CD8 T Effector Memory Cells Protect against Liver-Stage Malaria

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CD8+ T Effector Memory Cells Protect against Liver-Stage Malaria

Arturo Reyes-Sandoval,* David H. Wyllie,* Karolis Bauza,* Anita Milicic,* Emily K. Forbes,* Christine S. Rollier,*,† and Adrian V. S. Hill*

Identification of correlates of protection for infectious diseases including malaria is a major challenge and has become one of the main obstacles in developing effective vaccines. We investigated protection against liver-stage malaria conferred by vaccination with adenoviral (Ad) and modified vaccinia Ankara (MVA) vectors expressing pre-erythrocytic malaria Ags. By classifying CD8+ T cells into effector, effector memory (T EM), and central memory subsets using CD62L and CD127 markers, we found striking differences in T cell memory generation. Although MVA induced accelerated central memory T cell generation, which could be efficiently boosted by subsequent Ad administration, it failed to protect against malaria. In contrast, Ad vectors, which permit persistent Ag delivery, elicit a prolonged effector T cell and T EM response that requires long intervals for an efficient boost. A preferential T EM phenotype was maintained in liver, blood, and spleen after Ad/MVA prime–boost regimens, and animals were protected against malaria sporozoite challenge. Blood CD8+ T EM cells correlated with protection against malaria liver-stage infection, assessed by estimation of number of parasites emerging from the liver into the blood. The protective ability of Ag-specific T EM cells was confirmed by transfer experiments into naïve recipient mice. Thus, we identify persistent CD8 T EM populations as essential for vaccine-induced pre-erythrocytic protection against malaria, a finding that has important implications for vaccine design. The Journal of Immunology, 2011, 187: 1347–1357.

Major efforts are currently being made at developing an effective malaria vaccine, because infection by Plasmodium falciparum continues to be the most common cause of mortality in children younger than 5 in a number of countries, mainly in sub-Saharan Africa (1). Vaccines based on the pre-erythrocytic Ags circumsporozoite protein (2) and thrombospondin-related adhesion protein (TRAP) (3) are the leading immunization strategies currently under development. Some vaccine regimens inducing large numbers of CD8+ T cells specific for pre-erythrocytic Ags can protect humans against experimental challenge (4). However, despite having vaccination schedules inducing extensive malaria-specific T cell responses, such as those using adenoviral (Ad) and poxviral vaccine vectors (5), our knowledge on how to use these tools efficiently to protect against malaria is hampered by an incomplete understanding of how they work. For example, it is known that circulating T cell frequencies, measured by ELISPOT, recognizing liver-stage Ags after vaccination did not predict protection in individual subjects.

Some vaccine regimens inducing large numbers of CD8+ T cells for vaccine design.

Vaccination with irradiated sporozoites is one of the most effective ways to induce sterile protection both in animal models and in humans (11, 12). This approach can be more efficacious with the use of mosquito-bite–delivered live sporozoites in recipients under concomitant treatment with chloroquine (13, 14). It has been demonstrated that persistent Ag presentation of sporozoite-derived Ags is important for optimal induction of CD8+ responses against the malaria liver-stage parasites (15). Viral vectors expressing liver-stage Ags are another promising malaria vaccine approach able to induce sterile protection in animal models (16, 17). Of these, Ad vectors have the ability to persistently express a transgene in vivo for at least 2 y to maintain an activated cohort of Ag-specific T cells (18), whereas modified vaccinia Ankara (MVA) expresses a transgene only for some hours and becomes undetectable within 48 h (19).

Differences in longevity or persistence of transgene expression have an impact on the generation of memory T cell responses (20). Three major subsets of Ag-experienced CD8+ T cells have been identified, based on the expression of CD62L (L-selectin) and CD127 (IL-7R α-chain): central memory T cells (T CM; CD62L+CD127+), effector memory T cells (T EM; CD62L−CD127+), and effector T cells (T E; CD62L−CD127−). Bachmann et al. (20) have shown that the protective ability of each subset varies depending on the context of the infection: T E and T EM have a greater ability to protect against peripheral organ infection with vaccinia virus than T CM because of the distinct anatomical distribution of these cell types (20).

In this study, we investigate T E/T EM/T CM generation by viral vectors. We show that different regimens differ in T cell induction.
protection, and unequivocally demonstrate for the first time, to our knowledge, that TeM induction is critical for protection against sporozoite challenge.

Materials and Methods

Mice and immunizations

Female BALB/c mice 6–8 wk of age were purchased from Harlan. All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee. Viral vectors were administered intradermally in endotoxin-free PBS (Sigma, Dorset, U.K.) at a concentration of $1 \times 10^8$ plaque forming units (PFU) for MVA and $5 \times 10^7$ viral particles (vp) for AdC9.

Viral vectors

Vectors expressing the transgene ME.TRAP have been previously described (21, 22). The insert ME.TRAP is a hybrid transgene of 2398 bp encoding a protein of 789 aa. The ME string contains the BALB/c H-2Kx epitope P99, among a number of other human B and T cell epitopes (23). AdC9 was constructed and propagated as described previously (24).

Cell staining and flow cytometry

For intracellular cytokine staining, ammonium chloride and potassium bicarbonate-buffer-treated splenocytes were incubated for 5 h in the presence of $1 \mu$g/ml P99 and 1 $\mu$g/ml Golgi-Plug (BD Biosciences). To assess hepatic T cell responses, we initially perfused livers with PBS (Sigma) to eliminate the circulating blood. T cells were then isolated from livers by mechanical disruption and incubation for 1 h at 37°C in FCS-free MEM supplemented with glutamine (4 mM) and penicillin/streptomycin (100 U penicillin/100 $\mu$g streptomycin), containing DNase at a final concentration of 0.03 mg/ml (Sigma) and collagenase at 0.7 mg/ml (Sigma). The reaction was stopped using MEM with 10% FCS, and after washing, mononuclear cells were purified with Ficoll-Paque Premium (GE Healthcare) and stimulated as described earlier.

