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Genetically Attenuated Parasite Vaccines Induce Contact-Dependent CD8+ T Cell Killing of Plasmodium yoelii Liver Stage-Infected Hepatocytes

Adama Trimnell,* Akihide Takagi,* Megha Gupta,* Thomas L. Richie,† Stefan H. Kappe,* and Ruobing Wang2*†

The production of IFN-γ by CD8+ T cells is an important hallmark of protective immunity induced by irradiation-attenuated sporozoites against malaria. Here, we demonstrate that protracted sterile protection conferred by a Plasmodium yoelii genetically attenuated parasite (PyGAP) vaccine was completely dependent on CD8+ T lymphocytes but only partially dependent on IFN-γ. We used live cell imaging to document that CD8+ CTL from PyGAP-immunized mice directly killed hepatocyte infected with a liver stage parasite. Immunization studies with perforin and IFN-γ knockout mice also indicated that the protection was largely dependent on perforin-mediated effector mechanisms rather than on IFN-γ. This was further supported by our observation that both liver and spleen CD8+ T cells from PyGAP-immunized mice directly killed naive mice against parasite challenge (12–14). Protection in humans conferred by irradiated Plasmodium falciparum spz is likewise dependent primarily on the activity of cytotoxic CD8+ lymphocytes (15). Furthermore, CSP-specific CD8+ T cells have been found to efficiently lyse infected hepatocytes in vitro (12, 16, 17). All of these data point toward a requisite role of CD8+ T cells in providing sterile protection against spz challenge in the immunized host. However, the T cell-mediated effector mechanisms that inhibit the development of liver stages of malaria parasites remain poorly understood. IFN-γ responses have been correlated with pre-erythrocytic-stage protective immunity for a long time, although the importance of CD8+ T cell-derived IFN-γ, in particular, is not yet fully established (18, 19). Sterile protection obtained with genetically attenuated P. berghei spz was found to correlate quantitatively with IFN-γ-producing CD8+ T cells (3). Protective immunity induced with P. falciparum circumsporozoite protein-based pre-erythrocytic-stage vaccine, RTS.S, was also found to be dependent on IFN-γ-producing CD4+ and CD8+ T cells (20). On the other hand, IFN-γ-independent CD8+ T cell-mediated protective immunity has been demonstrated (21), and Chakravarty et al. recently found that IFN-γ secretion by primed CD8+ T cells is not essential for the protection of mice against live P. yoelii spz challenge (22).

With these contrasting reports explaining the possible effector functions of protective CD8+ T cells in various malaria models, we were intrigued to explore the CD8+ T cell-mediated protective...
mechanisms contributing to sterile immunity in *P. yoelii* genetically attenuated parasite (PyGAP)-immunized mice. We had earlier shown that protracted protection mediated by either of two genetic knockout (KO) parasites *Pyus3*(-) and *Pyuis4*(-) was absolutely dependent on CD8+ T cells, whereas depletion of CD4+ T cells did not reverse the protection (5). Here we report that CD8+ T cells from PyGAP-immunized mice led to rapid apoptosis of infected hepatocytes in vitro in an IFN-γ-independent manner. However, PyGAP-induced protection was found to be partly dependent on IFN-γ in immunized mice. Interestingly, PyGAP spz failed to protect against wild-type (wt) spz challenge in most perforin KO mice (PKO), which led us to propose that contact-dependent cytotoxicity by Ag-specific CD8+ T cells is one of the crucial mechanisms leading to rapid clearance of infected hepatocytes in PyGAP-immunized mice, thereby conferring complete sterile protection. PyGAP successfully generated memory responses providing long-term protection, and a population of liver CD8+ T cells in their effector phase (TEm) directly correlated with protective immunity. Our findings have important implications in the design of future T cell-based vaccines against malaria and in the determination of correlates of protection.

