

Dendritic Cells Induce Immunity and Long-Lasting Protection against Blood-Stage Malaria despite an In Vitro Parasite-Induced Maturation Defect

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Dendritic cells (DC) suffer a maturation defect following interaction with erythrocytes infected with malaria parasites and become unable to induce protective malaria liver-stage immunity. Here we show that, by contrast, maturation-arrested DC in vitro are capable of the successful induction of antigen-specific gamma interferon (IFN- γ) and interleukin 4 (IL-4) T-cell responses, antibody responses, and potent protection against lethal blood-stage malaria challenge in vivo. Similar results were found with DC pulsed with intact parasitized *Plasmodium yoelii* or *Plasmodium chabaudi* erythrocytes. Cross-strain protection was also induced. High levels of protection (80 to 100%) against lethal challenge were evident from 10 days after a single immunization and maintained up to 120 days. Interestingly, correlation studies versus blood-stage protection at different time points suggest that the immune effector mechanisms associated with protection could change over time. Antibody-independent, T-cell- and IL-12-associated protection was observed early after immunization, followed by antibody and IL-4-associated, IFN- γ -independent protection in long-term studies. These results indicate that DC, even when clearly susceptible to parasite-induced maturation defect effects in vitro, can be central to the induction of protection against blood-stage malaria in vivo.

Malaria is a leading cause of death in the Third World. Unfortunately, an effective human malaria vaccine remains elusive. There has been a large number of promising malaria vaccine studies with mice, monkeys, and humans (24). However, while human immune responses to vaccines have been detected, there is little evidence of induction of solid long-term protection in the field.

Dendritic cells (DC) are central to the induction of immunity; however, their role in the induction of protection (naturally or by vaccination) against the blood-stage malaria parasite is unknown. Their failure to induce protective liver-stage malaria immunity has been largely taken as an indication that this impairment would also prevent the induction of protective blood-stage immunity (19). DC are potent antigen-presenting cells which stimulate both naïve and memory T cells and are key modulators of primary immune responses (31). DC are no longer viewed as a homogeneous cell type but as a complex of different populations and functions. Immature DC can induce T-cell tolerance or, after maturation, become immunogenic (4, 16). Mature DC subpopulations can be generated from immature populations by stimulation with lipopolysaccharide (LPS), CD40L, or tumor necrosis factor alpha (TNF- α) and are uniquely potent at stimulating T cells (16). Furthermore, DC promote the production of antibodies and protection from a range of infectious pathogens (30). Both T cells and antibodies

have been shown to contribute to protective immunity during blood-stage malaria infection (reviewed in reference 12). In addition, in mice (35) and humans (27, 28), DC have been successfully demonstrated to elicit protective cellular immunity against tumors (25). Advances in the understanding of molecular mechanisms regulating the functions of DC have aided the design of DC-targeted vaccines for the treatment of various diseases. A recently devised DC-based vaccine that employs ex vivo culturing of DC loaded and pulsed with tumor antigens is now the subject of a clinical trial (3). Vaccines that target DC in vivo include the delivery of antigens to resident-skin Langerhans cells (14), DC fused with cancer cells have also been used for immunization (35), and DC engineered to express CD40L and then pulsed with a tumor antigen exhibited up-regulated interleukin-12 (IL-12), CD80, CD86, and CD54 expression and induced specific CD8 T cells, T-cell proliferation, and protection from tumors in a mouse model (15).

T-cell responses to mitogens and antigens in vitro are usually poor in individuals living in areas where malaria is endemic, and a single malaria infection is usually unable to induce long-lasting protective immune responses (23). It has been noted that surface interactions between DC and malaria parasites can prevent human DC from maturing in vitro. A recent study in malaria-exposed children has suggested that DC in peripheral blood may have down-regulated major histocompatibility complex class II expression (39). Thus, DC function can be impaired in vivo during the acute stages of malaria infection. This observation led to the hypothesis that DC may have cytokines produced by T helper 1 (Th1) cells characterized by gamma interferon (IFN- γ), interleukin 12 (IL-12), and TNF- α production and Th2 characterized by IL-4 and IL-10 that have been shown to be protective in both liver and blood stage (36).

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Recent results suggest that protective immune responses to peptides from a sporozoite protein can be induced by immunization with DC transfected with a recombinant viral vector (5) and that some murine DC may not be negatively affected by interaction with parasitized red cells (29) and limited ability to induce protective T-cell responses to blood-stage malaria (40). Two recent papers from the same group showed, in contrast, that DC from mice infected with *P. yoelii* nonlethal and *P. chabaudi* AS strains did not have any inhibitory effects on costimulatory markers on DC and also supported naïve T-cell stimulation in vitro with potent cytokine IL-12 secretion (17, 21). Failure of in vitro maturation-arrested murine DC pulsed with sporozoite-derived antigen to stimulate protective liver-stage immunity, however, provides contradictory evidence (29).

Here we show that whole erythrocytes isolated from animals infected with *Plasmodium* spp. can be taken up by DC ex vivo and stimulate powerful protective blood-stage malaria immunity in vivo. A novel and potentially useful pattern of protection was observed, with induction of both Th1- and Th2-type cytokines, antibodies, cross-protection between *P. yoelii* and *P. chabaudi* murine malaria strains, and long-term protection (120 days) after a single immunization. These results suggest that DC may be involved in the induction of natural immunity against blood-stage malaria parasites and further indicate that targeting of parasite antigens to DC may provide an attractive new approach for the development of blood-stage malaria vaccines.

MATERIALS AND METHODS

Mice and immunizations. *H-2K^b* C57BL/6 and IL-12p40-deficient (C57BL/6, *H-2K^b* background) female mice, 6 to 8 weeks old, were used in the experiments. Mice were bred at the Austin Research Institute Biomedical Animal Research Lab. Bone marrow-derived DC, which express moderate levels of CD11c, CD40, CD80, and CD86, were pulsed with (10:1 ratio) malaria-parasitized erythrocytes. DC (10⁶) were injected intradermally into mice in the hind footpads. Mice were sacrificed 10 days later (or otherwise where indicated), and splenocytes were removed and processed for cytokine production, assessed by enzyme-linked immunosorbent assay (ELISPOT) assay. Antibody responses in serum were tested by enzyme-linked immunosorbent assay (ELISA) at various time intervals up to 120 days postimmunization.

