Interferon-γ-Producing CD4⁺ T Cells Drive Monocyte Activation in the Bone Marrow During Experimental Leishmania donovani Infection

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Ly6C⁹ inflammatory monocytes develop in the bone marrow and migrate to the site of infection during inflammation. Upon recruitment, Ly6C⁹ monocytes can differentiate into dendritic cells or macrophages. According to the tissue environment they can also acquire different functions. Several studies have described pre-activation of Ly6C⁹ monocytes in the bone marrow during parasitic infection, but whether this process occurs during experimental visceral leishmaniasis and, if so, the mechanisms contributing to their activation are yet to be established. In wild type C57BL/6 (B6) mice infected with Leishmania donovani, the number of bone marrow Ly6C⁹ monocytes increased over time. Ly6C⁹ monocytes displayed a highly activated phenotype from 28 days to 5 months post infection (p.i.), with >90% expressing MHCII and >20% expressing iNOS. In comparison, in B6.Rag2⁻/⁻ mice <10% of bone marrow monocytes were MHCII⁺ at day 28 p.i., an activation deficiency that was reversed by adoptive transfer of CD4⁺ T cells. Depletion of CD4⁺ T cells in B6 mice and the use of mixed bone marrow chimeras further indicated that monocyte activation was driven by IFNgamma produced by CD4⁺ T cells. Under all of the above conditions, monocyte activation was associated with greater control of parasite load in the bone marrow. Through reinfection studies in B6.Ili10⁻/⁻ mice and drug (AmBisome®) treatment of B6 mice, we also show the dependence of monocyte activation on parasite load. In summary, these data demonstrate that during L. donovani infection, Ly6C⁹ monocytes are primed in the bone marrow in a process driven by CD4⁺ T cells and whereby IFNgamma promotes and IL-10 limits monocyte activation and that the presence of parasites/parasite antigen plays a crucial role in maintaining bone marrow monocyte activation.

Keywords: visceral leishmaniasis, mouse models, monocytes, CD4⁺ T cells, interferon-gamma, IL-10, bone marrow
INTRODUCTION

The bone marrow (BM) is the major site for hematopoiesis in adult mammals, producing all major cell lineages from a pool of committed precursors. Ly6C^hi monocytes are derived from a common myeloid progenitor, through intermediates that include monocyte-dendritic cell progenitors and granulocyte-monocyte progenitors (1), and with cell fate specified by the transcription factors PU.1, IRF1 and Klf4 as well as by the action of key hematopoietic growth factors such as CSF-1 (2). Ly6C^hi monocytes are released into the bloodstream and migrate to peripheral organs in normal and inflammatory conditions where they contribute to a wide range of physiological and pathological processes including innate and adaptive immune responses, tissue remodeling and tissue repair [reviewed in (3)].

Ly6C^hi monocytes are highly plastic and depending on the environment, they can differentiate into a variety of cells including macrophages and dendritic cells (DCs) or maintain a monocyte phenotype (4, 5). It is unclear, however, whether such terminal differentiation only occurs once in the peripheral tissues or is initiated earlier during BM residency. For example, specialized monocyte progenitors have been demonstrated in the BM and reflect later polarization of monocyte function in the periphery (6). In addition, Ly6C^hi monocytes have the capacity to respond to microbial stimuli during their development in the BM before their egress into the peripheral circulation and their tissue-specific functions are in part pre-programmed (7). Specifically, infection with Toxoplasma gondii led to the secretion of interferon-γ (IFNγ) by NK cells and this cytokine was responsible for monocyte priming and the development of regulatory capacity (7, 8). These and other studies (1, 6, 9, 10) collectively suggest that Ly6C^hi monocytes may become functionally committed prior to their arrival at sites of tissue inflammation or infection.

