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Phenotypic characterization of *Plasmodium berghei* responsive CD8+ T cells after immunization with live sporozoites under chloroquine cover

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Abstract

Background: An effective malaria vaccine remains elusive. The most effective experimental vaccines confer only limited and short-lived protection despite production of protective antibodies. However, immunization with irradiated sporozoites, or with live sporozoites under chloroquine cover, has resulted in long-term protection apparently due to the generation of protective CD8+ T cells. The nature and function of these protective CD8+ T cells has not been elucidated. In the current study, the phenotype of CD8+ T cells generated after immunization of C57BL/6 mice with live *Plasmodium berghei* sporozoites under chloroquine cover was investigated.

Methods: Female C57BL/6 mice, C57BL/6 mice B2 macroglobulin -/- [KO], or invariant chain-/- [Ic KO] [6-8 weeks old] were immunized with *P. berghei* sporozoites and treated daily with 800 µg/mouse of chloroquine for nine days. This procedure of immunization is referred to as "infection/cure". Mice were challenged by inoculating intravenously 1,000 infectious sporozoites. Appearance of parasitaemia was monitored by Giemsa-stained blood smears.

Results: By use of MHC I and MHC II deficient animals, results indicate that CD8+ T cells are necessary for full protection and that production of protective antibodies is either CD4+ T helper cells dependent and/or lymphokines produced by CD4 cells contribute to the protection directly or by helping CD8+ T cells. Further, the phenotype of infection/cure *P. berghei* responsive CD8+ T cells was determined to be KLRG1^{high} CD27^{low} CD44^{high} and CD62L^{low}.

Conclusion: The KLRG1^{high} CD27^{low} CD44^{high} and CD62L^{low} phenotype of CD8+ T cells is associated with protection and should be investigated further as a candidate correlate of protection.

Background

While reduction in malaria cases has been reported in many countries, malaria remains among the world's most prevalent and fatal infectious disease. In 2011, it was estimated there were 216 million malaria episodes and 655,000 malaria deaths [1]. With the majority of the deaths occurring in children less than five years of age, and with almost half of the world population at risk, an effective vaccine against malaria is urgently needed [2].

Natural exposures to malaria infections do not immediately induce immunity leaving infants and young children

in endemic areas susceptible to multiple episodes of the disease. Eventually, partial immunity is acquired in older children and adults, affording them protection against clinical symptoms and/or severe disease. However, protection is not sterile [3] and the immune responses to *Plasmodium* parasites are short-lived [4]. This is attributed to short half-life of protective antibodies [4] and to a cellular response [5-7] too weak to grant protection [8-11]. Sterile protection has however been achieved experimentally in both animal models of malaria and in malaria-naive humans immunized with whole live sporozoites [12].

Intravenous administration of irradiated sporozoites renders long-lasting protection [13-17] by a mechanism mediated largely by CD8+ T cells [18-23]. Although there is data in favour of other mechanisms [24] and different mice strains show different susceptibility to malaria [25,26],

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CD8+ T cell remain the main player in this model of protection. Evidence of protection conferred by immunization with sporozoites under chloroquine (CQ) cover was initially demonstrated in mice and rats using *Plasmodium berghei* [27,28]. More recently, infection under CQ cover has also been shown to induce long-lasting protection in malaria-naïve human [29,30] and murine models [31,32]. In these models, CD8+ T cells appear to play an important role in protection [24,29-32]. Taken together, these data suggest that while natural exposure elicits weakly protective humoral and cellular immunity, strategies such as inoculation of irradiated sporozoites or viable sporozoites under CQ cover induce CD8+ T cells which, alone or in combination with antibodies, provide long-lasting protection. A better understanding of the mechanics of these protective cells is fundamental to vaccine development efforts.

In this study, *P. berghei* murine model was used to characterize the phenotype of CD8+ T cells generated under CQ cover and to associate these phenotypes with protection from a lethal challenge.

