HLA Class II (DR0401) Molecules Induce Foxp3$^+$ Regulatory T Cell Suppression of B Cells in *Plasmodium yoelii* Strain 17XNL Malaria

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Unlike human malaria parasites that induce persistent infection, some rodent malaria parasites, like *Plasmodium yoelii* strain 17XNL (Py17XNL), induce a transient (self-curing) malaria infection. Cooperation between CD4 T cells and B cells to produce antibodies is thought to be critical for clearance of Py17XNL parasites from the blood, with major histocompatibility complex (MHC) class II molecules being required for activation of CD4 T cells. In order to better understand the correspondence between murine malaria models and human malaria, and in particular the role of MHC (HLA) class II molecules, we studied the ability of humanized mice expressing human HLA class II molecules to clear Py17XNL infection. We showed that humanized mice expressing HLA-DR4 (DR0401) molecules and lacking mouse MHC class II molecules (EA^0) have impaired production of specific antibodies to Py17XNL and cannot cure the infection. In contrast, mice expressing HLA-DR4 (DR0402), HLA-DQ6 (DQ0601), HLA-DQ8 (DQ0302), or HLA-DR3 (DR0301) molecules in an EA^0 background were able to elicit specific antibodies and self-cure the infection. In a series of experiments, we determined that the inability of humanized DR0401.EA^0 mice to elicit specific antibodies was due to expansion and activation of regulatory CD4$^+$ Foxp3$^+$ T cells (Tregs) that suppressed B cells to secrete antibodies through cell-cell interactions. Treg depletion allowed the DR0401.EA^0 mice to elicit specific antibodies and self-cure the infection. Our results demonstrated a differential role of MHC (HLA) class II molecules in supporting antibody responses to Py17XNL malaria and revealed a new mechanism by which malaria parasites stimulate B cell-suppressogenic Tregs that prevent clearance of infection.

Malaria is an *Anopheles* mosquito-borne infectious disease caused in humans by five different members of the protozoan genus *Plasmodium* (i.e., *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*). *P. falciparum* is the most virulent and deadly malaria parasite and infects 1 billion to 2 billion people annually. The number of *P. falciparum* deaths reported by the WHO for 2010 was 665,000, while the Institute of Health Metrics and Evaluation reported 1.24 million deaths for the same year (1).

Infection is initiated upon a bite of an infected *Anopheles* mosquito and inoculation of sporozoites into skin, which rapidly invade the bloodstream to infect hepatocytes and develop into liver stage parasites. The liver stage of infection is asymptomatic and lasts 5 to 7 days for human malaria species and 2 to 3 days for rodent malaria species (2). Mature liver stage parasites are then released into the bloodstream to invade red blood cells and to initiate the asexual erythrocyte cycles responsible for the pathological manifestations of malaria. During the course of blood infection, parasites may differentiate into female and male gametocytes that are taken up by the mosquito, where they undergo sexual reproduction and meiosis in the gut and generate sporozoites that migrate to the salivary glands to perpetuate the life cycle (3). Morbidity and mortality associated with *P. falciparum* occur mostly during first-time infection of infants and pregnant women living in areas where malaria is endemic, as these groups are at the highest risk for development of severe malaria syndromes such as lactic acidosis and anemia as a consequence of hyperparasitemia or cerebral and placental malaria due to sequestration of parasites in organs (1). Travelers are also very vulnerable to severe malaria. Naturally acquired immunity to malaria develops slowly upon repetitive episodes of infection and represents a state of semi-immunity where parasitemia is under control and prevents severe malaria (4).

The major histocompatibility complex (MHC) (HLA in humans) proteins are highly polymorphic glycoproteins consisting of two noncovalently associated α and β chains. A peptide binding groove is generated by the assembly of α1-β1 domains of the MHC class II (MHC-II) heterodimer, which allows binding of peptides of 13 to 17 amino acids in length derived from the exogenous pathway of antigen processing (5). MHC molecules are major players in generating immune responses to microorganisms, since their primary role is to present peptides for activation and differentiation of CD4 T cells. Among the various CD4 T cell subsets, CD4 T helper cells (Th1, Th2, and Th17) are required to orchestrate cellular and/or humoral responses needed to clear infections (6), while regulatory CD4$^+$ Foxp3$^+$ T cells (Tregs) downregulate cellular and/or humoral responses through direct cell-cell interactions or by secretion of suppressive cytokines such as inter-

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leukin-10 (IL-10) and transforming growth factor β (TGF-β) (7, 8). Recent studies indicated that some pathogens stimulate Tregs as an immune evasion mechanism (9). When it comes to malaria, studies in rodent models (10–20) and humans (21–23) have demonstrated an increase in Treg frequency shortly upon blood stage infection, although Treg frequency during the course of infection did not correlate with disease severity. Furthermore, attempts to deplete Tregs or to increase Treg frequency in mice prior to infection with a variety of parasite strains (*Plasmodium yoelii*, *P. berghei*, or *P. chabaudi*) have led to conflicting results. Depending on the parasite/mouse strain combination, Tregs have been shown to have either beneficial (10, 17, 18) or deleterious (14–16, 19) effects or no significant effect (12, 16). This has been attributed to the variety of severe malaria syndromes associated with each rodent parasite strain. While Tregs may be deleterious by inhibiting immune responses to malaria parasites, leading to hyperparasitemia, anemia, and metabolic derangement, Tregs may also be beneficial by suppressing strong inflammatory responses that lead to immunopathology (i.e., cerebral malaria). Importantly, Tregs in these models have not been characterized for their ability to downregulate cellular versus antibody (Ab) responses. This is important in considering the different role of cellular versus humoral responses in clearance of various rodent malaria strains. For instance, while clearance of *P. yoelii* parasites is thought to be mediated by antibodies (24), clearance of *P. chabaudi* is less dependent on antibodies but more dependent on CD4 T cells (25).

The differential ability of mouse MHC-II molecules versus human HLA class II (HLA-II) molecules to present epitopes to CD4 T cells is a major drawback of preclinical rodent models, which may explain in part why the immunogenicity and/or protective efficacy of many malaria vaccine candidates tested in rodent models did not translate into efficacy in clinical trials. The advent of humanized mice expressing HLA class II molecules and lacking mouse MHC class II molecules (EA^0) has significantly advanced the understanding of HLA-II molecules in infectious diseases and autoimmunity. We and others demonstrated that HLA class II molecules expressed in mice function similarly to those in humans, as they present immunodominant epitopes of foreign antigens and self-antigens to the same extent as they do in humans (26, 27). Humanized HLA-II mice also express a diverse set of T cell receptor beta (TCR Vβ) chains similar to that observed in humans and develop clinical and histological similarities of human autoimmune syndromes linked to HLA-II (28). Here we have investigated the role of HLA-II molecules in *P. yoelii* strain 17XNL (Py17XNL) malaria. Our results indicate that mice expressing HLA-DR0401 molecules, unlike C57BL/6, BALB/c, and humanized mice expressing other HLA-II molecules, succumb to Py17XNL malaria infection due to expansion and activation of CD4$^+$ Foxp3$^+$ T cells that suppress antibody responses by direct cell-cell contact with B cells.

