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Regulation of the Development of the Hepatic B Cell Compartment during *Schistosoma mansoni* Infection

Keke C. Fairfax, Bart Everts, Amber M. Smith, and Edward J. Pearce

During infection with the helminth parasite *Schistosoma mansoni*, Ab regulates hepatic inflammation, and local production of Ig in the liver appears to play a role in this process. Exploring the development of the B cell response during infection, we found that parasite-specific IgG1-secreting plasma cells appeared first in the hepatic and mesenteric lymph nodes (LNs) and then at later times in the spleen, liver, and bone marrow. The LN B cell population peaked between weeks 10 and 12 of infection, and then contracted at a time that coincided with the expansion of the hepatic IgG1+ B cell compartment, suggesting that B cells migrate from LNs to liver. CXCL9 and -16 expression in the liver increased during the time frame of B cell recruitment. Expression of the CXCL16 receptor CXCR6 was increased on B cells within the hepatic LNs, but not the mesenteric LNs. CXCR3, the receptor for CXCL9, was broadly expressed on IgG1+ B cells in LNs and liver during infection. Increased hepatic expression of CXCL9 and -16 failed to occur if the IL-10R was blocked in vivo, an intervention associated with decreased liver B cell infiltration and the development of severe disease. Hepatic LN IgG1+ cells migrated toward CXCL9 and -16 in vitro and to the liver in a pertussis toxin–sensitive fashion. Our data suggest that the coordinated expression of CXCL9 and -16 in the liver and of CXCR6 and CXCR3 on responding B cells within the hepatic LNs underpins establishment of the hepatic B cell infiltrate during chronic schistosomiasis. The Journal of Immunology, 2013, 191: 000–000.

Schistosomiasis is a neglected tropical chronic disease caused by infection with helminth parasites of the genus *Schistosoma*. These parasites infect >200 million people and are a major cause of morbidity in endemic countries (1–4). Mature *S. mansoni* worms live in the portal vasculature, where females release eggs that pass from the bloodstream into the intestinal lumen, from which they leave the body with the feces, thereby permitting transmission of the infection. However, ~50% of the eggs are carried by the blood flow into the liver, where they become trapped and stimulate a Th2 response (5, 6). This immune response fails to clear the infection, but orchestrates granuloma formation around trapped eggs and induces the alternative activation of macrophages, both of which are essential for host survival (7–10). As Th2-mediated inflammation continues, ongoing production of IL-13 leads to progressive fibrosis that can become life threatening (11, 12). The magnitude of the Th2 response and related hepatic inflammation typically declines later during infection, although in ~5–10% of untreated individuals this modulatory process fails and the severe hepatosplenic form of the disease, linked to excessive fibrosis and portal hypertension, develops (13).

Immune modulation during chronic schistosomiasis is at least in part accounted for by a decline in the proliferative capacity of Th2 cells through a process reminiscent of adaptive tolerance, and which is effectively similar to the T cell exhaustion that is observed in chronic viral and bacterial infections (6, 14–16). Although not instrumental in suppressing Th2 cell responses, IL-10 nevertheless has a role in modulating inflammation during chronic infection (17–20). We recently reported that blocking IL-10R during chronic infection resulted in more severe disease, with the development of portal hypertension in the absence of increased fibrosis (19). This was linked to the loss of IgG1-secreting plasma cells from within the liver, and mirrored the outcome of infection in mice genetically incapable of secreting class-switched Abs (19, 21–23). These findings suggested that the recruitment of IgG1-secreting B cells to the liver during schistosomiasis plays a vital part in regulating inflammation within this organ. However, little is known about the factors controlling B cell entry into hepatic tissues, or the role of IL-10 in this process. In this article, we describe the results of studies that aimed to identify the lymphoid organ of origin of liver-infiltrating B cells during schistosomiasis, and the chemokines responsible for B cell recruitment. Our data support the view that the B cell response that seeds the liver develops within the hepatic lymph node (LN) and suggest that the IL-10–regulated expression of the chemokines CXCL16 and CXCL9 plays an important role in the recruitment of B cells into the liver.

**Materials and Methods**

**Mice and parasites**

BALB/c, BALB/c 4gettKN2 (24, 25), and GFP [BALB/c CByJ.B6-Tg (UBC-GFP)30Scha/J, which express GFP under the direction of the human ubiquitin C promoter in all hematopoietic cells (26)], were bred in-house. All experimental procedures with mice were approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis, MO. Male mice, maintained under specific pathogen–free conditions, were infected at 8–12 wk of age by percutaneous exposure to 35 *S. mansoni* (Puerto Rican strain, Naval Medical Research Institute) cercariae. Soluble egg Ag (SEA) was prepared from isolated schistosome eggs, as previously described (15, 22).

