Loss of Dendritic Cell Migration and Impaired Resistance to *Leishmania donovani* Infection in Mice Deficient in CCL19 and CCL21[1]

Manabu Ato,[2]* Asher Maroof,[3]* Soombul Zubairi,[3]* Hideki Nakano,[4†] Terutaka Kakiuchi,[†] and Paul M. Kaye[3,5]*

The encounter between APC and T cells is crucial for initiating immune responses to infectious microorganisms. In the spleen, interaction between dendritic cells (DC) and T cells occurs in the periarteriolar lymphoid sheath (PALS) into which DC and T cells migrate from the marginal zone (MZ) along chemokine gradients. However, the importance of DC migration from the MZ into the PALS for immune responses and host resistance to microbial infection has not yet been elucidated. In this study, we report that following *Leishmania donovani* infection of mice, the migration of splenic DC is regulated by the CCR7 ligands CCL19/CCL21. DC in *plt/plt* mutant mice that lack these chemokines are less activated and produce less IL-12, compared with those in wild-type mice. Similar findings are seen when mice are treated with pertussis toxin, which blocks chemokine signaling in vivo. *plt/plt* mice had increased susceptibility to *L. donovani* infection compared with wild-type mice, as determined by spleen and liver parasite burden. Analysis of splenic cytokine profiles at day 14 postinfection demonstrated that IFN-γ and IL-4 mRNA accumulation was comparable in wild-type and *plt/plt* mice. In contrast, accumulation of mRNA for IL-10 was elevated in *plt/plt* mice. In addition, *plt/plt* mice mounted a delayed hepatic granulomatous response and fewer effector T cells migrated into the liver. Taken together, we conclude that DC migration from the MZ to the PALS is necessary for full activation of DC and the optimal induction of protective immunity against *L. donovani*. *The Journal of Immunology,* 2006, 176: 5486–5493.

The encounter between Ag-bearing dendritic cells (DC)[6] and naive T cells is one of the most important events for inducing immune responses (1). This event mainly occurs in the T cell area of secondary lymphoid organs, and localization of both DC and T cells is regulated by chemokine signaling (2, 3). In the spleen, the chemokines that attract T and B cells are differentially expressed by stromal cells in the T or B cell zone, respectively. Stromal cells in the T cell area constitutively express both CCL19 (formerly EBV-induced molecule 1 ligand chemokine) and the serine isoform of CCL21-Ser (formerly secondary lymphoid organ chemokine) (4). Both of these chemokines bind to CCR7, which is expressed on naive/central memory T cells and mature DC (5–7). Thus, following gradients of these chemokines, both DC and T cells migrate into the T cell area, where they interact and T cell priming occurs (8). The importance of chemokine-dependent migration is exemplified in mice with genetic deficiency in either chemokines or their receptors (9, 10). The paucity of lymph node T cell (*plt*) mutation, which arose as a spontaneous recessive mutation in mice, was recently mapped to the chemokine locus on chromosome 4 and results in loss of functional CCL19 and CCL21-Ser genes and in an aberrantly formed lymphoid T cell zone (11). As a consequence of lack of migration in response to these chemokines, *plt/plt* mice have impaired ability to recruit naive T cells and DC into the T cell areas of secondary lymphoid organs (9, 10).

Infection of mice with amastigotes of *Leishmania donovani* is a well-established experimental model of visceral leishmaniasis (12). Injected amastigotes are mostly phagocytosed by macrophages in the marginal zone (MZ) of the spleen, and perhaps rarely by DC (13). We and other groups have shown previously that early production of IL-12, localized by immunochemistry to DC in the T cell area of the spleen (the periarteriolar lymphoid sheath (PALS)), plays a key role in the innate and Ag-specific immune response against *L. donovani* (14, 15). IL-12 plays a key role in skewing naive T cells into IFN-γ-producing Th1 cells, and IFN-γ is essential for the activation of macrophages and elimination of parasites (16, 17). These findings suggest that DC in the MZ, which either acquire parasites or parasite-derived Ags, are stimulated to produce IL-12p40 and IL-12p70, migrate into the PALS, and induce effector T cells. However, the sequence of such events, at which the site of IL-12 induction is initiated, which cytokines regulate DC migration in this context, and the requirement for DC migration in the priming of host protective T cells all remain unknown.

To address some of these questions, we have now investigated the course of *L. donovani* infection using CCL19/21-deficient *plt/*

*Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom; and †Department of Immunology, Toho University School of Medicine, Tokyo, Japan

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[2] Current address: Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-0864, Japan.

[3] Current address: Immunology and Infection Unit, Hull York Medical School, and Department of Biology, University of York, P.O. Box 373, York YO10 5YW, U.K.

[4] Current address: Department of Immunology, Duke University Medical Center, Durham, NC 27710.

[5] Address correspondence and reprint requests to Dr. Paul M. Kaye, Department of Biology, University of York, P.O. Box 373, York YO10 5YW, U.K. E-mail address: pmk2@york.ac.uk

[6] Abbreviations used in this paper: DC, dendritic cell; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LCMV, lymphocytic choriomeningitis virus; MZ, marginal zone; MZM, MZ macrophages; PALS, periarteriolar lymphoid sheath; p.i., postinfection; PTX, pertussis toxin; STAg, soluble *Toxoplasma* Ag.

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plt mice. We demonstrate that *plt/plt* mice are more susceptible to *L. donovani* infection than normal mice. DC activation is limited early after infection, accompanied by lack of DC migration from the MZ to the PALS. These defects in early DC activation in *plt/plt* mice were mirrored by enhanced susceptibility to infection and elevated IL-10 mRNA accumulation and, in the liver, granuloma formation was delayed and effector $CD4^+$ and $CD8^+$ T cell recruitment limited. Taken together, these data indicate that chemokine-dependent encounters between DC and T cells are critical for optimal protection against *L. donovani* infection.

