IL-27 Receptor Signaling Regulates Memory CD4+ T Cell Populations and Suppresses Rapid Inflammatory Responses during Secondary Malaria Infection

Emily Gwyer Findlay, Ana Villegas-Mendez, Noelle O’Regan, J. Brian de Souza, Lisa-Marie Grady, Christiaan J. Saris, Eleanor M. Riley, Kevin N. Couper

Interleukin-27 (IL-27) is known to control primary CD4+ T cell responses during a variety of different infections, but its role in regulating memory CD4+ T responses has not been investigated in any model. In this study, we have examined the functional importance of IL-27 receptor (IL-27R) signaling in regulating the formation and maintenance of memory CD4+ T cells following malaria infection and in controlling their subsequent reactivation during secondary parasite challenge. We demonstrate that although the primary effector/memory CD4+ T cell response was greater in IL-27R-deficient (WSX-1-/-) mice following Plasmodium berghei infection than in wild-type (WT) mice, there were no significant differences in the size of the maintained memory CD4+ T population(s) at 20 weeks postinfection in the spleen, liver, or bone marrow of WSX-1-/- mice compared with WT mice. However, the composition of the memory CD4+ T cell pool was slightly altered in WSX-1-/- mice following clearance of primary malaria infection, with elevated numbers of late effector memory CD4+ T cells in the spleen and liver and increased production of IL-2 in the spleen. Crucially, WSX-1-/- mice displayed significantly enhanced parasite control compared with WT mice following rechallenge with homologous malaria parasites. Improved parasite control in WSX-1-/- mice during secondary infection was associated with elevated systemic production of multiple inflammatory innate and adaptive cytokines and extremely rapid proliferation of antigen-experienced T cells in the liver. These data are the first to demonstrate that IL-27R signaling plays a role in regulating the magnitude and quality of secondary immune responses during rechallenge infections.
memory CD4⁺ T cells (reviewed in reference 18), owing to its ability to promote expression of CD127, which is critically required for the maintenance of memory CD4⁺ T cells (reviewed in reference 19). A role for direct gamma interferon (IFN-γ) signals in inducing conversion of effector cells into memory CD4⁺ T cells has also been suggested (20). As the pathogen load is lower in WSX-1⁻/⁻ than in wild-type (WT) mice during primary infection with a number of different pathogens, but levels of IL-2, IFN-γ, and IL-12 are, in general, increased (1, 2), we hypothesized that WSX-1 signaling may play a major role in controlling the establishment of memory CD4⁺ T cells during infection and subsequently affecting their reactivation following secondary infection.

In this study, we have investigated the role of IL-27 in shaping memory CD4⁺ T cell responses following infection using Plasmodium berghei NK65 as a model proinflammatory infection. We have previously shown the important role of WSX-1 signaling in regulating the development of Th1 responses during primary malaria infection (1, 9). Of relevance, the signals that control memory CD4⁺ T cell development, maintenance, and function during malaria infection are, at present, poorly defined, and there remains significant debate regarding whether malaria-induced memory CD4⁺ T cell responses are defective (21, 22). We show that following drug clearance of primary malaria infection, there are subtle differences in the phenotype and function of memory CD4⁺ T cell populations in IL-27R⁻/⁻ mice. Importantly, we also demonstrate that WSX-1⁻/⁻ mice develop significantly decreased parasite burdens compared with WT mice following parasite rechallenge, but despite this, they exhibit increased weight loss. WSX-1⁻/⁻ mice develop rapid and exaggerated proinflammatory immune responses following reinfection with homologous parasites, associated with significantly increased IFN-γ production by antigen-experienced CD4⁺ T cells. These data are the first to identify a role for IL-27 in modulating the development, maintenance, and reactivation of memory CD4⁺ T cells during infection and increase our understanding of the factors that influence the memory CD4⁺ T cell molecular program.

MATERIALS AND METHODS

Mice and parasites. C57BL/6 mice were purchased from Harlan UK. Breeding pairs of IL-27R-deficient (WSX-1⁻/⁻) mice on a C57BL/6 background (23) were provided by Amgen, Inc. (Thousand Oaks, CA, USA). Animals were maintained under barrier conditions in individually ventilated cages. Cryopreserved P. berghei NK65 parasites were passaged once through C57BL/6 mice before being used to infect experimental animals.

Six- to ten-week-old mice were infected by intravenous injection of 10⁸ parasitized red blood cells (pRBCs). The course of infection was monitored by measuring weight loss and peripheral parasitemia every second day. Parasitemia was assessed by examination of Giemsa-stained thin blood smears. On days 8 to 12 of infection, mice were treated intraperitoneally (i.p.) with 15 mg of chloroquine/kg of body weight (Sigma UK) in phosphate-buffered saline (PBS), after which no parasites were visible in the blood. Blood was checked twice a week for reemergence of parasites until the termination of experiments at 2, 4, 8, or 20 weeks postinfection (p.i.).

