 cysts on day 0 (four mice per group). Survival was monitored daily. Results represent three experiments.

RESULTS

Neutrophils are required for resistance during early- but not late-stage infection. It has been demonstrated that neutrophils are essential at the time of infection for survival in a murine model (5, 33, 35). To better define when they are required during the acute stage of disease, we depleted mice of granulocytes by MAb administration at different times. Mice required during the acute stage of disease, we depleted mice of murine model (5, 33, 35). To better define when they are

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Neutrophil-depleted mice display a weaker type 1 immune response. Equal numbers of splenocytes from uninfectected, control infected, and depleted infected mice were cultured in medium for 3 days. When constitutive cytokine levels in cell-free supernatants were assessed, we found profound decreases in the control group at all time points tested (Fig. 2). Nevertheless, IFN-γ production by splenocytes from these mice recovered by day 6 postinfection. Levels of IL-12 in splenocyte supernatants from depleted infected animals were substantially lower than levels in the control group at all time points tested (Fig. 2).
Similarly, levels of TNF-α from depleted infected splenocyte cultures were always lower than levels in control samples, and indeed, TNF-α was not detected at any time point (Fig. 2). The basis for this dramatic effect is under investigation. Expression of IL-10 typically follows production of proinflammatory cytokines and is thought to control the tissue-destructive activities encountered in an inflammatory reaction (23, 26). We found that IL-10 levels in splenocyte cultures from depleted infected animals were consistently lower than those measured in control samples (Fig. 2). Levels of IL-4 and IL-5 were below the level of detection (data not shown). In contrast, the frequencies of IFN-γ1 cells at 6 days postinfection in depleted mice. While the percentages of IFN-γ+ CD4+ cells out of the total CD4+ population were similar in depleted and control groups (22 and 23%, respectively), fewer CD8+ cells were IFN-γ+ relative to the total CD8+ population in the depleted group than in the control group (22 and 35%, respectively; P < 0.05). In general, effects on the CD8+ subset were more pronounced at all time points (data not shown). In contrast, the frequencies of NK1.1+ cells and IFN-γ+ NK1.1+ cells were similar between the control-infected and depleted-infected groups (Fig. 5A).

At the time of euthanasia, it was noted that the spleens from depleted mice were grossly smaller than those from either uninfected or control-infected mice. The differences in size were reflected in total cell counts. In Fig. 5B, the total numbers of cells in each subset are shown. By day 6 postinfection, there was an expansion in the numbers of CD4+, CD8+, B220+, and NK1.1+ cells from control mice. When the number of IFN-γ+ cells in each subset was plotted, a similar expansion was noted for CD4+ and CD8+ cells (Fig. 5C). There were approximately one-fifth the number of IFN-γ+ CD4+ cells and IFN-γ+ CD8+
cells in the spleens of depleted animals. At this stage of infection, there were similar levels of NK1.1\(^+\) and B220\(^+\) cells staining for IFN-\(\gamma\). It has been noted that the Ab used to deplete mice of neutrophils, which recognizes Gr-1 or Ly-6G, may cross-react with Ly-6C, an epitope found on CD8\(^+\) T cells (9, 24). Montes de Oca et al. (24) reported an approximately 25 to 30% loss of CD8\(^+\) T cells in uninfected spleen cell populations upon administration of RB6C6.8C5, a figure consistent with our own studies (data not shown). While this Ab may contribute to the loss in CD8\(^+\) T cells, it is unlikely to account for the nearly 80% loss of these cells in infected populations. As demonstrated by the data in Fig. 5, the total numbers of IFN-\(\gamma\)-producing cells differed greatly by group. Overall, these data indicate a decrease in the number of cell types known to be important in mediating protection against \(T.\ gondii\) when mice are depleted of neutrophils at the time of infection. The loss of CD8\(^+\) T cells itself is unlikely to account for the inability to survive early infection, since mice negative for this T-lymphocyte subset, through either Ab treatment or gene deletion, are capable of surviving acute \(Toxoplasma\) infection (10, 34).