**Flow cytometric analysis**

To analyze hepatic cell populations, livers were removed from PBS-perfused animals, mashed, and incubated in RPMI 1640 (Mediatech) containing 250 μg/ml Collagenase D (Roche) at 37°C for 60 min. The
resulting suspension was disrupted through a 100-µm metal cell strainer and centrifuged through 40-60% isotonic Percoll/RPMI 1640 gradient. The resulting pellet was washed and used for analyses. Spleen and LN cells were harvested and counted as previously described (15). Surface staining with mAbs, acquisition, and analyses were performed essentially as described previously (15). Samples were acquired using a FACSCanto II flow cytometer (BD) and analyzed with FlowJo software (TreeStar). The following mAbs (BD, eBioscience, BioLegend, R&D Systems, or Invitrogen) against mouse Ags were used as PE, PE-Cy5, PE-Cy7, allophycocyanin, phycoerythrin-Cy7, and PE-Cy5: CD4 (RM4-5), CD19 (3D3), CD138 (281-2), IgG1 (Ab5-1), IgD (J43), and IgM (11/41), CXCR6 (221002), CXCR3 (220803), CXCR4 (247506), HuCD2 (RPA-2.10), PD-1 (J43), and CXCR5 (2G8). Biotinylated Abs were secondarily stained with allophycocyanin-Cy7-conjugated streptavidin. Fc-block (anti-mouse CD16/32 clone 93) was used in all experiments to minimize nonspecific signal. Pots shown are on a Logicle scale.

ELISA and ELISPOT

SEA-specific serum IgG1 endpoint titer was determined by ELISA using the IgG1-specific mAb × 56 (BD). Immunol 4HBX plates (Thermo Fisher Scientific) were coated overnight at 4°C with 2 µg/mL SEA, blocked with PBS, and incubated with serial dilutions of sera, followed by a peroxidase-conjugated anti-mouse IgG and ABTS substrate. For ELISPOTs, single-cell suspensions of lymphoid organs or liver-infiltrating cells from infected or control mice were dispersed in RPMI 1640 supplemented with FCS for 24 h in MultiScreen-HA plates (Millipore, Billerica, MA) coated with 2 µg/mL SEA. Bound Abs were detected with HRP-labeled anti-mouse IgG (SouthernBiotech). Bound Ab was detected with the AEC Chromogen Kit (Sigma-Aldrich) per the manufacturer’s instructions, and spots were counted using an ImmunoSpot analyzer (v4.1; Cellular Technology). For ELISPOTs, single-cell suspensions were stained with mAbs, acquired, and analyzed on a FACSCanto II according to the manufacturer’s instructions.

RNA isolation and purification and RT-PCR

Livers were perfused, and 10–20 mg of liver and 10–20 mg of spleen were immediately placed in 0.5 ml TRIzol (Invitrogen). Total RNA was isolated and purified using TRIzol Reagent (Invitrogen), and the resulting supernatant was imaged on a Leica TCS SPE microscope (all staining with mAbs, acquisition, and analyses were performed essentially as described previously (15). Samples were acquired using a FACSCanto II flow cytometer (BD) and analyzed with FlowJo software (TreeStar).

Immunochemistry

Small pieces (0.5 cm³) of PBS-perfused livers were immediately frozen in optimal cutting temperature (OCT) embedding compound (Sakura Fine-tek) over liquid nitrogen, and 10-µm sections were cut on a Leica cryostat. Sections were fixed in ice-cold 75% acetone/25% ethanol for 5 min. Sections were blocked with PBS containing 2% normal mouse serum and 2% normal donkey serum for 1 h at 4°C. Sections were stained with rat anti-mouse VCA-M1 (FITC; Invitrogen), rat anti-mouse F4/80 (BMS, allopheocyanin; BioLegend), and anti-CXCL6, PE-CR3 (R&D Systems) in blocking buffer overnight at 4°C, and then washed with PBS three times before being coverslipped. Slides were imaged on a Leica TCS SPE microscope using Leica LAS AF 2.1.1 software and a 20× objective 0.7 NA at a resolution of 1024 × 1024.

