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Apoptotic Deletion of Th Cells Specific for the 19-kDa Carboxyl-Terminal Fragment of Merozoite Surface Protein 1 During Malaria Infection

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Immunity induced by the 19-kDa fragment of merozoite surface protein 1 is dependent on CD4+ Th cells. However, we found that adoptively transferred CFSE-labeled Th cells specific for an epitope on Plasmodium yoelii 19-kDa fragment of merozoite surface protein 1 (peptide (p)24), but not OVA-specific T cells, were deleted as a result of P. yoelii infection. As a result of infection, spleen cells recovered from infected p24-specific T cell-transfused mice demonstrated reduced response to specific Ag. A higher percentage of CFSE-labeled p24-specific T cells stained positive with annexin and anti-active caspase-3 in infected compared with uninfected mice, suggesting that apoptosis contributed to deletion of p24-specific T cells during infection. Apoptosis correlated with increased percentages of p24-specific T cells that stained positive for Fas from infected mice, suggesting that P. yoelii-induced apoptosis is, at least in part, mediated by Fas. However, bystander cells of other specificities also showed increased Fas expression during infection, suggesting that Fas expression alone is not sufficient for apoptosis. These data have implications for the development of immunity in the face of endemic parasite exposure.


Materials and Methods

Experimental animals and parasites

Six- to 8-wk-old male and female BALB/c mice were purchased from Animal Resources Center (Willetton, Australia). P. yoelii YM (lethal strain) was used.

The rmSP1 19 protein and Ags

MSP1 19 of P. yoelii was produced in Saccharomyces cerevisiae as described previously (16). A dominant T cell epitope on MSP1 19 was synthesized as described previously (4). OVA was used as a control Ag (Sigma, St. Louis, MO).

Generation of T cell lines

T cell lines specific to p24, OVA, or P. yoelii YM were generated as described previously (4). Briefly, BALB/c mice were immunized in hind footpads with Ag (30 μg of peptide, 100 μg of OVA, or 3 × 107 parasitized RBC (PRBC) lysate) emulsified in CFA. Nine to 10 days later, popliteal and inguinal lymph nodes were removed, and a single-cell suspension was made. Cells were washed and cultured at a concentration of 2 × 10^6 cells/ml in complete medium. After 5 days, the cells were transferred to medium containing IL-2 at 10 U/ml.

Abbreviations used in this paper: MSP1 19, 19-kDa fragment of merozoite surface protein 1; p, peptide; FasL, Fas ligand; PRBC, parasitized RBC.
with 0.25 μCi of [3H]thymidine, and incorporation of radio-label was estimated 18–24 h later by β-emission spectroscopy.

**Lymphoproliferation assay**

Spleen or lymph node T cells were cultured in a volume of 200 μl in MEM supplemented with 50 μM 2-ME and 10% heat-inactivated normal mouse serum at 2 × 10^5 cells/ml in flat-bottom 96-well plate. Cells were cultured with different concentrations of Ags for 72 h and were then pulse labeled with 0.25 μCi of [3H]thymidine, and incorporation of radio-label was estimated 18–24 h later by β-emission spectroscopy.

**Cell surface phenotype characterization**

Single-cell suspensions of T cell lines or spleen cells were stained with PE- or FITC-conjugated mAbs specific for mouse CD4, CD3, TCRαβ, TCRγδ, CD120b (TNFR-p75), CD120a (TNFR-p55) (Caltag Laboratories, Burlingame, CA), Fas, FasL (BD PharMingen, San Diego, CA). Cells were incubated for 30 min at 4°C, washed twice with FACS buffer (0.1% BSA, 0.1% sodium azide, and PBS) and resuspended in 250 μl of 1% paraformaldehyde. The percentage of positive cells was measured by FACS (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences).

**Bioassay for IFN-γ, IL-2, and IL-4**

Culture supernatants were collected 24, 48, and 72 h after stimulation. IFN-γ, IL-2, and IL-4 activity was determined as described previously (15). IFN-γ activity was determined by measurement of inhibition of WEHI-279 cell proliferation. IL-2 and IL-4 activities were determined by using the growth-dependent CTL-2 and CT.4S cell lines, respectively. The concentrations were calculated from standard cytokine titers in the assays.