Methods

Mice

Female C57BL/6 mice, C57BL/6 mice B2 macroglobulin -/- (KO), or invariant chain -/- (Ic KO) (6–8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). These animals were housed at the Walter Reed Army Institute of Research (WRAIR) animal facility and handled according to institutional guidelines. All procedures were reviewed and approved by the WRAIR Animal Care and Use Committee (IACUC) and were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Euthanasia methods were compliant with the approved IACUC protocol, with frequent supervision by the institute's veterinary team. After the animals were challenged, strict humane endpoints criteria were adhered to by frequently monitoring the health of the animals in order to avoid or terminate unrelieved pain and/or distress. Animals positive for parasitaemia for two consecutive days were euthanized before showing distress and/or pain by inhalation of CO₂ from a pressurized tank in a chamber. This was followed by cervical dislocation prior to disposal of the dead mice.

Sporozoites

Sporozoites (Spz) from *P. berghei* ANKA strain were extracted from salivary glands of *Anopheles stephensi* mosquitoes 16–21 days post blood meal as previously described [19]. Mosquitoes were maintained in the WRAIR Entomology Branch Insectary facilities.

Reagents, antibodies and chemicals

Cell media and CFSE were purchased from Life Technologies (Carlsbad, CA). Purified antibodies or fluorochrome

labeled antibodies were purchased from eBioscience (San Diego, CA). Brefeldine A, Giemsa stain and CQ were purchased from Sigma (St. Louis, MO).

Immunization and treatment

Experimental animals were inoculated two or three times intravenously with 10,000 or 20,000 Spz (in 100 µl PBS), ten days apart and treated daily with 800 µg/mouse (100 µl in PBS) of CQ, administered intraperitoneally (i.p.), starting from the day of the first inoculation until nine days post last inoculation. This procedure of immunization is referred to as “infection/cure”. For the CQ control group, the same regimen of CQ as that of the experimental group was administered with the exception that these animals were not inoculated with Spz. The absolute control group received PBS only.

Challenge and assessment of protection

For challenge experiments, mice were inoculated intravenously with 1000 infectious Spz. Challenge was performed at two weeks post suspension of CQ treatment. Appearance of parasitaemia was monitored by Giemsa-stained blood smears. Animals free of parasitaemia for two weeks were considered protected.

Lymphocyte preparation

Mice were euthanized by CO₂ inhalation as described above. Livers and spleens were removed, and livers were perfused with 10 ml PBS. Single cell suspensions of lymphocytes were made from both organs (liver infiltrating lymphocytes and splenocytes). Cells were re-suspended in PBS and used for analysis or transfer. Peripheral blood for blood lymphocyte analysis was collected from the tail vein in a heparinized vial.

Serum and splenocyte transfer

Recipient mice in transfer experiments received either 500 µL/mouse of undiluted serum intraperitoneally, 20 × 10⁶ splenocytes re-suspended in 500 µL of PBS by injection into the tail vein, or a combination of both.

Flow cytometry analysis

Analysis was performed with an LSR II cytometer (Beckon Dickinson San Diego, CA) and data were analysed with Flow-Jo software (BD). The panel of fluorochrome conjugated antibodies for flow cytometry included: CD3- PrcP, CD4-Pacific Blue, CD8-V500 or Pacific Orange, CD45RB or CD44- Alexa fluoro -700, CD27-APC, CD127 -PE, KLRG-1 FITC. When the intracellular cytokine IFN γ was tested, the panel used was: CD8-PO, KLRG-1-FITC, CD62-PrCPcy5.5, CD44 Alexa-700, CD27-APC, IFN- γ -PE. In all assays UV-viability dye was included. Flow cytometry antibodies were purchased from Life Technologies (Carlsbad, CA).

Abbreviation for fluorochrome

Allophycocyanin (APC), Pyridine-chlorophyll proteins-Cy-5 (PrCP-Cy5), Phycoerythrin (PE), Fluorescein (FITC). Antibodies are indicated by the marker recognized-fluorochrome, e.g. anti CD3 Fluorescein-conjugated was CD3-FITC.