MATERIALS AND METHODS

Mice. All animal procedures were conducted under IACUC protocols approved by the WRAIR/NMRC and the USUHS in compliance with the Animal Welfare Act and in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (29). DR0401.EA^0 (C57BL/6) mice, expressing HLA-DR0401α1β1/IEα2β2 molecules and lacking expression of mouse MHC-II molecules (Abb knockout [KO] mutation, EA^0), were previously described (30), and they were purchased from Taconic (Hudson, NY). The chimeric DR0401-IE molecules expressed by DR0401.EA^0 mice contain the HLA-DR0401α1β1 peptide

binding site, with the remaining domains derived from murine IEα2β2 to allow binding to the mouse CD4 coreceptor. DR0402.EA^0 (C57BL/10) mice express full-length human molecules with mutations at positions β110 and β139 to allow mouse CD4 binding (31). DR3.EA^0, DQ8.EA^0, and DQ6.EA^0 (C57BL/10) mice express full-length human molecules that bind to mouse CD4 (32–34). DR0402.EA^0, DR3.EA^0, DQ8.EA^0, and DQ6.EA^0 mice were generated at Mayo Clinic, Rochester, MN. The HLA class II molecules expressed in EA^0 mice are functional, as they mediate thymic negative (Vβ5, Vβ11, and Vβ12) and positive (Vβ6 and Vβ14) selection, similarly to mouse I-E molecules, and rescue development of CD4 T cells (reviewed in reference 35). BALB/c and C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). EA^0 (C57BL/6) mice were generated by breeding DR0401.EA^0 mice with C57BL/6 mice, intercrossing the F1 mice, and screening F2 mice for the absence of HLA-DR0401αβ transgenes and the presence of the Aβ KO mutation by PCR. Primers used for screening of the HLA-DR0401αβ transgenes were previously described (36). Primers used to identify the Aβ KO mutation were forward primer CCGCAGGGCATTTCGTGTA and reverse primer GCCGCCGCAGGGAGGTGTG. To generate F1 hybrid mice coexpressing HLA-DR0401 molecules and mouse MHC-II molecules, the DR0401.EA^0 mice were crossed with BALB/c or with C57BL/6 mice. F1 control mice (BALB/c.C57BL/6) lacking expression of HLA-DRB1*0401 molecules were generated by crossing EA^0 (C57BL/6) mice with BALB/c mice.

Parasites. *Plasmodium yoelii* (17XNL strain) sporozoites were obtained by dissecting salivary glands of infected *Anopheles stephensi* mosquitoes as previously described (37). Py17XNL-infected red blood cells (Py-iRBCs) were obtained from BALB/c.RagKO mice challenged with Py17XNL sporozoites when parasitemia reached >30%. Mice were challenged intravenously with 200 sporozoites or with 5 × 10^4 Py-iRBCs. Parasitemia was measured using Giemsa-stained thin blood smears by counting an average of 3,000 RBCs.

Measurement of antibody titers by immunofluorescence assay (IFA). Teflon printed slides (12-well; Electron Microscopy Sciences, Hatfield, PA) were coated with 10^4 RBCs/well from infected BALB/c.RagKO mice when parasitemia reached >30%. Slides were air dried and stored at −80°C until use. Upon thawing, slides were blocked with phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA) for 30 min at 37°C. Twenty microliters of plasma at various dilutions was added to the wells and incubated for 1 h at 37°C. Slides were washed three times with PBS, incubated with fluorescein isothiocyanate (FITC)-labeled F(ab′)$_2$ goat anti-mouse IgG1, IgG2a, IgG2b, IgG2c, or IgG3 (Southern Biotechnologies, Birmingham, AL) for 30 min at 37°C, washed, and mounted with Vectashield-DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA).

Hematocrit count. Blood collected with heparin-coated capillaries was diluted 1:5,000 in PBS, and erythrocytes were counted with hemocytometers.

Chloroquine treatment. Mice were injected intraperitoneally with 25 mg/kg of body weight chloroquine diphosphate (Sigma, St. Louis, MO) in PBS, as described in figure legends.

Immunization with Py17XNL irradiation-attenuated sporozoites. Py17XNL-infected *Anopheles stephensi* mosquitoes were irradiated (10,000 rads) by using a cobalt irradiator (gamma irradiator; J. L. Shepherd), and sporozoites were purified from the salivary glands. Mice were injected intravenously with three doses of 30,000 irradiation-attenuated sporozoites (Irrd-spz) at 2-week intervals and challenged 2 weeks after the last immunization with 200 infectious Py17XNL sporozoites.

Fluorescence-activated cell sorter (FACS) analysis. Isolation and staining of splenocytes were carried out as described previously (38), using anti-human HLA-DR (clone TU39) and anti-mouse IA/IE (clone 2G9), CD3 (clone 145-2C11), CD4 (clone RM4-5), CD25 (clone PC61) (BD Biosciences, San Jose, CA), and Foxp3 (eBiosciences, San Diego, CA). Cells were gated and analyzed on mononuclear forward scatter/side scatter (FSC/SSC).
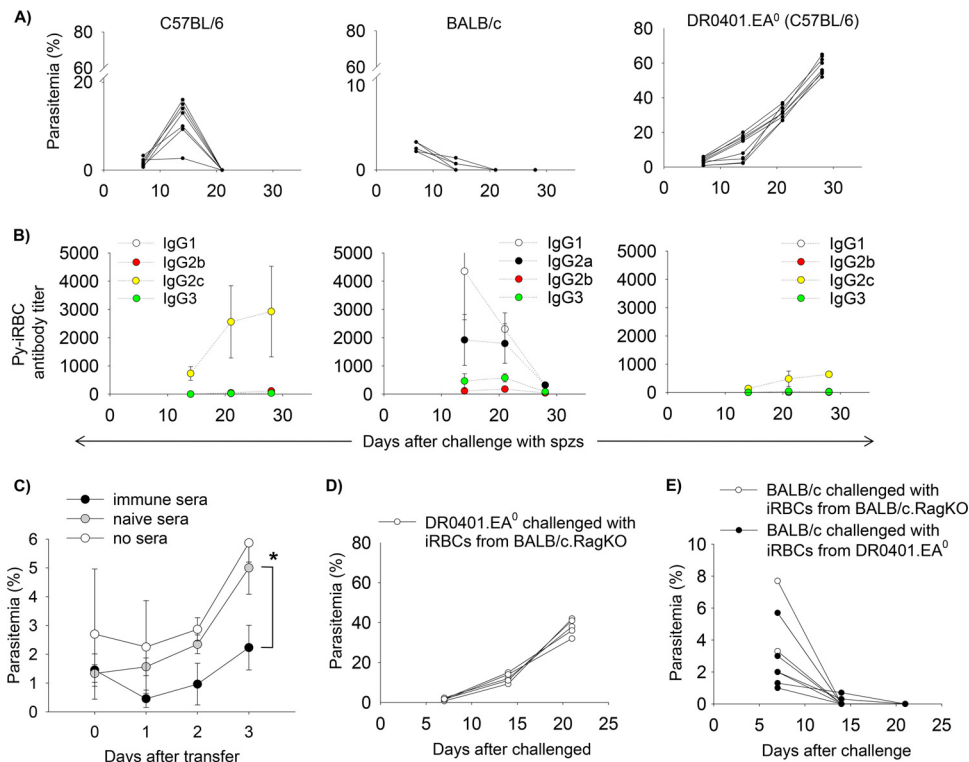
FIG 1 Mice expressing HLA-DR0401 as the only MHC class II molecule fail to self-cure Py17XNL malaria infection. (A and B) Groups of C57BL/6 ($n = 7$), BALB/c ($n = 5$), and DR0401.EA^0 ($n = 10$) mice were challenged intravenously with 200 Py17XNL sporozoites. (A) Parasitemia (iRBCs among 3,000 total RBCs) in individual mice, counted in Giemsa-stained thin blood smears. (B) Kinetics of IgG antibodies to Py17XNL-infected red blood cells (Py-iRBCs), as measured by IFA. Data represent means ± standard deviations for mice analyzed individually. (C) DR0401.EA^0 mice were challenged with 200 Py17XNL sporozoites, and 6 days later, they were injected intraperitoneally with immune sera (500 μl/mouse) from self-cured C57BL/6 mice or with an equal volume of sera from naive C57BL/6 mice or were left untreated. Mice were monitored for parasitemia by using thin blood smears. Data represent means ± standard deviations for three mice analyzed individually (*, $P < 0.05$ [determined by Student's t test]). (D) DR0401.EA^0 mice ($n = 5$) that were challenged with Py-iRBCs (5×10^4 per mouse) developed very high parasitemia and succumbed to infection. Data show parasitemia values for individual mice. (E) Kinetics of parasite clearance in BALB/c mice that were injected with Py-iRBCs (5×10^4) from infected DR0401.EA^0 mice ($n = 5$ recipients) or with the same number of Py-iRBCs from infected BALB/c.RagKO mice ($n = 3$ recipients). Both groups of recipient BALB/c mice cleared parasitemia similarly ($P = 0.4$ [determined by Student's t test]).