**Priming mice with p24**

Normal BALB/c mice were primed s.c. with PBS or 20 μg of p24 emulsified in CFA (Sigma). Two weeks later, mice were challenged i.p. with 20 μg of MSP1_{19} in IFA. Sera were collected for determining Ab to MSP1_{19}.

**Immunization of T cell-transfused mice with MSP1_{19}**

Nude mice were administered 5 × 10^6 p24- or OVA-specific T cells i.v., and 24 h later, they were then immunized with PBS or 20 μg of MSP1_{19} emulsified in CFA. Sera were collected and analyzed by ELISA.

**Ab assay**

Serum Ab levels were analyzed by ELISA as described previously (1). Briefly, Maxisorb immunoplates (Nalge Nunc International, Naperville, IL) were coated overnight at 4°C with 100 μl of 0.3 μg/ml MSP1_{19}. After three washes with 0.05% Tween 20PBS, wells were blocked with 200 μl of 1% BSA/PBS and incubated for 1 h at 37°C. Supernatants were discarded, and 100-μl serial dilutions of sera in 1% BSA in Tween 20PBS were added. After incubation at 37°C for 1 h, wells were washed, and 100 μl 1/3000 dilution of HRP-conjugated sheep anti-mouse IgGs (SILENUS Labs, Boronia, Australia) was added. After incubation at 37°C for 1 h, wells were washed, and 100 μl of substrate solution (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma) was added, and wells were incubated at room temperature for 30 min. The absorbance was read at 405 nm.

**In vivo study of CFSE-labeled cells**

Parasite-, p24-, or OVA-specific T cell lines were labeled with CFSE as described previously (17). Briefly, viable resting T cells were adjusted to 1 × 10^6 cells/ml in PBS and were stained with CFSE at a final concentration of 10 μM. Cells were incubated at 37°C for 30 min, washed twice with cold MEM, and resuspended in MEM. Labeled T cells were administered to nude mice, and some of these mice were challenged 4 h later with 10^5 P. yoelii YM PRBC. Mice were sacrificed 4 and 6 days after challenge, and spleen, lymph nodes, peripheral blood, and bone marrow were collected. Single-cell suspensions were prepared, washed, and CFSE staining was analyzed by FACS.

**Apoptosis study**

**Annexin staining.** Externalization of phosphatidylserine was detected by FITC-conjugated annexin V (Boehringer Mannheim, Mannheim, Germany) or PE-conjugated annexin V (Bender MedSystems, Vienna, Austria). In brief, 10^6 cells were washed with binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 5 mM CaCl_2). Cells were incubated for 10–15 min at room temperature with annexin V in binding buffer containing propidium iodide to exclude necrotic cells and were then analyzed by FACS.

**TUNEL assay.** Detection of cleavage of genomic DNA during apoptosis was performed by using the cell death detection kit (Boehringer Mannheim). Briefly, cell suspensions were incubated with FITC-conjugated anti-CD4 mAb at room temperature for 30 min. Cells were washed twice with FACS buffer and then fixed with 4% paraformaldehyde for 1 h at room temperature. Cells were washed once and permeabilized with FACS permeabilizing solution (BD Biosciences) for 10 min at room temperature. Cells were washed twice and then incubated with TUNEL reaction mixture for 1 h. Cells were washed twice, resuspended in FACS buffer, and analyzed by flow cytometry.

**Statistics**

Student’s t test for unpaired observations was used to determine differences between groups.