Experimental infections. *P. yoelii* YM and *P. chabaudi* AS erythrocytes were generated in mice from frozen stock (kindly provided by Ross Coppel, Monash University, Melbourne, Australia) and freshly harvested for intraperitoneal (i.p.) injection into naïve mice. Mice were monitored for percent parasitemia daily, and a collection of low-parasitic (5 to 10%) and high-parasitic (40 to 50%) blood was obtained by eye bleed. Mice with >60% parasitemia were culled. The blood was washed twice in sterile phosphate-buffered saline (PBS), and packed red blood cells (RBCs) were used at 10 RBCs per 1 cultured DC. Intradermal injection of DC with parasitized red cells did not induce detectable levels of parasitemia as analyzed by a daily Giemsa stain of blood smears in mice prechallenge (days 10 to 14). In long-term protection or immunogenicity experiments, mice were further confirmed to be parasite free with twice weekly tests up to day 120 (data not shown).

Generation and adoptive transfer of mature murine bone marrow-derived DC. Bone marrow cells from C57BL/6 female mice were cultured at 10⁶ cells/ml in tissue culture. Petri dishes contained conditioned RPMI 1640 medium (CSL, Parkville, Victoria, Australia) (RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 mg of streptomycin per ml, and 100 U of penicillin per ml [Gibco, Mt. Waverly, Victoria, Australia]) supplemented with 1,000 U of granulocyte-macrophage colony-stimulating factor (GM-CSF)/ml, 10 ng of IL-4/ml, 10% heat-inactivated fetal calf serum, 4 mM L-glutamine, 100 U of penicillin/ml, 100 mg of streptomycin sulfate/ml, and 100 mM β-mercaptoethanol. At day 6 the cells were washed, resuspended in the same cultured media at 10⁶ cells/ml, and incubated overnight with either normal or parasitized RBCs from either *P. yoelii* or *P. chabaudi* murine species of malaria. Pulsed DC were then washed thoroughly and resuspended at 10⁷

cells/ml in PBS, and 100 μl was injected intradermally into experimental mice. To mature DC, LPS at 1 μg/ml and TNF-α were added to DC cultures for 3 h.

Immunological assays. (i) **ELISPOT assay.** One million spleen cells were incubated in conditioned RPMI media alone, normal red cell lysate (50 μg/ml), or parasitized red cell lysate (50 μg/ml) for 18 h on mixed acetate plates (MAHA; Millipore) coated with anti-murine IFN-γ (clone R4 from the American Type Culture Collection, Manassas, Va.) or anti-murine IL-4 monoclonal antibody (MAb) (BVD4-1D11; Pharmingen, San Diego, Calif.) as described previously (22). Triplicate wells were set up for each condition. Cells were discarded and plates incubated 2 h with anti-murine IFN-γ MAb-biotin (XMG.21-biotin; Pharmingen) or anti-murine IL-4 MAb-biotin (BVD6-24G2-biotin; Pharmingen), respectively, followed by extravidin-alkaline phosphatase at 0.1 μg/ml (Sigma). Spots of activity were detected by using a colorimetric alkaline phosphatase kit (Bio-Rad, Hercules, Calif.) and counted using a dissection microscope. Data are presented as mean spot-forming units (SFU) per million cells ± standard deviations (SD) of the mean.

(ii) **ELISA.** Mice were bled by eye bleed and serum was collected. Polyvinyl chloride microtiter plates coated with homologous malaria lysate (100 μg/ml in 0.2 M NaHCO₃ buffer, pH 9.6) were blocked with 2% bovine serum albumin-PBS for 1 h at 37°C, washed with PBS-0.2% Tween five times, and incubated for 2 h at room temperature with mouse sera, and then washed 15 times, incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Ig) (Silenus, Parkville, Victoria, Australia) for 1 h at room temperature, and washed again before adding developing buffer [5 ml of 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS) buffer, 100 μl of ABTS stock, and 4 μl of H₂O₂] for 30 min. Plates were read on an EL312e microplate reader at 405 nm. Experimental titer was defined as the serum dilution at which the optical density at 405 nm was above the mean plus 3 SD of control naïve sera values in the same assay.

Challenge experiments. In all challenge experiments, frozen stocks of parasitized erythrocytes were injected into naïve C57BL/6 mice i.p., and parasitaemia was monitored until <10% could be detected in blood films. These mice were eye bled, and the fresh parasitized erythrocytes at 10⁶ were injected i.p. into experimental mice.

T-cell purification. T cells were purified using glass bead mouse T-cell immunocolumns (Cytovax Biotech, Alberta, Canada) according to the company protocol. T-cell preparations were >90% CD3⁺ cells.

Statistical analysis. Assays were set up in triplicate. Mean values were compared using the two-tailed unpaired *t* test. Mann-Whitney statistical analysis was used to compare percent parasitemia and percent survival. This compares survival data from two sets of data over a time course period; therefore, the *P* values represents the entire percent survival data set. Two *P* value thresholds are noted in the text for both protection and immunogenicity assays: *P* of <0.0001, to indicate a highly significant difference, and *P* of <0.05, to indicate a significant difference. Protection data used in correlation analyses was the percentage of mice surviving lethal challenge and remaining aparasitemic in a 30-day follow-up in a series of individual experiments. Briefly, before challenge, three to four mice from each experimental group were sacrificed to perform immunogenicity assays (ELISPOT and ELISA) which were then correlated to the percentage of surviving mice from the parallel challenge with mice that had received the same treatment, with standard linear regression analysis. Five to 10 mice were used for individual challenge experiments, and each experimental challenge condition was repeated two to six times. Correlation analyses had 6 to 15 separate replicate data points from individual experiments. Covariance of two data sets divided by the product of their SD was calculated to determine their degree of correlation (*r*) and its square (*r*²). *P* values were interpolated from the following formula: $r^2 = t^2 / (t^2 + n + 2)$.