Results

Three cycles of infection/cure results in protection

To establish the infection and cure model of C57BL/6 mice, animals were inoculated with infective Spz in the presence of CQ as described in the method section, referred to as infection/cure. Different dose and frequency regimens of infection/cure were compared (Table 1). Three groups of animals were challenged 4, 8 or 16 weeks post the last inoculation. Animals whose blood was negative for parasitaemia at 14 days post challenge were declared protected. One hundred percent of the animals that received three-inoculations of 20,000 Spz remained fully protected when challenged eight weeks post third inoculation, whereas those challenged 16 weeks post third inoculation exhibited only 40% protection (Figure 1). All animals treated with CQ (but not inoculated with Spz) and challenged two weeks post suspension of the CQ treatment became parasitaemia confirming a lack of a residual protective effect of CQ following suspension of the drug. This data indicates infection/cure immunization with three, 20,000 Spz inoculations resulted in protection.

Protection requires both CD8+ T cells and antibodies

To investigate the relative role played by the cellular and humoral immune response in protection from the challenge, spleens and serum were collected from infection/cure animals two weeks after suspension of CQ treatment and transferred into naïve animals. Groups of 10 naïve C57BL/6 mice were given either splenocytes, serum or both as described in the methods section. Recipient animals were challenged 24 h post-transfer and parasitaemia monitored for two weeks. Ninety percent of the animals treated with both splenocytes and immune serum were protected. Animals that received splenocytes or immune serum alone showed 50% and 60% protection respectively (Figure 2A). This data points to an additive protective effect of cellular and humoral immunity. Having demonstrated that cellular immunity

Table 1 Immunization regimen with Spz used for infection/cure of mice (10 animals/group)

Spz (dose)	Immunizations (n)	Protection (%)
10,000	3	80
20,000	2	40
20,000	3	100

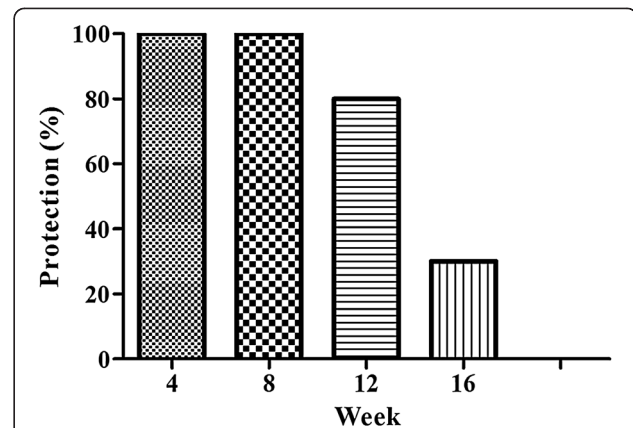


Figure 1 Infection with sporozoites under chloroquine cover induces long lasting protection. Infection with sporozoites under CQ cover induces long lasting protection. Three groups of ten mice each were inoculated three times, two weeks apart with 20,000 Spz. Each group of animals was challenged once with 1,000 sporozoites-at week 4, 8, 12 and 16 post the last inoculation as shown. For each group five naïve mice were used as infectivity controls. Appearance of parasitaemia was followed by Giemsa staining of blood smears.

was required to grant 100% protection, the role of CD4+ T cells and CD8+ T cells in protection using *Ic-/-* (MHC II KO, CD4+ cell deficient) and $\beta 2$ microglobulin-/- (MHC I KO, CD8 + cell deficient) mice was investigated. Immunized or non-immunized groups of 10 C57BL/6, MHC II KO or MHC I KO mice were challenged two weeks after suspension of the CQ treatment. As expected, wild type C57BL/6 mice were fully protected whereas MHC II KO and MHC I KO animals were only partially protected (Figure 2B). These results indicate that CD8+ T cells are necessary for full protection and that production of protective antibodies is either CD4+ T helper cells dependent and/or lymphokines produced by CD4 cells contribute to the protection directly or by helping CD8+ T cells.