***In vivo* depletion of Tregs.** Rat anti-mouse CD25 antibodies were purified from supernatants of PC61 hybridoma cells (ATCC, Manassas, VA), using MAR18 (mouse anti-rat) affinity chromatography columns. Mice were injected intraperitoneally with 250 μg of rat anti-mouse CD25 or the rat IgG isotype control daily for 3 days.

T cell responses. Splenocytes were cultured *ex vivo* for 4 days or stimulated with CD3/CD28 Abs (1 μg/ml each; BD Biosciences) or with concanavalin A (ConA) (2.5 μg/ml; Sigma-Aldrich, St. Louis, MO) for 48 h. Cytokine secretion in supernatants was measured by using Luminex kits (Invitrogen, Carlsbad, CA).

B cell responses. Negatively isolated (untouched) splenic B cells (Dynabeads mouse CD43; Invitrogen) from two mice (pooled) were stimulated with anti-mouse CD40 Abs (3 μg/ml; BD Biosciences), IL-4 (100 U/ml), and IL-2 (15 U/ml) (Invitrogen) or were left unstimulated. CD4$^+$ CD25$^+$ Tregs from 10 to 15 mice (pooled) were positively isolated with Dynabeads Flowcomp mouse CD4$^+$ CD25$^+$ Tregs (Invitrogen). Graded numbers of Tregs were added to B cell cultures in the presence of CD3/CD28 Abs (1 μg/ml each). To prevent cell-cell contact, B cells and Tregs were cultured in 0.4-μm transwells. At day 7 of culture, half of the medium was replaced by fresh medium. Immunoglobulin secretion in supernatants at day 16 of culture was measured by an enzyme-linked immunosorbent assay (ELISA) (mouse IgG ELISA quantitation set; Bethyl Laboratories, Montgomery, TX).

Passive transfer of sera. Pooled sera from C57BL/6 mice that had self-cured (day 21 postchallenge with Py17XNL sporozoites) or from na-

ive C57BL/6 mice were injected intraperitoneally into infected DR0401.EA^0 mice (0.5 ml serum/recipient).

Statistical analysis. Data were analyzed by using an unpaired (2-tailed) Student t test. For multiple comparisons, we used 2-way analysis of variance (ANOVA) followed by Tukey's *post hoc* comparisons of multiple conditions. For protection experiments, we used the Fisher exact test.

RESULTS

Mice expressing HLA-DR0401 molecules in the EA^0 background (DR0401.EA^0) have impaired antibody responses to Py17XNL blood stage parasites and cannot self-cure infection. Consistent with previous reports (39, 40), we found that C57BL/6 and BALB/c mice challenged with 200 infectious Py17XNL sporozoites develop mild blood stage parasitemia, and they self-cure the infection within 14 to 21 days postchallenge (Fig. 1A, left and middle). In contrast, humanized DR0401.EA^0 (C57BL/6) mice developed hyperparasitemia and succumbed to infection within 25 to 30 days (Fig. 1A, right). The cause of death was most likely severe anemia, since the infected DR0401.EA^0 mice had a 47% hematocrit reduction at day 21 postchallenge compared to hematocrit at prechallenge (4.5 million ± 1.6 million versus 8.5 million ± 1.4 million red blood cells [RBCs]/μl, respectively; $P < 0.0001$).
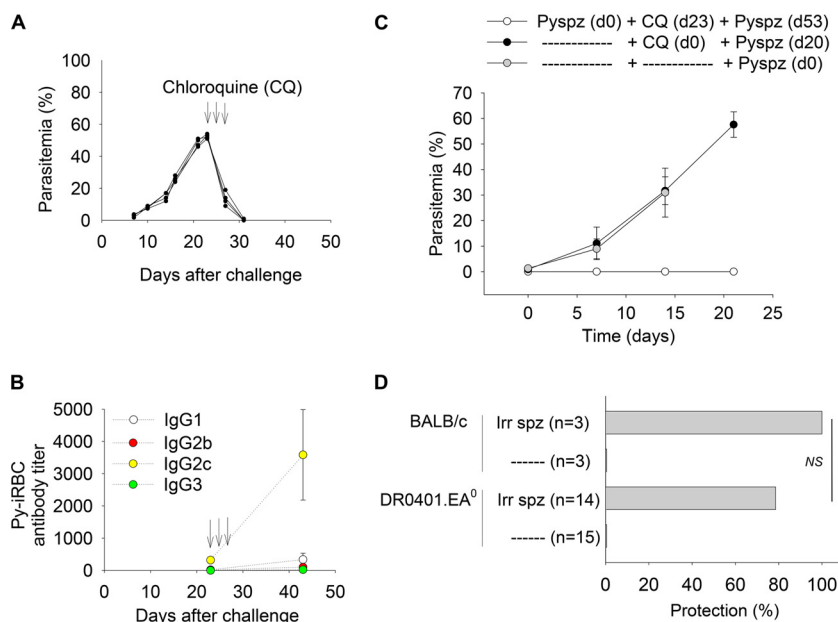
C57BL/6 mice have a deletion of the IgG2a gene but a func-

FIG 2 Chloroquine treatment allows Py17XNL-infected DR0401.EA^0 mice to elicit antibodies. (A to C) DR0401.EA^0 mice ($n = 5$) were challenged with 200 Py17XNL sporozoites and treated with chloroquine (CQ) (three doses of 25 mg/kg body weight) at days 23, 24, and 25 postchallenge (arrows). (A and B) Kinetics of parasitemia in individual mice (A) and mean titers $\pm$ standard deviations of Py-iRBC antibodies before and after treatment with chloroquine (B). (C) Mice infected and treated with chloroquine ($n = 4$) were protected against a second challenge with 200 Py17XNL sporozoites (Pyspz), whereas noninfected mice treated with chloroquine ($n = 4$) or infectivity controls ($n = 4$) were unprotected. (D) BALB/c ($n = 3$) and DR0401.EA^0 ($n = 14$) mice were immunized three times at 2-week intervals with Py17XNL Irrd-spz (30,000/dose) and challenged 2 weeks later with 200 infectious Py17XNL sporozoites. Shown is the percentage of protected (nonparasitemic) mice ($P = 1.0$ [determined by a Fisher exact test]).

tional IgG2c gene, whereas BALB/c mice have a deletion in the IgG2c gene and a functional IgG2a gene (41, 42). As measured by IFA, C57BL/6 and BALB/c mice developed high titers of antibodies to the infected RBCs (Py-iRBCs) (Fig. 1B, left and middle). The isotype profile of Py-iRBC antibodies differed between C57BL/6 and BALB/c mice, since C57BL/6 mice elicited mainly IgG2c antibodies, while BALB/c mice elicited IgG1 and IgG2a antibodies. Considering that IgG2a and IgG2c isotypes represent the signature of Th1-mediated antibody responses, whereas IgG1 represents the signature of Th2-mediated responses, these results indicated that C57BL/6 mice mounted a predominant Th1 response to the malaria parasites, whereas BALB/c mice mounted a mixed Th1/Th2 response. In contrast, infected DR0401.EA^0 mice elicited very low titers of specific antibodies (Fig. 1B, right), which may explain the lethality of Py17XNL infection in these mice. To further determine the protective role of antibodies in Py17XNL malaria, we carried out a passive transfer experiment. As illustrated in Fig. 1C, transfer of immune sera (0.5 ml) from self-cured C57BL/6 mice into infected DR0401.EA^0 mice significantly reduced the level of parasitemia compared to that of DR0401.EA^0 mice receiving sera from malaria-naive C57BL/6 mice, which indicated that antibodies are important for clearance of Py17XNL parasites. The DR0401.EA^0 mice also succumbed to infection when challenged with Py-iRBCs instead of sporozoites (Fig. 1D), which ruled out a possible tolerogenic effect on the blood stage parasites induced during liver stage infection.