Phenotypic analysis of CD8+ T cells was performed by intracellular cytokine staining using previously described Ab clones (17) (Bioscience), specifically anti-CD8 clone (clone 53-6.7), anti–IFN-γ (clone XMG1.2), and anti-CD127 (clone A7R34). Nonspecific binding of Abs was prevented by incubating with anti-CD16/CD32 FcγRII receptor (2.4G2; BD Phar-mingen) before staining. When using anti-CD62L (clone MEL-14; Bioscience), stimulated cells were incubated with TAPI-2 peptide (Peptides International) at a final concentration of 250 $\mu$M to prevent CD62L shedding from the cell surface. The P99 tetramer was produced by the National Institutes of Health (NIH) tetramer facility (MHC tetramer core facility, Vaccine Research Center, National Institutes of Health, Bethesda, MD) using the transgenic parasites (PbGFP-Luccon) used in the study. The transgenic P. berghei parasites (PbGFP-Luc++) and Pb9 tetramer were produced by the transgenic parasites (PbGFP-Luccon) used in the study. Mice and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee. Viral vectors were administered intradermally in endotoxin-free PBS (Sigma, Dorset, U.K.) at a concentration of $1 \times 10^8$ plaque forming units (PFU) for MVA and $5 \times 10^7$ viral particles (vp) for AdC9.

Adoptive cell transfer

For adoptive transfer of Te and TeM into naive recipient mice, donor BALB/c mice ($n = 15$) were immunized with AdC9 ME.TRAP (5 x $10^5$ vp) and then boosted with MVA ME.TRAP (1 x $10^8$ PFU) 8 wk later. Mice were bled a week after the final immunization, and T cells were sorted using a MoFlo cell sorter (DakoCytomation) after staining with anti-CD8 FITC, anti-CD62L PE, and anti-CD127 PE-Cy7 using similar clones to those previously described (17) to obtain three separate populations: CD8+ Te, TeM, and TeM. A separate staining with the P99 tetramer was used to calculate the total number of FcεRI+ Te, TeM, and TeM in every sorted subpopulation, and these were adjusted to contain equal numbers of P99-specific cells. A total of 43,500 P99-specific either Te or TeM were transferred to individual mice ($n = 3$). The yield of TeM cells was very low, and it was technically not possible to transfer them into recipient mice. Cells were transferred immediately after challenging mice with 1000 sporozoites. Blood smears were stained with Giemsa on days 5, 6, and 7 after challenge, and percentage of parasitemia was calculated in these three consecutive samples.

Parasite challenge

Plasmodium berghei (ANKA strain clone 234) sporozoites were isolated from salivary glands of female Anopheles stephensi mosquitoes. Parasites were resuspended in RPMI 1640 media with each mouse receiving a total of 1000 sporozoites i.v. Blood samples were taken daily from days 5–20; blood smears were stained with Giemsa and observed under a light microscope for the presence of parasites within the RBCs. In Table I, survival was defined as complete absence of parasites in blood. For investigation of a correlate of protection, a Kaplan–Meier analysis was conducted to compare the parasite growth rate, and protection was measured as a delay in reaching 0.5% parasitemia, as described elsewhere.

The transgenic P. berghei parasites (PbGFP-Luc++) used in the study expressed fusion GFP (mutant 3) and firefly luciferase (LucI) genes under the control of constitutive EF1a promoter (25). The parasites were generated by a stable double-crossover homologous integration of the transgene into P230p locus in the reference line of P. berghei ANKA line (cl2v5). The transgenic parasites were kindly provided by Dr. Oliver Bilker (Wellcome Trust Sanger Institute, Hinxton, U.K.).

In vivo imaging after challenge

Bioluminescent luciferase signal was detected through imaging the whole animals using the in vivo IVIS 200 imaging system (Caliper Life Sciences) as described previously (26). In brief, 44 h after the i.v. injection with 5000 transgenic P. berghei sporozoites, the C57BL/6 mice were anesthetized in batches of three using isoflurane, bellies shaved, and n-luciferin (Synchem Laborgemeinschaft OHG, Germany) was injected into the neck at a concentration of 100 mg/kg using sterile PBS (Sigma) as a diluent. Animals were imaged for 120 s at binning value of 8 and FVO of 12.8 cm, 8 min after the injection of n-luciferin. Mice were kept anesthetized throughout the whole procedure. Quantification of bioluminescence signal was performed using Living Image 4.2 software (Caliper Life Sciences).

Regions of interest were set around the liver area of mouse body and kept constant for all of the animals. The measurements were expressed as a total flux of photons per second of imaging time.

Statistical model for protection

Relationships between CD8 phenotype and protection were computed after challenging mice with 1000 P. berghei sporozoites. Blood parasite counts were obtained every day for 3 d from day 5 after challenge, blood stains stained with Giemsa, and percentages of parasitemia calculated in all animals. Relationships between log (percentage parasitemia) and time after challenge were plotted for mice developing parasitemia. Potential influence of vaccine on blood stage growth was assessed visually. As expected for a vaccine containing only pre-erythrocytic Ags, all infected mice exhibited similar exponential blood stage growth regardless of vaccination regimen (see Results); however, not all mice became parasitemic. We used survival analysis to assess vaccine efficacy, using time to 0.5% parasitemia (although any other level of parasitemia could also be used with equivalent results) as an outcome. This approach has been previously used; because time to parasitemia reflects number of parasites erupting from the liver provided, there is no blood stage immunity efficacy (27).

Differences between strata were assessed using log-rank or trend tests. We considered the time at risk to start on day 5 (when counting started) and to end on day 7 (when counting stopped). We obtained maximal likelihood estimates of time each mouse reached 0.5% parasitemia by modeling log ($b = k + c_i$, where $k$ is the growth rate (which is assumed to be constant for all mice, and estimated from the data), $t$ is the time after eruption from the liver, and $c_i$ is an intercept for each mouse and is proportional to the number of parasites erupting from the liver). Statistical analyses to determine differences in protection after prime–boost regimens (Table I) were performed using a Kaplan–Meier survival plot, and survival curves were compared using the log-rank test in Prism 5 (GraphPad software). Survival was considered as the complete absence of parasites in blood.