The phenotype of infection/cure *P. berghei* responsive CD8+ T cells is KLRG1^{high} CD27^{low} CD44^{high} and CD62L^{low}

In order to identify the phenotype of CD8+ T cells generated during this infection/cure under CQ cover regimen, splenocytes and peripheral blood from mice three weeks post last inoculation for the presence of CD8+, CD3+, CD11a, CD127, CD27, KLRG1, CD44 and CD62L cells by flow cytometry was analysed. Circulating peripheral lymphocytes of infection/cure mice had about six-fold higher percent of CD8+ T cells containing KLRG1^{high}, CD27^{low} compared to controls (Table 2) with p value of control group vs. infection/cure group at <0.0001. Figure 3 shows a representative experiment where splenocytes and peripheral blood lymphocytes were analysed. Analysis of the CD8+ KLRG1^{high}, CD27^{low} cell population had high expression of CD44 and low expression of CD62L (Figure 4).

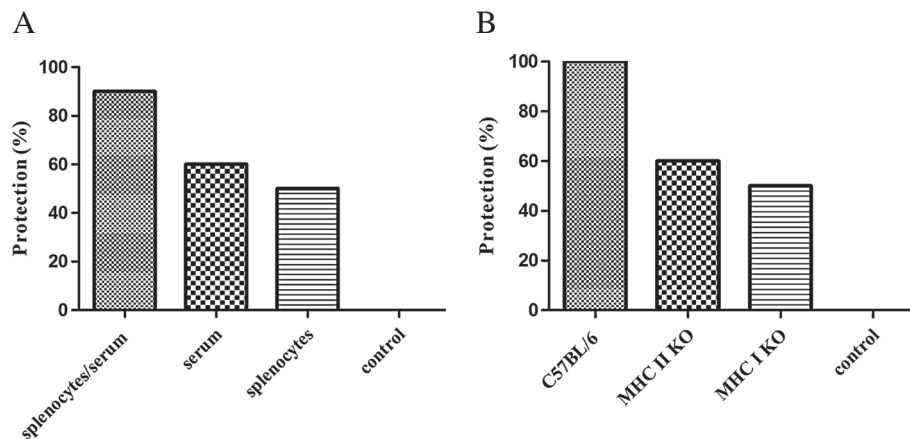


Figure 2 CD4+ T cells and CD8+ T cells are required for full protection. **A)** Infection/cure animals were sacrificed two weeks post suspension of CQ treatment. Splenocytes and/or serum from immunized or non-immunized animals were harvested and transferred into naive C57BL/6 mice. Groups of ten recipient mice received 2×10^7 cells, 100 μ L serum or a combination of both. Twenty-four hours later animals were challenged with 1000 Spz and appearance of parasitaemia was monitored for two weeks (representative of two experiments). **B)** Ten C57BL/6 wild type, or *Ic-/-* (MHC II KO), or $\beta 2$ macroglobulin *-/-* (MHC I KO) were infected/cured under CQ cover as described, group of animals received CQ alone as a control (results from one experiment). Two weeks post suspension of the CQ treatment animals were challenged with 1,000 Spz and the appearance of parasitaemia monitored for two weeks.

Infection/cure under CQ cover was thus associated with increased populations of effector memory CD8+ T cells. Results in Figures 3 and 4 are representative of three experiments with a pool of three spleens (splenocytes) or three experiments with 5–7 animals per group for the peripheral blood lymphocytes (peripheral blood). Statistical analysis for peripheral blood is reported in Table 2.

Identifying T cell phenotype associated with protection

To investigate whether the phenotype CD8+ KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} is associated with protection, partial protection conferred by a two immunization regimen with 20,000 Spz (Table 1) was used. Twelve C57BL/6 mice were immunized twice with 20,000 Spz. For controls, three animals were injected with CQ only. Two weeks after suspension of CQ treatment, circulating peripheral blood was collected from individual mice. The presence of CD8+ KLRG1^{high}, CD27^{low} cells was analysed. Animals were challenged and the appearance of parasitaemia monitored for two weeks. Expression of

KLRG1^{high}, CD27^{low} was significantly higher in protected animals compared to non-protected or controls with a p-value of 0.0087 and 0.024 respectively (Table 3), suggesting that CD8+, KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} cells were associated with protection.