Since the virulence of malaria parasites is controlled by genetic and epigenetic factors that can be modulated within the host (43), we also investigated whether the Py17XNL parasites in DR0401.EA^0 mice could have undergone increased virulence

from a nonlethal to a lethal stage. For this, recipient BALB/c mice were injected with 5×10^4 Py-iRBCs isolated from infected DR0401.EA^0 mice, and the recipients were monitored for parasitemia. As a control, BALB/c mice were injected with the same number of Py-iRBCs isolated from infected BALB/c.RagKO mice. As illustrated in Fig. 1E, both groups of BALB/c recipients cleared parasitemia within the same period of time. Finally, we also investigated whether Py17XNL parasites, which infect immature red blood cells (reticulocytes) (44), could have evolved to infect mature red blood cells (erythrocytes) in DR0401.EA^0 mice. As illustrated in Fig. S1 in the supplemental material, the Py17XNL parasites in DR0401.EA^0 mice infected reticulocytes. In aggregate, our results indicated that lethality of Py17XNL infection in DR0401.EA^0 mice is not due to an increased virulence of Py17XNL parasites or a change in tropism from reticulocytes to erythrocytes but rather to the inability of DR0401.EA^0 mice to elicit specific antibodies needed to clear the infection.

Chloroquine treatment allows Py17XNL-infected DR0401.EA^0 mice to elicit antibodies. Chloroquine is a potent schizonticide commonly used to treat malaria infection (reviewed in reference 45). Chloroquine treatment also facilitates immune responses to blood stage parasites, since low-dose injection of iRBCs under chloroquine cover induces protective immunity in mice (46). We thus investigated whether chloroquine treatment of infected DR0401.EA^0 mice enables antibody responses to blood stage parasites. For this, DR0401.EA^0 mice were challenged with Py17XNL sporozoites and treated with three doses of chloroquine (25 mg/kg of body weight) when parasitemia reached >40%. As illustrated in Fig. 2A, treatment with chloroquine led to a drastic decrease in parasitemia, and mice were cured by day 7 posttreatment. Inter-
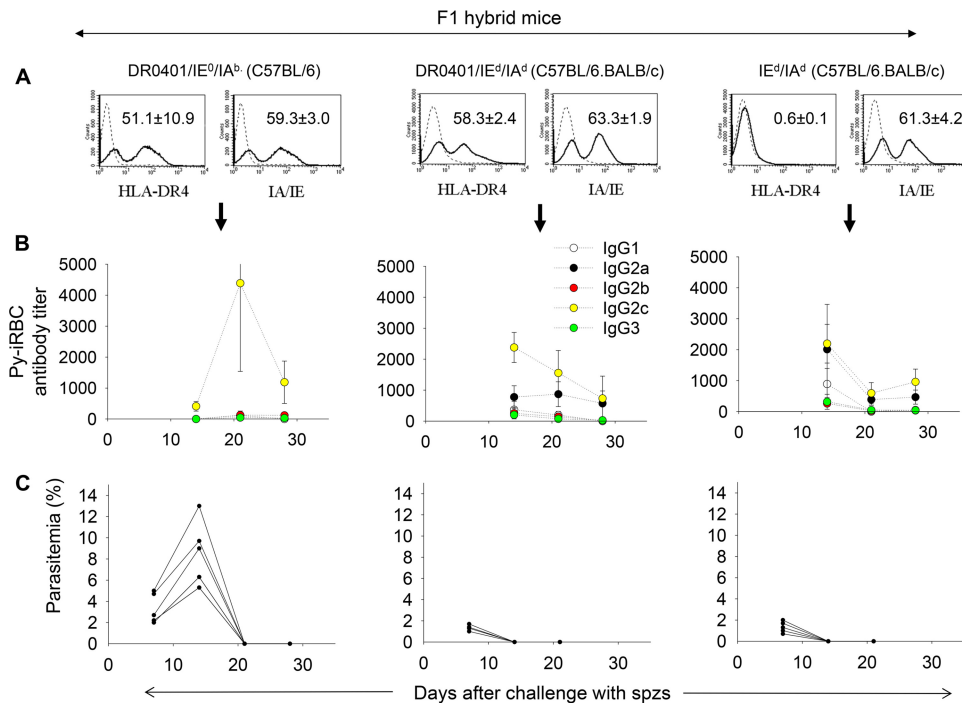
FIG 3 Mice coexpressing HLA-DR0401 and mouse MHC-II molecules clear Py17XNL malaria infection. (A) Expression of HLA-DR0401 and mouse MHC-II (IA/IE) molecules in spleens of F1 hybrid mice, as measured by FACS analysis. Data represent means ± standard deviations for three mice analyzed individually. (B and C) Groups of F1 hybrid mice ($n = 7$) were challenged with 200 infectious Py17XNL sporozoites and monitored for development of specific antibodies (B) and parasitemia (C). Data show that all F1 hybrid mice elicited antibodies and were able to clear infection by day 21 postchallenge.

estingly, by day 20 after treatment with chloroquine, all mice developed high titers of Py-iRBCs antibodies (Fig. 2B) that were comparable to those elicited by C57BL/6 and BALB/c mice upon first-time infection (Fig. 1C).

Since chloroquine treatment of infected DR0401.EA⁰ mice resulted in high titers of specific antibodies, we next investigated whether these mice could be protected against reinfection upon a second challenge with Py17XNL sporozoites. As illustrated in Fig. 2C, none of these mice developed parasitemia. In contrast, control (noninfected) DR0401.EA⁰ mice treated with chloroquine and challenged with Py17XNL sporozoites became parasitemic, which ruled out that residual levels of chloroquine in blood prevented parasite development. These results demonstrated that chloroquine treatment during infection allowed DR0401.EA⁰ mice to elicit antibodies to Py17XNL blood stage parasites and protected mice against reinfection.

Immunization with irradiation-attenuated sporozoites (Irrd-spz) protects animals and humans against liver stage malaria (reviewed in reference 47). We next investigated whether DR0401.EA⁰ mice can mount protective immune responses upon vaccination with Py17XNL Irrd-spz. For this, groups of DR0401.EA⁰ and BALB/c mice were injected three times with 30,000 Py17XNL Irrd-spz at 2-week intervals and challenged 2 weeks later with 200 infectious Py17XNL sporozoites. As shown in Fig. 2D, immunized-DR0401.EA⁰ mice were protected to the same extent as immunized-BALB/c mice. Together, these results demonstrated that DR0401.EA⁰ mice are immunocompetent and can elicit protective immune responses to liver or blood stage parasites upon vaccination, although they are unable to control acute (first-time) Py17XNL malaria infection.

Mice coexpressing HLA-DR0401 and mouse MHC-II molecules self-cure Py17XNL malaria infection. We next investigated in mice coexpressing HLA-DR0401 and mouse MHC class II molecules whether the HLA-DR0401 molecule plays a dominant negative role in Py17XNL malaria. DR0401.EA⁰ mice were crossed with C57BL/6 mice (IE⁰/IAᵇ) or BALB/c mice (IEᵈ/IAᵈ) to generate F1 hybrids expressing DR0401/IE⁰/IAᵇ (C57BL/6) or DR0401/IEᵈ/IAᵈ (C57BL/6.BALB/c), respectively. Expression of HLA-DR0401 and mouse MHC-II molecules in F1 hybrids is shown in Fig. 3A (left and middle). As a control, we generated F1 hybrids (C57BL/6.BALB/c) expressing IEᵈ/IAᵈ and lacking expression of HLA-DR0401 (Fig. 3A, right) by crossing EA⁰ (C57BL/6) mice with BALB/c mice. As illustrated in Fig. 3B and C, all F1 hybrid mice challenged with Py17XNL sporozoites elicited high titers of specific antibodies to Py-iRBCs and self-cured the infection by day 21 postchallenge. Of note, F1 hybrid mice lacking expression of HLA-DR0401 molecules (Fig. 3B and C, right) elicited antibodies and cleared the infection as efficiently as mice expressing HLA-DR0401 molecules in the same genetic background (Fig. 3B and C, middle). This indicated that HLA-DR0401 molecules do not play a dominant negative role in malaria when coexpressed with mouse MHC-II molecules.