Materials and Methods

Mice and parasites

The *plt*/*plt* mice backcrossed to the C57BL/6 (B6) background were provided by H. Hengartner and T. Junt (University of Zurich, Switzerland) and bred at the London School of Hygiene and Tropical Medicine under barrier conditions. B6 mice were purchased from Charles River Laboratories and were housed under specific pathogen-free conditions. *L. donovani* (LV9) amastigotes were isolated from infected hamsters, as previously described (18). Mice were infected at 6–8 wk of age by injecting 2×10^7 or 2×10^8 amastigotes i.v. via the lateral tail vein. Mice were killed by cervical dislocation and parasite burden in livers and spleens determined from Giemsa-stained impression smears. Parasite burden was expressed in Leishman-Donovan units (18). A total of 500 ng of pertussis toxin (PTX; List Biological Laboratories) in saline was administrated by i.p. injection twice, at 1 and 3 days before *L. donovani* infection. All animal procedures were approved by the London School of Hygiene and Tropical Medicine Animal Procedures Ethics Committee and under license from the U.K. Home Office.

Flow cytometry

Spleens were harvested and digested in RPMI 1640 (Invitrogen Life Technologies) containing 0.05% collagenase (Worthington Biochemical) and 100 μg/ml DNase I (Sigma-Aldrich) at 37°C for 30 min. After washing with calcium-free medium, cells were stained for FITC-, PE-labeled, or biotinylated CD11c (HL3), $I\text{-}A^b$ (M5/117 and 2G9), CD40 (3/23), CD80 (16-10A1), and CD86 (GL1) (BD Pharmingen), followed by incubation with allophycocyanin-labeled streptavidin. Cells were analyzed using a FACSCalibur (BD Biosciences).

Immunohistochemistry

Immunohistochemistry was performed on 6-mm frozen sections, as described before (19). Primary Abs were purified or biotinylated HL3 (mouse CD11c), 53-6.7 (CD8a), RM4-5 (CD4), Gr-1 (RB6-8C5) (BD Pharmingen), CI:A3-1 (anti-F4/80), FA-11 (CD68), 3D6.112 (anti-CD169) (Serotec), C17.8 (anti-IL-12p40), ER-TR9 (anti-specific ICAM-3 grabbing non-integrin-related 1; a gift from G. Kraal, Free University, Amsterdam, The Netherlands), or hamster antisera to *L. donovani* amastigotes. Secondary Abs were biotinylated rabbit anti-rat IgG (Vector Laboratories), 10% mouse serum-containing goat anti-hamster IgG (for *L. donovani*; Vector Laboratories), biotinylated rabbit anti-rat Ig (for confocal microscope; DakoCytomation), Alexa 488-conjugated goat anti-hamster IgG, or Alexa 546-conjugated streptavidin (Molecular Probes). As appropriate, sections were developed with Vector Elite-ABC kit, followed by Vector 3,3′-diaminobenzidine substrate kit (Vector Laboratories), or directly viewed using a Zeiss LSM510 confocal microscope. In some experiments, mice were injected i.v. with 200 μl of 5% (v/v in 0.9% NaCl) india ink (Rowney) to allow visualization of MZ macrophages in the spleen.

Restimulation assay

Spleens from mice infected for 7 days were digested with collagenase, as described above. Dead cells and RBC were removed using a Histopaque 1083 (Sigma-Aldrich) density gradient. A total of 2×10^5 spleen cells was incubated with or without 2×10^6 paraformaldehyde-fixed *L. donovani* amastigotes in 10% FCS (Sigma-Aldrich) containing RPMI 1640 medium in a 24-well plate at 37°C for 72 h. For proliferation assays, the cells were pulsed with 1 μCi of [^{3}H]thymidine for final 6 h of culture and then harvested onto glass fiber filters. The incorporated [^{3}H]thymidine was determined by liquid scintillation spectrometry (20). Supernatants from the assays were collected after a 72-h incubation. IFN-γ-specific ELISA was performed using IFN-γ ELISA kit (R&D Systems), as described previously (20).

Evaluation of granulomatous response

Paraffin sections of liver tissue, stained with H&E, were prepared by conventional methods. In some cases, frozen sections of liver tissue were stained with hamster anti-*L. donovani* serum. The granuloma response in the infected livers was graded as follows: 1) an infected Kupffer cell without cellular infiltrate; 2) an immature granuloma composing an infected Kupffer cell surrounded by a few inflammatory cells, but without organization; 3) a mature granuloma having an organized structure; or 4) an empty granuloma, in which amastigotes had been killed as a result of effective antileishmanial immunity (21). At least 25–50 high magnification fields per mouse were counted and evaluated.

Real-time RT-PCR

RNA was isolated from spleen tissue using an RNeasy Mini Kit with on-column DNase digestion (Qiagen), according to the manufacturer's instructions. RNA was reverse transcribed into cDNA, as described previously (22). Oligonucleotides (5′-3′) used for specific amplification were: IL-4, CCTCACAGCAACGAAGAACA (sense) and TGGACTCATTC ATGGTGCAG (antisense); IL-10, AGGGTTACTTGGGTTGCCAA (sense) and CACAGGGGAGAAATCGATGA (antisense); IL-12p40, GGAAGCACGGCAGCAGAATA (sense) and AACTTGAGGGAGAAG TAGGAATGG (antisense); IFN-γ, CCTCCTGCGGCCTAGCTC (sense) and TAACAGCCAGAAACAGCCATG (antisense); and for amplification of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were GTTGGATACAGGCCAGACTTTGTTG (sense) and GAT TCAACCTTGCGCTCATCTTAGGC (antisense). The number of cytokines and HPRT cDNA molecules in each sample was calculated by real-time RT-PCR using QuantiTect SYBR green master mix (Qiagen) and an ABI prism 7000 (Applied Biosystems), according to the manufacturers' instructions. Standard curves were constructed with known amounts of cytokines and HPRT cDNA, and the number of cytokine molecules per 1000 HPRT molecules in each sample was calculated.