Statistical analysis of cell phenotypes

We compared proportions of cells expressing a particular phenotype after a single immunization using either unpaired homoscedastic t tests (e.g., T effector phenotype) for two groups or ANOVA and a Bonferroni posttest. Exceptions (described in Results) were small populations with highly non-Gaussian distribution (as assessed visually or with Shapiro–Wilk testing), where we used the Mann–Whitney U nonparametric tests. Analyses used GraphPad Prism or Stat 9 software.

Results

MVA vaccine induces accelerated development of memory CD8+ T cells

After a single immunization with either adenovirus (Ad) or MVA encoding the ME.TRAP Ag, we found a striking difference between
the two viral vectors in the kinetics of Ag-specific CD8\(^+\) T cell expansion, contraction, and generation of memory phenotype (Fig. 1). Ag (Pb9 epitope)-specific CD8\(^+\)IFN-\(\gamma\) responses in spleen after MVA vaccination peaked within a week after immunization and contracted rapidly by week 3. In contrast, immunization with Ad resulted in delayed kinetics, with the peak of CD8\(^+\)IFN-\(\gamma\)-producing Ag-specific T cells using CD27, CD62L, and CD127 as memory T cell markers and CD43 as an effector cell marker. These markers enabled us to distinguish three main subsets of Ag-experienced CD8\(^+\) T cells: TEM (CD62L\(^-\)CD127\(^-\)), TCM (CD62L\(^-\)CD127\(^+\)), and T EM (CD62L\(^+\)CD127\(^+\)) (20).

Vaccination with Ad induced a high proportion (>90%) of Ag-specific CD8\(^+\)IFN-\(\gamma\)CD43\(^+\) cells that gradually declined over time, with >40% of the IFN-\(\gamma\)-producing cells retaining the CD43\(^+\) effector status by week 8 (Fig. 1B). A concomitant decrease or absence of memory markers CD27 and CD62L was observed on Ag-specific CD8\(^+\) T cells early after Ad administration (Fig. 1C–F). In contrast, the initial peak of ~80% of Ag-experienced CD8\(^+\) IFN-\(\gamma\)-producing cells expressing CD43\(^+\) at week 1 postimmunization with MVA was followed by a sharp loss of CD43 expression and an early and steady increase in expression of all memory markers assessed: CD27\(^+\) (Fig. 1C), CD62L (Fig. 1D), IL-2 (Fig. 1E), and CD127 (Fig. 1F). CD127 (IL-7R\(\alpha\)) was the only memory marker that increased in expression after Ad immunization and followed a similar trend to the MVA responses, albeit lower at all time points tested. These results suggest that, in contrast with Ad, a single injection of a poxviral vector MVA can result in a fast transition to CD8\(^+\) T cell memory responses, which develop as early as 2 wk after immunization.

To investigate whether these observations remain true in the absence of in vitro Ag stimulation, we used an H-2K\(^b\) Pb9 tetramer and analyzed ex vivo the kinetics of memory T cell generation in the spleen. Immunization with Ad induced a substantial number of CD8\(^+\) T cells shortly after vaccination (47% of the Pb9-tetramer-positive cells), and these slowly contracted, leaving a residual population of 15% of tetramer-positive cells 60 d after vaccination (Fig. 1G). The TEM compartment gradually expanded over time from 52%, reaching 78% of tetramer-positive cells by day 60. The compartment containing the lowest proportion of cells was the T CM, which reached a maximum of 5.7% on day 60 post-vaccination. In contrast, immunization with MVA induced a striking expansion of T CM cells, which by day 60 represented a third of all tetramer-positive cells. This was paralleled by a complete contraction of the early T EM population by day 60 (Fig. 1G). The proportion of T EM remained stable during the time tested.

An MVA boost 8 wk after Ad prime provides the highest degree of protection against malaria challenge but does not correlate with frequency of CD8\(^+\) IFN-\(\gamma\)-producing Ag-specific CD8\(^+\) T cells in spleen

We asked whether the increased CD8\(^+\) memory generation by MVA could be translated into a more efficient vaccination regimen by altering the order of the viral-vectorized vaccines and the prime-boost interval. To this end, we chose several vaccination regimens and assessed their protective efficacy against malaria 2 and 8 wk after the last vaccination, using the malaria parasite *Plasmodiumberghei* in a mouse challenge model (Table I). This stringent challenge model consists of an i. v. (i. v.) administration of 1000 sporozoites, and protection is usually measured as a complete absence of parasites in blood (sterile protection). Frequencies of IFN-\(\gamma\)-producing Ag-specific CD8\(^+\) T cells in the mouse spleen were used as a measure of the immunogenicity of the different regimens.

Regardless of the length of the prime–boost interval, vaccination regimens involving Ad prime and MVA boost showed a trend toward a more sustained protection against malaria as compared with MVA only or MVA primed Ad-boosted regimens (Table I): the highest degree of long-term sterile protection (96% survival of animals challenged at 2 wk and 59% survival of animals challenged at 8 wk) was achieved when animals were primed with Ad and boosted with MVA 8 wk later (Table I). This protection was significantly higher than the MVA-Ad regimen on a challenge performed both at 2 wk (hazard ratio, 12.02; 95% confidence interval [CI], 3.34–43.34; \(p = 0.0001\)) and at 8 wk after the last vaccination (hazard ratio, 7.79; 95% CI, 1.79–33.87; \(p = 0.0062\)). Importantly, we observed that this protective efficacy was not associated with higher Ag-specific T cell responses in the spleen before challenge (Table I). For most prime–boost intervals, the MVA-Ad regimen was superior to Ad-MVA at eliciting strong Ag-specific IFN-\(\gamma\) production in the spleen but indicated a trend toward less protection against malaria challenge at both 2 and 8 wk after vaccination. To further confirm these results, we vaccinated BALB/c mice (\(n = 17–24\)) with both regimens (AdC9-MVA and MVA-AdC9) in an 8-wk interval, and Ag-specific responses were assessed long after vaccination (14 wk) to determine the memory immune responses of every regimen using blood as a different compartment (Table I, bottom two rows). We observed a significantly higher immunogenicity by the MVA-Ad regimen than the Ad-MVA (30.18 versus 23.24% of CD8\(^+\)IFN-\(\gamma\); 95% CI, 1.87–12.02; \(p = 0.0086\)). Despite inducing higher Ag-specific frequencies of T cells, protection induced by MVA-Ad in these mice showed a trend toward less protective efficacy.