Cell transfers

IgG1+ B cells were sorted from 12-wk infected GFP or BALB/c mice based on surface expression of CD19 and IgG1, using a BD FACSaria, and collected in complete media. Sorted cells were > 95% pure. Cells were washed five times in phenol-red-free HBSS, and 150,000-200,000 cells (depending on the experiment) were transferred i.v. into 12- or 13-wk infected BALB/c recipient mice. For BALB/c donors, cells were incubated with CellTrace Violet (Invitrogen) according to the manufacturer’s instructions and washed before transfer. In some experiments, cells were incubated with 200 ng/ml pertussis toxin (List Biological Laboratories) for 1 h before transfer. Recipients were euthanized 40 h post transfer, and livers were perfused with PBS and processed as described above. Single-cell suspensions were stained with CD19 and IgG1, and either fixed and stained with anti-FITC or immediately acquired on a BD FACScanto II. Data were analyzed as described above.

Statistical analyses

Data were analyzed with the unpaired Student t test, a one-way, or a two-way ANOVA via Prism 6.0 (GraphPad Software). All data shown represent mean ± SEM, and p ≤ 0.05 was considered statistically significant.

Results

The B cell response due to infection with S. mansoni peaks between weeks 10 and 12 post infection

In previous work, we had noted increases in numbers of liver-infiltrating IgG1+ B cells and serum titers of schistosome Ag-specific IgG1 between weeks 10 and 16 of infection (19). To begin to establish where IgG1-producing B cells are being generated during infection, we used flow cytometry to assess numbers of CD19+IgG1+ B cells and IgD−CD138+ plasma cells in lymphoid organs that drain sites of schistosome infection. In this regard, we focused on the mesenteric LNs and additionally the hepatic LNs (a LN that has not been extensively studied in the context of schistosomiasis). Because schistosomes are intravascular parasites, we also examined the spleen. We compared uninfected mice to infected mice at 2-wk intervals between weeks 8 and 16 post infection. Week 8 of infection is generally acknowledged to represent the time point at which numbers of IL-4-producing Th2 cells peak and granulomatous inflammation is at its most intense, whereas by week 16 of infection, the Th2 immune response and granulomatous inflammation are fully modulated (15). We found that the overall cellularity of the hepatic LNs changed significantly over time post infection (p = 0.001; ANOVA). The LN size increased over baseline at week 8 of infection but underwent further expansion by weeks 10 and 12 (Fig. 1A). Thereafter, the cellularity of this LN decreased until by week 16 it had almost returned to baseline (Fig. 1A). The mesenteric LNs and spleen also underwent significant changes in cellularity over time (p = 0.003 and p = 0.0001, respectively; ANOVA), and both peaked in size between weeks 10 and 12 post infection and decreased thereafter, albeit at different rates, with the mesenteric LN losing cellularity at a faster rate than the spleen, which remained greatly enlarged over baseline at week 16 (Fig. 1A). The kinetics of cellular accumulation in the liver were different, in that over the same period the splenic mononuclear cell population dramatically increased and then remained stable at a level that was > 6-fold higher than in naive mice (these changes were significant at p = 0.0003; ANOVA). We next focused our analysis of the B cell compartment on the development of the IgG1 response. This isotype is most abundant during S. mansoni infection (28, 29) and has been implicated in the modulation of liver inflammation in this disease (19, 22, 30). As would be expected, the frequency of IgG1+ B cells was low in the hepatic organs of naive mice (Fig. 1B). Analysis of IgG1+ B cells and plasma cells in the hepatic and mesenteric LNs, as well as the liver, revealed sequential increases first in surface IgG1+ B cells, peaking at week 10, followed by plasma cells, peaking at week 12 (Fig. 1B–D; changes in these cellular populations over time were all significant at p ≤ 0.05; ANOVA). In contrast, numbers of both cell types increased progressively within the spleen between weeks 8 and 14 (Fig. 1B–D; p = 0.003; ANOVA). Representative FACS plots for the key time points of
weeks 10 and 12 are shown in Fig. 1B and 1C, where the transition from a B cell compartment dominated by CD19⁺IgG1⁺ B cells to one dominated by IgD⁺CD138⁺ plasma cells in LNs, and the accumulation of plasma cells in the liver between weeks 10 and 12, are illustrated.