Although it is known that Ly6C^hi monocytes play an important role against various intracellular parasites, the extent to which they contribute to the immune response against different species of Leishmania is still unclear, with the data often seemingly contradictory. During L. major infection, Ly6C^hi monocytes have been demonstrated to have a dual role, on the one hand aggravating during primary infection and on the other hand being protective during secondary infection (11). This latter function reflects their ability to facilitate rapid recruitment of CD4^+ T cells at the secondary site of infection and hence to enhance parasite elimination. Others have demonstrated, however, that monocyte-derived DCs are essential for priming protective Th1 response in L. major infected mice (12). The situation is further complicated in models of visceral leishmaniasis, where parasites accumulate predominantly in spleen, liver and BM (13). Monocytes accumulate in the spleen throughout the course of L. donovani infection and play a role in tissue remodeling (14). Several studies have shown that in absence of Ly6C^hi monocytes, B6.Ccr2^-/- mice fail to generate an effective early Th1 response allowing for a rise in tissue parasite burden load. More recently, a pathogenic role for Ly6C^hi monocytes in promoting parasite survival was demonstrated, a finding supported by the observation that emergency hematopoiesis during L. donovani infection leads to the differentiation of Ly6C^hi monocytes into regulatory monocytes in the BM that contribute to parasite survival (15). Given that the BM acts as a site of infection, T cells are also recruited in high numbers and we have previously demonstrated that TNF-dependent CD4^+ IFNγ^+ T cells accumulate in significant numbers in the BM resulting in progressive hematopoietic stem cell exhaustion (16) and erosion of the erythropoietic niche (17). However, the impact of these highly pathogenic CD4^+ T cells on local monocyte activation has not previously been determined.

In this study, therefore, we used a combination of gene targeted mice, mixed chimeras, antibody deletion and drug treatment to explore the mechanisms underpinning BM Ly6C^hi monocyte activation during L. donovani infection, and uncover its relationship to BM CD4^+ T cells, the production of the cytokines IFNγ and IL-10, and parasite load.

MATERIALS AND METHODS

Animals and Infection

B6.CD45.1 (B6.Ptprc<sup>b</sup>), B6.CD45.2 (B6.Ptprc<sup>a</sup>) and B6.CD45.2.Rag2<sup>2/3</sup> mice used in this study were bred and maintained under specific-pathogen free (SPF) conditions at the Biological Services Facility, University of York. BM cells from mice lacking the Ifngr1 gene (B6.Ifngr1<sup>-/-</sup>) on a B6 background were obtained from the Jackson Laboratory. All mice were between 5–8 weeks of age at the start of experimental work. Mice were infected via the lateral tail vein with 3x10<sup>7</sup> amastigotes of the Ethiopian strain of L. donovani (LV9). To assess the impact of drug-induced parasite clearance, mice were treated once with 10 mg/kg Amphotericin B (AmBisome<sup>®</sup>) at day 28 post-infection and killed 72 hours later. Animals were killed by CO<sub>2</sub> asphyxia and cervical dislocation at the time points specified. Spleen and liver parasite burden was expressed as Leishman-Donovan units (LDU), where LDU was equal to the number of parasites/1000 host nuclei multiplied by the organ weight in milligrams. BM parasite burden was determined by limiting dilution assay (LDA). Briefly, two-fold serial dilutions in 96-well flat bottom microtitrator plates were performed in OMEM medium supplemented with 20% FCS. The plates were scored microscopically for growth and the number of parasites in each tissue was determined from the highest dilution at which parasites could be grown out after 7–14 days incubation at 26°C.

Cell Extraction

The spleen was mechanically disrupted and incubated in RPMI with 0.25 mg/ml Collagenase IV and 0.1 mg/ml DNase 1 for 30 min at RT. Spleen tissue was then forced through a 70μm strainer to obtain a single cell suspension. Cell pellets were resuspended in ACK Lysing buffer (5min at RT) then washed with RPMI. BM cells from tibias were flushed with a 26g needle and cold RPMI. To obtain a single cell suspension, cells were run through a 70μm cell strainer and spun down. Cell pellets were resuspended in ACK lysing buffer for 5min at RT and washed. Cells were resuspended in RPMI 1640 medium (Life
Technologies) supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin and kept on ice. Cells were counted with trypan blue under a light microscope.

**Flow Cytometry and Cell Sorting**

For immunolabelling, cells were washed and labelled with LIVE/DEAD Fixable Dead Cell Stains (Thermo Fisher Scientific) to exclude dead cells. Cells were incubated with anti-Fc III/II (CD16/32) receptor Ab (2.4G2), followed by surface staining with various combination of the following antibodies for 30 min at 4°C in the dark: CD11b (M1/70), Ly6G (1A8); Sca1 (D7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD3e (145-2C11 and UCHT1), CD4 (RM4-5 and 4SM95), CD8β (H35-17.2), TCR-β (145-2C11), CD11c (N418), Ly6C (HK1.4), MHC-II (M5/114.15.2), MerTK (2B10C42), CD40 (XX), CD80 (XX), CD86 (GL-1), CD64(X54-5/7.1). Staining for CCR2 employed AlexaFluor 700 anti-CCR2 (475301) and was done prior to surface staining at 37°C for 20-30 min.