RESULTS

Murine DC show deficient maturation in vitro after interaction with parasitized erythrocytes. To confirm that murine DC maturation was affected by interaction with parasitized erythrocytes, bone marrow-derived DC were left untreated or were incubated in vitro with intact, freshly isolated *P. yoelii*- or *P. chabaudi*-parasitized erythrocytes, prior to adding maturing stimuli (TNF-α and LPS). As expected, the GM-CSF- and IL-4-grown bone marrow cells tested were consistently >90% CD11c⁺ (DC phenotype) on gated cells based on forward versus side scatter (Fig. 1A). Upon maturation with LPS-TNF-α, DC showed increased levels of costimulatory mole-

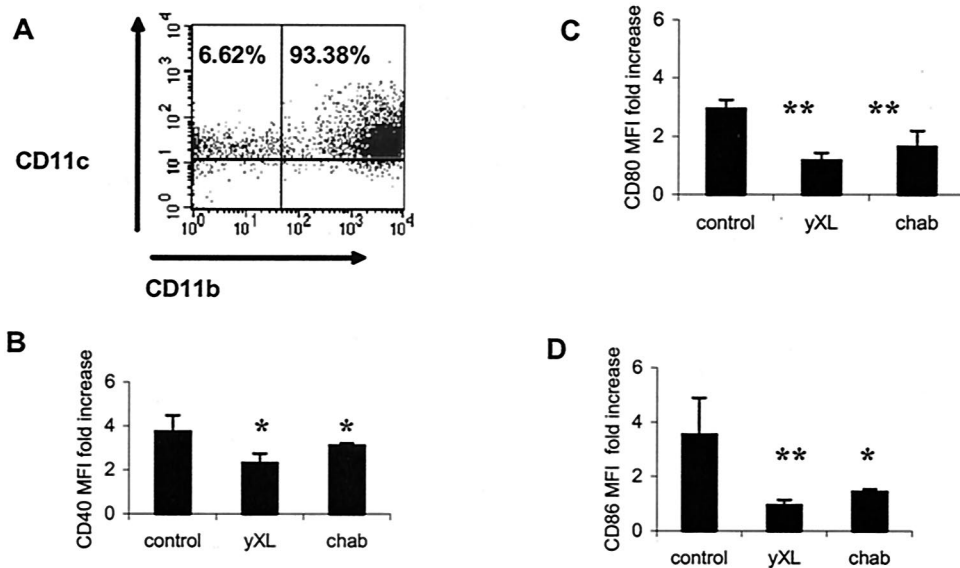


FIG. 1. Maturation arrest of DC after interaction with parasitized erythrocytes in vitro. (A) Representative FACS analysis ($n = 6$) showing DC grown from murine bone marrow in vitro in the presence of GM-CSF and IL-4 were routinely $>90\%$ CD11c⁺, with the majority also CD11b⁺, confirming a myeloid lineage. Forward versus side scatter gates for analysis included all live large and granular cells. In the figure shown, regions were set up based on control isotype directly conjugated stain controls (not shown). (B) DC were pulsed with intact nonparasitized erythrocytes (control) or parasitized *P. yoelii* (yXL) and *P. chabaudi* (chab) erythrocytes for 16 h and then matured with LPS and TNF- α for 3 h, followed by staining with phycoerythrin-MAB to specific costimulatory molecules CD40 ($n = 5$), CD80 ($n = 7$) (C), and CD86 ($n = 8$) (D) and analyzed by FACSscan. The data are shown as the n -fold increase in MFI of staining for costimulatory molecules after LPS and TNF- α treatment. The n -fold increase is calculated by dividing MFI after stimulation by MFI without stimulation. The mean MFI increase \pm SD in five to eight independent experiments is presented for each marker. The differences in up-regulation of surface expression of costimulatory molecules on DC by LPS and TNF- α exposed to intact parasitized erythrocytes compared to nonparasitized erythrocytes was statistically significant as noted at P of < 0.05 (*) and P of < 0.0001 (**) by two-tailed unpaired t test analysis.