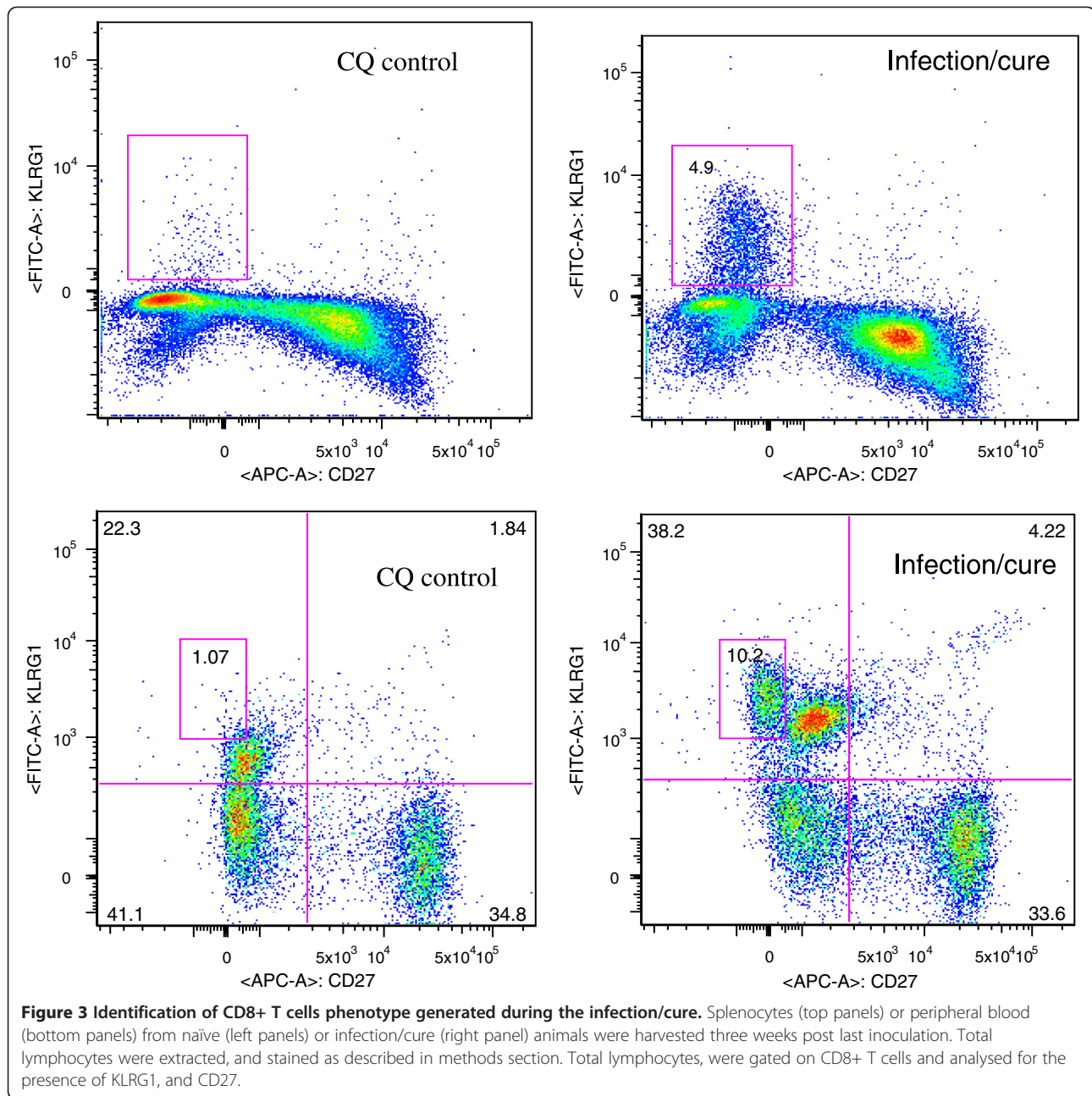
Discussion

Little progress has been made in elucidating the requirement for immune protection against malaria infection. Protection has not been achieved even with high titers of antibodies against known liver antigen [33], blood stage antigen [34,35] or antigen specific CD8+ T cells produced with adenovirus immunization [36]. In addition, human immunization with irradiated sporozoites has shown limited success unless sporozoites are injected intravenously [37]. Conversely, immunization with irradiated sporozoites or live sporozoites under CQ cover induces protection [29,31]. In the current study, the infection/cure C57BL/6 mouse model was used to elucidate the immune protection conferred during malaria infection by characterizing