Results

L. donovani *infection in* plt/plt *mice*

First, we determined the outcome of infection with *L. donovani* in *plt/plt* mice compared with wild-type B6 mice. Fig. 1*A* shows the outcome of infection in the liver, an organ associated with acquisition of cell-mediated immunity to *L. donovani* and thus with the capacity to clear intracellular amastigotes (23). Compared with B6 mice, liver parasite burdens were significantly higher in *plt/plt*

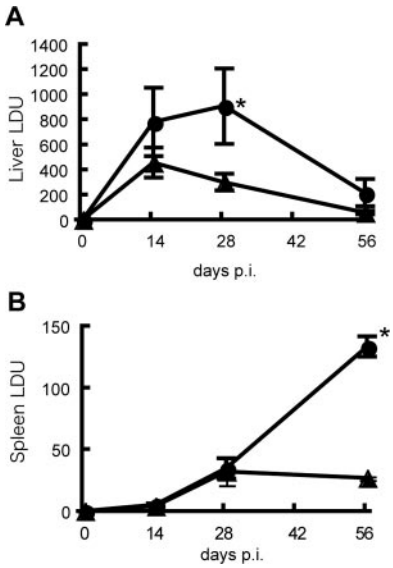

FIGURE 1. The course of *L. donovani* infection in B6 and *plt/plt* mice. B6 (▲) and *plt/plt* (●) mice were injected with 2×10^7 *L. donovani* amastigotes, and parasite burden was measured in the liver (*A*) or the spleen (*B*) at the time points indicated. The data are expressed as mean ± SEM for four to eight mice pooled from two experiments. *, $p < 0.05$.

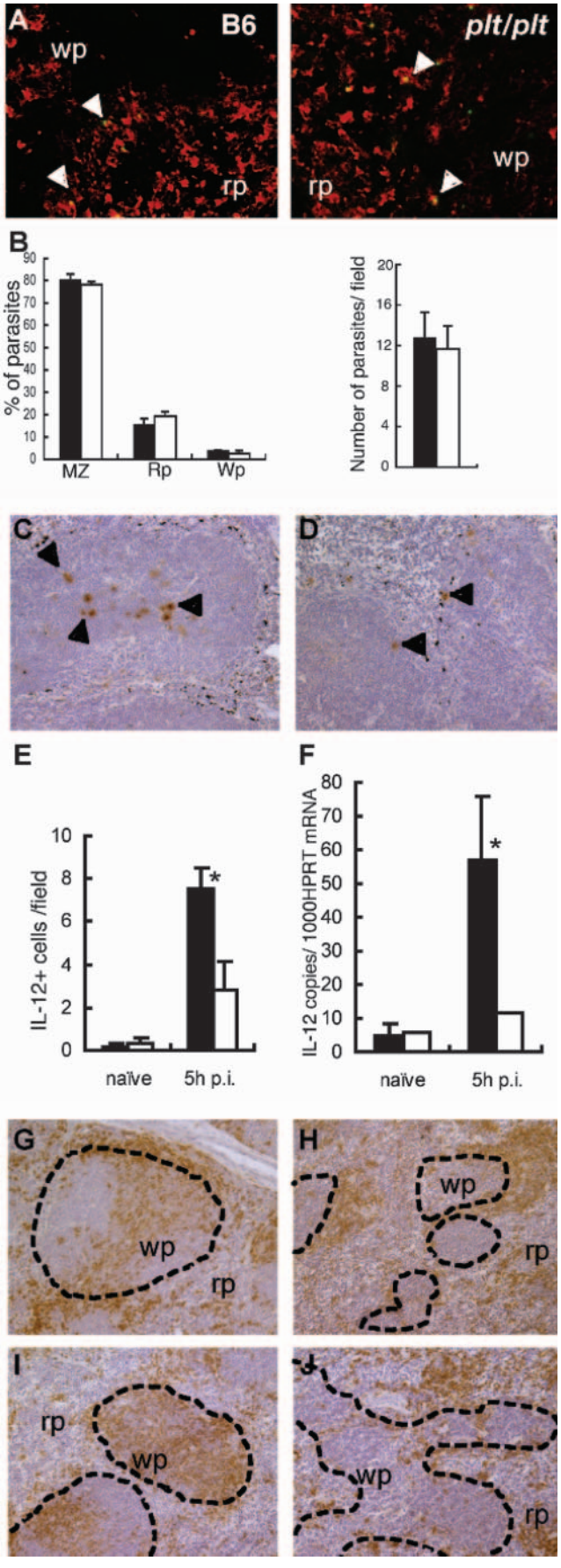

FIGURE 2. Distribution of parasite-infected macrophages, IL-12p40 production, and DC following *L. donovani* infection of B6 and *plt/plt* mice. The distribution of *L. donovani* amastigotes (green; arrowheads) and CD68$^+$ macrophages (red) in the spleens of B6 and *plt/plt* mice (*A*) at 1 h p.i. Original magnification is ×800. *B*, The distribution and number of parasites in B6 (■) or *plt/plt* (□) mice were determined from multiple spleen sections of individual mice. Data are expressed as mean ± SEM for three to five mice per strain. The distribution of IL-12p40-producing cells in the spleens of B6 (*C*) and *plt/plt* (*D*) mice at 5 h p.i. IL-12p40 (arrowhead) was visualized with immunohistochemistry (brown), and MZ macrophages were labeled with india ink (black). Original magnification is ×200. *E*, The number of IL-12p40-producing cells in B6 (■) or *plt/plt* (□) mice was determined. Data are expressed as mean ± SEM for three to five mice per strain. ∗, $p < 0.05$. *F*, IL-12p40 mRNA was detected by real-time RT-PCR in the spleen of naive and 5-h infected B6 (■) or *plt/plt* (□) mice. The values are expressed with mean ± SEM for three to five mice. ∗, $p < 0.05$. *G–J*, DC were stained using CD11c in naive B6 (*G*) and *plt/plt* (*H*) and in B6 (*I*) and *plt/plt* (*J*) mice at 5 h p.i. Dotted lines indicated the border of the MZ. Original magnification is ×200. wp, White pulp; rp, red pulp. Data are representative of one of three experiments.

mice at day 28 postinfection (p.i.), and although these mice eventually were able to resolve hepatic infection, even at day 56, amastigote numbers in the tissue remained somewhat higher than seen in B6 mice. In contrast to the curing response observed in the liver, the spleen is a site of parasite persistence, extending over the 56-day time period studied. Strikingly, although parasite numbers were equivalent in these two mouse strains at days 14 and 28, *plt/plt* mice ultimately failed to exert control over parasite growth, and at day 56 spleen parasite burden was ~5-fold higher in *plt/plt* mice than in B6 mice (Fig. 1*B*). These data indicate that *plt/plt* mice are more susceptible to *L. donovani* infection than B6 mice.