Py17XNL fosters CD4⁺ Foxp3⁺ T regulatory cell expansion and prevents clearance of infection in DR04010.EA⁰ mice. CD4⁺ Foxp3⁺ T regulatory cells (Tregs) maintain a state of self-tolerance by suppressing immune responses to self-antigens (7). Some pathogens evade the immune system by stimulation of preexistent Tregs (9). To determine whether Tregs could prevent DR0401.EA⁰ mice from self-curing Py17XNL malaria infection, we carried out a cross-sectional study to compare the splenic frequency of Tregs in naive (uninfected) mice and in mice infected
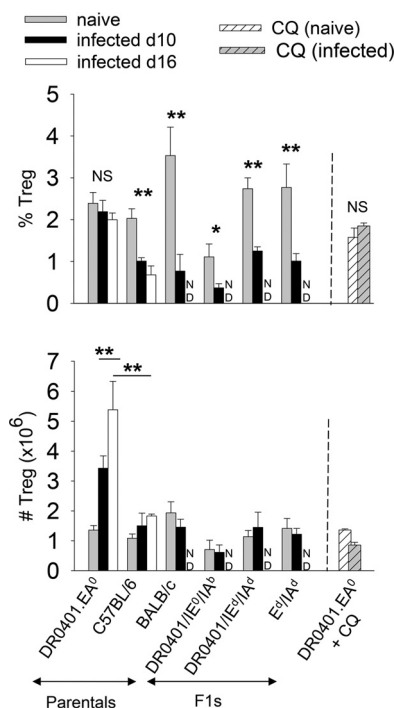
FIG 4 Py17XNL fosters expansion of Foxp3+ regulatory T cells (Tregs) in DR0401.EA0 mice. Groups of naive (noninfected) mice, infected mice at day 10 or 16 after challenge with 200 infectious Py17XNL sporozoites, infected mice treated with chloroquine (CQ) (as in Fig. 2A) at day 20 after chloroquine treatment, or naive mice treated with chloroquine at day 12 posttreatment were examined for the frequency of splenic Tregs (CD3+ CD4+ Foxp3+) by FACS analysis. The top panel shows Treg frequency, and the bottom panel shows total Treg numbers per spleen. Data represent means ± standard deviations for three mice analyzed individually (∗ P < 0.05; ∗∗, P < 0.005; NS, not significant [determined by Student's t test]). ND, not done.

with Py17XNL. As shown in Fig. 4 (top), at day 10 after challenge with Py17XNL sporozoites, the frequency of Tregs in infected C57BL/6, BALB/c, and F1 hybrid mice (all able to elicit antibodies and self-cure infection) significantly decreased compared to that in naive (noninfected) mice. C57BL/6 mice examined at day 16 postchallenge also showed a reduction in Treg frequency similar to that of mice examined at day 10. Despite the decrease in Treg frequency, the total numbers of Tregs per spleen in infected C57BL/6, BALB/c, and F1 hybrid mice (able to self-cure infection) were similar to those in naive mice (Fig. 4, bottom), which relates to the fact that Py17XNL infection induced splenomegaly and hypercellularity (see Table S1 in the supplemental material). In contrast, the frequency of Tregs in infected DR0401.EA0 mice at day 10 or 16 postchallenge was not reduced, and the total numbers of Tregs per spleen were significantly increased compared to those in naive DR0401.EA0 mice or infected C57BL/6, BALB/c, and F1 hybrid mice (Fig. 4, bottom). Thus, these results indicated that Py17XNL infection fostered Treg expansion in DR0401.EA0 mice but not in mice able to self-cure the infection.

To determine whether Tregs could prevent DR0401.EA0 mice from eliciting antibodies and self-curing the infection, DR0401.EA0 mice were depleted of Tregs by treatment with rat anti-mouse CD25 antibodies (PC61). CD4+ Foxp3+ Tregs express high levels of CD25 (IL-2Rα), as they are dependent on IL-2 for activation (7). Daily injection of CD25 Abs (250 μg) for three consecutive days, but not
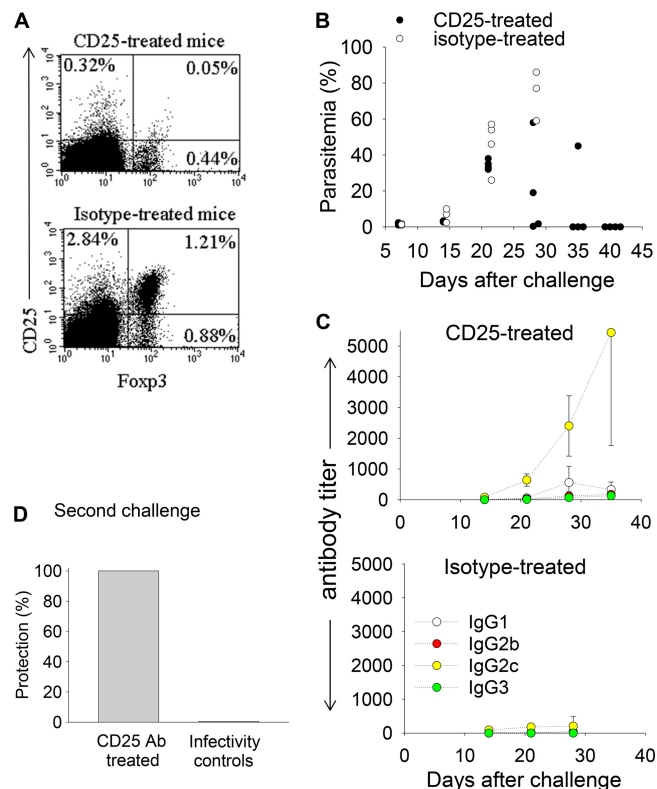

FIG 5 Py17XNL-stimulated Foxp3+ regulatory T cells (Tregs) prevent clearance of infection in DR0401.EA0 mice. (A) Groups of DR0401.EA0 mice (n = 3) were injected intraperitoneally three times at 1-day intervals with 250 μg of rat anti-mouse CD25 or the rat isotype control and analyzed 7 days after the last injection for the frequency of CD25+ Foxp3+ Tregs in spleens. Data represent mean values for pooled spleens. (B and C) Parasitemia and kinetics of specific antibodies in groups of DR0401.EA0 mice (n = 4) that were injected with CD25 antibodies or the rat isotype control as in panel B and then challenged with 200 Py17XNL sporozoites 3 days after the last injection. The CD25-treated mice but not the rat isotype-treated mice elicited antibodies and self-cured the infection. Parasitemia values are from individual mice, and antibody titers represent means ± standard deviations for mice analyzed individually. (D) Two months after the first challenge, CD25-treated mice (n = 4) from panels C and D were completely protected against a second challenge with 200 infectious Py17XNL sporozoites. In contrast, all the infectivity control mice (n = 4) became parasitemic.

injection of isotype control Abs, resulted in a significant (4-fold) depletion of splenic Foxp3+ T cells at day 7 postinjection (Fig. 5A). Additional groups of DR0401.EA0 mice (n = 4) were injected with CD25 Abs or control rat isotype Abs as described above, and 3 days later, mice were challenged with Py17XNL sporozoites. As illustrated in Fig. 5B and C, the anti-CD25-treated mice self-cured the infection and elicited antibodies, while the isotype control-treated mice were unable to elicit antibodies and succumbed to the infection. Furthermore, 2 months after the first challenge, the anti-CD25-treated mice were completely protected upon a second challenge with Py17XNL sporozoites, while all infectivity control mice became parasitemic (Fig. 5D). These results demonstrated that Tregs from infected DR0401.EA0 mice suppressed antibody responses to Py17XNL malaria parasites and prevented clearance of first-time (acute) infection.