A wave of SEA-specific Ab-secreting cell development in reactive LNs precedes the accumulation of SEA-specific Ab-secreting cells in the liver and bone marrow

There are two recognized fates for class-switched (surface IgG1⁺) B cells in germinal center reactions: differentiation into memory B cells, which remain surface IgG1⁺ and enter the circulation, or differentiation into plasmablasts that either become short-lived plasma cells in the LN or enter the circulation and migrate to the bone marrow to become long-lived plasma cells (31, 32). On the basis of the sharp increase in plasma cell numbers in the LNs, liver, and bone marrow following prior peaks in IgG1⁺ B cells (Fig. 1B–D), it seemed likely that the contraction of the IgG1⁺ B cell populations in the hepatic and mesenteric LNs between weeks 10 and 12 of infection reflected differentiation into plasma cells. To further address these possibilities, we examined the kinetics of the development of the SEA-specific IgG1 Ab-secreting cells (ASCs) following week 10 of infection. At week 10, we detected small numbers of SEA-specific ASCs in the hepatic LNs (Fig. 2A) and, to a lesser extent, in the mesenteric LNs (Fig. 2B) and spleen (Fig. 2C). These numbers increased significantly by week 12 in the LNs (p ≤ 0.05 comparing week 10 to week 12; Student t test) and trended in the same direction in the spleen, and declined thereafter back to week 10 levels by weeks 14–16 of infection, consistent with these cells being short-lived plasma cells. Overall, a higher percentage of plasma cells in the hepatic LNs were SEA-specific IgG1-secreting ASCs than was the case in the mesenteric LNs (Fig. 2D; p = 0.003; ANOVA), suggesting that greater priming for SEA-specific responses occurs in the hepatic LN.

Between weeks 14 and 16, we detected a significant increase in the number of SEA-specific ASCs in the bone marrow (Fig. 2E;
The SEA-specific B cell population undergoes significant expansion in the hepatic and mesenteric LNs between weeks 10 and 12 post infection, coincident with the expansion of Tfh cell (TFH) populations in these organs, and SEA-specific ASCs subsequently accumulate in the liver and bone marrow. Single-cell suspensions of hepatic and mesenteric LNs, bone marrow, and perfused livers were assayed for total SEA-specific IgG1-secreting cells by ELISPOT at the indicated weeks post infection (A-C, E and G). Data shown are the mean and SEM of 6–10 individual mice. (D) Percentages of total plasma cells in the hepatic and mesenteric LNs that are positive for SEA-specific IgG1 at the indicated time points. (F) Serum SEA-specific IgG1 Ab titers at indicated weeks post infection. Data shown are from individual mice with mean values ± SEM. (H) Cells isolated from livers of 14-wk infected mice were stained for surface IgG1, CD19, IgD, and CD138 and analyzed by flow cytometry; data shown are gated on IgD− cells and concatenated from four to five mice. (I and J) Isolated hepatic and mesenteric LN cells from infected BALB/c-4get/KN2 mice were analyzed by flow cytometry for expression of the markers indicated. Data shown in (I) are from gated CD4+ T cells or CD4+GFP+ T cells. Data shown are concatenated from three mice per group. Numbers show the percentages of cells that fall within indicated gates. (J) Numbers of GFP+HuCD2+ and GFP+CXCR5+PD-1+CD4+ T cells within the hepatic and mesenteric LNs of infected mice at times indicated. Data represent mean ± SEM of results from three mice per group. Experiments in (A)–(H) were performed at least three times.

\[ p = 0.01, \text{Student } t \text{ test}, \] which is consistent with the view that the wave of B cell responsiveness in the major reactive LNs between weeks 12 and 14 (Fig. 2A, 2B) seeds the establishment of a population of long-lived plasma cells within the bone marrow. This process is consistent with an ongoing increase in titers of SEA-specific IgG1 over this time (Fig. 2F). During approximately the same time period, we also observed a marked increase in the number of SEA-specific ASCs in the liver (Fig. 2G; \( p = 0.0004; \) Student \( t \) test). We found that > 50% of the IgG1+ CD19+ cells in the liver at week 14 post infection expressed the plasma cell marker CD138, which was in contrast to the situation in reactive LNs, where cells costaining for these markers were rare (Fig. 2H). These data suggest that IgG1+CD19+ cells in the liver may be B cells in the process of becoming plasma cells in situ. The development of peak IgG1-producing \( S. \) mansoni–specific B cell population between weeks 10 and 14 post infection is late relative to the reported kinetics of the development of the infection-induced Th2 response, which reaches a maximum at week 8.
We reasoned that this could reflect a discordance between the peak Th2 cell response and T follicular helper cell (Tfh cell) response, because the latter cells are responsible for helping B cells to class-switch, and therefore we analyzed the numbers of Tfh cells in the hepatic and mesenteric LNs at week 8, and again at week 10, the time point at which the B cell numbers in these organs begin to increase significantly. For these experiments, we took advantage of 4get/KN2 mice, in which transcription of the IL-4 gene is marked by GFP production and secretion of IL-4 protein is indicated by surface expression of human CD2 (24, 25). In these mice, CD4⁺PD1⁺CXCR5⁺ Tfh cells responding to SEA are also committed to IL-4 production, and marked by GFP and huCD2 expression (33). Consistent with the timing of increases in IgG1-producing B cell numbers in reactive LNs, we found significantly increased frequencies (Fig. 2I) and numbers (Fig. 2J) of IL-4–secreting Tfh cells in the hepatic LNs and, to a lesser extent, the mesenteric LNs, at week 10 compared with week 8.