In some experiments, cells were stimulated with Brefeldin A at 10 µg/ml alone or in combination with Phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) for 4 h at 37°C, then fixed and permeabilized with the eBioscience™ Intracellular Fixation & Permeabilization Buffer Set according to the manufacturer instructions and stained for IL-10 (JES5-16E3), NOS2 (CXNFT), TNF (MP6-XT22) and IFNγ (XMGl1.2).

All Abs were from eBiosciences, BD Biosciences, BioLegends or R&D systems. Data were collected using FACS Diva software on BD LSR Fortessa X-20 (BD Biosciences), and analyzed using FlowJo software (TreeStar). Forward-scatter and side-scatter width was employed to exclude cell doublets from analysis. Cell sorting to > 95% purity was performed on a MoFlo Astrios (Beckman Coulter).

**Mixed Bone Marrow Chimeras and Adoptive T Cell Transfer**

BM cells were purified as described previously. B6.CD45.1 recipient mice were irradiated with 850 rad from an X-ray source using a two split dose regimen and reconstituted with 10⁶ cells from B6.CD45.1 and B6.Iifgr1+/-CD45.2 mice admixed in a 50:50 ratio. Mice were maintained on oral antibiotics for the first 6 weeks post reconstitution. Mice were infected at 7 weeks of chimerism and killed 28 days after the infection. For T cell adoptive transfer, B6.Rag2+/- CD45.2 mice infected with 21 days received 10⁶ CD4+ or CD8+ T cells isolated from the BM of 28 day-infected B6.CD45.1 mice. Mice were killed 7 days post-transfer for analysis.

**Treatment With Anti-IL10R, Anti-CD4 Monoclonal Antibody**

CD4+ T cells were depleted by administering 400 µg of InVivoMab anti-mouse CD4 (clone GK1.5) intra-peritoneally, twice weekly beginning on day 14 post-infection and for 2 weeks. Control mice were injecting with the same amount and at the same frequency with InVivoMab rat IgG2b isotype control (anti-keyhole limpet hemocyanin; LTF-2). IL-10 signaling was blocked by injecting 250 µg of InVivoMab anti-mouse IL-10R (clone 1B1.3A; CD210) intraperitoneally every 3 days for 14 days starting on day 1 post-infection. InVivoMab rat IgG1 Isotype control (anti-trinitophenol; TNP6A) was administered to control mice. All antibodies and isotype controls were purchased from BioXcell (Lebanon, USA).

**Statistical Analysis**

Prior to applying appropriate statistical tests, parametric test assumptions were assessed. Normality of data distribution was based on Shapiro-Wilks test. Further, due to small sample n, data skewness and kurtosis were analyzed to confirm or dispute results from Shapiro-Wilks test. Where data did not comply with a normal distribution, a Log10-transformation was applied prior to test application, if that resulted in data normality. Homogeneity of sample variance was analyzed by F test (2 samples) or by Levene’s test (>2 samples). All groups analyzed were independent of one another, including data in time courses as different mice were harvested at each time point. To conform with the assumption of data continuity, proportional data (%) were converted by the arcsin conversion prior to test application as proposed by Sokal and Rohlf (18). Experimental group n refers to the number of mice per group and are stated in the respective figure legends. Where unequal sample size occurred, the smallest n is stated as the power calculation always relies on the smallest group n.

For two sample tests, Welch’s version of the t test was chosen for its greater robustness. As a non-parametric alternative, a Mann-Whitney test was applied. For >2 sample one-way tests, Welch’s version of the ANOVA was chosen for its greater robustness under minor departure from variance homogeneity. Alternatively, Brown-Forsythe’s version of the ANOVA was chosen where also mild to moderate data skewness was observed. As post hoc multiple comparison test, we used Dunnett’s T3 test. As a non-parametric alternative, the Kruskal-Wallis test was chosen. As a post hoc test, we used Dunn’s MCT test. For non-parametric two-way factorial design analysis, we used the ‘t2wat’ function from the ‘WRS2’ package in R (19) which used trimmed means for greater robustness. As a non-parametric alternative, a WRS2 version of the ANOVA was chosen for its greater robustness under minor departure from variance homogeneity. Alternatively, Brown-Forsythe’s version of the ANOVA was chosen where also mild to moderate data skewness was observed. Where unequal sample size occurred, the smallest n is stated as the power calculation always relies on the smallest group n.