Table 2 CD8+ T cells containing KLRG1^{high}, CD27^{low} phenotype

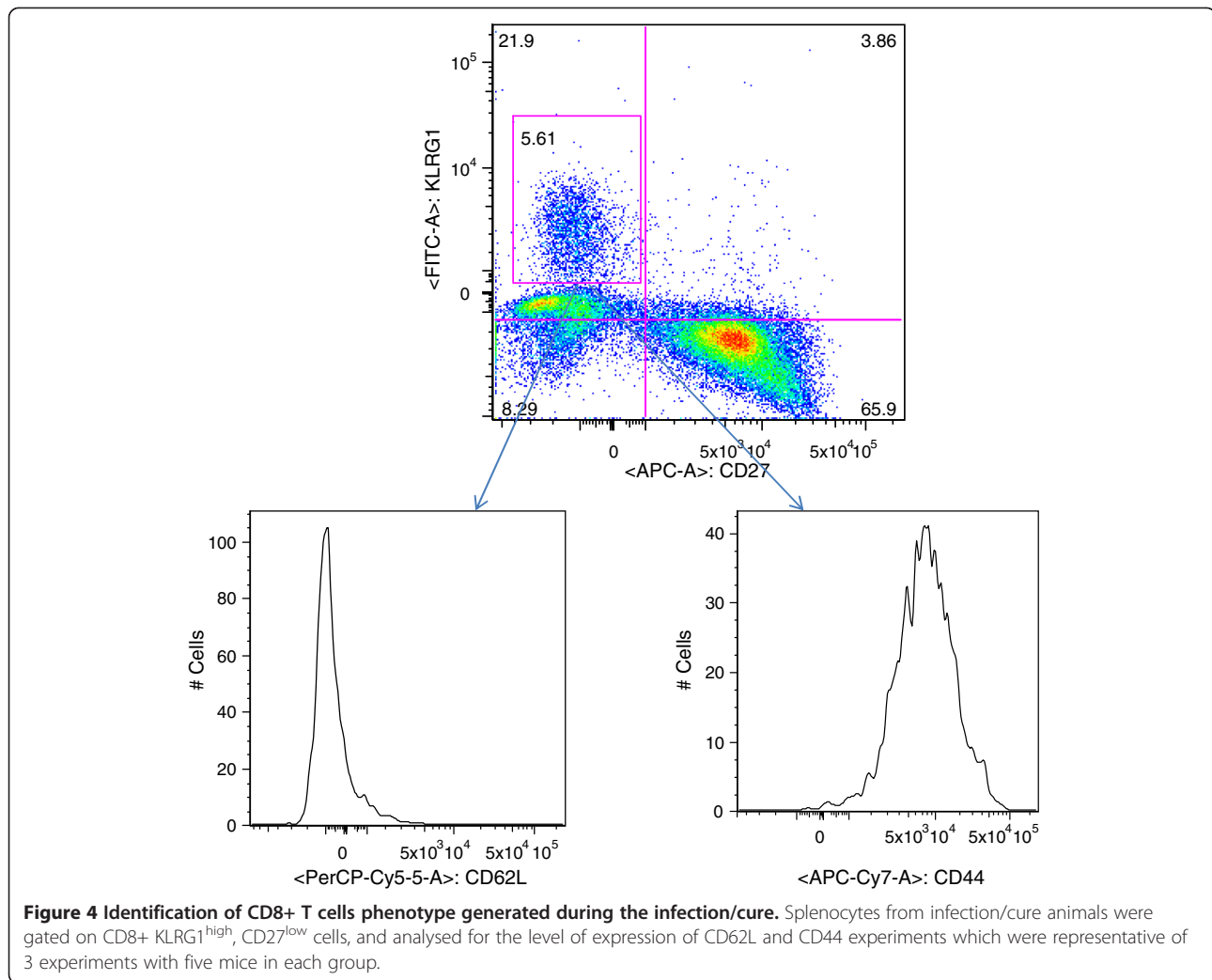
Group	Mean CD8+ KLRG1 ^{high} , CD27 ^{low} cells (%)	95% CI of mean		Number of lymphocytes/ml $\times 10^3$	Absolute number of CD + KLRG1 ^{high} , CD27 ^{low} T cells $\times 10^3$ /ml of blood
		Lower	Upper		
Control (15)	1.04	0.73	1.36	15,409	160.168
Post-3 (22)	5.72	4.41	7.04	14,894	841.32
Post-1 (5)	1.30	0.95	1.64	16,518	214.147