**IL-10–dependent expression of CXCL16 and CXCL9 in the liver as a result of infection**

Our data show that B cells infiltrate the liver during infection, resulting in an accumulation of SEA-specific ASCs. The chemokines CXCL9, -10, -11, -12, and -16 have been implicated in either B cell trafficking or the infiltration of immune cells into the liver (34–39), so to begin to understand how B cells enter the liver during schistosomiasis, we asked whether expression of any of these is induced in the liver during infection. We found that expression levels of CXCL16 increased significantly as a result of infection (p = 0.0042; Fig. 3A). The small elevations in CXCL9 and 10 were not statistically significant. In contrast, expression of CXCL12 was decreased as a result of infection (p = 0.0045; Fig. 3A), and there was no significant change in CXCL11 expression (Fig. 3A), indicating that these chemokines are not playing a role in leukocyte recruitment. The decrease in CXCL12 expression is especially interesting, as this chemokine is typically thought to drive activated B cell migration (35, 36, 40).

On the basis of the extent of the increase in CXCL16 expression in the liver owing to infection, we used microscopy to identify the cellular source of this chemokine. In fresh-frozen sections of naive liver, CXCL16 was evident as punctate staining in VCAM-1–, positive cells that presumably are endothelial cells (Fig. 3B). In contrast, during chronic infection, expression was clearly increased and largely localized throughout the cytoplasm of F4/80⁺ cells with macrophage-like morphology (Fig. 3B), numbers of which are significantly increased during infection (19, 41). Macrophages have also been implicated in the production of CXCL16 in the lungs (42) and synovial fluid (43) of rheumatoid arthritis patients. Because CXCL9 transcripts were also elevated during infection, we measured chemokine protein levels in liver tissue before and after infection. We found that CXCL9 was elevated significantly (p ≤ 0.0001) by 16 wk post infection (Fig. 3C). We were unable to localize CXCL9 in frozen sections, so at this time we are unclear about whether the same cells make both CXCL16 and CXCL9.

We reported previously that IL-10 plays a crucial role in the recruitment of B cells into the liver during chronic schistosomiasis (19). We therefore asked whether IL-10R blockade had an effect on the expression of chemokines within the liver. As expected, Ab-mediated blockade of IL-10R decreased the frequency of IgG1⁺ B cells in the liver during chronic infection, although it did not affect the frequency of these cells within the mesenteric LNs (Fig 3D). We reasoned therefore, that if CXCL9, -10, and -16 are...
playing a role in recruitment of B cells into the liver, their expression might be affected by IL-10R blockade. We found that treatment with anti-IL-10R Ab inhibited infection-induced increases in expression of CXCL9 and CXCL16 (p = 0.0037 and p = 0.0119 respectively; Fig. 3E) but did not significantly affect CXCL10 expression, whereas CXCL11 and CXCL12 were not detectable in either group. Taken together, these data implicate CXCL9 and -16 in the IL-10R–dependent recruitment of B cells into the liver.