Py17XNL does not attenuate CD4 T-cell function but stimulates Tregs to directly suppress B cell function. Tregs can downregulate antibody responses indirectly by suppressing CD4 T
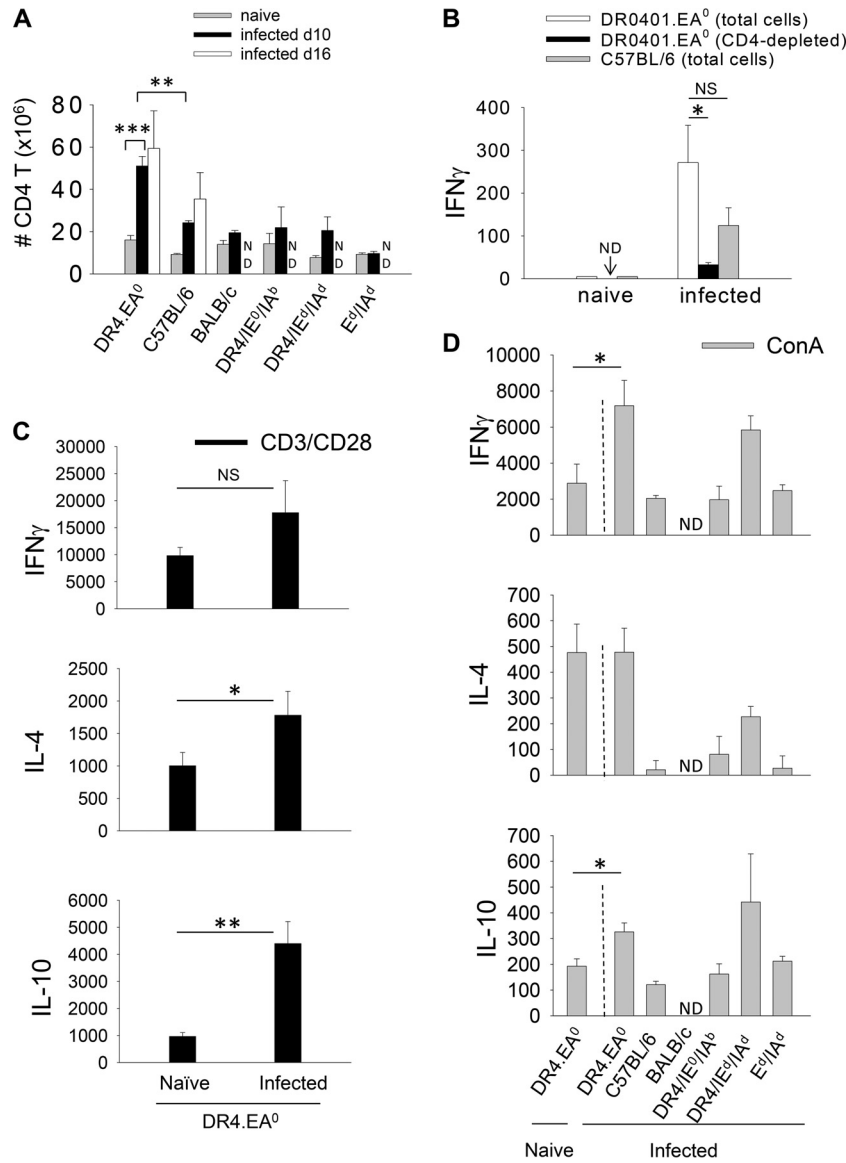
FIG 6 CD4 T cells from infected DR0401.EA[0] mice are not suppressed during Py17XNL malaria infection. (A) Total numbers of CD4 T cells in spleens of naive and infected mice. (B) *Ex vivo* IFN-γ response (pg/ml) of splenic T cells from infected DR0401.EA[0] and C57BL/6 mice at day 10 after challenge with Py17XNL sporozoites or from naive (noninfected) mice. No IL-4 or IL-10 secretion was detected in cell culture supernatants (data not shown). Depletion of CD4 T cells from spleens of infected DR0401.EA[0] mice abrogated the IFN-γ response. No CD4 T cell depletion was carried out in spleens of naive (uninfected) DR0401.EA[0] mice (ND, not done). (C) Cytokine response (pg/ml) of splenic T cells from infected (day 10 after challenge with sporozoites) and naive DR0401.EA[0] mice stimulated with CD3/CD28 Abs. (D) Cytokine response (pg/ml) upon stimulation with ConA. Data represent means ± standard deviations for three mice analyzed individually (∗, $P < 0.05$; ∗∗, $P < 0.005$; ∗∗∗, $P < 0.0005$; NS, not significant [determined by Student's t test]). ND, not done.

helper cells or by direct suppression on B cells (9, 48, 49). Thus, we examined CD4 T cells in infected DR0401.EA[0] mice. At day 10 after challenge with Py17XNL sporozoites, the CD4 T cell numbers in spleens of infected DR0401.EA[0] mice were significantly higher than those in spleens of noninfected DR0401.EA[0] mice and also higher than those in spleens of infected C57BL/6 mice, BALB/c mice, and F1 hybrid mice (Fig. 6A), which indicated that CD4 T cells in DR0401.EA[0] mice rapidly expanded during Py17XNL infection. *Ex vivo* culture of splenic T cells from infected DR0401.EA[0] or C57BL/6 mice resulted in secretion of gamma interferon (IFN-γ), whereas IFN-γ was not detectable in cultures of naive (uninfected) mice (Fig. 6B). Depletion of CD4 T cells

from spleens of infected DR0401.EA[0] mice abrogated the IFN-γ response, which indicated that this cytokine was secreted by activated CD4 T cells during infection. No detectable levels of IL-4 or IL-10 were detected in the *ex vivo* cultures from infected DR0401.EA[0] or C57BL/6 mice (data not shown). Upon polyclonal stimulation with CD3/CD28 Abs (Fig. 6C) or ConA (Fig. 6D), splenic T cells from infected DR0401.EA[0] mice were proficient at secreting IFN-γ, IL-4, and IL-10 compared to splenic T cells from naive DR0401.EA[0] mice or to splenic T cells from infected C57BL/6 and F1 hybrid mice. In aggregate, these results indicated that the effector CD4 T cell function in DR0401.EA[0] mice was not suppressed during acute Py17XNL infection.
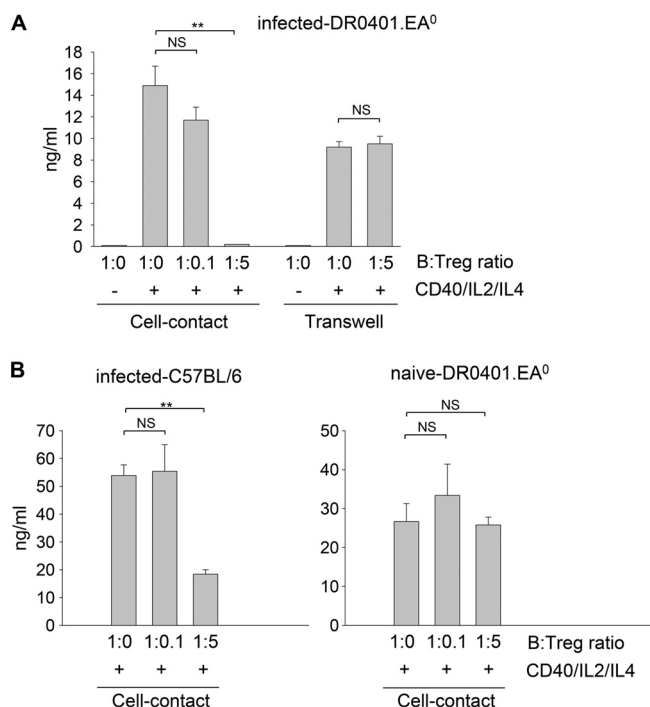
FIG 7 Py17XNL stimulates Tregs to suppress B cell function by direct cell contact with B cells. Splenic B cells (3×10^5) from naive DR0401.EA^0 mice were stimulated with CD40 Abs plus IL-2 and IL-4 or left unstimulated (see Materials and Methods) and cultured in the absence or presence of graded numbers of Tregs isolated from DR0401.EA^0 mice at day 10 after challenge with Py17XNL sporozoites (A) or Tregs isolated from naive DR0401.EA^0 mice (B, right). To prevent direct cell-cell interactions, cultures were also carried out in 0.4-μm transwell plates (A). Likewise, splenic B cells (3×10^5) from naive C57BL/6 mice were stimulated, as described above, in the presence of Tregs isolated from infected C57BL/6 mice (B, left). IgG secretion in cell culture supernatants was measured by ELISA at day 16 of culture in triplicate wells. Data were analyzed by 2-way ANOVA followed by Tukey's test (**, $P < 0.005$; NS, not significant).