**Neutrophil-depleted mice display extensive pathologic changes in lymphoid and nonlymphoid tissue.** Tissues from all major organs were collected from depleted-infected, control-infected, and uninfected mice at the time when neutropenic mice...
became clinically ill (usually day 8). Tissues were fixed, and sections were stained with hematoxylin and eosin. Figure 6 demonstrates typical lesions found in the mice. In the spleens of neutrophil-depleted animals, there was extensive lymphoid follicular depletion due to marked lymphoid necrosis (Fig. 6B). In addition, there was moderate myeloid hyperplasia in peri-follicular areas and red pulp (data not shown). Intracellular and extracellular tachyzoites were abundant (Fig. 6B). In contrast, lesions present in infected control mice were much less severe, and parasites were less numerous (Fig. 6A). Severe granulomatous and necrotizing lymphadenitis with marked disruption of architecture and small numbers of organisms were found in the mesenteric lymph nodes of these animals (Fig. 6D). Lesions in the mesenteric lymph nodes from control infected mice were less severe (Fig. 6C). The uninfected mice displayed no evidence of pathology (data not shown).

In general, lesions in nonlymphoid tissues of depleted infected mice were more extensive and severe than in infected control mice. There was moderate to locally-extensive alveolitis with moderate numbers of tachyzoites in the pulmonary tissue of depleted mice (Fig. 7B). Pulmonary tissue from control infected mice exhibited less severe inflammation, and tachyzoites were less abundant (Fig. 7A). The livers from depleted infected mice demonstrated moderate to marked hepatocyte cord dissociation with moderate cytoplasmic vacuolar degeneration (Fig. 7D). Additionally, there were small, multifocal areas of lymphoplasmacytic infiltrates accompanied by few macrophages (i.e., granulomas). The livers from control infected mice had similar multifocal granulomas and individual cell necrosis (Fig. 7C). In the brains of infected depleted mice, there were locally extensive areas of gliosis and a few parasites (Fig. 7F). Lesions in the control nervous system were rare in control infected mice (Fig. 7E). No pathologic changes were noted in uninfected mice (data not shown).

**Uncontrolled tachyzoite replication occurs in neutrophil-depleted mice.** Quantitation of parasite burden in the brain, lung, liver, and spleen was performed by RT-PCR using an ABI Sequence Detector. Levels of p22 (SAG 2) and HPRT (housekeeping gene) mRNA were measured. Normalized levels of p22 in control infected mice were arbitrarily set at 1. Levels of normalized p22 from depleted-infected mice were then compared with control levels (Fig. 8). In all organs examined, there was a 1.6- to 14-fold increase in p22 expression in tissues from depleted infected mice, indicating a greater parasite burden in neutrophil-depleted animals.

**DISCUSSION**

Within the context of toxoplasmosis, the ability of neutrophils to release cytokines upon infection (3–5, 19), the occurrence of neutrophilia in vivo (19), the inability of neutropenic mice to survive infection (5, 33, 35), and the presence of increased numbers of neutrophils at the site of infection (3) all suggest that these cells play an important role in vivo. Neutrophils have also been implicated as immunomodulators in other infections. Perhaps the best-studied model is that of *C. albicans*, where neutrophil IL-12 production is associated with a protective type 1 response, while IL-10 production leads to a type 2 response and exacerbated disease (30–32). An immunomodulatory role for neutrophils has also been proposed for infections with *M. tuberculosis* and *L. monocytogenes* (7, 9, 27, 29). A recent paper by Tacchini-Cottier et al. (38) demon-
strated that neutrophil depletion at the time of infection abrogated the early burst of IL-4 that otherwise occurs in the draining lymph nodes of mice infected with Leishmania major. Subsequently, development of a type 2 response was inhibited and there was partial resolution of footpad lesions, suggesting an early nonprotective role for neutrophils in susceptible BALB/c mice. Depletion of neutrophils 1 day after infection had no effect. Similarly, neutrophil depletion after day 3 of infection with a C. albicans vaccine strain did not enhance disease (31). In fact, mice appeared to benefit from late depletion, and this was attributed to an inhibition of inflammation. In accord with these results, we found that neutrophils are required for survival only during the first few days of T. gondii infection (Fig. 1). In the case of Plasmodium berghei infection, neutrophil depletion improves the clinical course of disease. Prevention of cerebral malaria with decreased central nervous system hemorrhages and sequestration of monocytes was attributed to impaired type 1 immune response development in the brain when neutrophils were absent (6). While neutrophils appear to be generally protective during infections with T. gondii and C. albicans, they may exacerbate disease in other situations, highlighting the paradoxical nature of neutrophil function. Thus, neutrophil function can be harmful or beneficial to the host, depending upon the character and magnitude of the response induced by these cells.