Materials and Methods

**Mice and parasites**

Female 6- to 8-wk-old BALB/c mice and C.129S7(B6)-Ifngtm1Ts1J mice were purchased from The Jackson Laboratory for immunization experiments. Perforin-deficient BALB/c mice (PKO) were provided by Dr. J. T. Harty (University of Iowa). Female 6- to 8-wk-old Swiss Webster (SW) mice purchased from Charles River Laboratories were used for maintenance of *P. yoelii* parasite life cycle. Animal handling and care were in accordance with Institutional Animal Care and Use Committee-approved protocols. The *P. yoelii* 17X NL (a nonlethal strain) clone 1.1 *Pyus3*(-) and *Pyuis4*(-) parasites were cycled between *Anopheles stephensi* mosquitoes and SW mice as previously described (5).

**Immunization studies**

Groups of BALB/c mice (five mice per group) were immunized by i.v. injections of 50,000 or 10,000 *Pyus3*(-) or *Pyuis4*(-) spz via the tail vein. Booster injections were administered 2 wk apart. Immunized mice were challenged 14 days postimmunization by i.v. injection with 10,000 wt *P. yoelii* spz.

Separate sets of immunization experiments were conducted with genetic KO mice, PKO and IFN-γ KO mice in a BALB/c background were injected with 50,000 *Pyuis4*(-) spz i.v. via tail vein. Immunized mice were boosted 2 wk apart.

**Wild-type *P. yoelii* challenges**

All immunized mice were challenged with 10,000 wt *P. yoelii* spz after 7 days of immunization(s). Protection was determined by the absence of parasites in Giemsa-stained blood smears taken daily from day 2 to day 6 postchallenge.

Naive BALB/c mice, as a nonprotective model, were also challenged at the same time with a single dose of 10,000 wt *P. yoelii* spz and the parasitemia levels were checked thereafter. Splenocytes and liver-infiltrated lymphocytes were used as controls for cellular assays from these mice 4 wk after the challenge.

**CD8+ T cell and IFN-γ depletion studies**

CD8+ T cells and IFN-γ were depleted in immunized mice. They were injected i.p. daily, with 0.5 mg per dose of anti-CD8 mAb 2.43 (TIB210; American Type Culture Collection) for 4 consecutive days before challenge. Depletion of IFN-γ, mice were administered 1 mg per dose of mAb XMG6-6 (anti-IFN-γ) for 4 consecutive days before challenge (23). They were administered anti-IFN-γ mAb on the day of challenge and 3 days postchallenge as well. All treated and naive control mice were challenged with 10,000 wt spz 14 days after the last immunization. Control mice received equivalent doses of control rat IgG. These doses and regimens were found to be optimal for >95% depletion efficiency of CD8+ T cells and IFN-γ in mice (data not shown).

**Cellular inhibition of LS development assay**

Primary hepatocytes were isolated from BALB/c mouse as previously described except for the use of a steady-state manual liver perfusion instead of a mechanical pump (24). Isolated hepatocytes were diluted and dispensed at 100,000 cells in 250 μl per well in Lab-Tek chamber coverslip slides or 40,000 cells in 100 μl per well in 96-well tissue culture plates (Nunc) and incubated overnight at 37°C in an atmosphere of 5% CO2 in air. The next day, hepatocyte cultures were infected with 80,000 GFP-tagged *P. yoelii* wt spz in 100 μl of medium per chamber well and 30,000 in 50 μl of medium per well of 96-well plate. Infected cultures were further incubated for 16 h at 37°C in an atmosphere of 5% CO2 in air. Each well was washed three times with 300 μl of Eagle’s complete MEM to remove unattached spz. Fresh medium plus dexamethasone was added thereafter to each well and cultures were incubated for 8 h at 37°C in an atmosphere of 5% CO2 in air for hepatic stage development. The infected hepatocytes were then exposed to enriched splenic or intrahepatic CD8+ T cells from immunized or control mice. To introduce physical separation between lymphocytes and infected hepatocyte population, intrahepatic lymphocytes were incubated with infected hepatocytes in presence or absence of filter in a 96-transwell plate (Corning).

Enriched CD8+ T cells from liver and spleen were isolated 5 days postimmunization of mice as previously described (23). Lymphocytes were also isolated from naive mice 4 wk postchallenge with wt *P. yoelii* spz. Splenocytes were isolated from sacrificed mice by passing spleens through a 70-μm cell strainer (BD Biosciences), and erythrocytes were lysed with RBC lysis buffer (eBioscience). Liver cells were collected by passing the liver tissue through a 100-μm cell strainer, and leukocytes were separated by Percoll gradient centrifugation (Sigma-Aldrich) followed by lysis of erythrocytes. Spleen and liver CD8+ T cells from these leukocyte fractions were purified using the MACS system negative selection beads according to the manufacturer’s instructions (Miltenyi Biotec). Twenty million splenocytes and 2 million liver infiltrate cells were used to purify and negatively select CD8+ T cell populations.