In some experiments, mice were reinfected by intravenous injection of 10⁹ parasitized red blood cells at 20 weeks p.i. The course of infection was monitored by assessing weight loss, and thin blood smears were performed daily until termination of the experiment at 3 or 7 days postchallenge.

Preparation of tissues. Blood was obtained by cardiac puncture and spun at 7,870 × g for 8 min, and serum was removed and stored at −80°C. Single-cell suspensions of spleen and liver were prepared by homogenization with a 70-μm cell strainer (BD Biosciences). Red blood cells (RBCs) were lysed by using RBC lysis buffer (BD Biosciences). Leukocytes were enriched from liver homogenates by resuspension of the cell pellets in a solution containing 32% Percoll–3% PBS–65% Hanks balanced salt solution (HBSS) and centrifugation at 600 × g for 10 min. Floating hepatocytes were removed, and the cell pellet was collected. To obtain bone marrow cells, femurs were cleaned, the ends were snapped off, and the bone marrow was flushed out with 1 ml of HBSS. The resulting cell suspension was passed several times through a 25-gauge needle. Absolute cell numbers were calculated by microscopy using a hemocytometer, and live/dead cells were differentiated by trypan blue exclusion.

Flow cytometry. Phenotypic characterization of cell populations was performed by surface staining with anti-mouse CD4 (clone GK1.5), anti-mouse CD8 (53-6.7), anti-mouse CD44 (IM7), anti-mouse CD62L (MEI-14), anti-mouse KLRG1 (2F1), anti-mouse CCR7 (4B12), anti-mouse CD27 (LG7E9), and anti-mouse CD127 (A7R34), according to previously reported protocols (1, 24). For intracellular staining of T-bet (4B10), Gata3 (TWAJ), and Foxp3 (FJK-16S), mice were first stained with antibodies to surface markers, washed, and permeabilized by overnight incubation with the Foxp3 fixation/permeabilization buffer (eBioscience). The cells were then washed and incubated for 30 min with antibodies against the intracellular targets. All antibodies were purchased from eBioscience or BD Biosciences.

Intracellular staining of IFN-γ (XMG1.2), tumor necrosis factor (TNF) (MP6-XT22), and IL-4 (11B11) was performed by incubating 1 × 10⁶ cells for 4 h at 37°C with 200 μg/ml phosphoryl myristate acetate (PMA) (Sigma UK) and 1 μg/ml ionomycin (Sigma UK) in the presence of brefeldin A (eBioscience). Cells were then stained according to the intracellular protocol described above. All flow cytometry acquisition was performed by using an LSR II instrument (BD Systems, United Kingdom). All analyses were performed by using FlowJo software (Treestar, Inc., OR, USA). Flow cytometry controls included antibody titrations, single-stained and unstained samples, and fluorescence-minus-one (FMO) controls.

Quantification of plasma cytokines. Cytometric bead arrays were used to assess cytokine concentrations in the serum. Beads and buffers to detect IL-1α, IL-12p70, IL-2, IL-10, IL-1A, IFN-γ, and TNF were purchased from BD Biosciences and used according to the manufacturer’s instructions.

Malaria-specific antibody ELISA. Malaria pRBC lysate was prepared by purifying mature pRBC trophozoites/schizonts by positive selection using LD columns (Miltenyi), followed by three cycles of freeze-thawing and sonication (10 times for 30 s). Ninety-six-well microtiter plates were coated overnight at 4°C with pRBC lysate antigen at a concentration of 1 μg/ml. To quantify antibody levels, plasma samples were added at an initial 1:100 dilution, followed by 5× serial dilutions. Reciprocal endpoint titers were calculated as the final dilution before antibody levels reached a plateau. The capture enzyme-linked immunosorbent assay (ELISA) was performed as previously described (25).

Real-time PCR. IL-2, IL-7, IL-15, and IFN-γ mRNA levels were quantified by real-time PCR (TagMan), using validated gene expression assays from ABI Biosystems (Warrington, United Kingdom). cDNA was extracted (RNasey kit; Qiagen) and DNase I treated (ABI, Austin, TX), and cDNA was synthesized. cDNA expression was standardized by using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin. Cycling conditions were as follows: initialization for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Histology. To examine splenic and hepatic architecture and pathology, tissues were removed from mice and placed into 10% Formalin saline. Preserved samples were then sectioned and stained with hematoxylin and eosin (H&E). The presence of necrosis, inflamed and damaged blood vessels (including occluded vessels), and disrupted architecture was then examined by microscopy (×20 magnification).