Uptake of L. donovani *amastigotes in the spleen of* plt/plt *mice*

In the spleen, *L. donovani* amastigotes were phagocytosed mainly by macrophages in the MZ (13). However, we have demonstrated previously that *plt/plt* mice are deficient in MZ macrophages (MZM), a highly phagocytic subset of macrophages in the MZ (24). To determine whether the lack of MZM influenced initial splenic infection in *plt/plt* mice, we determined the number and location of amastigotes in the spleen at 1 h postinfection, when blood clearance is essentially complete (13). Double immunohistochemistry to identify amastigotes within subsets of splenic macrophages illustrated that CD68$^+$ macrophages in the MZ, but not F4/80$^+$ red pulp macrophages, are mainly responsible for uptake of *L. donovani* in *plt/plt* mice (Fig. 2*A* and data not shown). The localization (Fig. 2*B*, *left panel*) and absolute number (Fig. 2*B*, *right panel*) of amastigotes in the spleen of *plt/plt* mice were not altered compared with wild-type mice, with uptake being predominantly within the MZ in both strains. Thus, the deficiency of MZM in *plt/plt* mice does not compromise initial splenic infection, and most amastigotes are taken up by alternate CD68$^+$ macrophages in the MZ.

Early IL-12p40 responses and migration of DC are impaired in plt/plt *mice*

The pattern of infection observed in *plt/plt* mice was remarkably similar to that previously described in studies in which IL-12p40 was targeted by neutralizing Abs (14) or in IL-12p40-deficient mice (15). As IL-12p40 is produced solely by DC in the early phase of immunity to *L. donovani* (13), we examined whether IL-12p40 responses were intact in *plt/plt* mice, using immunohistochemistry to identify both the number and localization of cells producing this cytokine. IL-12p40$^+$ cells were rarely observed in naive mice of either strain. Five hours after infection of B6 mice, IL-12p40$^+$ cells were readily observed in the spleen, mainly localizing, as previously described (13), to the deep periarteriolar

region (Fig. 2*C*). In contrast, few IL-12p40$^+$ cells were identified in the spleens of *plt/plt* mice, and those that were seen were localized in the MZ (Fig. 2*D*). To quantify this observation in more detail, we scored the number of IL-12p40$^+$ cells in multiple tissue sections from multiple mice. As shown in Fig. 2*E*, the frequency of IL-12p40$^+$ cells per white pulp section was significantly reduced in *plt/plt* mice compared with B6 mice. As an independent means of evaluating the IL-12p40 response, we used real-time RT-PCR to determine the accumulation of IL-12p40 mRNA in spleen samples from naive and infected B6 and *plt/plt* mice. This analysis confirmed at the level of mRNA expression that the IL-12p40 response of *plt/plt* mice was indeed significantly reduced (Fig. 2*F*).

To examine whether altered localization of IL-12-producing cells in *plt/plt* mice is associated with abnormal distribution of DC, we visualized DC in the spleen of B6 and *plt/plt* mice, with or without *L. donovani* infection. DC in noninfected B6 mice distributed mostly in the MZ, with a few found in the PALS, as previously reported (25) (Fig. 2*G*). In contrast, DC in *plt/plt* mice were absent from the PALS and accumulated in the MZ and red pulp (9) (Fig. 2*H*). After 5 h of infection, most DC in B6 mice had migrated into the PALS (Fig. 2*I*), but the distribution of DC in *plt/plt* mice was not altered following infection (Fig. 2*J*). These data demonstrate that DC in *plt/plt* mice fail to migrate into the PALS and rather accumulate in the MZ.

Impaired IL-12 production in PTX-treated mice

To further define the importance of chemokine-dependent migration of DC in the introduction of IL-12 production, we treated B6 mice with PTX, which blocks chemokine signaling (26). DC in PTX-treated mice failed to migrate from the MZ into the PALS after 5 h of *L. donovani* infection, and showed a scattered distribution throughout the white pulp and red pulp (data not shown). As shown in Fig. 3, IL-12 p40 mRNA accumulation did not increase in the spleen of infected B6 mice treated with PTX as compared with control infected mice. These findings further support the notion that chemokine signaling at the early stage of *L. donovani* infection is critical for DC migration and optimal IL-12 production from splenic DC.

DC activation is impaired in plt/plt *mice*

IL-12p40 production is only one of a number of alterations that accompany the activation and maturation of splenic DC. To extend this analysis, we evaluated the expression of CD80, CD86, and CD40 on splenic CD11c^{high}MHC-II^{high} DC isolated from naive and infected B6 or *plt/plt* mice. As shown in Fig. 4,

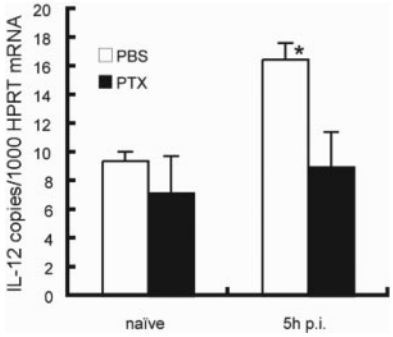

FIGURE 3. IL-12p40 expression in *L. donovani*-infected mice treated with PTX. IL-12p40 mRNA accumulation was determined by real-time RT-PCR in PBS-treated B6 mice (□) or B6 mice treated with 500 ng of PTX at 1 and 3 days before infection (■). The values are expressed with mean ± SEM for three to five mice. Data are representative of one of three experiments. *, $p < 0.05$.