All statistical tests were applied either in GraphPad PRISM v.9.1.0 or in R v.4.1.0 using RStudio v. 1.4.1717. In addition to the previously cited ‘WRS2’, ‘emmeans’, ‘pwr’ and ‘pwr2’ packages, the following R packages were used: ‘readxl’ (23), ‘xlsci’ (24), ‘rlstat’ (25), ‘janitor’ (26), ‘rstatix’ (27), ‘car’ (28), ‘moments’ (29), ‘reshape2’ (30), ‘effectsize’ (31) and ‘tidyverse’ (32).
Results

Inflammatory Monocytes Are Primed in the BM of B6 but Not B6.Rag2−/− Mice During L. donovani Infection

The activation of monocytes in the BM during infection has yet to be explored in the context of L. donovani infection. In contrast to infection of immunocompetent B6 mice, infection in B6.Rag2−/− mice was characterized by delayed hepatosplenomegaly and unchecked parasite growth in spleen, liver and BM over a 5 month period (Figures 1A–C). Inflammatory monocytes (iMo), defined as CD45+CD11b+Ly6G Ly6C+CCR2+ cells (Figure 1D), decreased in number early after infection, as noted by others (15), but then transiently increased in number in the BM of B6 mice being ~2-fold higher in absolute number at 28 day p.i compared to day 0 and ~3.5 fold increased compared to d4. In contrast in B6.Rag2−/− mice, no early decline in absolute monocyte numbers was noted early after infection and a transient ~2 fold increase was observed at d28 (Figure 1E). To determine whether these iMo were found in a primed state, we analysed expression of MHCIi and iNOS (7) over the course of infection. MHCIi expression was low in both strains of mice for the first 14 days p.i. and increased from d28 p.i., with marked differences between B6 and B6.Rag2−/− mice (Figures 1F, G). In B6 mice, MHCIi was abundant on almost all BM monocytes from d28 p.i. onwards whereas in B6.Rag2−/− mice, only 20% of monocytes expressed MHCIi at later time points, with statistical difference reached at day 28, day 56 and day 152 (p<0.0001) between B6 and B6.Rag2−/− mice. This lack of activation in immunodeficient mice was even more evident when measuring intracellular iNOS (Figure 1H). These data suggest that iMo are primed in the BM during L. donovani infection and that adaptive immunity is required to drive this response. Furthermore, as infection is controlled in B6 mice, the extent of BM monocyte activation as measured by iNOS generation wanes, despite maintenance of high MHCIi expression.

CD4+ T Cells Are the Main Producers of IFNγ and Drive BM Monocyte Activation in an IFNγ-Dependent Manner

IFNγ has been described as a key cytokine controlling monocyte activation. To identify probable sources of IFNγ in the BM of L. donovani-infected mice, we stimulated BM leucocytes at different times p.i. with PMA and Ionomycin. Approximately 60-70% of CD4+ T cells had the capacity to produce IFNγ at d28 and these cells persisted to 8wks p.i ([16] and Figure 2A). In contrast, ~30% of CD8+ T cells were capable of producing IFNγ at 28 p.i. and this frequency declined thereafter. No IL-10 production was detected in both the BM of B6 mice at d28 (p < 0.0001) (Figure 2B). Of note, the expansion of IFNγ-producing CD4+ T cells preceded that of MHCIi expression on iMo (Figure 2C), suggesting a causal relationship. To address this directly, we used an adoptive transfer model in B6.Rag2−/− recipients (Figure 2D). Adoptively-transferred CD4+ isolated from d28-infected B6 mice retained their capacity to produce IFNγ in B6.Rag2−/− hosts (Figure 2E) and induced MHCIi expression on recipient BM iMo as shown by the significant increase in MHCIi expression in infected B6.Rag2−/− mice receiving CD4+ T cells vs infected B6.Rag2−/− mice (p < 0.0001) (Figures 2F, G). In contrast, CD8+ T cells produced less IFNγ after adoptive transfer and no significant differences were found between the percentage of MHCIi+ iMo cells in infected B6.Rag2−/− mice receiving CD8+ T cells vs infected B6.Rag2−/− (Figures 2E–G). Importantly, transfer of CD4+ T cells into a naïve B6.Rag2−/− mice did not lead to BM iMo activation, ruling out an effect due to homeostatic T cell expansion (Figure S1) and demonstrating a requirement for cognate antigen recognition in vivo. The origin of CD4+ T cells (naïve versus d28 infected B6 mice) did not change the outcome of the experiments (Figure S1).