These data indicate that high numbers of Ag-specific, CD8\(^+\) IFN-\(\gamma\)–producing T cells are not a simple predictor of the vaccine’s protective ability and highlight the importance of dissecting vaccination-induced immune responses to find better correlates of protection.

The highly protective Ad-MVA regimen predominantly induces CD8\(^+\) cells with TEM phenotype in the spleen

To identify the mediators of vaccine-induced protection, we investigated the phenotypic and functional profile of the Ag-specific T cells in the spleen using two of the most protective regimens consisting of prime–boost intervals of 8 wk (Table I): Ad-MVA and MVA-Ad regimens. Two weeks after priming with Ad the majority of Ag-experienced T cells were TEM (61.8%), with an overall hierarchy TEM\(\rightarrow\)TEM\(\rightarrow\)TEM (Fig. 2A). In contrast, priming with MVA induced a predominant (56.1%) TEM phenotype and a small proportion of TEM\(\rightarrow\)TEM\(\rightarrow\)TEM (Fig. 2A). Two weeks after the boost injection, the proportion of Ag-specific cells with a TEM phenotype was comparable between the two regimens (mean for AdC9-MVA = 7.9 versus MVA-AdC9 = 9.2; 95% CI, −5.46 to 2.94). However, the TEM population was significantly higher in the Ad-MVA regimen (mean of 86.1% compared with 77.6% for MVA-Ad; 95% CI, 4.4–12.5; \(p = 0.0022\) by \(t\) test), whereas the opposite was true of the TEM population (mean of 5.9% compared with 13.0% for MVA-Ad; 95% CI, −12.13 to −1.99; \(p = 0.014\) by \(t\) test, Fig. 2A).

It is recognized that different subsets of memory CD8\(^+\) T cells vary in their ability to acquire effector functions and circulatory patterns. TEM cells, for instance, rapidly acquire effector functions (28) and have a preferential circulatory pattern to the peripheral organs, such as the liver and lung (29). To further confirm the predominant induction of Ag-specific TEM cells by Ad-MVA, we analyzed in the same mice the circulatory properties of Ag-specific CD8\(^+\) T cells in the liver, a peripheral organ, as well as the target organ for our malaria vaccines (Fig. 2C). A single
FIGURE 1. Accelerated CD8 T cell memory generation by MVA and delayed memory induction by AdC9. BALB/c mice were immunized intradermally with AdC9 (5 × 10⁹ vp) or MVA (1 × 10⁶ PFU) expressing ME.TRAP. A–F, Spleen T cells were assessed for expression of IFN-γ and effector/memory cell surface markers in response to Pb9 peptide (CS252–260) stimulation, at different intervals after immunization with AdC9 (○) or MVA (●) viral vectored vaccines. A, Kinetics of Ag-specific CD8⁺ T cells expressing IFN-γ⁺. This cell subset was further characterized for the presence of the Tₑ marker CD43⁺ (B), and memory cell markers CD27⁺ (C), CD62L (L-selectin) (D), IL-2 (E), and CD127 (IL-7Rα) (F) at the same time points. Asterisks indicate statistically significant differences between the AdC9 and the MVA immunizations at the given time point: **p < 0.01, ***p < 0.001. G, Unstimulated Ag-specific CD8⁺ T cells in the spleen were identified using the H-2Kd Pb9 tetramer at the indicated days postimmunization and analyzed for Tₑ, T_CM, and T_EM phenotype, based on the expression of memory markers CD62L or CD127. Bottom left quadrants represent Tₑ, bottom right quadrants represent TEM, and top right quadrants represent T_CM. Numbers in quadrants represent the percentage of Pb9 tetramer-positive cells classified as Tₑ (CD62L⁺ CD127⁻), T_EM (CD62L⁻ CD127⁺), or T_CM (CD62L⁺ CD127⁺). Data are representative of two independent experiments with between three and six mice per experiment.
immunization with an Ad vector induced a stronger Ag-specific CD8+ response in the liver compared with MVA prime (mean of Ad of 45.4% [95% CI, 30.96–45.56] compared with 7.1% for MVA; p < 0.001 by t test) 2 wk postimmunization. The T cell responses in the liver were also higher on week 2 after the boost in the Ad-MVA regimen compared with MVA-Ad (mean of 35.9% [95% CI, 2.46–31.44] compared with 18.9% for MVA-Ad; p = 0.029 by t test). Thereafter, analysis of the full kinetics of the Ag-specific CD8+ T cells in the liver revealed that, 8 wk postboost (week 16) onward, the proportion of CD8+IFN-γ+ PB9-specific cells in the liver is the same in both Ad-MVA and MVA-Ad vaccination regimens (Fig. 2D). The capacity of the Ag-specific CD8+ T cells in the liver to produce multiple immune defense cytokines was also assessed for the two vaccination regimens (Fig. 2E). However, phenotypic differences in vaccine-induced hepatic T cells were also observed when measuring Ag-induced IFN-γ/TNF-α/JL2+ secretion by hepatic T cells with significantly higher IFN-γ/TNF-α+ (mean of 20.4% compared with 11.4% for MVA-Ad; 95% CI, 2.9–32.2; p = 0.030 by t test; Fig. 2E) with the protective regimen, suggesting a persistent alteration in phenotype induced by Ad-MVA.