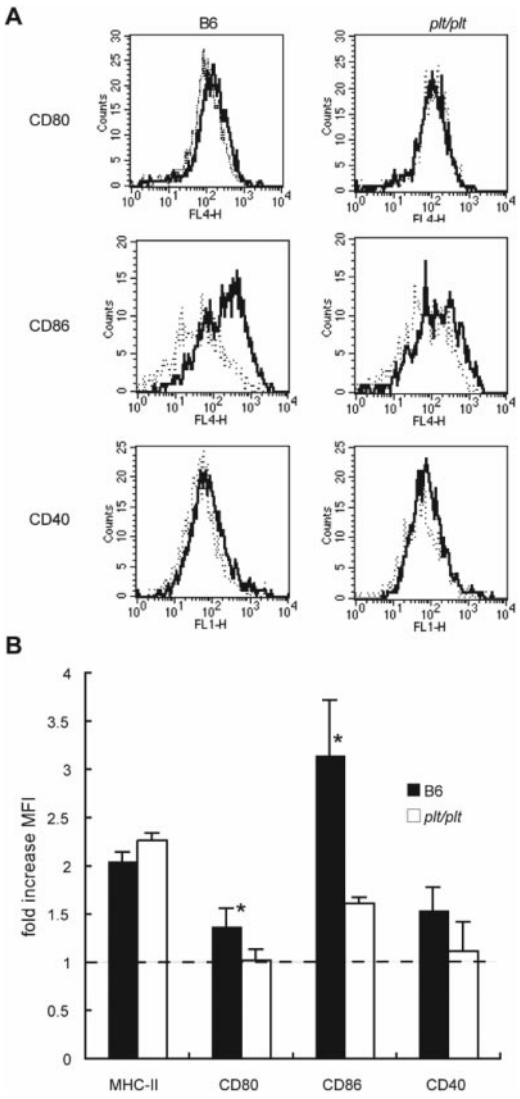

FIGURE 4. DC activation during early *L. donovani* infection of B6 and *plt/plt* mice. *A*, Spleen DC from B6 (*left panels*) and *plt/plt* (*right panels*) were identified as CD11c$^+$ MHC class II^{high} and then analyzed for expression of CD80 (*top*), CD86 (*middle*), and CD40 (*bottom*). Histograms show representative staining of naive (dotted line) and 5-h infected (solid line) mice. Data are representative of three mice from three independent experiments. *B*, Mean fluorescence intensities (MFI) of MHC-II, CD80, CD86, and CD40 on splenic DC from infected B6 (■) and *plt/plt* (□) mice. Data are shown as fold increase over DC in naive mice. The values are expressed with mean ± SEM for three independent experiments. *, $p < 0.05$.

CD11c^{high}MHC-II^{high} DC isolated from B6 mice 5 h p.i. have slightly, but reproducibly increased levels of expression of CD80 and CD40 compared with naive B6 mice. In contrast, CD86 shows a more significant response in these mice. DC in *plt/plt* mice were comparable in their expression of CD11c and MHC-II with those in B6 mice (data not shown, Fig. 4*B*). However, following infection, no alteration in expression of CD80 and CD40 was observed on DC from *plt/plt* mice. Furthermore, although CD86 was upregulated, the increase was relatively weak compared with that observed in B6 mice (Fig. 4*B*). Thus, by various criteria, DC in *plt/plt* mice appear to be muted in their response to *L. donovani* infection compared with those in B6 mice.

Immune deviation in plt/plt *mice*

To evaluate whether the restricted activation of DC observed in *plt/plt* mice was translated into defective T cell priming (27), we

isolated spleen cells from naive and infected B6 and *plt/plt* mice and restimulated them with *L. donovani* Ags in vitro. We observed that both the proliferative response (Fig. 5*A*) and the Ag-dependent production of IFN-γ (Fig. 5*B*) were comparable in these two mouse strains. To extend this analysis to other cytokines and to determine without any in vitro bias whether any form of immune deviation had occurred in these mice, we used real-time RT-PCR to evaluate the accumulation of mRNA for IFN-γ, IL-4, and IL-10 in naive and infected B6 and *plt/plt* mice. At day 14 p.i., the level of IFN-γ mRNA accumulation was identical in both strains (Fig. 5*C*), mirroring the data obtained from in vitro cultured spleen cells. IL-4 has also been shown to be coexpressed at the early stages of *L. donovani* infection (28), and although IL-4 mRNA was detected in infected mice, no difference was observed between B6 and *plt/plt* mice (Fig. 5*D*). In contrast, when we measured IL-10 mRNA accumulation, it was evident that expression of this cytokine was significantly enhanced in *plt/plt* mice compared with B6 mice (Fig. 5*E*). Thus, *plt/plt* mice show immune deviation toward production of a cytokine with known ability to inhibit antileishmanial immunity (29).

Delayed hepatic granuloma formation in plt/plt *mice*

Expression of immunity to *L. donovani* is most evident in the liver, and studies with asplenic mice indicate that T cell priming and differentiation to effector T cells in the spleen contribute significantly to the hepatic immune response (C. Engwerda, A. Stanley, C. Alexander, and P. Kaye, unpublished observations). We therefore wished to determine whether potential changes to T cell priming in the spleen of *plt/plt* mice were translated into reduced hepatic immunity as expressed by granuloma function. Tissue sections from the livers of infected B6 and *plt/plt* mice were evaluated throughout the time course of infection, and granuloma maturation was quantitated. As shown in Fig. 6, granuloma maturation was significantly retarded in *plt/plt* mice compared with B6 mice. Although amastigotes were abundant in the liver of both strains of mice at day 14 p.i. (Figs. 1 and 6, *A* and *D*), in *plt/plt* mice there was minimal initiation of a granulomatous response around infected Kupffer cells (Fig. 6*D*). At day 28 p.i., granuloma formation was detected in both B6 and *plt/plt* mice (Fig. 6, *B* and *E*), and by day 56, empty granulomas were observed in B6 mice, but less frequently in *plt/plt* mice (Fig. 6, *C* and *F*). Although absolute number of inflammatory foci was similar (Fig. 6*G*), the delay in maturation was observed throughout the time course studied, and at day 56 p.i., almost 20% of infected foci in *plt/plt* mice had still failed to generate a significant histologic response (Fig. 6*H*). Immunohistochemistry indicated that the defect in granuloma formation was associated with a lack of infiltration of both $CD4^+$ and $CD8^+$ T cells, whereas the accumulation of $F4/80^+$ macrophages was comparable (Fig. 7). These data suggest therefore that although IFN-γ production is equivalent in B6 and *plt/plt* mice, this response fails to efficiently drive granuloma maturation in *plt/plt* mice, possibly as a consequence of the elevated IL-10 response.