In certain experiments, infected hepatocytes were pretreated with 2 μg/ml PyCSP peptide (SVYPSQAEP; purchased from Sigma-Aldrich at >95% purity) or with ECFp control pool of 23 peptides from human influenza virus (purchased from SynPep at >90% purity) for 4 h at 37°C before coculturing with 50,000 enriched spleen or liver CD8+ T cells per chamber or well for 16 h. Peptide-stimulated infected cultures were thoroughly washed with complete medium before adding spleen or liver CD8+ T cells. Live imaging or apoptosis assays were performed on these cultures.

**In vitro live cell imaging**

To study live cellular interactions, liver- or spleen-enriched CD8+ T cells from immunized or control mice were loaded with the lysomotropic stain LysoTracker Red (Invitrogen), according to the manufacturer’s recommendations. These enriched CD8+ T cells were added to primary hepatocyte cultures infected with GFP-tagged wt *P. yoelii* spz, and images were captured using a Delta Vision microscope (Applied Precision). Samples were recorded at 2-min intervals in multiple z-layers with a double wavelength excitation of 488 nm for GFP expression by LS-infected hepatocytes and 555 nm for LysoTracker Red; differential interference contrast images for each z-plane and time frame were recorded and data were analyzed using the softWoRx software (Applied Precision). Relative distance traveled by each CD8+ T cell toward the LS-infected hepatocyte was assessed per sample.

**Apoptosis assay and indirect immunofluorescence**

At 48 h after spz invasion and coculturing with spleen/liver-enriched CD8+ T cells, live apoptosis assays were performed using enzymatic assays for caspase-3 and caspase-7 according to the manufacturer’s recommendations (FLICA Red from Immunochemistry Technologies). FLICA Red reagent that permeabilizes the cells and deposits a red fluorescent compound at the site of enzyme activity was used to detect CTL-induced apoptosis of the hepatocytes. For the enzymatic detection assay, 0.5 μl of 1/5 dilution of the FLICA Red reagent was added to 100 μl each of supernatant medium containing adherent LS-infected hepatocyte culture and incubated for 40 min at 37°C, 5% CO2. Samples were washed five times each with 1× FLICA Red wash buffer or PBS. This was followed immediately by indirect immunofluorescence assay to stain LS parasites in the culture. After the final wash step for each apoptosis assay, samples were blocked with 5% BSA in PBS followed by incubation with anti-MAp4 (anti-CSP/spz17) Ab or NYS1.5 mAb (for Hsp17) at room temperature for 1 h. After five washes in PBS, samples were incubated for 45 min at room temperature with the anti-rabbit IgG Alexa Fluor 488-conjugated Ab at a dilution of 1/1000 in 2.5% BSA.
in PBS. Samples were then washed five times with PBS. To detect apoptotic nuclei, samples were then treated with the Hoechst stain (as recommended by the manufacturer, Immunochemistry Technologies). Treated samples were viewed using a fluorescence-inverted microscope (Eclipse TE2000-E, Nikon) and images were recorded and analyzed using the MetaMorph Office version 7.0 software.

Results

GAP-induced protection is partially dependent on IFN-γ and perforin

In protected models of irradiated spz, CD8+ T cell-derived IFN-γ has been found to be responsible for complete protection (17, 19, 25). To determine whether such an effector mechanism plays an important role in PyGAP-induced protection, groups of BALB/c mice (n = 5/group) were immunized with three doses of 10,000 or 50,000 of Pyuis3(−) or Pyuis4(−) spz as previously described (5). CD8+ T cells or IFN-γ were depleted in immunized mice before challenging them with 10,000 wt P. yoelii spz.