Statistical analysis. The normality of data was tested by using the D’Agostino-Pearson omnibus normality test. For comparisons between 2 groups, sta-
RESULTS

Recovery from primary drug-cured malaria infection is largely unimpaired in IL-27R<sup>−/−</sup> mice. We have previously shown that anomalous CD4<sup>+</sup> T cell responses develop in WSX-1<sup>−/−</sup> mice during primary <i>P. berghei</i> NK65 infection, which leads to excessive production of IFN-γ, increased accumulation of effector CD4<sup>+</sup> T cells in nonlymphoid tissues, and resultant immunopathology (1, 9). To expand on these data and investigate the role of IL-27 in controlling memory CD4<sup>+</sup> T cell responses during infection, we infected C57BL/6 (WT) and WSX-1<sup>−/−</sup> mice with <i>P. berghei</i> NK65. As WSX-1<sup>−/−</sup> and WT mice normally succumb to <i>P. berghei</i> NK65 infection on day 14 and days 25 to 30 postinfection (p.i.), respectively (1), mice were treated with chloroquine on days 8 to 12. This reduced the level of tissue pathology in WSX-1<sup>−/−</sup> mice (results not shown) and allowed the mice to survive and subsequently develop memory immune responses. Drug treatment successfully cleared the infection (Fig. 1A). Interestingly, WSX-1<sup>−/−</sup> mice still lost significantly more weight than did wild-type mice (Fig. 1B) during and after clearance of parasites, despite displaying equivalent peripheral parasitemia prior to drug treatment. Disruption of splenic architecture with significant expansion of the white pulp was observed in WT and WSX-1<sup>−/−</sup> mice at 2 months postinfection but appeared to largely revert to normal in both WT and WSX-1<sup>−/−</sup> mice by 20 weeks postinfection, although hemozoin was still visible in the spleens of WT mice (results not shown). No gross pathology or inflammation was apparent in the livers of WT or WSX-1<sup>−/−</sup> mice at 2 months or 20 weeks postinfection, although there was some evidence of hemozoin or bile pigment deposition at 20 weeks postinfection (results not shown).

In agreement with previously reported data (1), significantly higher numbers of activated (effector/memory) CD4<sup>+</sup> CD44<sup>+</sup> T cells were found in the livers of WSX-1<sup>−/−</sup> mice during the early stages of infection (Fig. 1C). Significantly increased numbers of CD4<sup>+</sup> CD44<sup>+</sup> T cells were also observed in the spleen—the main site of immunological priming during malaria infection (26)—of WSX-1<sup>−/−</sup> mice compared with WT mice (Fig. 1D). Following successful drug treatment, the effector/memory CD4<sup>+</sup> T cell populations contracted, and the numbers of intrahepatic and splenic CD4<sup>+</sup> CD44<sup>+</sup> T cells were similar in WSX-1<sup>−/−</sup> and WT mice by 8 weeks and 20 weeks, respectively (Fig. 1C and D). Combined, these results show that the exaggerated primary CD4<sup>+</sup> T cell response generated in WSX-1<sup>−/−</sup> mice during primary infection is transient and does not persist long term postinfection.

Absence of WSX-1 signaling does not affect the generation of Tem and Tcm CD4<sup>+</sup> T cell populations after a primary malaria infection. To examine how WSX-1 signaling modulates the development and maintenance of effector/memory CD4<sup>+</sup> T cells during infection, we first broadly subdivided the CD4<sup>+</sup> CD44<sup>+</sup> T cell population identified in the spleen, liver, and bone marrow post-parasite clearance into effector/effector memory (Tem) (CD4<sup>+</sup> CD62L<sup>−</sup>) and central memory (Tcm) (CD4<sup>+</sup> CD62L<sup>+</sup>) T cell subsets (Fig. 2A). As expected, the frequencies and numbers of effector/Tem CD4<sup>+</sup> T cells increased significantly in the spleen, liver, and bone marrow of WT and WSX-1<sup>−/−</sup> mice on weeks 2 and 4 postinfection, before contracting and stabilizing by week 20 postinfection (Fig. 2B to G). Only minor differences in the frequencies of effector/Tem CD4<sup>+</sup> T cells were observed in the tis-
WSX-1 signaling does not play a major role in regulating the generation and maintenance of Tem and Tcm CD4+ T cell populations after primary malaria infection. WT and WSX-1−/− mice were infected with 10⁴ P. berghei NK65 pRBCs on day 0 and treated with chloroquine on days 8 to 12. At 0, 2, 4, 8, and 20 weeks p.i., spleen, liver, and bone marrow cells were removed. (A to G) Phenotypic characterization of naive (CD44+CD62L−), effector/effector memory (CD44+CD62L−), and central memory (CD44+CD62L+) cells. (A) Representative plots demonstrating gating of cells within the spleen. (B to M) Frequencies (B to D and H to J) and numbers (E to G and K to M) of effector/effector memory CD4+ T cells (B to G) and central memory CD4+ T cells (H to M) within the spleen (B, E, H, and K), liver (C, F, I, and L), and bone marrow (D, G, J, and M) of WT and WSX-1−/− mice postinfection. Results are representative of two separate experiments with 4 to 5 mice per group (*, P < 0.05 between WT and WSX-1−/− mice; **, P < 0.01 between WT and WSX-1−/− mice).
Lack of WSX-1 signaling leads to minor alterations in the memory CD4+ T cell compartment following resolution of primary malaria infection. WT and WSX-1−/− mice were infected with 10^6 P. berghei NK65 pRBCs on day 0 and treated with chloroquine on days 8 to 12. At 0, 2, 4, 8, and 20 weeks p.i., spleen, liver, and bone marrow cells were removed. Shown is a detailed phenotypic characterization of memory CD4+ T cell subsets within the spleen of WT and WSX-1−/− mice at 20 weeks p.i. (A) Representative plots demonstrating the gating strategy of splenic CD4+ T cells. (B to D) Contribution of each memory CD4+ T cell subtype to the total CD4+ CD44− T cell population within the spleen (B), liver (C), and bone marrow (D) of WT and WSX-1−/− mice at 20 weeks p.i. Results are representative of two separate experiments with 4 to 5 mice per group (+, P < 0.05 between WT and WSX-1−/− mice; **, P < 0.01 between WT and WSX-1−/− mice).