To independently confirm the role of CD4+ T cells in iMo activation, we used the alternate approach of depleting CD4+ T cells from immunocompetent mice. We depleted CD4+ cells in infected B6 mice from day 14 p.i to d28 p.i. with anti-CD4 depleting antibody, the time frame over which iMo MHCIi expression increases (Figure 2C). After CD4+ T cell depletion, the frequency of total BM cells producing IFNγ in the BM, measured directly ex vivo, was reduced by approximately 70% (0.8% vs 2.8% in anti-CD4 treated vs. control mice (p=0.0079); Figure 3A). Although depletion was incomplete, we observed a reduction in MHCIi expression in iMo reflected in both the percentage of MHCIi+ cells, (p=0.0027 and p=0.0036 respectively) (Figures 3B, C) and in median fluorescent intensity of expression (Figure 3D). Of note, parasite load in the BM of the mice treated with anti-CD4 mAb was increased compared to control mice, (p=0.0082) (Figure 3E), indicating that the reduction in CD4+ T cells and presumably IFNγ production had a significant impact on host resistance to L. donovani. This is supported by the observation of a significant decrease of parasite number in the BM of adoptively transferred B6.Rag2−/− mice as compared to their control (Figure S2).

Finally, in order to verify that BM iMo respond directly to IFNγ, we used a mixed chimera model. Irradiated B6.CD45.1 mice were reconstituted with B6.CD45.1 and B6.Ifngr1−/−.CD45.2 BM cells in a 1:1 ratio (Figure S3A). At day 28 p.i, CD45.1+ (wild type) but not CD45.2+ (Ifngr1−/−) iMo were primed in the BM, as determined by expression of MHCIi (p=0.0079; Figures 3F, G). Ifngr1−/− iMo also produced minimal levels of NOS2 and TNF compared to wild type iMo in these IFNγ-sufficient mixed chimeras (Figure 3H). As expected, Ifngr1−/− and wild type CD4+ T cells produced equivalent amount of IFNγ in the BM (Figure S3B). Collectively, these data indicate that BM CD4+ T cells produce IFNγ and that IFNγ signaling on BM iMo is necessary to induce their activation in L. donovani-infected mice.

IL-10 Inhibits iMo Activation and Restraints Parasite Elimination

Having demonstrated the main pathway related to iMo activation in the BM of L. donovani-infected mice, we next wished to determine whether this was constrained in any way by regulatory cytokine production. Although IL-10 has been long...
Figure 1: Monocytes are activated during L. donovani infection in B6 but not in B6.Rag2<sup>-/-</sup> mice. (A, B) Weight (left panel) and Leishman Donovan Unit (right panel) in the spleen (A) and liver (B) of B6 mice and B6.Rag2<sup>-/-</sup> at different time points during the course of the infection. Two-way factorial analysis applied to LDUs in (A, B): Spleen P=0.001, Liver P=0.001; statistical power: Spleen 94%, Liver 99%; post hoc test EMM: statistical difference reached in spleen and liver at day 56 and day 152 (P<0.0001; **); (C) Parasite load per one million cells in the bone marrow measured by limiting dilution assay. Two-way factorial analysis applied: P=0.09; statistical power: 32.3%; n≥3; post hoc test EMM: statistical difference reached at day 152 (P<0.0001; ***); (D) Gating strategy to identify Ly6C monocytes in BM. (E) Absolute number of iMo cells per femur. Two-way factorial analysis applied: P=0.165; statistical power: 98.5%; n≥7; post hoc test EMM: statistical difference reached at day 28, day 56 and day 152 (*; P=0.014, P=0.036 and P=0.046), respectively. (F, G) MHCII expression on BM Ly6C<sup>CCR2<sup>+</sup></sup> cells shown as percentage (F) and as representative flow histogram plots (G) in B6 and B6.Rag2<sup>-/-</sup> mice during the course of the infection. Two-way factorial analysis applied in (F): P=0.001; statistical power: 99%; n=4; post hoc test EMM: statistical difference reached at day 28, day 56 and day 152 (P=0.0001; ****). (H) iNOS and MHCII expression on Ly6C<sup>CCR2<sup>+</sup></sup> monocytes. Data are derived from analysis of 8 to 10 individual mice (4 to 5/experiment) of each strain at each time point pooled from two independent experiments and are shown as mean ± SD. Data in (G, H) are representative plots, including lower and upper range of MHCII expression at day 14. In (A–F) median with 95% CI is shown.
implicated as a negative regulator of macrophage activation in multiple other settings (33), its role in iMO activation is not known. As BM CD4+ T cells did not produce demonstrable IL-10 after in vitro restimulation (Figure 2A), we used B6.IIl0−/− mice to evaluate the possible role of IL-10 in regulating IFNγ-mediated iMO activation (Figure 4). B6.IIl0−/− mice have previously been shown to be resistant to L. donovani infection in terms of liver and spleen parasite loads (34). In BM, B6.IIl0−/− mice had reduced parasite load at all time points measured (Figure 4A) and at d56 parasites were undetectable in the BM of B6.IIl0−/− mice when measured by LDA. We then measured iMO activation by MHCII and iNOS expression. At d14 p.i., 18.9% ± 11.2 of iMo in B6.IIl0−/− mice were NOS2+ compared to 0.85% ± 1.54 in B6 mice (p=0.0077; Figure 4F). Similarly, 98.5% ± 6.0 of iMo in B6.IIl0−/− mice expressed MHCII compared to 42.85% ± 12.73 in B6 mice (p<0.0001; Figure 4E). MHCII MFI was also significantly increased in B6.IIl0−/− mice (p=0.0003; Figure 4D). Together, these data indicate a more rapid kinetic for iMO activation in the absence of IL-10 (Figures 4B–F). Nevertheless, at d28 p.i., iMO activation for iNOS production was similar in the presence or absence of IL-10 (22.7 ± 15.0 vs. 19.8 ± 13.4, respectively; p=0.989) as was MHCII expression (97.8 ± 2.7 vs. 90.5 ± 10.6; p=0.522; Figure 4E, F). Surprisingly, whereas iMo activation was sustained in wild type mice at d56 p.i., it was reduced at this later time point in IL-10-deficient mice, as determined by frequency of...
MHCII+ cells (94.7 ± 6.0 vs. 40.0 ± 13.9; p<0.0001; Figure 4E). In contrast, iNOS production declined at a similar rate in both B6 mice and IL-10-deficient mice (8.0 ± 5.1 vs. 1.5 ± 5.1% respectively; p=0.73; Figure 4F).