CXCR6 is preferentially expressed on the surface of IgG1+ B cells in the liver and hepatic LNs

We reasoned that if CXCL16 and CXCL9 are responsible for recruitment of B cells to the liver during infection, then expression of their receptors CXCR6 and CXCR3, respectively, should be measurable on IgG1+ B cells. We found that in naive mice B cell expression of CXCR6 is restricted to those cells recovered from the liver (Fig. 4). By 10 wk of infection, IgG1+ B cells from the liver, spleen, and hepatic, and mesenteric LNs expressed CXCR6, but by weeks 12 to 14, expression of CXCR6 was most prominent in subsets of IgG1+ B cells in the hepatic LNs (46% and 30% of the population positive for surface CXCR6) and liver (70% and 53% of the population positive for surface CXCR6) (Fig. 4), compared with these populations in the mesenteric LNs (32% and 17%) and spleen (33% and 11%) at these time points. These data suggest that CXCR6 is an important chemokine receptor for B cell recruitment and retention in the liver, and indicate that cells from the hepatic LNs may play a dominant role in seeding this liver population. The expression pattern of CXCR3 in naive mice was the inverse of that of CXCR6, with cells from the lymphoid organs, but not the liver, expressing this receptor. In infected mice, at all times examined, CXCR3 expression persisted in IgG1+ B cells from liver and lymphoid organs, with the cells from the LNs and spleen being indistinguishable in their staining for this receptor. Because CXCR4 (the receptor for CXCL12) has previously been shown to be important for recruiting plasma cells to the bone marrow and activated Ag-specific B cells to inflamed tissues, we also examined the kinetics of expression of this receptor, and found that it essentially mirrored that of CXCR6, with uniform upregulation at 10 wk post infection in all examined organs, followed by downregulation in the mesenteric LNs and spleen, and a biphasic expression in the hepatic LNs and liver that persists at 14 wk post infection (Fig. 4). CXCR4 is not expressed on IgG1+ B cells from naive mice in any organ.

IgG1+ B cells from reactive hepatic, but not mesenteric, LNs are capable of entering the liver in infected mice

On the basis of the findings outlined above, we reasoned that the B cell response that seeds the liver with IgG1-producing cells develops primarily in the hepatic and mesenteric LNs, and that cells are subsequently recruited to the liver via the interaction of CXCL9 and -16 made in the liver with CXCR3 and -6 on the surface of IgG1+ B cells. To directly address the abilities of IgG1+ B cells from the hepatic and mesenteric LNs to migrate into the liver during infection, we sorted CD19+IgG1+ cells from these organs from 12-wk infected GFP mice and transferred them into infection-matched or naive BALB/c mice and used flow cytometry to measure the accumulation of GFP+ cells in the liver 40 h later. GFP+IgG1+ B cells sorted from the hepatic LNs were detected in liver tissues of infected, but not naive, recipients, whereas by comparison few cells from the mesenteric LNs were detected in the liver following transfer into either infected or naive recipients (Fig. 5A, 5B). To specifically ask whether the lack of migration of IgG1+ B cells from the mesenteric LNs is due to chemokine nonresponsiveness, we assayed the ability of CD19+IgG1+ cells from either the hepatic or the mesenteric LNs of 12-wk infected mice to migrate across a Transwell toward either CXCL16 (Fig. 5C) or -9 (Fig. 5D). IgG1+ B cells from the hepatic, but not the mesenteric, LNs were able to migrate toward both CXCL16 and -9. The lack of migration of the mesenteric LN cells toward CXCL9 is surprising, as these cells upregulate CXCR3 (Fig. 4), and suggests that they may be responsive to another ligand for this receptor (CXCL10 or -11) that is not as highly expressed in the liver during infection. To determine whether the ability of IgG1+ cells to migrate to the liver of infected mice was chemokine receptor dependent, we sorted IgG1+ B cells from the hepatic LNs of GFP mice at 11.5 wk post infection and incubated them with or without pertussis toxin for 1 h before transferring them into infection-matched recipients. Pertussis toxin blocks G-coupled protein-dependent signaling and has previously been used to show that cellular migration is chemokine receptor dependent (44, 45). At 40 h post transfer, the blood and livers of recipient mice were analyzed for the presence of GFP+ cells. IgG1+ B cells incubated with PBS prior to transfer were detected in the liver (Fig. 5E, 5F) and, to a lesser degree, in the blood. In contrast, incubation with pertussis toxin prior to transfer resulted in a failure of the cells to reach the liver, as well as increased frequency of cells within the blood (Fig. 5E, 5F). The sensitivity of the migration of these IgG1+ B cells to pertussis toxin suggests that their migration is chemokine receptor dependent.