We next investigated whether Tregs from Py17XNL-infected DR0401.EA^0 mice could directly inhibit B cells to secrete antibodies. For this, purified B cells from naive DR0401.EA^0 mice were stimulated with CD40 Abs, IL-2, and IL-4 in the absence or presence of graded numbers of purified Tregs from infected DR0401.EA^0 mice, and the level of IgG secreted into cell culture supernatants was measured by ELISA. As illustrated in Fig. 7A, the IgG response was proportionally inhibited by increasing numbers of Tregs. To determine whether B cell suppression was mediated by direct cell-cell contact with Tregs, cells were cocultured in a transwell setting. The secretion of IgG was not inhibited in the transwell cultures (Fig. 7A), indicating that B cell suppression was mediated by direct cell contact with Tregs. Interestingly, the Tregs from naive (noninfected) DR0401.EA^0 mice failed to inhibit B cells to secrete IgG (Fig. 7B, right), which indicated that Py17XNL parasites stimulate Tregs in DR0401.EA^0 mice to suppress B cell function.

To further determine whether Py17XNL infection also stimulates B cell-suppressogenic Tregs in control mouse strains able to self-cure infection, we carried out a similar experiment using Tregs from infected C57BL/6 mice. As shown in Fig. 7B (left), the Tregs from infected C57BL/6 mice also suppressed B cells to secrete IgG. The fact that the B cell-suppressogenic Tregs expanded in infected DR0401.EA^0 mice but not in infected C57BL/6 mice (Fig. 4, bottom) accounts for the inability of DR0401.EA^0 mice to elicit specific antibodies and resolve Py17XNL infection.

Humanized mice expressing HLA-II molecules other than HLA-DR0401 elicit specific antibodies and clear Py17XNL malaria infection. Considering our findings that humanized DR0401.EA^0 mice cannot self-cure first-time (acute) Py17XNL infection, it was important to investigate the ability of humanized mice expressing other HLA class II molecules to elicit antibody responses and to clear Py17XNL infection. For this, groups of DR0301.EA^0 (C57BL/10), DQ6.EA^0 (C57BL/10), DQ8.EA^0 (C57BL/10), and DR0402.EA^0 (C57BL/10) mice were challenged with 200 sporozoites and monitored for parasitemia and development of specific antibodies. As illustrated in Fig. 8, DR0301.EA^0, DQ6.EA^0, DQ8.EA^0, and DR0402.EA^0 mice were able to elicit specific antibodies and to clear the infection. The DR0402.EA^0 and
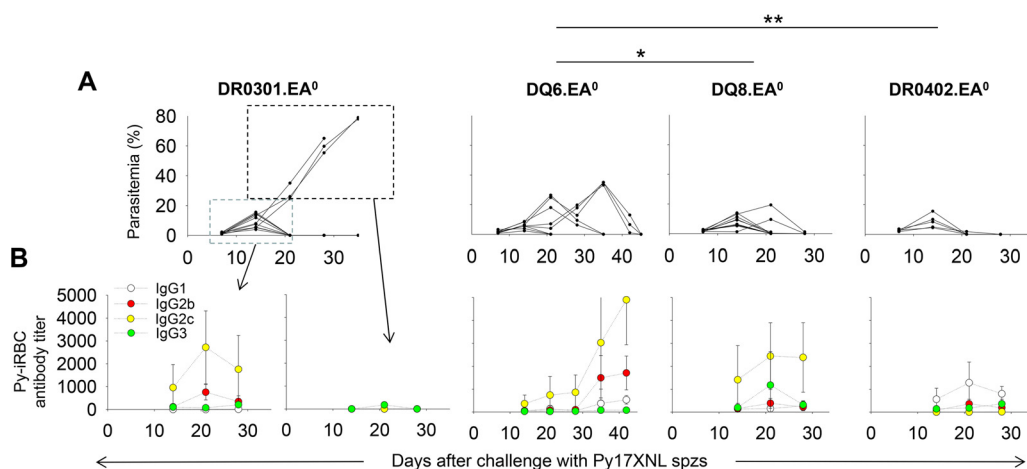

FIG 8 Humanized mice expressing HLA-II molecules other than HLA-DR0401 elicit specific antibodies and self-cure Py17XNL infection. Groups of DR0301.EA^0 ($n = 11$), DQ6.EA^0 ($n = 8$), DQ8.EA^0 ($n = 11$), and DR0402.EA^0 ($n = 5$) mice were challenged with 200 Py17XNL sporozoites and monitored for parasitemia and development of specific antibodies to Py-iRBCs. (A) Parasitemia values for individual mice. (B) Mean antibody titers ± standard deviations for mice analyzed individually (*, $P < 0.05$; **, $P < 0.005$ [determined by Student's t test]).

DQ8.EA^0 mice were more efficient in clearing parasitemia than the DQ6.EA^0 mice, since they self-cured by day 28 after challenge, whereas 5 out of 8 DQ6.EA^0 mice were still parasitemic by day 28 and needed an additional 12 days to self-cure. On the other hand, the DR0301.EA^0 mice showed a more heterogeneous response to the infection, since 8 out of 11 mice elicited antibodies and cleared the infection by day 21, whereas the remaining 3 mice were unable to elicit antibodies and succumbed to the infection. Thus, unlike the HLA-DR0401 molecules, HLA-DR0402, HLA-DQ6, HLA-DQ8, and, to some extent, HLA-DR0301 molecules are proficient at supporting antibody responses to Py17XNL malaria parasites and self-curing acute (first-time) infection.

DISCUSSION

This study shows that expression of HLA-DR0401 molecules in mice results in lethal malaria infection by Py17XNL, a parasite that otherwise induces a self-curing disease in inbred mouse strains. DR0401.EA^0 mice developed hyperparasitemia and severe anemia and succumbed to infection, thus resembling the lethal infection induced by Py17XL in inbred mouse strains. The lethality of Py17XNL infection in DR0401.EA^0 mice was not due to an increased virulence of Py17XNL malaria parasites but rather to the inability of DR0401.EA^0 mice to support antibody responses needed to clear the blood stage infection. These mice were, however, able to mount antibody responses and to clear the infection by *in vivo* depletion of Tregs, treatment with chloroquine, or coexpression with mouse MHC-II molecules.