In summary, vaccination with the Ad-MVA regimen, when compared with the less protective MVA-Ad, preferentially induced CD8+ TEM (CD8+IFN-γ+CD127+CD62L-) cells in the spleen and high frequencies of Ag-specific CD8+ cells in the liver, a key organ required by the sporozoite for replication and a target for our pre-erythrocytic vaccine. Blood CD8+ TEM responses correlate with protection against liver-stage malaria

Our results suggested that CD8+ TEM responses could play a role in protection against liver-stage malaria, so we set out to explore whether a preferential induction of blood Ag-specific TEM cells constitutes a correlate of protection at the individual level. We used the prime–boost interval of 8 wk, followed by a challenge with 1000 malaria sporozoites at week 22 (Fig. 3A). This late time point was chosen to ensure optimal conditions for comparing the influence of T cell phenotype, as we showed earlier that the proportion of Ag-specific CD8+IFN-γ+ T cells in the liver at week 22 is similar in the two regimens (Fig. 2D). We also tested the kinetics of peripheral blood PB9-specific responses between the two regimens, and at this time-point found lower levels in the protective Ad-MVA regimen compared with the less protective MVA-Ad (mean of 21.89% compared with 30.16% for MVA-Ad; 95% CI, 2.86; p = 0.0036 by t test; Fig. 3B), making enhanced protection by the Ad-MVA regimen because of higher CD8+IFN-γ+ T cell frequencies in the blood unlikely.

In an analysis of the relative sizes of the TE,T EM, and TCM subsets in peripheral blood at the time of malaria challenge, we found that, similar to our observations in the spleen, the Ad-MVA regimen induced significantly higher TEM cell proportions than MVA-Ad (54.4% compared with 47.1% for MVA-Ad; p = 0.003), TE,T proportions were significantly lower in the Ad-MVA regimen (p = 0.02, t test), whereas the TCM subsets were similar (Fig. 3C). After sporozoite challenge, mice were sampled on days 5, 6, and 7 for the analysis of parasitemia. These 3-d serial blood counts were available on all animals (n = 35). Five animals showed no parasitemia at any time point; therefore, growth curves for the 30 parasitemic animals are shown. As expected for a vaccine with no impact on blood stage growth, the parasitemias exhibited exponential blood stage expansion with similar growth rates (Fig. 3D). As a measure of disease outcome, we used the time taken to reach 0.5% parasitemia, which reflects parasite numbers erupting from the liver, a recognized measure for evaluating pre-erythrocytic vaccine efficacy (27). The Ad-MVA regimen showed a trend toward enhanced protection compared with the MVA-Ad, which was not significant at this time point (Fig. 3E). Next, having available several parameters calculated in blood samples from

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### Table I. Ag-specific CD8+ responses and protective efficacy against sporozoite challenge by Ad-MVA and MVA-Ad vaccination regimens with different prime–boost intervals

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Prime–Boost Interval (wk)</th>
<th>Ag-Specific CD8+ Responses in Spleen (% [95% CI]) (n = 3)</th>
<th>% Sterile Protection against P. berghei Sporozoite Challenge (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 wk Postboost</td>
<td>8 wk Postboost</td>
</tr>
<tr>
<td>No AdC9a</td>
<td>—</td>
<td>1.11 [0.8–1.4]</td>
<td>0.89 [0.4–1.4]</td>
</tr>
<tr>
<td>MVA-AdC9a</td>
<td>1</td>
<td>2.47 [0.9–4.75]</td>
<td>1.54 [0.7–2.31]</td>
</tr>
<tr>
<td>MVA-AdC9a</td>
<td>2</td>
<td>2.11 [0.6–3.61]</td>
<td>1.83 [−0.9–4.62]</td>
</tr>
<tr>
<td>MVA-AdC9a</td>
<td>4</td>
<td>1.39 [0.45–2.32]</td>
<td>1.54 [0.2–2.79]</td>
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<tr>
<td>MVA-AdC9a</td>
<td>8</td>
<td>2.69 [1.6–3.74]</td>
<td>2.36 [−0.0–4.78]</td>
</tr>
<tr>
<td>No MVA</td>
<td>0.15 [0.03–0.3]</td>
<td>0.07 [0.04–0.1]</td>
<td>0</td>
</tr>
<tr>
<td>AdC9-MVA</td>
<td>1</td>
<td>1.67 [0.7–2.61]</td>
<td>0.90 [0.54–1.27]</td>
</tr>
<tr>
<td>AdC9-MVA</td>
<td>2</td>
<td>1.75 [0.26–3.25]</td>
<td>1.00 [−0.4–2.49]</td>
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<tr>
<td>AdC9-MVA</td>
<td>4</td>
<td>1.93 [−0.55–4.40]</td>
<td>1.18 [0.18–2.17]</td>
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<tr>
<td>Naive</td>
<td>8</td>
<td>2.38 [0.38–4.37]</td>
<td>1.38 [1.05–1.72]</td>
</tr>
</tbody>
</table>

*BALB/c mice (n = 16/group) were immunized intradermally with AdC9 ME.TRAP (5 × 109 vp/mouse) or MVA ME.TRAP (1 × 109 PFU/mouse). A heterologous boost was administered 1, 2, 4, or 8 wk later using the same viral dose for each vector. Subsets of vaccinated mice (n = 3/group) were sacrificed on weeks 2 and 8 after the last vaccination, and their spleens harvested to assess the number of Ag-specific CD8+ T cells producing IFN-γ. Challenge was performed by i.v. administration of 1000 sporozoites P. berghei. Statistical analyses were performed using a log-rank test as described in Materials and Methods. Statistical differences are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and show comparison of individual regimens with the naive control. Immunogenicity was assessed using three mice per group, and challenge was performed using five mice per group (as indicated in the column headings) unless otherwise indicated (larger groups of n = 10, 17, 20, and 27 mice were used to confirm and strengthen the statistical comparisons).