Discussion

Although the kinetics of the CD4+ T cell response in murine schistosomiasis are well characterized (12, 15), to the best of our knowledge this is the first detailed report of the development of the IgG1 class-switched B cell response induced by schistosome infection. We found that the peak IgG1+ B cell response occurs between weeks 10 and 12, and is focused in the hepatic and

FIGURE 4. Differential chemokine receptor expression on IgG1+ B cells from different reactive organs during infection. Single-cell suspensions from the indicated organs from infected mice were stained with Abs against CXCR6, CXCR3, CXCR4, IgG1, and CD19 to quantify the level of chemokine receptor expression on class-switched B cells at the indicated weeks post infection. Plots are concatenated from data from three to five mice per group. Each experiment was performed at least three times. Gray lines on each plot delineate the positive population based on isotype control staining.
mesenteric LNs. These kinetics are quite different from those of the infection-induced Th2 cell response, which, based largely on analyses of splenic populations, has been reported to peak at week 8 post infection. However, when we focused more specifically on the IL-4–secreting Tfh cell compartment, which is presumably primarily responsible for providing help to B cells for IgG1 class switching, we discovered that Tfh cell numbers in hepatic and mesenteric LNs increased significantly between 8 and 10 wk post infection. The correlation between this expansion of IL-4–secreting Tfh cells and a subsequent increase in IgG1 class switching suggests that a threshold level of IL-4 protein in the LN may be necessary for optimal production of \textit{S. mansoni}–specific IgG1 + B cells, and that the conditions necessary to stimulate this IL-4 secretion are not present until \( \sim 10 \) wk post infection. This possibility is consistent with recent work showing that Ag dose controls the development of the Tfh cell compartment (46) and other work showing that IgG1+ cells develop in an IL-4 dose-dependent manner in vivo (47), which suggests that in the case of \textit{S. mansoni} infection, numbers of IL-4–secreting Tfh cells and a subsequent increase in IgG1 class switching suggest that a threshold level of IL-4 protein in the LN may be necessary for optimal production of \textit{S. mansoni}–specific IgG1+ B cells, and that the conditions necessary to stimulate this IL-4 secretion are not present until \( \sim 10 \) wk post infection. This possibility is consistent with recent work showing that Ag dose controls the development of the Tfh cell compartment (46) and other work showing that IgG1+ cells develop in an IL-4 dose-dependent manner in vitro (47), which suggests that in the case of \textit{S. mansoni} infection, numbers of IL-4–secreting Tfh cells sufficient to induce a strong IgG1 response may develop only after weeks of accumulating antigenic stimulation associated with ongoing infection. The timing of development of the peak Tfh cell/IgG1+ B cell response coincides with the well-documented downregulation of granulomatous inflammation that occurs during chronic schistosomiasis, suggesting that the two events may be linked (19).

We consistently found hepatic IgG1+ B cells prior to the development of a significant population of hepatic plasma cells. A recent report documented the recruitment of memory B cells into inflamed lung tissues during influenza reinfection, where they proceeded to differentiate into IgG- and IgA-secreting plasma cells (48). Our findings could reflect an analogous situation in schistosome-infected mice in which memory B cells, detectable as CD19+ surface IgG1+ cells in the liver, \( > 40\% \) of which are positive for the memory cell surface marker CD38 (data not shown), differentiate in situ into plasma cells. Plasmablasts, which are a transitional stage between memory B cells and plasma cells, can simultaneously express CD19 (normally downregulated on plasma cells) and the plasma cell marker CD138 (49, 50). We found that by week 14 post infection, the majority of surface IgG1+ B cells in the liver had also upregulated CD138, suggesting that they are in the process of differentiating into plasma cells, a situation that is not evident in the LNs at the same time of infection. We postulate that this difference reflects the distinction between plasma cell generation in a reactive germinal center and the generation of plasma cells from activated or memory B cells within the liver (48, 51). It is worth noting that the development of plasma cells in the lungs during influenza occurs within tertiary lymphoid structures, which to date have not been identified in the liver during schistosomiasis; this is an area that clearly requires further investigation. Nevertheless, the maintenance of SEA-specific ASCs within the liver during chronic infection, combined with our prior finding of plasma cells resident in the livers of naïve mice (19), raises the possibility that the liver may act as a repository for long-lived plasma cells. It is also important to note that we found surprisingly high numbers of surface IgG1+ B cells in the liver in the steady state, suggesting that memory B cells may reside in the liver in the absence of an inflammatory stimulus.