As parasites were no longer detectable at d56 p.i in B6.IL10−/− mice (Figure 4A), we hypothesized that the presence of parasites may be necessary to maintain iMo activation status, providing an antigen depot to allow continued stimulation of IFN-producing CD4+ T cells. To test this hypothesis, we measured the level of IFNγ production by CD4+ T cells at d14 and d56 p.i in the BM of B6 and B6.IL10−/− mice (Figures 4G-J). At d14 p.i., CD4+ T cells from B6.IL10−/− mice showed a trend towards greater IFNγ production compared to B6 mice after both polyclonal stimulation (Figures 4G, H) and more strikingly directly ex vivo (Figures 4I, J). In contrast, at d56 p.i., this situation was reversed, again more strikingly with direct ex vivo measurement of IFNγ (p=0.0003 and p=0.0032, respectively) (Figures 4H, J). These data suggest that IL-10 initially restraints iMo activation, but that at later time points, IL-10 may indirectly contribute to sustaining the level of iMo activation via maintenance of parasite load and ongoing effector CD4+ T cell IFNγ production.

To confirm whether early IL-10 restraints iMo activation, we used the alternate approach of blocking IL-10 signaling in B6 mice by administration of anti-IL-10 receptor antibody for 14 days, starting at day 1 p.i. (Figure 4K). Blockade of IL-10 signaling led to a decrease in parasite load in the BM, as expected from the results observed in B6.IL10−/− mice although not significantly (p=0.0717) (Figure 4A), as well as an increase in iMo activation, as measured by MHCII expression (Figures 4L, M).

The Presence of Parasites in the BM Contributes to iMo Activation

To determine whether parasite load/and or antigen availability plays a role in determining the level of iMo activation, we re-infected B6.IL10−/− mice at d56 p.i., a time at which they had cleared their primary infection (Figure 5A). One day post-re-infection, Leishmania parasites were detected in the BM of re-infected mice (p=0.006; Figure 5B). Strikingly, BM iMo from these re-infected mice were highly activated, returning to levels of MHCII expression seen at d14 p.i. (B6.wt vs B6.IL10−/− R1 (P=0.0006) and B6.IL10−/− vs B6.IL10−/− R1 (P=0.0002)) (Figures 5C-E).