FIG 3 Lack of WSX-1 signaling leads to minor alterations in the memory CD4+ T cell compartment following resolution of primary malaria infection. WT and WSX-1−/− mice were infected with 10^6 P. berghei NK65 pRBCs on day 0 and treated with chloroquine on days 8 to 12. At 0, 2, 4, 8, and 20 weeks p.i., spleen, liver, and bone marrow cells were removed. Shown is a detailed phenotypic characterization of memory CD4+ T cell subsets within the spleen of WT and WSX-1−/− mice at 20 weeks p.i. (A) Representative plots demonstrating the gating strategy of splenic CD4+ T cells. (B to D) Contribution of each memory CD4+ T cell subtype to the total CD4+ CD44− T cell population within the spleen (B), liver (C), and bone marrow (D) of WT and WSX-1−/− mice at 20 weeks p.i. Results are representative of two separate experiments with 4 to 5 mice per group (+, P < 0.05 between WT and WSX-1−/− mice; **, P < 0.01 between WT and WSX-1−/− mice).

Lack of IL-27R signaling modulates the maturation of the effector memory CD4+ T cell pool post-malaria infection. Although the overall sizes of the broadly defined CD4+ T cell Tcm and Tem pools were not majorly affected by a lack of IL-27 signaling, these broad populations represent a heterogeneous mix of memory cell subsets (27). Thus, expanding upon the gating strategy described previously by Stephens and Langhorne (22), we subdivided the CD4+ CD44− T cell compartment into central memory (Tcm) (CD44+ CD62L−), early effector memory (CD44+ CD62L− CD27− CD127+), late effector memory (CD44+ CD62L− CD27− CD127+), effector (CD44+ CD62L− CD27+ CD127− KLRG1−), and terminal differentiated effector (CD44+ CD62L− CD127− KLRG1+) cells and investigated whether there were specific imbalances in these subsets in WSX-1−/− mice compared with WT mice at 20 weeks postinfection (Fig. 3A).

In each site, approximately 30 to 50% of CD4+ CD44− T cells expressed the phenotypic profile of Tcm cells, and Tcm cell frequencies did not differ between WT and WSX-1−/− mice at this time point. Interestingly, significantly higher frequencies of Tem late cells were observed in the spleen and the liver of WSX-1−/− mice than in WT mice (Fig. 3A to C). Very few terminally differentiated effector cells were observed in any organs in either WT or WSX-1−/− mice. Populations of CD44+ CD44+ CD62L− CD127− CD27+ cells were identified in the spleen, liver, and bone marrow of both WT and WSX-1−/− mice (Fig. 3B to D). While the frequencies of these cells were comparable in the livers of both strains of mice, fewer CD44+ CD44+ CD62L− CD127− CD27+ cells were observed in the spleen and bone marrow of WSX-1−/− mice than in WT mice (Fig. 3B to D). This population of cells is believed to include regulatory T cells (28).