In conformity with our previous report, all immunized mice depleted of CD8+ T cells developed patent blood stage infections 1 day later than did nonimmunized control mice (Fig. 1). Immunized mice that received IgG as a control remained completely protected. Half (50%) of the IFN-γ-depleted mice that were immunized with 3 × 10,000 Pyuis4(−) spz, 3 × 50,000 Pyuis4(−) spz, or 3 × 50,000 Pyuis3(−) spz were completely protected (Fig. 1). This suggests that both Pyuis3(−) and Pyuis4(−)-induced protection is partially dependent on IFN-γ. However, immunization with 10,000 Pyuis3(−) spz led to parasitemia in all of the mice after IFN-γ depletion, unlike those immunized with 10,000 Pyuis4(−) spz. This is likely explained by the fact that Pyuis4(−) spz confers more robust protection as compared with Pyuis3(−) spz at low doses of immunization. Therefore, IFN-γ depletion might cause increased failure of protection in mice immunized with low dose of Pyuis3(−) spz.

Immunization of IFN-γ KO mice and subsequent challenge with wt P. yoelii spz also led to protection of 50% mice from parasitemia in two independent experiments (Fig. 2), further reiterating our finding that IFN-γ plays a partial role in providing protection to the immunized mice.

To probe the importance of perforin-mediated killing by CD8+ T cells in PyGAP-induced immunity, PKO mice were immunized with 3 × 50,000 Pyuis4(−) spz. It was found that at the end of 6 days, only 20% of immunized PKO mice were protected from the wt challenge (Fig. 2), indicating a partial role of perforin in the conferment of protection by PyGAP spz.

In contrast, the naive mice that were challenged with 10,000 wt spz were 100% infected. Parasitemia levels reached up to 50–60% ~7–10 days after challenge but were cleared out completely by 3 wk postchallenge.

CD8+ T cell mediates contact-dependent killing of LS-infected hepatocytes

These in vivo depletion studies indicated that PyGAP-induced protection is completely dependent on CD8+ T cells but only partially dependent on IFN-γ. This prompted us to further investigate if cytolytic activity of CD8+ T cells mediates the protection by killing LS-infected hepatocytes and whether CD8+ T cell-derived IFN-γ is involved in the process. Primary hepatocytes infected with GFP-tagged wt P. yoelii spz (26) were cocultured with CD8+ T cells isolated from mice immunized with three doses of Pyuis4(−) and loaded with fluorescent lysosomotropic probe LysoTracker Red. Fig. 3 and supplemental video 1 show the time-frame snapshots and live video of interaction between the target LS-infected hepatocyte and LysoTracker-stained Pyuis4(−)-induced CD8+ T cells, respectively, over a period of 1200 s at 120-s intervals, starting from 0 s after the addition of the CD8+ T cells. As seen in Fig. 3A, immediately after adding the Pyuis4(−)-specific CD8+ T cells into the infected hepatocyte culture (red arrow), one LysoTracker-stained T cell settled directly above the infected hepatocyte (green arrow) and another unstained T cell migrated toward the infected hepatocyte membrane (yellow arrow). Through a time course of 0–960 s, the infected hepatocyte cell membrane and LS parasite residing inside became larger in size and, subsequently, green fluorescence of the GFP-tagged LS parasite gradually diminished, indicating the death of both the parasite and the infected hepatocyte. The LysoTracker Red stained was rapidly released from the loaded CD8+ T cells and taken up by the targeted hepatocyte, suggesting the mobilization of lytic granules. At the 840-s time point after the CTL action, the T cell moved away from the LS-infected hepatocyte cell membrane (Fig. 3C, 840 s). In contrast, the CD8+ T cells from a mock control mouse that was immunized with mosquito salivary gland debris did not migrate toward the LS-infected hepatocyte, even after a time course of 1200 s (Fig. 3D and video not shown). This indicated that Pyuis4(−) CD8+ T cells specific for P. yoelii LS Ags recognized the infected hepatocytes and led to the cytotoxicity.

The online version of this article contains supplemental material.
To verify the movement of primed CD8+ T cells specifically toward infected hepatocytes, relative distance traveled by CD8+ T cells toward or away from the LS-infected hepatocyte was measured. Fig. 4 shows the overall dynamics of movement of PyGAP-specific spleen CD8+ T cells toward LS-infected hepatocyte at 24 h (Fig. 4A) and 48 h (Fig. 4B) post invasion with P. yoelii GFP-tagged spz. It shows that spleen CD8+ T cells from Pyuis4-(-) immunized mice are specifically attracted to LS-infected hepatocyte as compared with the nonspecific random movements of T cells from mock control or naive mice.