We then conducted the reciprocal experiment, using the antileishmanial drug AmBisome® to clear parasite load prematurely in day 28-infected B6 mice. Treatment with AmBisome® for 3 days reduced BM parasite load to less than 10 parasites/106 cells (p<0.0001; Figure 5F) and the level of activated iMo was reduced significantly, as determined by a reduction in the frequency of MHCII+ cells (99.37% ± 0.61 reduced to 71.73% ± 4.63 in control...
FIGURE 4 | Absence of IL-10 changes the kinetics of monocyte activation in the BM. B6 and B6.IL10−/− mice were infected with L. donovani amastigotes in the tail vein for 14, 28 and 56 days. Data from the BM are shown (A–G). (A) Parasites burden in the bone marrow determined by limiting dilution assay at the indicated time point. Two-way ANOVA applied on log10 transformed data: P<0.0001; statistical power: 11.9%; n=6; post hoc test Šidák’s MCT: statistical difference reached at day 28 and day 56 (P<0.0001; ***). (B) Dot plot representative of nos2 and MHCII expression by Ly6C+CCR2+ monocytes. (C–F) Histogram (C), bar graph showing MFI (D), percentage of MHCII (E) and percentage of nos2 (F) on BM Ly6C+CCR2+ monocytes. Two-way factorial analysis applied in (E): P=0.0013; statistical power: 6.1%; n≥4; post hoc test EMM: statistical difference reached at day 14 and day 56 (P<0.0001; ****); and two-way factorial analysis applied in (F): P=0.013; statistical power: 6.1%; n≥4; post hoc test EMM: statistical difference reached at day 14 (P<0.0001; ****); and two-way factorial analysis applied in (G–J) percentage of IFN-γ produced by CD4+ T cells after PMA-Ionomycin-BFA stimulation for 4h (I, J) and ex vivo (BFA stimulation only, I, J). Brown-Forsythe ANOVA applied in (H): P<0.0001; statistical power: 43.8%; n=4; post hoc test Dunnett’s T3: statistical difference reached at day 56 (P<0.0003; **); 95% CI: 8.16 – 30.46; and Brown-Forsythe ANOVA applied in (J): P=0.0002; statistical power: 47.5%; n=4; post hoc test Dunnett’s T3: statistical difference reached at day 56 (P<0.0032; **); 95% CI: 4.99 – 24.47. (K–M) B6 mice infected with L. donovani were injected with an anti-IL-10 depleting antibody or the isotype control for 14 days starting 1 day prior to the infection (K) Parasite load determined by limiting dilution assay in the BM at day 14 p.i. Welch’s t test applied: P=0.0717; 95% CI: -1.86 – 0.115; statistical power: 13.9%; n=5; (L) Percentage and (M) MFI of MHCII expressed on Ly6C+CCR2+ monocytes. Brown-Forsythe ANOVA applied in (L): P=0.0001; statistical power: 100%; n=5; post hoc test Dunnett’s T3: statistical difference reached in NA vs. aI10R (P<0.0001; ****); 95% CI: -116.93 – -83.79 and CLT vs. aI10R (P<0.0001; ****; 95% CI: -79.79 – -53.79); and Brown-Forsythe ANOVA applied in (M): P=0.0009; statistical power: 100%; n=5; post hoc test Dunnett’s T3: statistical difference reached in NA vs. aI10R (P<0.0034; **); 95% CI: -43.61 – -117.56; and Brown-Forsythe ANOVA applied in (M): P=0.0034; statistical power: 72.5%; n=5; post hoc test Dunnett’s T3: statistical difference reached in NA vs. aI10R (P<0.0034; **); 95% CI: -43.61 – -117.56. For (K–M), data are derived from analysis of 6 to 8 individual mice (2 to 4/experiment) of each strain at each time point pooled from two independent experiments and are shown as mean ± SD. Data in (B, C, G) and I are representative plots. For (K–M), data are the pool of 5 mice/group/time point. Mean ± SD is shown. Experiment was performed once. In (A, D–F) median with 95% CI is shown. For statistical details are found in Table S1.
vs drug-treated mice; p<0.0001; Figure 5G) and a reduction in MFI for MHCII (3619 ± 1360 to 616.3 ± 90.2 in control vs drug-treated mice; Figure 5H). Thus, forced early reduction in parasite load is accompanied by reduced iMo activation in IL-10-sufficient B6 mice.

Collectively, these data suggest a model whereby parasite load in the BM is an essential driver of CD4+ IFNγ T cell-dependent iMo activation, and that IL-10 serves to regulate this pathway via its capacity to regulate macrophage leishmanicidal activity.