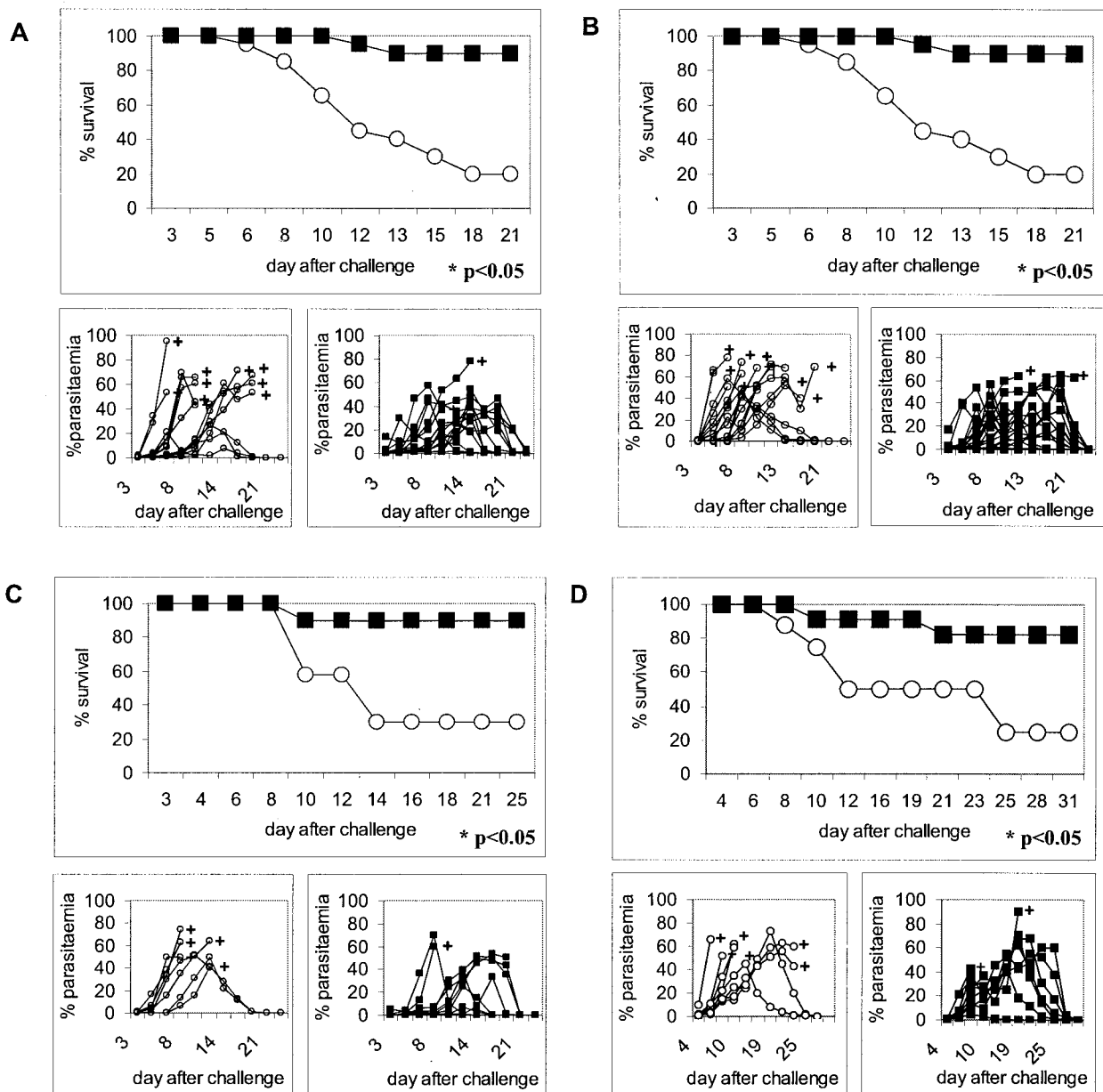
cules, CD40 (Fig. 1, panel B), CD80 (panel C), and CD86 (panel D), in the presence of normal autologous RBCs. Data are shown as the n -fold increase in mean fluorescence intensity (MFI) for each marker after LPS-TNF- α stimulation according to the following formula: MFI after stimulation/MFI before stimulation (6, 13). In contrast, preincubation of DC with *P. yoelii*- or *P. chabaudi*-parasitized intact erythrocytes (PRBC) prevented efficient up-regulation of costimulatory molecules CD40, CD80, and CD86 on DC by these maturation stimuli in vitro (Fig. 1). We confirmed that DC which have been incubated with PRBC have decreased stimulatory ability in mixed lymphocyte reaction (MLR) assays, with these results being consistent with those of Urban et al. when analyzing *Plasmodium falciparum* and human DC (data not shown) (38).

DC induce protection against blood-stage malaria. To test whether DC can play a role in the induction of protection against blood-stage malaria infection despite DC modulation, DC treated with parasites were used to immunize animals (after in vitro pulsing with intact freshly isolated untreated or *P. yoelii*-parasitized erythrocytes). These immunized animals were challenged 10 days later with 10^6 freshly isolated parasitized *P. yoelii* erythrocytes i.p. A single immunization of DC pulsed with intact parasitized erythrocytes (DC-PRBC) promoted high levels of survival against subsequent lethal parasite challenge (90%, 18 of 20), whereas few animals survived in the group immunized with DC pulsed with nonparasitized erythrocytes (DC-RBC) (17%, 3 of 18) (Fig. 2A; $P < 0.05$). Two immunizations 14 days apart with DC pulsed with intact parasitized erythrocytes also induced greater than 90% protection

compared to control mice with no significant difference in survival after single or multiple DC immunizations (data not shown). Due to the observed differences in the mechanisms of protective immunity between different species of malaria in mice, DC were also used to immunize C57BL/6 mice after pulsing them ex vivo with *P. chabaudi*-parasitized erythrocytes. Similar to what was observed using *P. yoelii*, this protocol induced significant protection against homologous *P. chabaudi* lethal parasite challenge compared to that of the control (DC-RBC-immunized) mice (13 of 15 versus 3 of 13) (Fig. 2B; $P < 0.05$).

Interestingly, cross-protection between different murine malaria species was evident after immunization with DC pulsed with parasitized *P. yoelii* erythrocytes and challenged with 10^6 *P. chabaudi*-parasitized erythrocytes (9 of 10 versus 1 of 7) (Fig. 2C; $P < 0.05$). The cross-protection effects by using parasitized red cells from *P. chabaudi* to induce cross-protection against *P. yoelii* infection were not examined in this study but will be part of a specific study on the extensive issue of cross-protection. Such cross-protection has not been reported for other vaccination protocols and could suggest that DC have unusual potential to induce such cross-protective immunity.

Induction of long-term protection. High levels of protection were found 10 days after a single immunization with DC pulsed with *P. yoelii*-parasitized erythrocytes. To assess their potential to induce long-term protection, animals were immunized once and challenged with *P. yoelii*-parasitized erythrocytes 120 days later. Highly significant protection was maintained 120 days after a single immunization, with 80% (8 of 10) of mice surviving compared to 20% (2 of 10) in the group immunized with