CD4 T cells are critical for resolving Py17XNL malaria infection, as demonstrated in mice depleted of CD4 T cells and in nude (T cell-deficient) mice that cannot resolve the infection (50, 51). Our results are consistent with those observations, since the ability of mice to self-cure or succumb to Py17XNL malaria was related solely to the expression of MHC-II (HLA-II) molecules, and the primary role of MHC-II (HLA-II) molecules is to present peptides to CD4 T cells. HLA-II molecules are highly polymorphic and differ in their ability to present different sets of peptides to CD4 T cells. For example, within the HLA-DR4 group, there are about 50 different allelic variants that differ in only 3 amino acids in the peptide binding groove. The HLA-DR0401 but not the HLA-DR0402 molecules predispose to rheumatoid arthritis by their differential ability to present arthritogenic peptides to autoreactive CD4 T cells (52, 53). Here we showed that HLA-DR0402, HLA-DQ6, HLA-DQ8, and, to some extent, HLA-DR0301 molecules sufficed for supporting antibody responses and self-curing Py17XNL infection, while HLA-DR0401 molecules failed to do so. Our results thus indicate that HLA class II restriction of malaria epitopes and, hence, CD4 T cells play a critical role in the clearance of Py17XNL parasites.

Since erythrocytes do not express MHC (HLA) molecules, and infected erythrocytes cannot be eliminated by CD4 T cells in a MHC (HLA) class II-restricted manner, the critical role of CD4 T cells in resolving Py17XNL infection is likely mediated by the ability of CD4 T cells to provide help for B cell differentiation and secretion of antibodies. The contribution of antibodies to the clearance of Py17XNL malaria has been demonstrated by the therapeutic and antiparasitic effects of hyperimmune sera (54, 55) as well as in B cell-deficient mice and mice depleted of B cells with anti-IgM antibodies that failed to resolve Py17XNL malaria infection (24, 46). In agreement with those observations, we found that passive transfer of immune sera from infected C57BL/6 mice (able

to elicit antibodies and clear infection) significantly reduced the level of parasitemia in DR0401.EA^0 mice. Thus, our study demonstrates a critical role of HLA-II molecules in supporting antibody responses and dictating susceptibility or resistance to Py17XNL malaria.

The Tregs from infected DR0401.EA^0 and C57BL/6 mice suppressed B cells to secrete antibodies through a cell-cell interaction, but Tregs from naive (uninfected) mice were unable to suppress B cell function. Thus, our study provides the first evidence for a new sidearm immune evasion mechanism by which Py17XNL malaria parasites stimulate Tregs that suppress the protective B cell function. During the second week of infection, C57BL/6 mice and other mouse strains that self-cure the infection, such as BALB/c and F1 hybrid mice, had total numbers of splenic Tregs comparable to those in spleens of uninfected mice, although the Treg frequency was reduced, since the infection induced splenomegaly. Our results for C57BL/6, BALB/c, and F1 hybrid mice are in agreement with results from previous studies by Couper et al. (12) and Berretta et al. (20), using nonlethal Py17XNL and *P. chabaudi* AS strains, indicating that Tregs expand during the first week of infection and that the Tregs are then deleted by apoptosis in such a way that the total numbers of Tregs return to basal levels. In contrast, the numbers of Tregs in spleens of infected DR04010.EA^0 mice expanded to reach a 5-fold increase over the Treg numbers in spleens of uninfected DR0401.EA^0 mice. The ability of Py17XNL parasites to stimulate and expand B cell-suppressogenic Tregs accounts for the inability of DR0401.EA^0 mice to elicit antibodies and to self-cure the infection. The role of B cell-inhibitory Tregs in preventing clearance of infection in DR0401.EA^0 mice was evident, since *in vivo* Treg depletion enabled the DR0401.EA^0 mice to elicit antibodies and to self-cure the infection.

In humans, B cell-suppressogenic Tregs have been found in T-B area borders within the germinal centers of lymphoid tissues and have been associated with development of systemic lupus erythematosus (48, 49), an autoimmune disorder mediated by exacerbated production of autoantibodies. When it comes to human malaria, the role of Tregs is supported by studies in volunteers challenged with *P. falciparum*, in which the expansion of CD4$^+$ FOXP3$^+$ T cells in blood was associated with enhanced parasite growth (21), as well as by studies in children and adults from areas where malaria is endemic which indicated an association between Treg frequency in blood and hyperparasitemia (22, 23). However, the ability of Tregs to suppress antibody responses to *P. falciparum* has not been investigated.

Interestingly, the Tregs from Py17XNL-infected DR0401.EA^0 mice did not alter the effector CD4 T helper compartment, since effector CD4 T cells were able to expand during infection, and they were proficient at secreting cytokines either *ex vivo* or upon polyclonal stimulation with ConA or CD3/CD28 Abs. Although there is overwhelming evidence that Tregs are able to directly suppress CD4 T helper function (reviewed in reference 9), to our knowledge, only studies by Lim et al. (48) and Iikuni et al. (49) and this study demonstrate the existence of a new Treg subset that is able to directly suppress B cells. The mechanisms by which Tregs can selectively suppress B cell function remain to be investigated.

Infected DR0401.EA^0 mice treated with chloroquine were able to elicit antibodies and were protected against reinfection. Chloroquine is a potent antimalaria drug able to inhibit parasite development *in vitro* and *in vivo* (45), although chloroquine can also enhance immune responses to malaria parasites, as indicated by

the following: (i) immunization with low-dose blood stage parasites under chloroquine cover protects mice against blood stage malaria (46), (ii) immunodeficient mice need significantly higher doses of chloroquine and longer treatment to clear parasitemia than immunocompetent mice (46), and (iii) parasitemic immunocompetent but not immunodeficient mice treated with chloroquine are protected against subsequent challenges with malaria parasites (46). However, there is debate on whether the ability of chloroquine to enhance immune responses to malaria parasites is due to its immunomodulatory effects or whether it may simply act by reducing the parasite mass, since blood stage parasites induce dendritic cell dysfunction and tolerance (56, 57). Infected DR0401.EA^0 mice developed splenomegaly (see Table S1 in the supplemental material), but upon treatment with chloroquine, their spleens returned to normal size and cellularity by day 20 posttreatment and contained similar numbers of Tregs as those in spleens of uninfected mice. To determine whether chloroquine can deplete Tregs, we treated naive (noninfected) DR0401.EA^0 mice with chloroquine ($n = 3$; three doses of 25 mg/kg at 1-day intervals), and mice were examined at day 12 posttreatment. The uninfected chloroquine-treated mice had Treg numbers similar to those in untreated mice ($P = 0.9$) (Fig. 4A, bottom), indicating that in the absence of infection, chloroquine does not deplete Tregs. However, at present, we cannot rule out whether chloroquine treatment could have had a direct immunomodulatory effect on the infected DR0401.EA^0 mice.

F1 hybrid mice coexpressing HLA-DR0401 and mouse MHC class II molecules elicited antibodies and resolved the infection, which indicated that HLA-DR0401 molecules do not play a dominant negative role in Py17XNL malaria. Studies with ethnic populations in Ghana and Gabon showed an association between HLA-DR4 expression and severe *P. falciparum* malaria (58, 59), although the HLA-DR4 alleles were not subtyped. The overall frequency of HLA-DR4 in sub-Saharan African populations is 10 times lower than that in Europe or North America (59), and it was undetectable in some other areas where malaria is endemic (60). This has been attributed to negative selection of HLA-DR4 by *P. falciparum* malaria. The results with the Py17XNL model indicating that coexpression of HLA-DR0401 molecules with other MHC-II molecules allowed mice to elicit antibodies and to resolve infection cannot account for an association between HLA-DR0401 and severe *P. falciparum* malaria, as most humans express at least two different HLA-II molecules. However, whether or not HLA-DR0401 molecules play a recessive or dominant role when it comes to *P. falciparum* malaria infection remains to be investigated.

In aggregate, our results demonstrate a differential role of MHC (HLA) class II molecules in supporting antibody responses to Py17XNL malaria and revealed a new immune evasion mechanism whereby Py17XNL parasites activate B cell-suppressogenic Tregs. Humanized HLA-II mice may represent a valuable tool to identify immunogenic or suppressogenic HLA class II-restricted CD4 T cell epitopes expressed by Py17XNL and *P. falciparum* and for testing of malaria vaccine candidates.

ACKNOWLEDGMENTS

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