**BALB/c mice were immunized intradermally with AdC9 ME.TRAP (5 × 109 vp/mouse) or MVA ME.TRAP (1 × 109 PFU/mouse). A heterologous boost was administered 8 wk later using the same viral dose for each vector. Ag-specific T cell responses were assessed 14 wk later in blood by flow cytometry after stimulation of PBMCs with PB9 peptide. % indicates cells producing IFN-γ within the CD8+ compartment. Statistical differences are indicated as **p < 0.01. Mice went through a challenge with 1000 sporozoites P. berghei per mouse, and sterile protection was assessed. Experiments were performed using 14 (MVA-Ad) and 21 (Ad-MVA) mice per group.

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### Materials and Methods

In an analysis of the relative sizes of the TE,T EM, and TCM subsets in peripheral blood at the time of malaria challenge, we found that, similar to our observations in the spleen, the Ad-MVA regimen induced significantly higher TEM cell proportions than MVA-Ad (54.4% compared with 47.1% for MVA-Ad; p = 0.003), TE,T proportions were significantly lower in the Ad-MVA regimen (p = 0.02, t test), whereas the TCM subsets were similar (Fig. 3C). After sporozoite challenge, mice were sampled on days 5, 6, and 7 for the analysis of parasitemia. These 3-d serial blood counts were available on all animals (n = 35). Five animals showed no parasitemia at any time point; therefore, growth curves for the 30 parasitemic animals are shown. As expected for a vaccine with no impact on blood stage growth, the parasitemias exhibited exponential blood stage expansion with similar growth rates (Fig. 3D). As a measure of disease outcome, we used the time taken to reach 0.5% parasitemia, which reflects parasite numbers erupting from the liver, a recognized measure for evaluating pre-erythrocytic vaccine efficacy (27). The Ad-MVA regimen showed a trend toward enhanced protection compared with the MVA-Ad, which was not significant at this time point (Fig. 3E). Next, having available several parameters calculated in blood samples from
FIGURE 2. Ad/MVA prime–boost regimens induce a predominant CD8$^+$ T$^{\text{EM}}$ phenotype in spleen and strong liver T cell responses. BALB/c mice were immunized with AdC9-MVA or MVA-AdC9 regimens using the same doses as in Fig. 1. Ag-specific immune responses to the H2-K$^d$ immunodominant Pb9 peptide were assessed in the spleen 2 wk after the boost using the Pb9 tetramer (A, B) and in the liver on stimulation with Pb9 peptide (C–E). A. Relative frequencies of CD8$^+$ Te, T$^{\text{EM}}$, and T$^{\text{CM}}$ subsets after a viral-vectorized prime or boost shown in representative plots for each vaccination regimen. Ex vivo splenocytes were stained with anti-CD8, -CD62L, -CD127, and H2-K$^d$ Pb9 tetramer. Levels of CD62L and CD127 expression within the Pb9 tetramer-positive (Tet$^+$) population are shown (gates were set using isotype controls). B. Bar graph representation of the plots in A after prime–boost, showing the group averages for the three T cell subpopulations (*$p < 0.05$). C. Ag-specific CD8$^+$ IFN-γ$^+$ responses in the liver generated by prime or prime–boost immunizations; representative plots are shown. Mononuclear cells were isolated from perfused livers 2 wk after the last vaccination and stimulated for 5 h with Pb9 peptide to identify Ag-experienced T cells induced by vaccination and homing to the liver. D. Kinetics of the Pb9-specific IFN-γ responses by CD8$^+$ T cells in the liver after prime–boost vaccination. BALB/c mice ($n = 33$) were vaccinated with either Ad-MVA or MVA-Ad regimens, and liver
every mouse, we used a mathematical model to investigate whether prechallenge TEM, TCM, and TEM proportions in blood correlate with protection against liver-stage malaria. After plotting the highest, middle, and lowest tertiles for each of the cell subsets, we found a significant difference in survival for the TEM cell subset (test for equality of survival between groups: \( \chi^2 = 7.44, p = 0.02 \)), with the lowest TEM numbers associated with lowest survival (Fig. 3E).

In conclusion, using a mathematical model to calculate parasite eruption from the liver, we found that CD8⁺IFN-γ⁺ TEM cells in blood are a correlate of protection against malaria sporozoite infection.

Parasite eruption from hepatocytes coincides with a CD8⁺ T cell phenotype change in the liver

The phenotype of Ag-specific CD8⁺ lymphocytes in the liver of the vaccinated animals was analyzed before and on day 7 after challenge (Fig. 4A). At both time points, <5% of all Ag-experienced CD8⁺ cells had the TCM phenotype. However, a striking difference was observed between the relative proportions of the TEM and TE subsets before and after challenge (\( p = 0.001 \) for both comparisons, Student t test). At the time of challenge, 68.2% of the Pb9-specific CD8⁺ T cells in the liver were from the TEM subset, whereas 7 d after challenge, the majority (65.1%) of the Pb9-specific CD8⁺ T cells displayed the TE phenotype (Fig. 4A). The causes of the altered phenotype are unclear but probably include a combination of proliferation and

**FIGURE 3.** Enhanced prechallenge TEM numbers in peripheral blood correlate with protection. A, Diagram showing the experimental design. BALB/c mice (\( n = 42 \)) were vaccinated at weeks 0 (prime) and 8 (boost), and challenged with 1000 *P. berghei* sporozoites at week 22. B, Relative proportion of Pb9-specific IFN-γ CD8⁺ T cell responses in peripheral blood, after prime-boost vaccination. C, Distribution of the TE, TEM, and TCM subsets in blood after Ad-MVA and MVA-Ad vaccination regimens, measured at the time of challenge. D, Growth curves of *P. berghei* blood-stage parasites from patent animals; animals were sampled from day 5 onward (onset of the disease blood stage). E, Protection against *P. berghei* disease by the two vaccination regimens, measured as time taken to reach 0.5% parasitemia. F–H, Survival curves for animals with the highest (red), middle (blue), and lowest (green) tertile of each cell phenotype: (F) TEM, (G) TE, or (H) TCM.
differentiation of T_{EM} into T_E cells (20) and an influx of T_E cells into the liver.