† indicates death

FIG. 2. Protection from lethal malaria parasite challenge in mice after immunization with DC pulsed with parasitized erythrocytes. (A) Protection against lethal *P. yoelii*. DC were pulsed overnight with nonparasitized erythrocytes (DC-RBC; $n = 18$) (○) or parasitized *P. yoelii* erythrocytes (DC-PRBC; $n = 20$) (■), washed, and injected into C57BL/6 mice intradermally at 10^6 cells per mouse. Ten days later, mice were challenged i.p. with 10^6 parasitized *P. yoelii* erythrocytes. Venous blood smears were collected daily from day 4 after challenge and parasitemia was scored on Giemsa-stained slides as described previously (7). Similar results were obtained challenging animals 30 days after immunization (not shown). (B) Protection against *P. chabaudi*. Mice were immunized with DC pulsed with intact nonparasitized erythrocytes (DC-RBC; $n = 13$) (○) or *P. chabaudi*-parasitized erythrocytes (DC-PRBC; $n = 15$) (■), challenged i.p. with 10^6 parasitized *P. chabaudi* erythrocytes, and monitored for survival and parasitemia as described above. (C) Cross-protection against lethal malaria challenge. DC were pulsed with intact nonparasitized erythrocytes (control; $n = 7$) (○) or *P. yoelii*-parasitized erythrocytes (DC-PRBC; $n = 10$) (■) and injected into C57BL/6 mice, and 10 days later mice were challenged with *P. chabaudi*-parasitized erythrocytes as described above. (D) Long-term protection against lethal malaria challenge. DC were pulsed with nonparasitized erythrocytes (DC-RBC; $n = 10$) (○) or *P. yoelii*-parasitized erythrocytes (DC-PRBC; $n = 10$) (■), and 10^6 cells were injected intradermally into C57BL/6 mice. One hundred twenty days later, mice were challenged with 10^6 *P. yoelii*-parasitized erythrocytes i.p. and monitored for survival and parasitemia as described above. Percent survival is statistically significant at P of <0.05 or very statistically significant at P of <0.0001 by Mann-Whitney statistical analysis.

DC pulsed with nonparasitized erythrocytes (Fig. 2D; $P < 0.0001$).

The mechanisms associated with protection change over time. The ability of DC pulsed with parasitized erythrocytes to induce Th1- and Th2-type responses was assessed by measuring IFN- γ and IL-4 responses by ELISPOT assay. GM-CSF- and IL-4-derived DC were pulsed with parasitized *P. yoelii* erythrocytes. There was a consistent increase in the number of both Th1 (IFN- γ) and Th2 (IL-4) *P. yoelii*-specific T cells in mice immunized with DC pulsed with parasitized *P. yoelii* erythrocytes (DC-PRBC) compared to control mice immunized with DC pulsed with nonparasitized erythrocytes (DC-RBC) (Fig. 3A and B; $P < 0.0001$). In addition, significant malaria parasite lysate-specific antibody responses above controls were detectable 30 (Fig. 3C; $P < 0.0001$) and 90 (Fig. 3D; $P < 0.0001$) days after immunization with DC pulsed with *P. yoelii*-parasitized erythrocytes (DC-PRBC). In contrast, analysis 10 days after a single immunization showed no detectable parasite lysate-specific antibody responses. At day 10 postimmunization, cross-reactive T-cell responses to both homologous *P. yoelii*- and *P. chabaudi*-parasitized red cell lysate, compared to nonparasitized lysate in vitro, were investigated. Specific IFN- γ responses were increased in mice immunized with DC-PRBC for *P. yoelii* and *P. chabaudi* red cell lysate with no significant responses to nonparasitized red cell lysate (Fig. 3E; $P < 0.0001$). These findings suggest that at the time of blood-stage challenge and the induction of protection, cross-reactive T cells were present and able to react to both *P. yoelii* and *P. chabaudi*, but cross-reactive antibodies were not detectable (Fig. 3F).

Maturation of DC induces protective immunity against a lethal *P. yoelii* malaria parasite challenge. GM-CSF-IL-4-grown DC were further matured with LPS and TNF- α for 3 h prior to injection into mice and compared to untreated DC at different immunization doses. Although, as described in the previous sections, maturation was strongly impaired in vitro for DC that had received parasitized erythrocytes, as reflected in an inability to up-regulate costimulatory molecules CD40, CD80, and CD86. Immunization with 10^6 matured DC was observed to induce maximal protection against *P. yoelii* challenge compared to 10^5 matured DC or immature DC at either dose, which were less effective (Fig. 4A and B; $P < 0.05$). It is possible that other DC costimulatory surface molecules, or soluble factors, are up-regulated normally and are sufficient to further promote the induction of protective immunity. Alternatively, the minor up-regulation of CD40, CD80, and CD86 may have been sufficient to cause an incremental benefit in the induction of blood-stage protection.

Correlative nature of the immune response versus protection induced by DC. Protection (percent survival) and immunogenicity data were collated, and correlation analysis was performed on each variable at different time points. At day 10 after immunization, there was no correlation between protection and antibody responses (total Ig) ($n = 30$), but there was a strong correlation with increased IFN- γ ($r^2 = 0.7$, $P < 0.0001$) and IL-4 ($r^2 = 0.61$, $P < 0.0001$) responses. There was a significant correlation between these responses, indicating that DC induced high levels of both cytokines in the same animals ($r^2 = 0.64$, $P < 0.0001$). Neither of the two cytokines