**Results**

**Characterization of T cell lines**

T cell lines specific to p24, OVA, and P. yoelii were generated by repeated cycles of stimulation with specific Ags in vitro. T cell lines specific to p24 and OVA were 99% CD4+ and 99% TCRβ+. When the lines were stimulated with specific Ags, they proliferated and secreted IFN-γ and IL-2 with no IL-4 detected as determined by bioassay (Fig. 1). p24 immunization can elicit T cell help for an MSP1_{19}-specific Ab response

Normal BALB/c mice were primed with PBS or p24 and then challenged 2 wk later with MSP1_{19}. There was no MSP1_{19}-specific Ab detected in baseline sera or sera collected before challenge (Fig. 2). The Ab level increased rapidly in mice that were primed with p24 compared with mice that were primed with PBS. The data suggest that p24 is recognized by p24-specific Th cells, which provide help to MSP1_{19}-specific B cells. The helper role of p24-specific T cells was thus investigated. Nude mice were administered p24-specific T cells and were then immunized with PBS or MSP1_{19}. Mice that received OVA-specific T cells were used as controls. Sera were collected and assayed for MSP1_{19}-specific Ab levels by ELISA. Baseline sera from mice that later received p24 T cells were and were immunized with MSP1_{19}. Did not contain detectable Abs; however, Ab tiers specific for MSP1_{19} increased after immunization with MSP1_{19} (Fig. 3). No significant level of MSP1_{19}-specific Abs was detected in p24 T cell-transfused nude mice that were immunized with PBS. No anti-MSP1_{19}-specific Abs were detected in nude mice that were given OVA-specific T cells and were then immunized with PBS or MSP1_{19} (data not shown). The results confirm that p24-specific T cells can provide help to MSP1_{19}-specific B cells.

T cell apoptosis in normal BALB/c mice during P. yoelii YM infection

To investigate whether P. yoelii infection induces T cell apoptosis, normal BALB/c mice were infected with 10^5 P. yoelii YM PRBC i.v. on day 0. Spleen cells were harvested on days 1, 2, 4, and 6 after infection, and CD4+ T cell apoptosis was assessed by dual staining with CD4-PE and annexin, which binds phosphatidylserine on the surface of apoptotic cells. A TUNEL assay was also performed. The
percentages of CD4$^+$ cells recovered from spleens of infected mice were consistently lower than those recovered from uninfected mice (uninfected mice - infected mice = 5.54, 6.8, and 5.38% on days 2, 4, and 6, respectively; Fig. 4A). Of the remaining CD4$^+$ cells in spleens from infected mice, a higher proportion stained positive for annexin on days 4 and 6 after challenge compared with CD4$^+$ cells from uninfected mice (Fig. 4B). This correlated with the results from the TUNEL assay (Fig. 4C), suggesting that *P. yoelii* infection induced apoptosis of CD4$^+$ cells. Spleen cells were also stained for apoptosis-related molecules. There was an increase of Fas, FasL, TNFR1, and TNFR2 expression on the surface of CD4$^+$ cells recovered from infected mice on days 4 and 6 (data not shown).

**Deletion of p24-specific T cells during malaria infection**

To investigate whether *P. yoelii YM* infection induces apoptosis of MSP1$_{19}$-specific T cells, T cell lines specific to p24, whole parasite, and OVA were generated, and resting T cells from the lines

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**FIGURE 1.** Characteristics of p24 and OVA T cell lines. T cell lines were analyzed for cell surface phenotype (A), IFN-γ (B), IL-2 (C), and IL-4 (D) production and for proliferative response to specific Ags (E). The concentration of IFN-γ and IL-2 were determined by estimation from a standard curve. The standard concentration of IL-4 was 1000 U/ml.
were tagged with CFSE and transferred into nude mice ($1 \times 10^7$ cells/mouse). Four hours later, the mice were infected with $10^4$ P. yoelii YM PRBC. Nude mice that received T cells alone but were not infected were used as controls. Mice were sacrificed 6 days after infection, and peripheral blood, spleen, lymph nodes, and bone marrow were collected for analysis of CFSE$^+$ cells. Fig. 5 shows the FACS profile of CFSE-labeled p24-specific T cells from spleens. There was a reduction in the number of p24-specific T cells in spleens of infected compared with uninfected mice. Fig. 6 is a summary of the absolute numbers of CFSE$^+$ cells recovered from various tissues. The numbers of OVA-specific T cells were not affected by infection. In contrast, the numbers of p24- and whole parasite-specific T cells were reduced by 6 days after infection. Infection resulted in a decrease of p24- and P. yoelii-specific T cells in all sites. Disappearance of CFSE$^+$ cells was not due to extensive division of T cells to the extent that the CFSE label was reduced beyond detection. This was confirmed by double staining of CFSE$^+$ cells with PE-conjugated anti-mouse CD4, which showed that there was no increase of CD4$^+$ cells (data not shown). Deletion of p24-specific T cells was comparable to the deletion of P. yoelii-specific T cells.