**FIGURE 5.** IgG1+ cells from the hepatic LNs, but not the mesenteric LNs, are able to migrate toward CXCL9 and CXCL16 in vitro, and to the liver in vivo, via a pertussis toxin–sensitive mechanism. CD19+IgG1+ cells from GFP mice infected for 12 wk were sorted from the hepatic and mesenteric LNs and either transferred to 12-wk infected or naïve BALB/c recipients (A and B) or assayed for the ability to migrate toward CXCL16 (C) or CXCL9 (D) in a Transwell assay. At 40 h post transfer, recipient livers were analyzed for the presence of GFP+ donor cells by flow cytometry (A), and total donor cells were quantified (B). (E and F) CD19+IgG1+ cells from GFP mice infected for 12 wk were sorted from the hepatic LNs and incubated with or without pertussis toxin and transferred to 13-wk infected BALB/c recipients. At 40 h post transfer, recipient blood samples (live) and liver single-cell extracts (fixed samples stained with an anti-GFP secondary Ab) were analyzed for the presence of GFP+ donor cells by FACS (E), and total donor cells were quantified (F). The transfer experiment in (A) and (B) was performed three times, whereas the transfer experiment in (E) and (F) was performed twice.
CXCR4 and its ligand CXCL12 have traditionally been thought to drive the homing of activated B cells and plasma cells to inflamed tissues and the bone marrow (35, 36, 40), so we were surprised to find that expression of CXCL12 was decreased in the liver as a result of infection, whereas expression of CXCL9 and -16 increased (Fig. 3). In light of this, it seems unlikely that CXCR4 could be responsible for recruiting B cells into the liver over the long term during infection. Our findings therefore suggest that B cell migration to the liver uses a program distinct from the process of B cell recruitment and retention in the bone marrow and lymphoid tissues identified previously (35, 36, 52, 53). Rather, the patterns of expression of CXCR3 and CXCR6 on B cells from the liver and LN s, along with the induced, IL-10R–dependent expression of CXCL9 and -16, suggest that these chemokine/receptor pairs are most important for the recruitment of IgG1+ B cells into the liver. Our ability to visualize CXCL16 expression in cells that are VCAM-1+ and underlie the vasculature in the livers of naïve mice provides a possible recruitment mechanism for IgG1+ B cells into hepatic tissues in the absence of overt inflammation. The finding that the cellular source for CXCL16 during infection is altered so that, in addition to VCAM-1+ cells, macrophage-like cells also produce CXCL16, can be interpreted as support for the importance of this chemokine in B cell recruitment during S. mansoni infection. Moreover, the findings that CXCR6 is expressed by IgG1+ B cells in the liver in the steady state, and that its expression increases as a result of infection, along with the reported importance of CXCR6 for NK cell recruitment into the liver (39), suggest that CXCR6 may specifically drive liver homing for all lymphocytes, and that chemokine-driven migration may be organ dependent rather than cell type dependent (34), so that for B cells CXCR3 expression and CXCR6 expression work in concert to drive class-switched B cell migration to the liver.

Our data indicate that, during chronic schistosomiasis, the hepatic and mesenteric LN s develop numerically similar populations of IgG1+CD19+ B cells and SEA-specific ASCs. Nevertheless, these populations of IgG1+ cells differ in that those from the hepatic LN s expressed CXCR6 and could migrate to the liver, whereas those in the mesenteric LN s did not express this receptor and could not migrate to this organ. We interpret these data to indicate that the hepatic B cell population that plays an important protective role during infection originates in the hepatic LN s. We postulate that IgG1+ B cells differentiate into plasma cells both within the hepatic LN s and after migration to the liver. The disposition of IgG1+ B cells originating in the mesenteric LN s remains unknown at present, but these cells possibly seed plasma cell populations in other organs, such as the bone marrow or intestine. The mechanism that underlies the difference in CXCR6 upregulation of B cells from the hepatic and mesenteric LN s remains to be determined.

In summary, the general and Ag-specific class-switched B cell response develops surprisingly late following S. mansoni infection. We believe this timing is due to the delayed development of sufficient IL-4–secreting Th2 cells in both the hepatic and mesenteric LN s. The B cell populations that develop in these two LN s have distinct migrational abilities and fates, possibly owing to differences in the Th2 cell populations in the two LN s, as only the cells primed in the hepatic LN s are competent to migrate to the liver during infection. Liver expression of CXCL9 and -16 is dramatically upregulated over the course of S. mansoni infection in an IL-10R–dependent manner. This upregulation underlies the development of the S. mansoni–specific hepatic B cell compartment from B cells that are primed in the hepatic LN s. This observation is in agreement with a growing body of literature that suggests the existence of tissue-specific B cell niches (48, 54).

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Disclosures
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