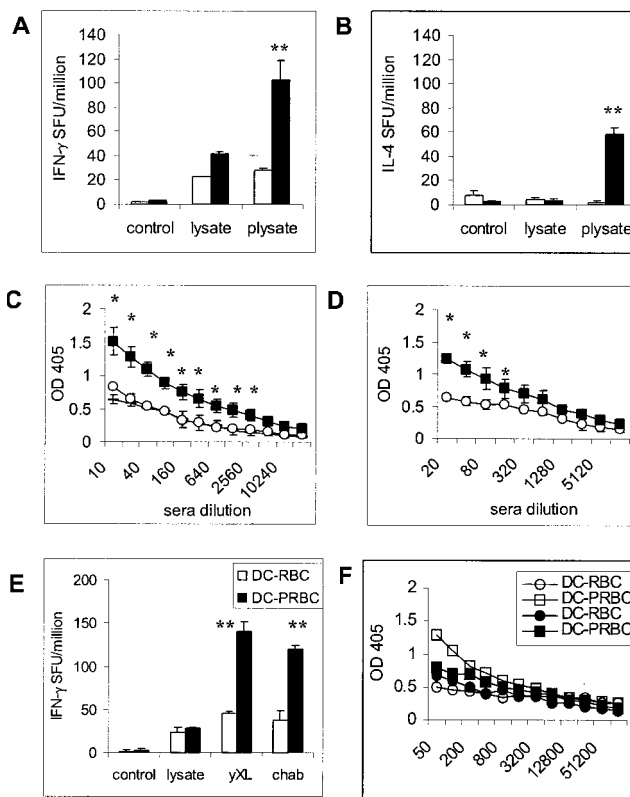
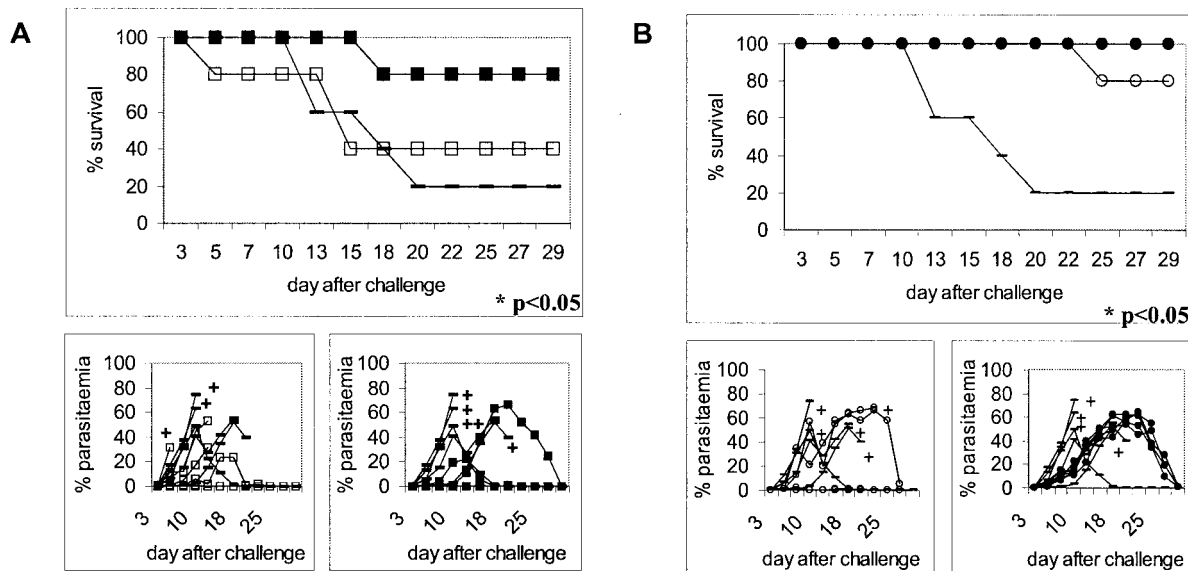


FIG. 3. Immune responses in mice immunized with DC. DC were pulsed with nonparasitized erythrocytes (DC-RBC; $n = 10$) (white bars) or *P. yoelii*-parasitized erythrocytes (DC-PRBC; $n = 10$) (black bars) and injected into C57BL/6 mice. Ten days later, IFN- γ (A) and IL-4 (B) responses from splenic T cells reactive to homologous malaria lysates, parasitized red cell lysate (plysate), nonparasitized red cell lysate (lysate), or to nothing (control) were measured by the ELISPOT assay. These responses were titratable (10 to 100 $\mu\text{g/ml}$) (not shown). Mean SFU \pm SD of triplicate samples of three mice are shown. A total of three experiments showed similar results. Total Ig levels to homologous parasitized malaria red cell lysate (plysate) in serum were assessed by the ELISA 30 (C) and 90 (D) days after immunization. Mean titrations \pm SD of triplicate samples of three animals are shown. The following mouse groups were tested: negative control mice, naive serum (-); DC pulsed with nonparasitized erythrocytes (DC-RBC) (\circ); and parasitized *P. yoelii* erythrocytes (DC-PRBC) (\blacksquare). (E) DC were pulsed with either intact nonparasitized erythrocytes (DC-RBC; $n = 3$) or *P. yoelii*-parasitized erythrocytes (DC-PRBC; $n = 3$) and injected into C57BL/6 mice. IFN- γ responses from splenic T cells were measured by the ELISPOT assay for homologous malaria red cell lysate, *P. yoelii* (yXL) and *P. chabaudi* (chab), nonparasitized red cell lysate (lysate), and nothing (control). Mean SFU \pm SD of triplicate samples are shown. One of two similar experiments is shown. (F) Serum from mice immunized with DC pulsed with nonparasitized and parasitized (*P. yoelii*) erythrocytes was taken 30 days after immunization and tested against homologous *P. yoelii*-parasitized red cell lysate (DC-RBC and DC-PRBC, open symbols) and heterologous *P. chabaudi*-parasitized red cell lysate (DC-RBC and DC-PRBC, filled symbols). Mean titrations \pm SD of triplicate samples for three mice are shown. Antibody titers (optical density [OD]) and T-cell responses (SFU) were statistically significant at P of < 0.05 (*) or highly statistically significant at P of < 0.0001 (**) by unpaired t test statistical analysis for ELISA and ELISPOT assays, respectively.

or their ratio was significantly correlated with antibody responses.

By contrast, at day 30 ($n = 6$), total Ig ($r^2 = 0.83$, $P < 0.05$), IFN- γ ($r^2 = 0.89$, $P < 0.05$), and IL-4 ($r^2 = 0.96$, $P < 0.0001$)



† indicates death

FIG. 4. Differences in DC maturation and quantity used to immunize mice affect the level of protection induced. (A) C57BL/6 mice were immunized with untreated 10^6 immature DC (untreated DC; $n = 8$) (—), 10^5 immature DC pulsed with parasitized *P. yoelii* erythrocytes ($n = 8$) (□), or 10^6 immature DC pulsed with parasitized *P. yoelii* erythrocytes ($n = 8$) (■) per mouse. (B) Similarly, C57BL/6 mice were immunized with untreated 10^6 matured DC (untreated DC; $n = 8$) (●), 10^5 matured DC pulsed with parasitized *P. yoelii* erythrocytes ($n = 8$) (○), or 10^6 matured DC pulsed with parasitized erythrocytes ($n = 8$) (LPS) per mouse. LS and TNF- α were added to immature DC overnight to mature them. Ten days later, mice were challenged with parasitized *P. yoelii* erythrocytes i.p. Cumulative parasitemia and survival data from two experiments are shown. Percent survival is statistically significant at P of <0.05 or very statistically significant at P of <0.0001 by Mann-Whitney statistical analysis.

responses all directly correlated significantly with protection. The association with IL-4 had the strongest predictive value. At this time point both IFN- γ ($r^2 = 0.87$, $P < 0.05$) and IL-4 ($r^2 = 0.9$, $P < 0.0001$) levels were strongly correlated with antibodies but not significantly with each other. The correlation between antibody levels and IL-4 was maintained at day 120 ($n = 7$) ($r^2 = 0.88$, $P < 0.0001$), at which time there was no significant correlation between IFN- γ and IL-4 or IFN- γ and antibodies. At this late time point, protection was strongly correlated with both IL-4 ($r^2 = 0.91$, $P < 0.0001$) and antibody ($r^2 = 0.83$, $P < 0.0001$) levels but not with IFN- γ . Therefore, IL-4 production was consistently correlated with protection, whereas antibodies and IFN- γ were only associated with protection at late or early time points after immunization, respectively.