Spleen cells recovered from infected OVA-specific T cell-transfused mice responded to specific Ag at the same level as spleen...
cells from uninfected mice. In contrast, spleen cells from p24-specific T cell-transfused mice proliferated significantly less in response to p24 or MSP1<sub>19</sub> (Fig. 7), indicating that deletion of p24 T cells interferes with the specific immune response.

**FIGURE 6.** Recovery of cells from various organs of infected (■) and uninfected (□) nude mice that had received CFSE-labeled OVA-, *P. yoelii* YM-, or p24-specific T cells 6 days earlier. Cells from individual spleens, whole-blood, lymph nodes, and bone marrow were assessed. Data are the number of CFSE<sup>+</sup> cells per spleen (A), per milliliter of blood (B), per two inguinal and popliteal lymph nodes (C), and per two femurs (D). The data show the mean ± SE of three mice.

**FIGURE 7.** Proliferative response of spleen cells recovered from uninfected (□) or infected (■) nude mice that received OVA- or p24-specific T cells 6 days after infection. The ∆cpm per spleen was determined ([(cpm in the presence of Ag − cpm in the absence of Ag) × total number of spleen cells]/4 × 10<sup>5</sup> cells). The data show the mean ± SE of three mice.

**FIGURE 8.** Annexin V staining of CFSE-labeled spleen cells 6 days after infection. Nude mice were administered CFSE-labeled OVA- or p24-specific T cells and were (■) or were not (□) infected with 1 × 10<sup>4</sup> *P. yoelii* YM PRBCs. The data show the mean ± SE of three mice.

**Apoptosis is responsible for deletion of p24 T cells**

To determine the mechanism of deletion of p24-specific T cells, spleen cells from OVA- or p24-specific T cell-transfused mice were stained with annexin V to identify apoptotic cells. As shown in Fig. 8, there was no significant difference in the percentage of annexin-positive CFSE-labeled OVA T cells between uninfected and infected mice.
and infected mice. In contrast, a higher percentage of CFSE-labeled p24-specific T cells stained positive with annexin V in infected compared with uninfected mice, and this approach suggested that apoptosis contributes to deletion of p24-specific T cells during infection.

Increase in percentages of Fas-positive malaria-specific and nonspecific T cells during infection

To investigate the pathway involved in apoptosis of p24-specific T cells during P. yoelii infection, CFSE-labeled T cells recovered from spleens 6 days after infection were stained with Abs specific for apoptosis-related molecules. There were higher percentages of CFSE-labeled p24-specific T cells that stained positive for Fas in infected compared with uninfected mice (Fig. 9). However, there was also an increase of Fas− CFSE-labeled OVA-specific T cells in infected mice compared with uninfected mice. Although these latter data did not reach significance (p = 0.07), the data suggest that Fas is up-regulated on CD4+ T cells during malaria infection. No significant increase of FasL, TNFR1, or TNFR2 on the surface of p24-specific T cells, but not OVA-specific T cells, was detected. It seems likely that activation-induced cell death requiring TCR engagement by peptide-MHC complex may be responsible for the deletion. The results in Fig. 5 indicate that transferred cells do not need to see the Ag to proliferate in the host, but they need to see the Ag to die. Activation of TCR cross-linking induces a rapid expression of FasL, which in turn up-regulates Fas, and the subsequent interaction activates the cell-death program (29, 30). In addition, repeated release of parasite Ags after P. yoelii infection may lead to death of p24- and whole-parasite-specific T cells. It has been demonstrated that prolonged conjugation between effector T cells and APCs can result in apoptosis of T cells (31). Nevertheless, Th1 cells have been shown to be more susceptible to either Fas-induced (32–34) or NO-induced (35) apoptosis than Th2 cells. Because T cell lines used in the present study exhibited Th1 type, it is of interest to determine whether Th2 cells would survive better following infection.