To better understand the effect of parasite burden on the CD8^+ T cell phenotype, we examined the relationship between the proportion of hepatic CD8^+ T_{EM} cells 7 d after challenge and the number of parasites that erupt from the liver, estimated by linear regression analysis on sequential blood counts (see Materials and Methods). Interestingly, we found that the proportion of liver T_{EM} cells decreased in challenged mice when compared with unchallenged mice (Fig. 4B). Furthermore, we noted that this phenotype switch was lowest in mice without parasitemia (i.e., high parasitemia), increased with the increasing number of parasites escaping from the liver, compatible with parasite burden driving the change of CD8^+ phenotype in the liver.

**CD8 T_{EM} protect against sporozoite challenge**

We adoptively transferred CD8^+ T_{EM} and T_E cells into naive mice and evaluated protection against malaria challenge. The donor mice were immunized using the Ad-MVA regimen with an 8-wk prime–boost interval, and 2 wk after the last immunization, PBMCs were isolated and stained with anti-CD8, -CD127, and -CD62L to be sorted in three populations: T_{CM} (CD62L^+ CD127^-), T_{EM} (CD62L^- CD127^-), and T_E (CD62L^- CD127^+). Part of the PBMC sample was stained with the H-2K^d Pb9 tetramer in addition to the phenotypic markers, to quantify the Ag-specific T cells and ensure identical absolute numbers of Pb9-specific cells were transferred for both T_{EM} and T_E cell populations. The harvested donor samples enabled transfer of 43,300 Ag-specific cells/mouse into three recipient animals for both the T_{EM} and T_E subsets (Fig. 5A). Because of a very low number of T_{CM} in the harvested donor sample, it was not possible to assess their protective efficacy. However, our earlier results suggested that this CD8^+ T cell subset plays no role in protection against malaria in this model (Fig. 3H). Nevertheless, we would not rule out the potential of the T_{CM} cells to contribute to protection because this phenotypic cell possesses the best proliferative ability and has been shown to protect mice in certain disease models, such as infection with lymphocytic choriomeningitis virus that replicates in lymphoid organs (20). Therefore, it will be important in the future to find an immunization approach that induces high levels of T_{CM} to allow a cell transfer and determine their protective efficacy against pre-erythrocytic malaria.

The recipient mice were challenged with malaria at the time of the transfer (1000 sporozoites/mouse, administered i.v.). Mice were screened for development of parasitemia in peripheral blood on days 5, 6, and 7 after challenge, and counts were analyzed as described earlier. Parasite growth curves in each recipient mouse confirmed similar growth rates for all parasites (Fig. 5B) and were used to calculate the initial parasite eruption from the liver. Using Kaplan–Meier survival analysis, we assessed the protective efficacy for each transferred population using the proportion of mice not reaching a defined parasitemia (Fig. 5C). Survival was enhanced in the T_{EM} group (T_{EM} versus others: \( \chi^2 = 3.90, p = 0.048 \)). From these results, we can conclude that the previously observed association between T_{EM} and survival (Fig. 3F–H) is causal, and that protection against malaria is not mediated by T_E. Nevertheless, with so few T_{CM} induced by our approach, so far we cannot rule out the contribution of these cells to protection against pre-erythrocytic malaria, and further studies will be required to assess this.

**Validation of the mathematical model using real-time in vivo imaging of luciferase transgenic parasites**

Mouse models permit assessment of the parasite burden directly in liver after a sporozoite challenge, whereas quantitation of blood-stage parasitemia is usually considered a surrogate end point for estimating the efficacy of pre-erythrocytic vaccines. The most adequate technique to accurately assess liver parasite burden in rodent models is the quantitative real-time PCR (qPCR) (30). We validated our mathematical model by correlating blood counts with liver parasite burden using an in vivo imaging system to visualize the parasite in the liver. Our mathematical model requires blood samples taken on days 5–7 after challenge, and a correlation with liver qPCR is incompatible because of the necessity to sacrifice the mice to take the liver 2 d postchallenge. Recently, transgenic P. berghei parasites expressing the reporter gene luciferase have been used to visualize and quantify the parasite development in the mouse liver using real-time luminescence imaging, and this technique has been shown to correlate well with established qPCR methods (26). We used this non-invasive technique to quantify the liver parasite burden at 40 h postchallenge whereas maintaining the mice alive for further quantification of parasites in blood on days 5–7, thus permitting a correlation analysis to validate our mathematical model. We visualized and quantified the parasites by challenging naive and vaccinated mice with luciferase transgenic sporozoites to induce different levels of protection. Vaccinated mice showed low levels of luciferase expression (Fig. 6A), whereas higher expression levels were observed in mock-vaccinated control mice (Fig. 6B). Luciferase expression correlated well with parasite blood counts on day 5 (\( r = +0.78, p = 0.008 \); Fig. 6C), and an even better
A correlation was observed using the time required to reach a 0.5% parasitemia, a variable calculated by a linear regression using percentage of parasites in blood on days 5, 6, and 7 ($r = 0.79$, $p = 0.008$; Fig. 6D).

FIGURE 5. CD8$^+$ T$_{EM}$ protect against malaria on transfer into naive recipient mice. A, BALB/c mice ($n = 15$) were immunized with the Ad prime–MVA boost regimen 8 wk apart. Mice were terminally bled 2 wk after the last immunization; blood PBMCs were stained with anti-CD8, -CD127, and -CD62L; and populations of T$_E$, T$_{EM}$, and T$_{CM}$ were sorted on a MoFlo sorter. A separate sample was used to quantify Pb9 tetramer-positive cells in each subset, and the number of transferred cells was adjusted to normalize the number of Pb9-specific CD8$^+$ cells in each recipient mouse. The T$_{CM}$ cells were not transferred because of an insufficiently low yield. B, Growth curves of P. berghei blood-stage parasites from patent animals; animals were sampled at the onset of the blood stage. Parasite growth was exponential and similar in all mice sampled. C, Kaplan–Meier survival analysis comparing the growth rates between the naive controls and the mice receiving T$_E$ and T$_{EM}$.