These correlations indicate the immune protective mechanisms induced by ex vivo pulsed DC can change over time, but further experiments will be required to substantiate any specific hypotheses as to the contribution, and potential synergies, of protective immune effector functions over time.

Further analysis of the mechanism of protection against lethal malaria in the absence of antibodies. T-cell immunity generally, and IFN- γ -secreting cells specifically, are promoted by IL-12 (11, 26). This cytokine is a major factor mediating protective immunity during the early stages of malaria blood-stage infection in mice (32, 33). GM-CSF- and IL-4 (IL-12p40-deficient)-grown DC were pulsed with nonparasitized (DC-RBC) or parasitized *P. yoelii* erythrocytes (DC-PRBC) and injected into either C57BL/6 or knockout IL-12p40-deficient mice. No protection (0% survival) was observed in the IL-

12p40-deficient mice after lethal *P. yoelii* malaria challenge 10 days postimmunization (Fig. 5A; P , not significant), whereas C57BL/6 mice were 85% protected (Fig. 5B; $P < 0.05$). As expected, IFN- γ responses in these immunized mice were absent (data not shown).

Given a strong correlation between IFN- γ and IL-4 cellular responses, but not antibodies, with protection levels at day 10 and IL-12p40-dependent induction of immunity, it was likely that T cells were mediating early protection. To test this directly, T-cell transfer experiments were performed. C57BL/6 mice were immunized with either DC pulsed with nonparasitized erythrocytes (DC-RBC) or *P. yoelii*-parasitized erythrocytes (DC-PRBC) and injected intradermally. T cells were isolated from the spleens of these mice 10 days after immunization through a T-cell column. Cells (4×10^7) were injected intravenously into naïve C57BL/6 mice that were challenged 48 h later. There was significant protection in recipient mice of splenic T cells from mice immunized with DC pulsed with *P. yoelii*-parasitized erythrocytes, but not with nonparasitized erythrocytes, after challenge (Fig. 5C; $P < 0.05$). The passive transfer of protective immunity by T cells further supported the contention of a cellular mechanism of protection early after immunization with DC pulsed with parasitized erythrocytes.

DISCUSSION

DC pulsed with whole intact *Plasmodium*-parasitized erythrocytes were potent inducers of protection that could be sustained for long periods, suggesting effective processing and

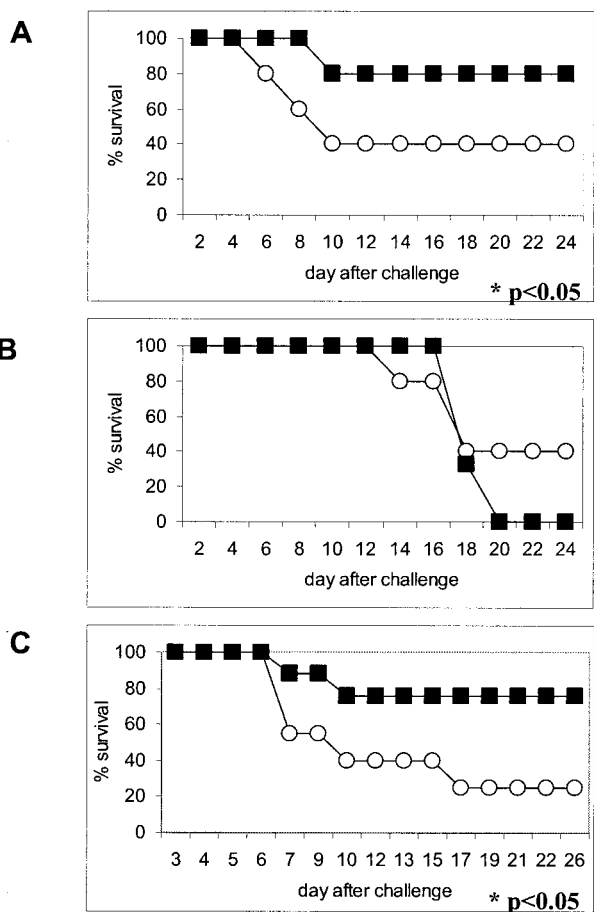


FIG. 5. Cell-mediated immunity plays an important role in induction of protective responses—assessment of protection in IL-12-deficient animals. DC from IL-12p40BL/6 mice were pulsed with nonparasitized erythrocytes (DC-RBC; $n = 8$) (○) or parasitized *P. yoelii* erythrocytes (DC-PRBC; $n = 8$) (■) and injected into C57BL/6 (A) or IL-12p40BL/6-deficient mice (B) intradermally. Ten days later, mice were challenged with 10^6 parasitized *P. yoelii* erythrocytes. (C) Adoptive transfer of T cells confers blood-stage protection. DC were pulsed with nonparasitized erythrocytes (DC-RBC; $n = 5$) (○) or parasitized *P. yoelii* erythrocytes (DC-PRBC; $n = 8$) (■) and injected intradermally into C57BL/6 mice. Ten days later, spleens were removed and whole CD3 T cells were purified on a T-cell column. T cells were injected intravenously into the tails of naïve C57/BL6 mice. Forty-eight hours later, mice were challenged with parasitized *P. yoelii* erythrocytes i.p. Percent survival is statistically significant at P of <0.05 or very statistically significant at P of <0.0001 by Mann-Whitney statistical analysis. In all experiments, percent parasitemia and percent survival were monitored daily.

presentation of immunogenic blood-stage antigens by DC. Direct rather than indirect processing and presentation (cross-priming via uptake of dead cell material) was suggested by the fact that the DC maturation stage had a direct effect on the level of protection induced as well as the number of injected DC.