As IL-2, IL-7, IL-15, and IFN-γ are known to regulate the maintenance of memory CD4+ T cells, we quantified the expression levels of IL-2, IL-7, IL-15, and IFN-γ in spleens or livers of naive WT and WSX-1−/− mice by real-time PCR. There were no differences in the expression levels of IL-2, IL-7, IL-15, and IFN-γ in spleens or livers of naive WT and WSX-1−/− mice, showing that there are no intrinsic differences in the cytokine signals directing memory cell development and/or maintenance in WSX-1−/− mice under homeostatic conditions. However, significantly higher levels of IL-2
transcripts were observed in the spleens and livers of WSX-1−/− mice than in WT mice at 2 weeks postinfection (see Fig. S1A and S1B in the supplemental material), when memory precursor cells begin to form; this may explain the higher numbers of CD4+ CD44+ T cells in the spleens and livers of WSX-1−/− mice at 2 weeks p.i. (Fig. 1C and D). IFN-γ levels were also higher in the liver, but not spleen, of WSX-1−/− mice on day 14 postinfection than in WT mice. Apart from a slightly higher concentration of splenic IL-2 transcripts in the spleens of WSX-1−/− mice than in WT mice, there were no biologically relevant or statistically significant differences in the concentrations of cytokine transcripts in the spleens or livers of WSX-1−/− mice compared with WT mice at 20 weeks p.i. (see Fig. S1A and S1B in the supplemental material).

In summary, these data show that WSX-1 signaling plays a modest role in modulating the cytokine signals required for development/maintenance of memory CD4+ T cell subsets following clearance of malaria infection and that the absence of WSX-1 leads to only minor perturbations in the memory CD4+ T cell compartment.

Malaria infection-induced memory CD4+ T cells from WSX-1−/− mice produce less IFN-γ than those from WT mice. We next examined whether WSX-1 signaling influenced memory CD4+ T cell functionality following clearance of primary malaria infection. Interestingly, effector CD4+ T cells from the spleen, both effector memory and central memory cells from the livers, and effector memory CD4+ T cells from the bone marrow of WSX-1−/− mice (at 20 weeks p.i.) produced significantly less IFN-γ than their WT counterparts (Fig. 4A to C). However, the absence of WSX-1 did not affect the frequencies of T cell subsets expressing T-bet in the spleen or bone marrow and led to only a slight increase in the central memory CD4+ T cell population within the liver (Fig. 4D to F). Interestingly, the majority of T cell subsets in the livers of WT and WSX-1−/− mice expressed high levels of T-bet, whereas very low T-bet expression levels were observed in the bone marrow (Fig. 4E and F).

Frequencies of FoxP3+ effector CD4+ T cells were significantly higher in spleens of WSX-1−/− mice (30%) than in WT mice (15%) (Fig. 4G). In contrast, significantly fewer central memory and effector CD4+ T cells expressed Foxp3 in the bone marrow of WSX-1−/− mice than in WT mice. Gata3 expression was low in all T cell subsets in the spleen, liver, and bone marrow of both WT and WSX-1−/− mice (results not shown). Moreover, there were no significant differences in the production of IL-4 or TNF by the T cell subsets from WT or WSX-1−/− mice (results not shown).

Combined, these data show that the lack of IL-27R signaling led to tissue-specific alterations in the capacity of individual mem-
ory CD4+ T cell populations to produce IFN-γ and minor modulations in Th1 versus regulatory T cell differentiation. WSX-1−/− mice are more resistant to secondary malaria infections and develop exaggerated recall responses. As the primary function of memory T cells is to promote protection upon reencountering the pathogen, we assessed whether WSX-1−/− mice were more or less protected than WT mice during secondary malaria infection. Unlike WT mice, WSX-1−/− mice reinfected with P. berghei NK65 at 20 weeks p.i. did not develop patent parasitemia (Fig. 5A) but suffered from severe weight loss (Fig. 5B), which was significantly more rapid than that during primary infection (Fig. 1) (1). The enhanced parasite control, and more severe pathology, in the WSX-1−/− mice was accompanied by very high serum concentrations of TNF, IL-17A, IFN-γ, IL-12p70, IL-2, and IL-10 on day 3 after reinfection (Fig. 5C to H), whereas cytokine levels were only modestly increased by reinfection in WT mice (Fig. 5C to H). Crucially, these very high levels of cytokines were not observed on day 3 of a primary infection in age-matched WSX-1−/− mice (results not shown), demonstrating that the cytokine storm was specifically related to reexposure to the parasite. Although marked, the exaggerated response to secondary infection in WSX-1−/− mice was very short lived, with serum cytokines returning to baseline levels by 7 days p.i. (Fig. 5).

IL-27 signaling constrains CD4+ T cell effector responses during secondary malaria infection. We next examined the dynamics of the CD4+ T cell response in WT and WSX-1−/− mice during secondary infection. The total numbers of splenic CD4+ T cells (Fig. 6A) and antigen-experienced CD4+CD44+ T cells (Fig. 6B) peaked at 3 days after rechallenge, with cell numbers on day 3 of secondary infection far exceeding those found on day 3 or week 20 of a primary infection (Fig. 6B and results not shown). Numbers of splenic CD4+ T cells and CD4+CD44+ T cells did not, however, differ between WT and WSX-1−/− mice on either day 3 or 7 of secondary infection (Fig. 6A and B). The numbers of intrahepatic CD4+ T cells increased and were significantly higher in WSX-1−/− mice than in WT mice on day 7 of secondary infection (Fig. 6C and D). In contrast, cell numbers were low and were similar in the livers of WT and WSX-1−/− mice on day 7 of primary infection (results not shown).