Infection/cure animals were inoculated three times (post-3) or one time with 20,000 Spz (post-1). The second column shows means of percent CD8+ cells expressing KLRG1^{high}, CD27^{low} phenotype in the circulating peripheral lymphocytes. Statistical significance was determined using Mann Whitney test. The numbers of animals used in each experiment per group are shown in brackets. There was significant difference between the control vs. post-3 groups ($p < 0.0001$) and between post-3 vs. post-1 ($p = 0.0013$). There was no significant difference between control vs. post-1 groups. The last two columns show the number of CD8+ KLRG1^{high}, CD27^{low} T cell $\times 10^3$ /ml of blood.



the nature of CD8+ T cells. Recent studies showed that both CD4+ T cells and CD8+ T cells are necessary to mediate immunity to liver stage malaria parasites [31,32]. This was demonstrated by immunizing BALB/c mice with *Plasmodium yoelii* iRBC under CQ cover and then treating with depleting antibodies against CD4+ T cells and CD8+ T cells. In this study, the infection/cure CD8+ T cells deficient (MHC I KO) mice resulted in only partial protection from lethal challenge, thus implicating CD8+ T cells in protection. Passive transfer with serum or cell alone also failed to confer 100% protection, thus indicating the cooperation of antigen specific cells and antibodies in

granting protection. MHC II animals were also only partially protected. The most likely explanation is that the protective antibodies generated during infection/cure need CD4+ T cell help, which is lost in CD4+ T cell deficient animals. However, the role of CD4+ T cell in this model cannot be excluded is also to support the generation of protective CD8+ T cells or that they are directly involved in parasite killing. It will be interesting to perform transfer experiments to test the protective effect of serum from infection/cure MHC II animals enriched with CD4+ T cells from infection/cure wildtype animals. This will be important to discriminate between the roles of CD4+ T cells as



pure ‘helper’ for antibodies production or more direct involved in anti-plasmodium activity. From this study and that by Belnoue *et al.*, data strongly suggest that both a humoral response and CD8+ T cells are required for immunity to liver stage *P. berghei* parasites [31,32].

Table 3 Mean CD8+ KLRG1^{high}, CD27^{low} T cell (%) expressed in circulating peripheral blood of control, protected and non-protected animals

Group	Mean CD8+ KLRG1 ^{high} , CD27 ^{low} cells (%)	95% CI of mean	
		Lower	Upper
Control (3)	1.117	0.3696	1.864
Protected(6)	5.772	3.759	7.785
Non-protected (6)	2.385	1.563	3.207

Twelve C57BL/6 mice were immunized twice with 20,000 Spz. Six mice were protected and six were not protected. Statistical significance was determined using Mann Whitney test. The numbers of animals used in each group are shown in brackets. There was significant difference between the control vs. protected groups ($p = 0.024$) and protected vs. non-protected ($p = 0.0087$). There was a marginal significant difference between control vs. non-protected ($p = 0.0476$).