Controversy has surrounded the role of DC in protective immunity to blood-stage malaria since the *P. falciparum* parasite has been shown to impair human DC maturation in vitro (38); however, this effect was not reproduced using *P. chabaudi* and murine DC (29). Notably, in the human DC studies noted

above, DC were grown with GM-CSF and IL-4, whereas the murine studies used DC grown with GM-CSF alone. Similar observations were seen in our own studies 8 days after mice were infected with *P. yoelii* and *P. chabaudi*, resulting in lower levels of costimulatory molecules on splenic DC in vivo (data not shown). Recent studies using GM-CSF-grown murine DC shows that they are susceptible to a similar maturation defect, which renders them incapable of effectively inducing liver-stage malaria immunity and protection (19). Our study shows conclusively that DC that have taken up parasitized erythrocytes are, in contrast, fully capable of inducing blood-stage malaria protective immunity against homologous challenge. These results using whole parasitized red cells as both regulator and antigen are further consistent with recent results by Perry and colleagues who demonstrated that DC from infected animals can still induce naïve T-cell proliferation to an unrelated antigen (21). DC which have been incubated with PRBC had decreased stimulatory ability in MLR assays (data not shown). The studies herein show that additional maturation of DC with LPS and TNF- α (despite the inability of these signals to induce normal increases in costimulatory surface molecule levels) enhances immunogenicity and protection in mice. One possibility to explain the efficacy of DC-PRBC immunization is that, although a maturation defect was evident in vitro, once returned to the in vivo environment, these DC can eventually develop into mature DC. Thus, there may be a delay in maturation rather than a maturation arrest.

Antibodies to *Plasmodium* antigens are accepted as the main effectors of malaria blood-stage protection (18); however, T cells have been shown to be necessary to induce and maintain blood-stage protection (2, 37). T helper cells can be divided into functionally distinct subsets, Th1 and Th2, depending on their lymphokine profiles, and the role of these lymphokines can have dramatically different effects during malaria infection. Th1-type lymphokines, such as IFN- γ and IL-12, are important in early malaria infection, and Th2-type lymphokines, IL-4 and IL-10, are important in parasite clearance during later infection (20). CD8⁺ T cells do not appear to be as important in blood-stage infection; however, other T-cell subsets, for example, gamma delta ($\gamma\delta$ T) cells, are significantly increased during malaria infection and have been implicated in the clearance of parasites (although not formerly shown) (41). In the present study, significant IFN- γ and IL-4 responses to malaria lysates were observed after immunization with DC pulsed with parasitized erythrocytes. Specific antibody responses were also induced and became detectable 30 days postimmunization. DC pulsed ex vivo with parasitized erythrocytes can thus induce both Th1- and Th2-type responses, which may help explain their unusually high protective efficacy against malaria infection. Correlation analysis suggested that DC vaccination is capable of inducing simultaneous Th1-like and Th2-like cellular immunity, potentially able to protect in the short term in the absence of antibodies and in the long term without IFN- γ -producing T cells. Thus, although DC vaccination induced rapid protection that is sustained at similar levels over time, the immune effector mechanisms involved can change over time. To note, the mechanism of long-term protection, although correlating directly with antibody titers, will need to be formally confirmed to be antibody mediated by antibody transfer experiments. It may also be interesting in this context to

explore differences in the immune effector mechanisms involved in short- versus long-term cross-strain protection, as cross-protection has rarely been observed using other, usually high-antibody-inducing immunization protocols. Our studies suggest short-term cross-protection may be mediated by cross-reactive T cells rather than antibodies. This mechanism needs to be further explored for long-term cross-protection studies.

In experiments where protection was observed 10 days postimmunization (in the absence of antibodies), it was likely to be cell mediated. This result would be consistent with previous studies suggesting IL-12 and IFN- γ can be protective during the acute stages of blood-stage malaria (9, 10). The action of IFN- γ on blood-stage parasites is thought to involve nitric oxide (NO); however, there is evidence suggesting that it may also promote protection by increasing the phagocytic activity of macrophages (42). Resident macrophages, notably in the spleen, have shown efficient parasite killing ability against particular species of murine malaria (34). The importance of IL-12 in blood-stage malaria protection is emphasized in this study. IL-12p40-deficient mice were not protected against a lethal challenge of *P. yoelii* parasites compared to wild-type C57BL/6. IL-12p40 is responsible for the early link between innate and adaptive immunity (reviewed in reference 1). IL-12p40-deficient mice demonstrate greater effects on Th1 immunity where IFN- γ and antigen-specific cellular immunity are greatly reduced in IL-12p40-deficient mice but not IL-12p35-deficient mice (8). It remains to be determined whether IL-12 acts directly or through the induction of IFN- γ , but this result clearly indicates the importance of the induction of cellular immunity for this protection to occur.

Understanding the effects of blood-stage malaria infection on the development of specific immune responses may provide important insights for vaccine development. In this study, we successfully induced protective immunity against malaria blood stages by immunization with DC pulsed ex vivo with *P. yoelii*- and *P. chabaudi*-parasitized erythrocytes. These results show that despite parasite modulation of DC function, DC and blood-stage malaria proteins can be successfully targeted for the development of malaria vaccines. Ex vivo pulsed DC vaccines have no practical use in areas where malaria is endemic, where isolation, culturing, and transport of DC for large numbers of humans are not feasible. However, specific targeting of DC in vivo may be investigated as an antimalaria strategy and may contribute to development of a vaccine that is not only immunostimulatory but induces long-lasting protection. It is hoped that as these and other vaccine approaches are developed in diverse medical areas, selected elements of their design will be utilized to provide an effective, long-lasting, stable, safe, and cheap vaccine for large-scale application in areas where malaria is endemic.

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