**PyGAP-specific CD8+ T cells mediate apoptosis of infected hepatocytes**

To ascertain the pathways leading to death in CTL-targeted infected hepatocytes, live enzymatic apoptotic assays were performed. Cocultures of CD8+ T cells and infected primary hepatocytes were stained to detect the classical apoptosis markers: caspase-3 and caspase-7. Sequentially, the cultures were stained with polyclonal anti-MAP4 Abs (stains CSP and Hep17) to detect the LS-infected hepatocytes. Both spleen and liver CD8+ T cells from Pyuis4-(-) immunized mice induced apoptosis of the infected hepatocytes (Fig. 5A–C). This was confirmed by the presence of numerous apoptotic bodies (white arrows) and fragmented nuclei (stained blue). Apoptotic LS-infected cells appeared detached and shrunken with the presence of apoptotic bodies. Fig. 5C shows an apoptotic cell harboring LS parasite next to an apoptotic nucleus. Despite membrane blebbing, the cells did not lose the membrane integrity, which confirms the occurrence of apoptosis rather than necrosis. In contrast, spleen or liver CD8+ T cells from
mock control mice did not trigger apoptosis of the infected hepatocytes (Fig. 5, D and E). Few background apoptotic cells were observed, possibly due to mechanical shearing while preparing the primary hepatocytes, but most of the infected cells were seen intact. Thus, physical contact of Pyuis4(H11002) CTLs with infected hepatocytes initiated a cascade of events that led to rapid apoptosis of infected cells. A general observation that the LS parasites (green) in the infected hepatocytes cocultured with Pyuis4(H11002)-specific CTLs (Fig. 5A) appeared smaller in size than those from samples incubated with mock control T cells (Fig. 5D) suggests that CD8+ T cells from Pyuis4(H11002)-immunized mice may also induce inhibition of parasite development inside the liver cells. These results give credence to the live imaging observations of PyGAP CD8+ T cell-induced contact-dependent killing of infected hepatocytes (supplemental video 1).

Surprisingly, none of the common cytokines (IFN-γ or TNF-α) was detected in the supernatants of the cultures from the infected hepatocyte in the presence of Pyuis4(H11002)-specific CTLs, as measured by ELISA (data not shown) or the ultrasensitive electrochemiluminescence MSD mouse multiplex cytokine assay (Fig. 6A). This shows that apoptotic activity of Pyuis4(H11002) CTLs leading to the clearance of LS parasites was independent of the secretion of IFN-γ. Results obtained by physically separating the lymphocytes and infected hepatocyte in cocultures by transwell experiment also reiterated the fact that killing by CD8+ T cells was indeed contact dependent and cytokines do not play a major role in cytotoxicity. There was an ~24% decrease in the number of LS parasites in wells where PyGAP-specific lymphocytes were physically separated from infected hepatocytes, whereas we observed almost 85% inhibition of LS parasites in wells without filters (Fig. 6B). Nevertheless, it does not rule out the possibility that there is potentiation of CD8+ T cell activity by IFN-γ in vivo.