Discussion

DC are APC that localize at peripheral tissues throughout the body and migrate into the T cell areas of secondary lymphoid organs for presentation of Ag to T cells. Therefore, migration of DC is thought to be one of the most important events for the induction of protective immunity to pathogens. In the spleen, most blood-borne pathogens are

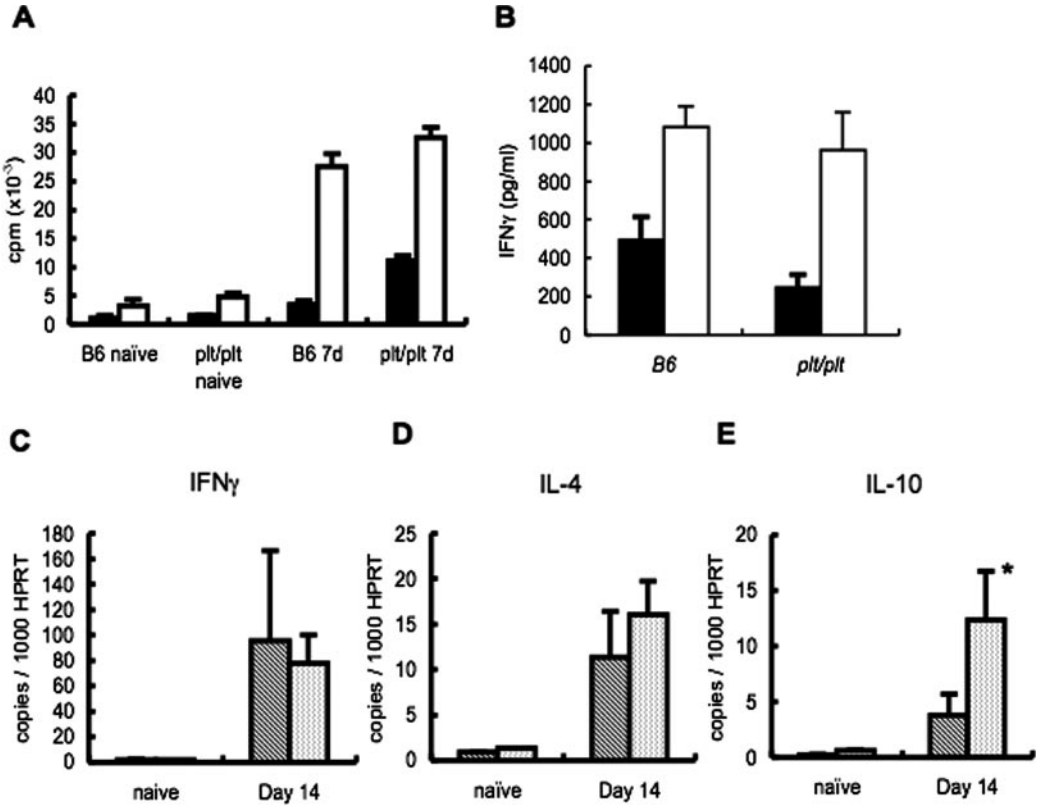

FIGURE 5. Cytokine responses in *L. donovani*-infected B6 and *plt/plt* mice. *A*, Spleen cells from naive and day 7 infected B6 and *plt/plt* mice were cultured for 72 h in the absence (■) or presence (□) of *L. donovani* amastigote Ag. Proliferation was determined by scintillation counting. *B*, IFN-γ was determined in culture dependents from restructured B6 and *plt/plt* mice in the absence (■) or presence (□) of *L. donovani* amastigote Ag. *C–E*, mRNA was extracted from the spleens of naive or day 14 p.i. B6 (dark hatch) or *plt/plt* (light hatch) mice, and accumulation of IFN-γ (*C*), IL-4 (*D*), and IL-10 (*E*) mRNA was detected by real-time RT-PCR. ∗, $p < 0.05$.