In an effort to further elucidate immune protection, CD8+ T cells in peripheral blood and splenocytes were characterized. The distinct populations of CD8+ T cells with phenotype KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} were shown to be associated with protection. This population is absent in the intrahepatic lymphocytes. In the classical model of protection by immunization with irradiated sporozoites, the putative protective CD8+ T cells are found mostly in the liver [18,19] and produce IFN γ . In a recent study, Nganou-Makandop *et al.* [21] reported comparable levels of hepatic CD8+ T cells with a CD44^{high}, CD62L^{low} phenotype that produce IFN γ in response to PMA/Iono. In this study, mice were either immunized with radiation-attenuated sporozoites or infected with *P. berghei* under CQ cover. In the current study, the CD8+ KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} phenotype that was associated with protection was not present in the liver of immunized animals. A possible explanation of the apparent discrepancy is that more than one population of CD8+ T cells contributes to protection. One can

speculate that the splenic CD8⁺ KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} cells and the intrahepatic CD8⁺ CD44^{high}, CD62L^{low} cells are the same antigen specific population whose phenotype and organ-homing is modulated by antigen exposure. In explorative experiments, it was observed that splenic and peripheral blood CD8⁺ KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} T cells respond to *in vitro* stimulation with anti CD3 and *in vivo* sporozoites infection by producing IFN γ (see Additional file 1: Figure S1 and Figure S2). This is consistent with CD8⁺ KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} T cells participating to protection by producing IFN γ . However, these cells do not account for all the IFN γ produced in response to *in vitro* stimulation with anti CD3 (see Additional file 1: Figure S1 and Figure S2), or *in vivo* stimulation with sporozoites see Additional file 1: Figure S1 and Figure S2). It is, therefore, conceivable that other CD8⁺ T cells with low expression of KLRG1 are generated during this infection/cure regimen. Such cells respond to sporozoite infection with IFN γ production and display an elevated ability to respond to T cell receptor triggered by releasing IFN γ . The role of this CD8⁺ T cell phenotype in protection remains to be determined. KLRG1 has been shown to down regulate T cell receptor signaling [38,39], thus suggesting that the acquisition of the KLRG1^{high} phenotype is important for effector memory population after antigen encounter and execution of effector function.

Conclusions

KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} are the circulating CD8⁺ T cells generated during infection which are associated with protection and thus represent a correlate of protection. Passive transfer of purified/enriched KLRG1^{high}, CD27^{low}, CD44^{high}, CD62L^{low} CD8⁺ T cells will be critical in demonstrating a direct correlation of this phenotype in protection.

Additional file

Additional file 1: Interferon produced by CD8⁺ KLRG1^{high}, CD27^{low}.

Infection/cure animals were challenged with 1,000 sporozoites three weeks post third immunization. Twenty four hours later, animals were injected with 100 μ g of Brefeldine A to prevent lymphokines secretion. Five hours later animals were euthanized and blood collected by cardiac puncture. Five naïve mice were used as controls. Blood from 5 naïve or 5 infection/cure animals was pooled together, red blood cells were lysed and the peripheral lymphocytes incubated in the presence of media control of 5 μ g/ml of anti CD3 antibody 2C11. Sixteen hours later cells were washed and stained for CD3, CD8, KLRG1, CD27 and IFN γ . Figure S1) Peripheral blood lymphocytes were gated on CD8⁺ KLRG1^{high}, CD27^{low} and analyzed for IFN γ produced by CD8⁺ KLRG1^{high}, CD27^{low}. Figure S2) Splenocytes from animal infection/cure were labeled with CFSE, transferred into naïve mice and then challenged with 1,000 Spz 24hr post transfer. Forty-eight hours post challenge animals were euthanized and splenocytes harvested and analyzed for the production of IFN γ by transferred (immune cells) and recipient's (naïve) cells. CFSE+ (transferred/immune) or CFSE- (naïve) cells were gated on CD8⁺, CD27^{low} and analyzed for KLRG1 and IFN γ .

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: EK, CB, CFO. Performed the experiments: CB, EK. Analysed the data: CB, EK. Provided reagents and support: CFO, JM. Wrote the Manuscript: EK, CB. Reviewed the Manuscript: CFO, JHR. All authors approved the final version of the manuscript.

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Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

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