Wild-type spz-induced CTLs trigger apoptosis of noninfected hepatocytes

To understand the basis of protection in PyGAP-immunized mice more clearly, we compared the activity of CD8+ T cells from a...
nonprotective model (nonimmunized mice that recovered from a single dose of 10,000 wt P. yoelii spz challenge) with the activity of CD8$^+$ T cells from a protective model (Pyuis4-immunized mice). Strikingly, the CD8$^+$ T cells from the wt spz-challenged mice did not recognize the infected cells but indeed caused apoptosis of surrounding uninfected cells (Fig. 7A), contrasting with Pyuis4 CTL activity that completely eliminated all of the infected cells (Fig. 7B). It has been shown in various studies that the surrounding uninfected cells are traversed by the spz before infecting a single hepatocyte (27, 28). While traversing, spz shed their surface protein, circumsporozoites in the traversed cells, which is processed and presented with MHC class I on the surface of hepatocytes (16, 28). Therefore, it is likely that the CD8$^+$ T cells from the wt spz-challenged mice mainly recognize CSP on the surface of uninfected traversed hepatocytes. To confirm this, the infected cell cultures were pulsed with CSP CTL epitope before coincubating with the CD8$^+$ T cells. There was no significant change in the activity of Pyuis4 CTLs even when added to the CSP-pulsed cultures of infected hepatocytes. Massive apoptosis of infected as well as uninfected hepatocytes was observed (Fig. 7D). In case of CD8$^+$ T cells from wt-challenged mice, there was an increase in the apoptosis of infected cells along with the death of uninfected cells (Fig. 7C). The infected cells were not recognized by these wt spz-primed CD8$^+$ T cells when LS-infected cultures were treated with a control peptide (Fig. 7E). These results strongly suggest that most CD8$^+$ T cells from wt spz-challenged mice were specific for CSP epitope and failed to recognize other LS Ags expressed on the surface of infected hepatocytes. Thus, more likely, non-CSP LS Ag-specific CTLs induced by GAP immunization play a crucial role in the complete elimination of LS parasites.

FIGURE 7. Cellular inhibition of LS parasites in presence of CD8$^+$ T cells from PyGAP-immunized mice and from wt-challenged mice. CD8$^+$ T cells from mouse that had recovered from challenge with a single dose of 10,000 wt P. yoelii spz (A) or intrahepatic CD8$^+$ T cells from mice immunized with three doses of 10,000 Pyuis4 spz (B) were cocultured with infected primary hepatocytes in a 96-well plate as described in Materials and Methods. Forty-eight hours later, cultures were sequentially treated with FLICA Red to detect caspase-3 and caspase-7 followed by staining with anti-Hep17 mAb (NYLS3). Staining with Hoechst reagent was done to detect apoptotic bodies. Stained slides were observed under inverted fluorescence microscope for the presence of apoptotic cells (stained red), LS parasites (stained green), and apoptotic nuclei (stained blue). In another experiment, infected hepatocytes were pretreated with 2 μg/ml PyCSP peptide (SYVPSAEQI) for 4 h at 37°C before coculturing with enriched spleen or liver CD8$^+$ T cells from wt-challenged mice (C) or PyGAP-immunized mice (D). Infected hepatocytes stimulated with control peptide ECFp and subsequently treated with CD8$^+$ T cells from wt-challenged mice were used as controls (E). LS parasites (stained green) in primary hepatocytes were manually counted per sample using the fluorescence microscope (F). Scale bar, 100 μm. Values shown are the mean (±SD) values from four independent experiments.
CTL-mediated killing of infected hepatocytes correlate with protective immunity in PyGAP-immunized mice

We wanted to determine whether the PyGAP-induced CTL activity against LS-infected hepatocytes in vitro correlates with the protection against spz challenge in vivo. LS-infected hepatocyte cultures were stained with mAb against LS specific Ag (Hep17) and quantified at the end of 48 h of CTL assay to assess the percentage inhibition of LS parasites. There were on average 31 LS parasites per well after incubation with T cells from liver of the mock control mouse that received noninfected mosquito debris, as compared with 24 and 6 LS parasites per well after coculturing with T cells from mice that recovered from wt spz challenge and mice immunized with Pyuis4(spz), respectively (Fig. 7F). The percentage inhibition of LS parasite was 22 and 80 for CD8+ T cells for wt spz-challenged and PyGAP-immunized mice, respectively. To determine the cytolytic activity induced by CSP-specific T cells, LS-infected hepatocyte cultures were sensitized with the CSP-derived CTL epitopes before initiating CTL assay. The cellular inhibition of LS development by CD8+ T cells from the wt spz-challenged or PyGAP-immunized mice was increased to 66% and 92%, respectively. A similar pattern of inhibition was observed for CD8+ T cells obtained from the spleen (Fig. 7F). The PyGAP-induced CTL killing of LS-infected hepatocytes was significantly greater than that induced by CTLs from wt spz-challenged mice (CSP-specific, p = 0.002 and non-CSP specific, p = 0.0009; Student’s t test). However, there was a 44% increase of the inhibition of LS-infected hepatocytes in the presence of CSP peptide in the cultures, indicating that the CTL killing of infected hepatocytes by the CTLs derived from P. yoelii wt spz challenge was mainly CSP-specific. Nevertheless, the in vitro killing data for CD8+ T cells from PyGAP-immunized mice was correlated with the protective immunity induced by PyGAP spz in vivo.