These results permitted a validation of the mathematical model that we have used with a real-time in vivo imaging system that permits quantification of the liver parasite burden without the requirement for qPCR.

FIGURE 6. Validation of the statistical model: correlation of liver parasite burden with percentage parasites in blood. A, C57BL/6 mice ($n = 10$ group) were vaccinated with (A) an Ad expressing the P. berghei circumsporozoite (AdC63 PbCS) or mock-vaccinated with (B) an empty AdC63. Two weeks later, both groups were challenged with transgenic luciferase P. berghei sporozoites. Parasite burden was assessed by detecting the luciferase signal in liver after 40 h and by blood screening for the presence of parasites from days 4–7 after challenge. Image shows an overlay of grayscale photo and luciferase bioluminescence image of 20 mice. Parasite counts on day 5 (C) and time to reach 0.5% parasitemia (D) were correlated with the bioluminescence signal expressed as a total flux of photons per second of imaging time.
Discussion

We show in this article that Ag-specific CD8+ TEM constitute a correlate of protection against liver-stage malaria that can be measured in peripheral blood and liver. Moreover, we demonstrate a preferential induction of a TEM after immunization with Ad-MVA regimen, which can efficiently deploy CD8+ T cells to the liver, the first potential target organ for sporozoite arrest and prevention of their egress into the bloodstream (31, 32). Our results suggest that the induction of the protective phenotype can be tailored by the choice of the viral vector used to prime or boost responses, because MVA tends to induce fast formation of the TCM response, whereas the persistent nature of Ad Ag expression (18) drives the adaptive immune system to maintain a TE/TEM response over a longer period.

It has been demonstrated that prolonged Ag presentation is required for the development of protective CD8+ T cell responses in a mouse malaria liver-stage model (15). In fact, this is considered an important mechanism behind the efficacy of the immunization with irradiated sporozoites in protection against liver-stage malaria (15). Cockburn et al. (15) showed that prolonged Ag presentation of malaria liver-stage Ags can be beneficial for protection because it increases the magnitude of the memory cell compartment and promotes development of T cells that can replicate on further encounter with the pathogen in a challenge.

Viral vectored vaccines are a leading vaccination strategy against malaria, and various regimens have been successfully tested in clinical trials, demonstrating various degrees of efficacy (3–5, 33). Little is known about either optimal routes for their administration or the kinetics of in vivo Ag expression and how this can be used to maximize the generation of T cell responses and protection. Using a real-time in vivo photon imaging technique, Gieben-Lynn et al. (19) showed a long-term expression of an adenovirus-delivered transgene. In contrast, transgene expression by recombinant vaccinia was lost after 4 d, and MVA was undetectable by day 2. We have previously established the concept of persisting vaccines by using replication-deficient Ad vectors of both human and simian origin (18). In that study, we demonstrated that Ad vectors have the ability to persist in vivo, similar to a replication-competent adenovirus (18).

All these previous observations are important to understand the results described in this article regarding the phenotypic changes of CD8+ T cells induced by either Ad vector or MVA. Although a single immunization with Ad vector induces predominantly CD8+ TE and TEM phenotypes, MVA rapidly generates a TCM phenotype, possibly as a consequence of the limited Ag availability/expression by the poxvirus. Moreover, responses initially elicited by Ad give rise preferentially to a TEM population after an MVA boost. Upon Ag encounter, the balance of these populations is determined by Ag availability and time. If the Ag is available, the cells further differentiate into TEM or in the absence of Ag, they become TCM (20).

The three cell subsets efficiently produce effector cytokines, such as IFN-γ, but both TE and TEM cells are more efficient in protecting against peripheral infection (20). This has been explained by the distinct anatomical distribution of these three populations. For instance, CD62L+ (TEM) cells have the ability to enter peripheral tissues, such as the ovaries (20) or the liver, and confer protection at the time of vaccine challenge. However, CD62L− TCM cells primarily home to secondary lymphoid tissues where they would need to be reactivated to be able to migrate to peripheral tissues (20, 34).

For malaria sporozoite challenge, a time delay in reaching the liver can be a fundamental factor in determining protection against liver-stage parasites. Hepatocyte infection represents a critical stage where the parasite is susceptible to recognition and elimination by CD8+ T cells. Its duration varies among different hosts and parasite strains, from approximately 2 d for rodent strains to 5–7 d for P. falciparum in humans (32). With such a short time to fight the parasite, it is easy to infer that previous or immediate presence of Ag-specific CD8+ T cells in the liver is necessary to protect against the disease. As shown by our results, the protective Ad-MVA regimen induces a preferential CD8+ TEM response, and this translates into high frequencies of liver CD8+ T cells. Using immunization with radiation-attenuated sporozoites, Schmidt et al. (35) recently demonstrated in mice that resistance or susceptibility to sporozoite infection does not result from differences in CD8+ frequencies in the host, but is instead related to TCM and TEM phenotypic differences among different mouse strains. Our results provide an explanation for these observations, as our model establishes a correlation with protection of TEM cells and a lack of correlation with either TE or TCM CD8+ cells. The study by Schmidt et al. (35), however, defines TEM cells by the lack of expression of CD62L in secondary CD8 cells long after the immunization, without taking into consideration the coexpression of CD127. We have shown that such CD62L− population can consist of a mixture of TEM/TE cells that can be defined by the expression or lack of expression of CD127, respectively. These two phenotypes, as shown in our study, possess different abilities to protect, with only TEM (CD62L−CD127+) responsible for protection.

To our knowledge, our study provides the first direct and compelling demonstration of CD8+ TEM as mediators of protection against pre-erythrocytic malaria and a vaccine correlate of protection measurable in blood. It also raises further questions about the mechanism by which the CD8+ TEM operate to induce protection, as well as a potential involvement of TEM in infection by other hepatic intracellular pathogens, such as viral hepatitis. Our results open a route to logical vaccine design, suggesting that other regimens inducing potent, persistent CD8+ TEM populations will induce protection against this important disease.

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Disclosures

The authors have no financial conflicts of interest.

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