Depletion of neutrophils at the time of infection with T. gondii was associated with decreased levels of IFN-γ, TNF-α, and IL-12, cytokines that are well known to be important in parasite control (Fig. 2, 3, and 4) (1, 10, 15, 36, 42). Indeed, in our studies, neutrophil-depleted mice displayed greatly increased parasite levels in tissue as determined by histopathology and RT-PCR (Fig. 6, 7, and 8). The majority of organs examined revealed lesions of enhanced severity in depleted infected mice, suggesting that these mice succumb to infection because of multiorgan system disease due to uncontrolled tachyzoite replication and associated tissue destruction. In particular, there was extensive destruction of splenic white pulp in depleted animals. This finding corresponded to decreases in the numbers of CD4⁺, CD8⁺, and NK1.1⁺ subsets (Fig. 5).

Previous studies have demonstrated that neutrophils can produce cytokines such as IL-12 and TNF-α (5, 11, 12, 32). Nevertheless, we think it unlikely that decreased cytokine levels in the neutrophil-depleted mice are a direct consequence of the loss of these cells. Thus, our hypothesis is that during early T. gondii infection, neutrophils play an instructive role in the development of immunity to the parasite.

Classically, naive T helper cells are thought to be activated in secondary lymphoid tissue (14). Previous studies show rapid neutrophil accumulation at the site of infection (3). Our hypothesis is that neutrophils exert their protective effect during
toxoplasmosis at least in part, through their ability to produce T-cell immunoregulatory cytokines. How do neutrophils exert their effects on T cells? One possibility is that neutrophils act indirectly through the actions of antigen-presenting cells, particularly dendritic cells. Numerous reports indicate a need for priming by various stimuli in order for dendritic cells to activate T cells (17, 37, 40). Neutrophils may provide these priming signals. Another possibility is that neutrophils enter secondary lymphoid tissue with phagocytosed antigen. Once there, they might influence T-cell differentiation by releasing instructive cytokines. In support of this hypothesis, Tacchini-Cottier et al. (38) found neutrophils in the subcapsular space of the draining lymph nodes after infection with L. major. Moreover, Harmsen et al. (18) demonstrated neutrophil antigen acquisition in the lung and subsequent migration to tracheobronchial lymph nodes. Antigen-containing neutrophils were found in lymphatic vessels and paracortical areas of the lymph nodes. Finally, major histocompatibility complex II expression on human neutrophils has been demonstrated, and it is possible that neutrophils act as antigen-presenting cells (13, 16, 20).

The studies presented here and elsewhere clearly demonstrate a previously unappreciated role for neutrophils as cytokine-producing immunoregulatory cells. Nevertheless, neutrophils are also well known for their phagocytic activity and release of microbial molecules during infection. Therefore, it is probable that both general functions contribute to the ability of these cells to protect against infection or, in certain situations, to exacerbate disease. The relative contributions of these activities to the outcome of infection likely depend upon the particular pathogen and, in the case of toxoplasmosis, are an area of ongoing study in our laboratory.

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REFERENCES


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