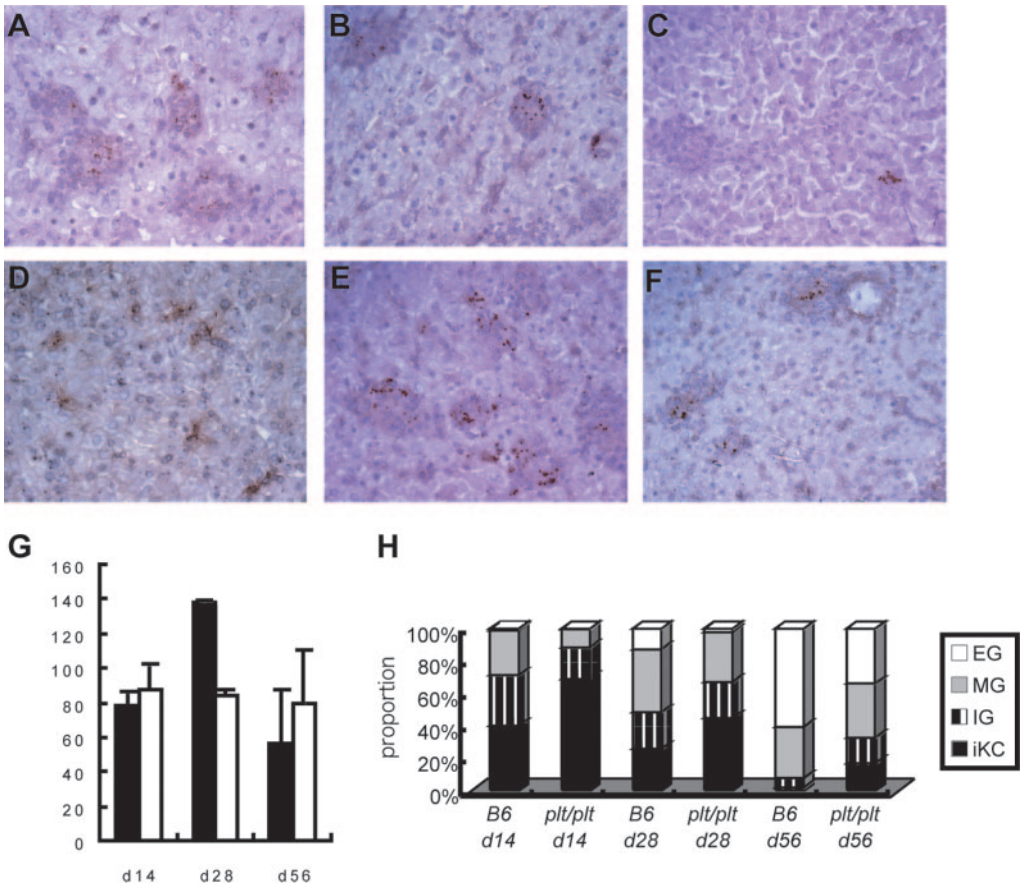

FIGURE 6. Granuloma formation in the *L. donovani*-infected livers of B6 and *plt/plt* mice. Liver sections at day 14 p.i. (*A* and *D*), day 28 p.i. (*B* and *E*), and day 56 p.i. (*C* and *F*), in B6 (*A–C*) or *plt/plt* (*D–F*) mice were stained with anti-*L. donovani* sera. Original magnification is ×400. *G*, The number of hepatic granulomas in infected B6 (■) or *plt/plt* (□) mice at each time point. *H*, Granuloma maturation during *L. donovani* infection of B6 or *plt/plt* mice. Data represent the frequency of infected Kupffer cells (iKC), immature granulomas (IG), mature granulomas (MG), and empty granulomas (EG) per mouse at each time point.

trapped within the MZ, where macrophages and DC are abundantly distributed (30). DC in the MZ, activated following i.v. infection of LPS or soluble *Toxoplasma* Ag (STAg), rapidly move to the T cell area and form tight clusters with T cells (31, 32). These movements of DC are tightly controlled by chemokines and the regulation of chemokine receptor expression. For example, DC migration after LPS administration was not seen in *plt/plt* mice (9), and injection of STAg into CCR5-deficient mice failed to recruit DC into the PALS (33). Other animal studies also showed that the distribution of DC in the spleen was altered when they were infected with malaria (34), *Toxoplasma* (35), *Salmonella* (36), or lymphocytic choriomeningitis virus (LCMV) (37). However, none of these studies with live infectious agents have defined which chemokines are responsible for the observed migration of DC, how altered migration affects the functional behavior of these DC, or the consequences for host protection.

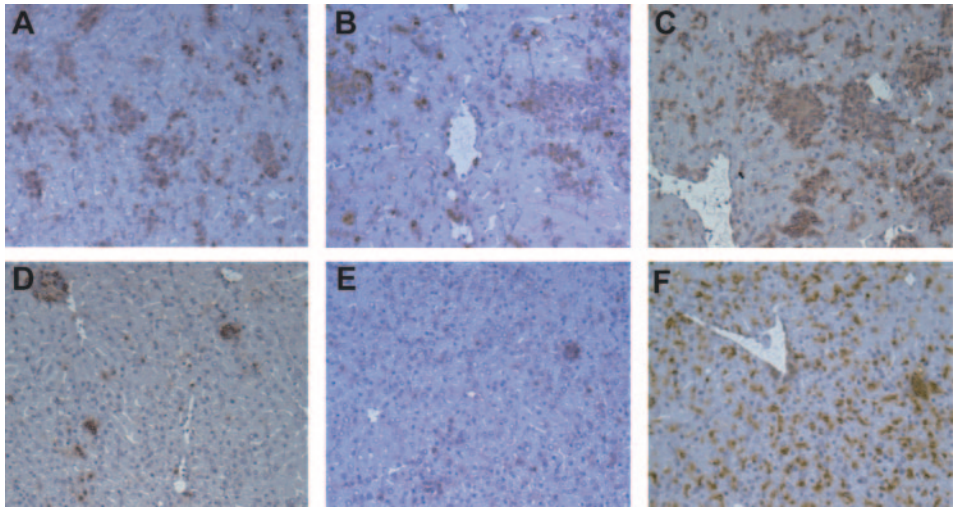

FIGURE 7. Hepatic T cell and macrophage recruitment during *L. donovani* infection of B6 and *plt/plt* mice. Liver sections from B6 (*A–C*) or *plt/plt* (*D–F*) mice at day 14 p.i. were stained for CD4 (*A* and *D*), CD8α (*B* and *E*), and F4/80 (*C* and *F*). Original magnification is ×200. Data are representative of one of three experiments.

In a mouse model of visceral leishmaniasis, we and other groups have demonstrated previously that macrophages in the MZ of the spleen phagocytosed the majority of parasites in the first hours after injection (13, 38), whereas IL-12-producing DC were observed deep in the T cell area of the spleen (13). These findings strongly suggested that either a small proportion of infected DC, or DC that had captured parasite-derived Ags in the MZ, migrated to the PALS, where they could encounter T cells for priming host-protective immune responses. In this study, we have demonstrated that the chemokines CCL19/21 are necessary for the migration of DC in this early phase of *L. donovani* infection. Many aspects of DC migration and activation in *Leishmania* infection appear different from that induced by LPS or STAg, however. In contrast to LPS or STAg administration, *L. donovani* infection induces migration of only a fraction of the DC population, and clustering of DC in the PALS is not as evident as with these other stimuli. Whereas DC in wild-type mice up-regulated both MHC class II and costimulatory molecules homogeneously at 5 h p.i., DC from *plt/plt* mice can up-regulate MHC class II to a similar extent, but a notably poor response was observed in terms of CD86 expression (Fig. 2). Another study of *L. donovani* infection using MyD88-deficient mice, which lack this common signaling pathway of TLR, has indicated that activation of DC was severely impaired, but that migration from the MZ to the PALS was not affected by loss of TLR signaling (39). These facts suggest there are at least two different stimuli acting on DC in *L. donovani* infection, which differentially affect DC migration and activation phenotype.