Having established that WSX-1−/− mice develop exaggerated systemic immune responses following secondary challenge infection, we investigated whether reactivated memory CD4+ T cells...
WSX-1 restricts IFN-γ production by CD4⁺ T cells during the early phase of secondary malaria infection. WT and WSX-1⁻/⁻ mice were infected with 10⁴ P. berghei NK65 pRBCs on day 0 and treated with chloroquine on days 8 to 12. At 20 weeks p.i., mice were reinfected with 10⁵ pRBCs. (A to D) Total numbers of CD4⁺ cells (A and C) and CD4⁺CD44⁺ T cells (B and D) in the spleen (A and B) and liver (C and D) in WT and WSX-1⁻/⁻ mice at the stated time points of primary and secondary infections. (E) Representative plots (gated on CD4⁺ T cells) demonstrating the expression of IFN-γ (following PMA and ionomycin stimulation) versus CD44 by CD4⁺ T cells from the spleen and liver of WT and WSX-1⁻/⁻ mice at the stated time points of primary and secondary infections. (F to I) Frequencies (F and H) and total numbers (G and I) of CD4⁺CD44⁺ cells producing IFN-γ in the spleen (F and G) and liver (H and I) of WT and WSX-1⁻/⁻ mice at the stated time points of primary and secondary infections. Results are representative of two separate experiments with 4 to 5 mice per group (*, P < 0.05 between WT and WSX-1⁻/⁻ mice; **, P < 0.01 between WT and WSX-1⁻/⁻ mice).
were a major source of IFN-γ. T cells were the overwhelmingly predominant source of IFN-γ in the livers of WT and WSX-1−/− mice throughout the experiment, contributing >85% to the total intrahepatic IFN-γ response (results not shown). Significantly increased frequencies of splenic CD4+ T cells expressed CD44 and IFN-γ on day 7 of secondary infection in WSX-1−/− mice compared to those in WT mice (Fig. 6E and F). Importantly, the overall frequency of CD4+ T cells expressing CD44 and IFN-γ was significantly higher in WSX-1−/− mice during secondary infection than during primary infection (results not shown). Total numbers of splenic CD4+ T cells expressing CD44 and IFN-γ were significantly higher in WSX-1−/− mice than in WT mice on day 3 of challenge infection, but owing to the dramatic contraction in total splenic CD4+ T cell numbers between day 3 and day 7 of challenge infection (Fig. 6A and G), which was slightly exacerbated in WSX-1−/− mice, there were no significant differences in numbers of splenic CD4+ T cells expressing CD44 and IFN-γ in WT and WSX-1−/− mice on day 7 of challenge infection (Fig. 6G).

The majority of intrahepatic CD4+ T cells in both WT and WSX-1−/− mice expressed CD44 and IFN-γ throughout the course of the experiment (Fig. 6E and H). Nonetheless, significantly higher frequencies of intrahepatic CD4+ T cells expressed CD44 and IFN-γ in WSX-1−/− mice than in WT mice on day 3 of rechallenge (Fig. 6H). Moreover, significantly higher numbers of CD4+ T cells expressing CD44 and IFN-γ were observed in WSX-1−/− mice on day 7 of secondary infection than in WT mice (Fig. 6I). These data show that WSX-1 plays a role in constraining CD4+ T cell effector function during secondary infections, inferring a role for WSX-1 in regulating memory CD4+ T cell reactivation and antiparasitic functions.

DISCUSSION

It is well established that IL-27 signaling regulates effector CD4+ T cell responses during primary infection (1–11). We therefore hypothesized that as environmental signals and CD4+ T cell functionality are intrinsically altered in WSX-1−/− mice during primary infection, this would lead to significant perturbations in the development and maintenance of memory CD4+ T cell populations postinfection. Somewhat surprisingly, however, we have shown that long-term survival of antigen-experienced CD4+ T cells following malaria infection is largely unaffected by the ablation of WSX-1 expression. Instead, we have identified a minor role for WSX-1 signaling in shaping the differentiation and maturation of the memory CD4+ T cell compartment and a larger role in regulating the strength of early secondary immune responses.