Previous studies in other Plasmodium species have suggested that malaria-induced apoptosis is mediated by Fas (12, 13, 36). Increased percentages of Fas+ CFSE-labeled p24 T cells from infected mice (Fig. 9) suggest that Fas may, at least in part, be responsible for P. yoelii-induced apoptosis. There was no major increase in the level of TNFR expression on the surface of p24 T cells, suggesting that TNF is probably not the major mediator of apoptosis. However, although the absolute number of OVA-specific T cells was not affected by P. yoelii infection (Fig. 6), there was an increase in percentage of Fas+ cells in infected mice (Fig. 9). This might result from cytokines such as TNF or IFN-γ being produced by the host after exposure to malaria parasites. The ability of IFN-γ and TNF to up-regulate Fas and FasL expression has been reported (37–40). It has been demonstrated that the requisite Fas/FasL interaction can occur on a single activated cell (30), and only T cells that receive TCR engagement at the time of Fas expression will undergo apoptosis (41–43). This may explain why bystander T cells, such as OVA-specific T cells, were not deleted.

CD4+ T cells (15). We now show that such deletion also occurs for T cells specific for a helper epitope present on MSP119. As shown above, p24-specific T cells act as Th cells, providing help to MSP119-specific B cells to produce anti-MSP119 Abs (Figs. 2 and 3). In preliminary experiments, nude mice given p24-specific T cells, but not mice given OVA-specific T cells, which were then immunized with MSP119, showed protection against lethal challenge infection (data not shown), indicating the importance of p24-specific Th cells in protective immunity. An increase in the percentage of CFSE-labeled p24 T cells that stained positive with annexin V indicated that apoptosis was involved in such deletion.

Apopoposis of responding T cells may be a mechanism used by pathogens to escape the host immune response. Activation-induced T cell death is responsible for exacerbation of Trypanosoma cruzi growth in macrophages (23). Toxoplasma gondii infection induces CD4+ T cell apoptosis, and as a consequence, a transient state of unresponsiveness occurs (24). The mechanism and the factors responsible for malaria-induced apoptosis remain to be established. It is possible that the parasite itself may induce apoptosis directly. The addition of P. falciparum schizont-rich extract induces lymphocyte apoptosis in vitro (14). It has been reported that P. yoelii has a superantigenic-like activity, which induces a preferential deletion of T cells expressing Vβ9 (25). The study of cerebral malaria induced by P. berghei shows that T cells bearing the TCR Vβ8 are overactivated during infection (26). In general, superantigens activate T cells through the appropriate Vβ chains, which result in the cells dividing before undergoing apoptosis (27, 28).

In the study reported here, only whole parasite- and p24-specific T cells, but not OVA-specific T cells, were deleted. It seems likely that activation-induced cell death requiring TCR engagement by peptide-MHC complex may be responsible for the deletion. The results in Fig. 5 indicate that transferred cells do not need to see the Ag to proliferate in the host, but they need to see the Ag to die. Activation of TCR cross-linking induces a rapid expression of FasL, which in turn up-regulates Fas, and the subsequent interaction activates the cell-death program (29, 30). In addition, repeated release of parasite Ags after P. yoelii infection may lead to death of p24- and whole-parasite-specific T cells. It has been demonstrated that prolonged conjugation between effector T cells and APCs can result in apoptosis of T cells (31). Nevertheless, Th1 cells have been shown to be more susceptible to either Fas-induced (32–34) or NO-induced (35) apoptosis than Th2 cells. Because T cell lines used in the present study exhibited Th1 type, it is of interest to determine whether Th2 cells would survive better following infection.
after malaria infection, but only parasite Ag-specific T cells that become susceptible to death are killed.

In summary, we found that T cells specific for a Th cell epitope on MSP119 were deleted via apoptosis during P. yoelii infection. Deletion or apoptosis of MSP119-specific Th cells might be beneficial for parasite growth, because it could limit the degree of Ab response, thus hindering the development and maintenance of memory responses. Understanding the mechanism and factors responsible for deletion may enable us to devise strategies to enhance immune following immunization.

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