In *plt/plt* mice, although IL-12-producing cells were seen after 5 h of infection, the total amount of IL-12p40 mRNA was markedly decreased compared with that induced in B6 mice. In addition, all IL-12p40-producing DC were localized at the MZ in *plt/plt* mice. These findings indicate that partially activated DC can produce only small amounts of IL-12p40, whereas DC that have migrated into the PALS may become more fully activated and produce large amounts of IL-12p40. Although not studied in this work, this result may also imply that IL-23, which uses the IL-12p40 submit (40), may have differential expression in the MZ and PALS. Although *plt/plt* mice are deficient in MZM (24), our data demonstrate that $CD68^+$ macrophages in the MZ substitute as the main phagocytes for amastigotes in *plt/plt* mice (Fig. 2), and also suggest that reduced parasite uptake in the spleen does not underlie the defect in DC IL-12p40 production seen in these mice. Animals rendered MZM deficient by clodronate treatment (data not shown), and mice treated with PTX, an inhibitor of chemokine receptor signaling, also showed markedly decreased IL-12 production from DC at 5 h p.i. (Fig. 3). Thus, DC may be unable to become fully activated until they encounter $CD4^+$ T cells in T cell area. The precise molecular interactions involved in this cell-cell cooperation remain to be elucidated.

In our previous studies, IL-12 production in the early period after infection was shown to determine the course of infection in the spleen, and it is striking that *plt/plt* mice appear similar to B6 mice in which IL-12 has been neutralized and to B6 IL-$12^{-/-}$-deficient mice (14, 15). The data reported in this work might, therefore, suggest that the increased susceptibility of *plt/plt* mice is a direct consequence of the lack of IL-12 production early after infection. The relative importance of IL-12 in determining disease outcome could thus explain why there are discrepancies between the responses of *plt/plt* mice to *Leishmania* (this work), murine hepatitis virus (9), and LCMV infection (41). IL-12 is not required for induction of protective immunity for LCMV (42), and expansion of Ag-specific effector $CD8^+$ T cells in LCMV-infected *plt/plt* mice is comparable to that of wild-type mice. LCMV can also directly activate DC (37), whereas amastigotes of *L. donovani* infect very few DC in vivo (13) and DC migration is necessary to achieve full activation (Figs. 2 and 4). Furthermore, loss of CCR7-depending migration of DC is one of the critical factors associated with the chronic stage of *L. donovani* infection (43), whereas this event is less important for the host-protective response against LCMV infection (41). Alternatively, reduced IL-12 production may merely serve as another indicator of poor activation of DC in *plt/plt* mice, because our data do not directly address whether decreased IL-12 production is the most significant defect in DC activation, in terms of the ultimately lowered resistance of *plt/plt* mice to *L. donovani* infection. Surprisingly, in preliminary experiments, we have been unable to enhance resistance in either *plt/plt* mice or wild-type mice by a single administration of 1 μg of rIL-12 at 5 h postinfection (M. Ato and P. Kaye, unpublished observations). These data contrast with the host-protective effects of sustained (7-day) administration of rIL-12, as reported by others (44), suggesting that either IL-12 produced at later times during infection is also important for host resistance, or that other defects in DC activation in *plt/plt* mice, as noted above, play an important role in determining the outcome of infection.

Given the above findings, it was also surprising to observe that the induction of IFN-γ and IL-4, key cytokines involved in optimal host resistance and granuloma development following *L. donovani* infection (16, 45), was similar in both wild-type and *plt/plt* mice. Thus, the defects reported in this study in *plt/plt* mice clearly do not block T cell priming completely, as anticipated from other studies (46). In contrast, the accumulation of mRNA for IL-10, a cytokine with notable inhibitory effects on host resistance (28, 47), was increased in *plt/plt* compared with wild-type mice. IL-10 has multiple cellular sources during active infection with *L. donovani*, with IL-10 mRNA being most abundant (on a cell per cell basis) in $CD4^+$ T cells, NK cells, DC, and macrophages, and to a lesser extent in $CD8^+$ T cells, B cells, and neutrophils (A. Maroof, M. Svensson, S. Stager, and P. Kaye, unpublished observations). However, the broad expression of IL-10, difficulties associated with identifying IL-10-producing cells directly ex vivo, and a similarly wide cellular distribution of IL-10R expression together pose significant challenges for identifying functionally relevant cellular interactions mediated through IL-10. These are only likely to be addressable in vivo by the future development of mice, allowing cell-specific and regulated targeting of IL-10 and its receptor.

Resolution of hepatic infection is dependent on granuloma formation, in which various effector cells are recruited and produce cytokines. Both $CD4^+$ and $CD8^+$ T cells are required to induce granuloma formation in the liver (21). *plt/plt* mice have delayed granulomatous responses and an associated reduction in the recruitment of effector T cells. The lack of T cell recruitment is unlikely to be due to a lack of CCL19/21 in the liver, because CCL21 is detected in afferent lymphatics in the liver of *plt/plt* mice to the same extent as in the livers of wild mice (data not shown) (11). Rather, our data suggest that this lack of granuloma maturation is directly linked either to the altered functional development of effector $CD4^+$ and/or $CD8^+$ T cells, or their homing potential in an IL-10-rich environment. These speculations may be supported by the fact that IL-10 suppresses granuloma formation and recruitment of effector cells to the infected liver, independently of IFN-γ production (47).

In conclusion, *plt/plt* mice have a deficiency in DC activation resulting from impaired CCL21/CCL19-dependent migration of DC in these mice, which, most likely acting in concert with established defects in T cell migration (9), leads to a demonstrable increase in susceptibility to *L. donovani* infection. This study,

therefore, reveals for the first time the potential link between migration-dependent DC activation and protection against *L. donovani* infection, substantiating the importance of an appropriate chemokine environment for the generation and expression of optimal host-protective immune responses.

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