We have shown that slightly increased frequencies and numbers of late effector memory CD4+ T cells develop and are maintained in WSX-1−/− mice following malaria infection and that these cells, which maintain T-bet expression, underscore their developmental history, produced lower levels of IFN-γ. In addition, hepatic central memory T cells from WSX-1−/− mice produced less IFN-γ than the corresponding population in WT mice following clearance of malaria infection. Combined, these results suggest that IL-27 plays a role in suppressing the terminal differentiation of effector memory CD4+ T cells following malaria infection and concomitantly/consequently maintains the capacity of TEM and TCM cells to produce IFN-γ in specific anatomical locations. The lack of concordance between T-bet expression and IFN-γ production by effector/memory CD4+ T cells was described previously (29).

It was reported recently that STAT3, when activated downstream of IL-10 and IL-21 receptor signaling, programs the transcriptional landscape required to maintain the memory CD8+ T cell phenotype and function (30, 31). As IL-27 is also a strong activator of STAT3, it is foreseeable that IL-27R-driven STAT3 activation may limit the maturation of the memory CD4+ T cell pool. However, as the memory CD8+ T cell population was only marginally affected by ablation of WSX-1 signaling (see Fig. S2 and S3 in the supplemental material), our data reveal some differences in the pathways that maintain memory CD4+ T cells and CD8+ T cells post-malaria infection. Of note, apart from an increase in the expression level of CD25 by hepatic CD4+ CD44+ T cells in WSX-1−/− mice compared with WT mice at 20 weeks p.i., there were no global changes in the repertoire of costimulatory (CD27, CD28, ICOS, 4-1BB, OX40, and CD40L) and regulatory (PD-1, BTLA, and CTLA-4) receptor expression by memory CD4+ T cells in WSX-1−/− mice post-malaria infection that could obviously modulate cellular functionality (results not shown).

Moreover, there were no differences in the numbers of lymphoid tissue inducer cells, in either the spleen or bone marrow of WSX-1−/− mice compared with WT mice, either at homeostasis or at 20 weeks p.i. (results not shown).

In contrast to the above-described results, we have shown an important role for IL-27R signaling in regulating immune responses during the early stages of secondary malaria infection. WSX-1−/− mice exhibited significantly enhanced innate immune responses on day 3 of secondary infection, including heightened levels of production of IL-12p70, TNF, and IFN-γ. Crucially, this cytokine storm was not observed in WSX-1−/− mice during the early phases of primary infection, indicating that the elevated pro-inflammatory response was specifically imprinted by prior parasite exposure and memory immune responses. In agreement, we have shown that antigen-experienced CD4+ T cells produced significantly larger amounts of IFN-γ in the spleen and liver of WSX-1−/− mice and accumulated to higher numbers in the liver of WSX-1−/− mice than in WT mice during rechallenge infection. The increased accumulation of IFN-γ-producing effector/memory CD4+ T cells in the livers of WSX-1−/− mice may reflect either increased in situ proliferation or reduced apoptosis of CD4+ T cells within the inflamed tissue or may reflect changing dynamics of migration from the spleen. To our knowledge, this is the first evidence that IL-27 regulates secondary innate and CD4+ T cell responses and provides new information on the immunological factors that control memory immune responses following malaria infection. As we were unable to measure IL-4 or IL-5 levels in plasma or production by CD4+ T cells, further work is required to determine if Th2-type responses are also amplified in WSX-1−/− mice during secondary malaria infections.

The observation that memory CD4+ T cells produce less IFN-γ in WSX-1−/− mice during the maintenance phase post-primary infection yet produce much higher levels of IFN-γ during secondary infection are, at first impression, contradictory. However, it was shown very recently that pathogen-specific memory CD4+ T cells that produce lower levels of IFN-γ following clearance of primary influenza virus infection are able to mount the strongest IFN-γ responses during secondary infection (32).

We attempted to define whether CD4+ T cell-intrinsic IL-27R signaling regulates the memory CD4+ T cell compartment post-primary infection and/or constrains innate and CD4+ T cell responses during challenge infection. Unfortunately, however, we
were unable to purify sufficient numbers of memory CD4\(^+\) T cells from WT and WSX-1\(^{-/-}\) mice at 20 weeks p.i. for adoptive transfer into naïve and/or infected WT and WSX-1\(^{-/-}\) mice to definitively answer these questions. This, combined with the lack of availability of validated anti-WX-1 antibodies for in vivo use, meant that we were also unable to determine whether IL-27 represses secondary immune responses during challenge infection through active signals mediated during recall and/or whether it preprograms memory CD4\(^+\) T cells for low-level IFN-\(\gamma\) production following reactivation during the maintenance phase. Of relevance, however, it was suggested very recently that following interaction with memory CD4\(^+\) T cells, DCs produce IL-27, which subsequently suppresses naïve T cell activation and proliferation (33). While these data may suggest that priming of naïve CD4\(^+\) T cells may also be modified in WSX-1\(^{-/-}\) mice during secondary infection, contributing to the heightened numbers of CD4\(^+\) CD44\(^+\) IFN-\(\gamma\)-T cells observed in the liver of WSX-1\(^{-/-}\) mice on day 7 of secondary infection, our results also imply that memory CD4\(^+\) T cells may be subject to IL-27R-mediated regulation exerted by conditioned DCs/APCs during malaria infection.