Increase of liver TEM cells is a potential indicator of protective immunity

The importance of memory T cell responses for long-term protection has been described for malaria vaccines (29–31). TEM were detected in mice immunized with P. berghei irradiated-spz and PbGAP vaccines (3, 32). Here, we investigated the generation of memory T cell phenotypes in PyGAP-immunized mice. Substantial populations of TEM and central memory T cells (T CM) were detected in spleen and liver of immunized mice. We correlated these T cell populations with protection induced after each dose of immunization with Pyuis4(spz) as compared with that in control mice that received salivary gland lysate from noninfected mosquitoes. Five days after primary immunization with 10,000 Pyuis4(spz) spz, there was decline in liver CD8+ TEM and proportional increase in TEM cells, whereas T CM and TEM pools in spleen were comparable with those in control mice (Table I). However, those immunized mice were not protected when challenged with 10,000 wt spz on day 7 after primary immunization (Table I), indicating that the magnitude of TEM cells was insufficient to mount protective immunity.

Following a larger primary immunization dose of 50,000 Pyuis4(spz), which resulted in complete protection, we observed an increased differentiation of T CM cells to TEM cells in both liver and spleen (Table I). This was seen by CD62L down-regulation and CD44 up-regulation of CD8+ T cells 5 days after immunization. The increase in the ratio of TEM to T CM was more prominent in the liver than in the spleen. There was no further increase in the ratio of the T cell memory phenotypes when boosted with 50,000 Pyuis4(spz), indicating that the magnitude of TEM cells may have reached its maximum level after a single high-dose immunization. Thus, quantifying the ratio of TEM to T CM termed the protection correlation index (PCI), may be a viable measure of protective immunity.

Discussion

The focus of our study was to elucidate the CD8+ T cell-mediated effector mechanisms that contribute to the sterile immunity induced by PyGAPs, Pyuis4(spz), and Pyuis3(spz) in BALB/c mice. Previously, we reported that CD8+ T cell responses were indispensable for PyGAP-induced protracted sterile protection (5). Apart from the release of IFN-γ, other potential effector mechanisms deployed by CD8+ T cells have not been explored thoroughly. To the best of our knowledge, this is the first report that provides direct evidence of contact-dependent cytotoxicity as one of the important methods used by GAP-specific CD8+ T cells to eliminate LS parasites. Partial loss of protection in PKO and IFN-γ KO mice indicated that PyGAP-induced protection is partly dependent on IFN-γ as well as perforin. However, noticeably, CD8+ T cell-mediated cytotoxicity of infected hepatocytes in vitro was found to be independent of IFN-γ release.

Live imaging studies of time-lapse microscopy using enriched liver or spleen CD8+ T cells from Pyuis4(spz)-immunized mice showed rapid movement of CD8+ T cells toward hepatocytes infected with P. yoelii spz. Swelling of the hepatocyte membrane was observed as CD8+ T cells made direct contact and dispersed the lysomotrophic probe into the infected cells, indicating the discharge of lytic granules. Caspase-3 and caspase-7 staining suggested apoptosis of infected target hepatocytes. This was similar to that reported in detail by Wiedemann et al. demonstrating the polarization of lytic granules of CTL toward the target cell (33). The signaling molecules that are involved in specific movement of PyGAP-specific spleen CD8+ T cells, however, remain to be elucidated. Lyubchenko et al. reported that calcium influx is involved at the point of contact for granule exocytosis (34). More detailed studies are needed to determine the precise requirements for granule exocytosis in PyGAP-induced CTL activity. Another recent study also described similar contact-dependent apoptotic activity

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### Table I. PCI: ratio of TEM/T CM correlated with protection induced by GAP Pyuis4(-) immunization

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<thead>
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<th>Experimental Group (BALB/c mice)</th>
<th>Spleen</th>
<th>Liver</th>
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<tr>
<td></td>
<td>CD8+ T Cells*</td>
<td>PCI</td>
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<td>Five days after first immunization with 50,000 Pyuis4(-)</td>
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<td>Five days after second immunization with 50,000 Pyuis4(-)</td>
<td>100</td>
<td>64.6</td>
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* No. indicates percentage of each subpopulation at the time point; TEM, CD44<sup>hi</sup>CD62L<sub>lo</sub>; TCM, CD44<sup>lo</sup>CD62L<sub>hi</sub>.