We therefore propose two not necessarily mutually exclusive scenarios that potentially explain the development of heightened anamnestic immune responses in WSX-1\(^{-/-}\) mice. In the first scenario, memory CD4\(^+\) T cells interact with DCs during the very early stages of secondary malaria infection, which, perhaps in combination with other infection-modified environmental signals, leads to accelerated and heightened IL-27 production by DCs/APCs, in comparison to primary infection. Loss of cell-intrinsic WSX-1 expression by memory CD4\(^+\) T cells and consequent loss of IL-27-mediated regulation may then lead to enhanced memory CD4\(^+\) T cell reactivation, including increased IFN-\(\gamma\) production and excessive inflammation. In this scenario, it is foreseeable that direct IL-27R signaling inhibits NF-κBp50 or Zap70 signaling, both of which are necessary for rapid IFN-\(\gamma\) production by memory CD4\(^+\) T cells (34, 35). In the second scenario, loss of WSX-1 receptor expression within the innate system, rather than on memory CD4\(^+\) T cells, drives the heightened anamnestic immune responses observed in WSX-1\(^{-/-}\) mice. In this case, memory CD4\(^+\) T cells, or other memory components, instruct the production of IL-27 by innate cells, which subsequently exerts autocrine or paracrine regulatory activity to limit innate proinflammatory cascade(s), ultimately resulting in the downstream limitation of memory and effector CD4\(^+\) T cell activity. Of note, Foxp3\(^+\) regulatory T cell frequencies and numbers are unimpaired in WSX-1\(^{-/-}\) mice during challenge infection (results not shown), indicating that the enhanced proinflammatory immune responses evident in WSX-1\(^{-/-}\) mice during secondary infection are not due to a deficit in Foxp3\(^+\) T cells. Further work using more tractable systems will be required to fully elucidate how IL-27 controls memory immune responses.

Perhaps most importantly, we have also shown that parasite control was significantly enhanced in WSX-1\(^{-/-}\) mice during re-challenge malaria infection albeit at the cost of increased morbidity, demonstrated by increased weight loss. Acquired immunity to blood-stage malaria infection is believed to depend on CD4\(^+\) T cells (with their ability to secrete IFN-\(\gamma\) making a contribution to their protective role) and antibody (36–38). As we were unable to purify memory CD4\(^+\) T cells from WT and WSX-1\(^{-/-}\) mice for transfer into naïve mice, we are unable to separate the contribution of the memory CD4\(^+\) T cells to protection during secondary infection from any effect of humoral immunity. Of note, however, significantly fewer B cells were present in the spleens and livers of WSX-1\(^{-/-}\) mice than in WT mice at 20 weeks p.i., and malaria-specific IgG levels were similar in WT and WSX-1\(^{-/-}\) mice at all time points (see Fig. S4 in the supplemental material), suggesting that differences in protection of WT and WSX-1\(^{-/-}\) mice against secondary infection are not due to differences in humoral immunity. Moreover, as we have previously shown that increased parasite control in WSX-1\(^{-/-}\) mice during primary infection is CD4\(^+\) T cell dependent but IFN-\(\gamma\) independent (1), our data may indicate that memory CD4\(^+\) T cells confer protection through IFN-\(\gamma\)-independent mechanisms during secondary infection. Conversely, our data might suggest that there are different roles for CD4\(^+\) T cell-derived IFN-\(\gamma\) during primary and secondary malaria infections. Nonetheless, as macrophages are required to control primary Plasmodium yoelii infections (39), and nitric oxide is required for vaccination-induced protection in the Plasmodium chabaudi model (40), it is also probable that the heightened innate immune responses observed in WSX-1\(^{-/-}\) mice contribute to protection during secondary infection.

In summary, we have demonstrated a minor role for IL-27R signaling in regulating the development of the central and effector memory CD4\(^+\) T cell pools following malaria infection and in subsequently controlling proinflammatory antiparasitic immune responses during secondary infection. These data further expand our understanding of the immunoregulatory activities of IL-27R and may implicate an important role for IL-27 in determining the effectiveness of vaccination strategies. Our study provides the foundation for further analysis of the mechanisms by which IL-27 modulates anamnestic immune responses.

ACKNOWLEDGMENTS

The study was supported by the BBSRC (grants 004161 and 020950) and by a Medical Research Council career development award to K.N.C. (G0900487).

We thank Christopher Hunter (University of Pennsylvania) for critically reviewing the manuscript.

REFERENCES


Downloaded from http://iai.asm.org on October 16, 2017 by guest