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of CSP-specific CD4+ T cell clones (35). CD8+ T cell-mediated lytic activity would require the recognition of processed liver stage Ags in complex with MHC class I on the surface of infected hepatocytes. It is quite likely that professional APCs like dendritic cells may be involved during initial priming of CD8+ T cells in the immunized mice as discussed elsewhere (36–38). The fact that spleen-derived CD8+ T cells also exhibited rapid lysis of infected hepatocytes suggests that such immune-responsive tissues can be potential sites for CD8+ T cell priming, as the parasite traverses multiple tissues and sheds its surface proteins before invading the liver (39). Another study supports a similar notion where the authors observed that the first cohort of CD8+ T cells is primed in cutaneous lymph nodes, which subsequently travels to the liver during malaria infection (36). IFN-γ and other inflammatory cytokines released during immunization or infection can possibly direct the CTLs to the site of action. It is noteworthy that the in vitro apoptotic activity of CD8+ T cells was independent of CD8+ T cell-derived IFN-γ, but the likelihood of potentiation of the CTL activity by IFN-γ from other sources in vivo cannot be ruled out. Our finding corroborates recent studies that found protection induced by CD8+ T cells against LS parasite was independent of CD8+ T cell-derived IFN-γ (22). Our in vivo studies, however, indicated that IFN-γ was partially required for the generation of protective immunity in PyGAP-immunized BALB/c mice. Apart from direct cytotoxic effects on infected cells, IFN-γ may also be involved in complex cellular interactions facilitating the activation of immune cells, including CD8+ T cells. IFN-γ has been used as a surrogate marker for immunity in evaluating potential of vaccines against LS malaria infection (20, 40–42). Our findings warrant the search for more definite correlates of protective immunity in addition to this pleiotropic cytokine.

Interestingly, the liver CD8+ T cells from naive mouse that survived wt P. yoelii spz (10,000 spz) challenge did not kill the infected hepatocytes in vitro. On the contrary, they recognized and lysed the surrounding noninfected hepatocytes. This may be indicative of an immune evasion strategy adopted by wt spz to divert the host immune system toward unaffected cells. This idea has been proposed previously but no evidence has been put forward until now (43). This kind of condition is likely to operate among the populations that live in malaria endemic areas, thereby explaining the absence of protective immune responses against the infection even after repeated exposures to the parasite. It has also been shown earlier that infected cells are protected from apoptosis by hepatocyte growth factor/MET signaling (44, 45). Our results are also supported by the findings of Bongfen et al., who demonstrated that CSP, an immunodominant spz surface protein, is processed and presented by both traversed and infected primary hepatocytes and that they were both efficiently lysed by CSP-specific CTLs, apparently via perforin/granzyme mobilization (16). Since in vitro cell traversal and hepatocyte invasion are very similar between wt and GAP spz (our unpublished data), it can be very well envisaged that in wt infection, since the infected cells are protected from apoptosis (46) and thus the presentation of various LS Ags is inhibited (37), most of the CD8+ T cell population from wt spz-challenged mice is primed to CSP, which is presented by traversed hepatocytes. In contrast, Pyuis4(-)-induced CD8 T cells rapidly recognize infected hepatocytes in addition to traversed hepatocytes. However, Pyuis4(-)-specific CD8 T cells are capable of eliminating both spz-traversed and LS parasite-infected hepatocytes after incubation of infected hepatocyte cultures with CSP-specific CTL epitope peptide. Hypothetically, growth-arrested GAP spz break down early in their development in the hepatocyte and stimulate immune responses against a plethora of LS Ags in the immunized mice (5). As a consequence, heterogeneous mix-
References

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