**DISCUSSION**

Our data extend a previous study of BM monocytes that also demonstrated an elevation of MHCII expression on Ly6Chi iMo in the BM early during *L. donovani* infection (15) by conducting analysis into the chronic period of infection and through analysis of the mechanisms responsible for this finding. Notably, we demonstrate that the change seen in B6 mice in terms of MHCII expression is a direct consequence of IFNγ-signaling and in turn that this IFNγ is produced predominately by CD4+ T cells and opposed by IL-10.

Through independent experimental approaches using gene targeted mice and mAb blockade, we have provided evidence that IL-10 impacts on the activation status of BM monocytes during *L. donovani* infection. Although IL-10 production by splenic CD25+Foxp3+ CD4+ T cells has been reported to correlate with disease progression in experimental VL (35, 36), in the BM we found no evidence that CD4+ (or CD8+) T cells produced appreciably levels of IL-10 over 8 weeks course of infection (Figure 2A). This finding is in accord with the notion that the CD4+ T cell population recruited to the BM during infection may be selected for IFNγ production (16). In the absence of IL-10 production by T cells, other cellular sources are therefore implicated. In spleen and liver, these have been shown to include NK cells (37) and CD11c+ conventional dendritic cells (36) in addition to macrophages and monocytes themselves (38–41). Furthermore, innate activation of B cells to produce IL-10 has

**FIGURE 5** | The presence of *L. donovani* parasites in the BM is necessary to maintain monocyte activation. (A–E) B6.IL10−/− mice were re-infected (RI) at d56 with 3x107 *L. donovani* amastigotes and killed 1 day post re-infection. (A) Experimental strategy. (B) Parasite load per one million cells determined by limiting dilution assay in the BM at day 56 or 1 day post RI. Mann-Whitney test applied; P=0.006 (**); 95% CI: 87.73 – 200; statistical power: 100%; n=7. (C) Percentage of MCHII on Ly6CCCR2+ monocytes in the BM. Brown-Forsythe ANOVA applied only to day 56 data; P=0.0019; statistical power: 99.5%; n=3; post hoc test Dunnett’s T3: statistical difference reached in B6.wt vs. B6.IL10−/− (P=0.0073; **); 95% CI: 5.06 – 44.58) and B6.IL10−/− vs. B6.IL10−/− RI (P=0.0024; **); 95% CI: -51.32 – -12.13). (D) Level of MHCII expressed by the BM Ly6CCCR2+ monocytes represented on histogram. (E) MFI of MHCII on Ly6CCCR2+ monocytes in the BM of the indicated mice. Brown-Forsythe ANOVA applied only to day 56 data; P=0.0019; statistical power: 26.7%; n=3; post hoc test Dunnett’s T3: statistical difference reached in B6.wt vs. B6.IL10−/− RI (P=0.0006; ***); 95% CI: -4297 – -1310) and B6.IL10−/− vs. B6.IL10−/− RI (P=0.0002; ***); 95% CI: -5099 – -2266). (F–H) B6 mice infected for 28 days were treated with 10mg/kg of Ambisome®. Data were collected 3 days post-treatment. (F) Parasite load measured by limiting dilution assay in the BM. Welch’s t test applied in (F) P<0.0001; ****; 95% CI: -3.31 – -1.67; statistical power: 100%; n=6. Percentage (G) and MFI (H) of MHCII expressed on Ly6CCCR2+ monocytes. Brown-Forsythe ANOVA applied in (G) P<0.0001; statistical power: 100%; n=6; post hoc test Dunnett’s T3: statistical difference reached in all pairs (P<0.0001; ****), and Kruskal-Wallis test applied in (H) P=0.0002; statistical power: 100%; n=6; post hoc test Dunn’s MCT in Na+Amb vs. Inf (P=0.0071; **). In (A–E), data are from 3 to 5 mice per group/experiment. Experiments were performed twice and pooled data are shown. In (F–H), data were derived from 3 to 4 mice/group. Mean ± SD is shown except in A Median with 95% CI is shown.
been demonstrated in the context of *L. donovani* infection (42) and plasma cells producing IL-10 have been implicated in directing myeloid cell differentiation under homeostatic conditions (43). Further investigation with for example IL-10 transcriptional reporter mice will be required to confirm the identity of the IL-10-producing cells in the BM of *L. donovani*-infected mice and their relative roles in regulating monocyte activation.

In this study, we did not aim to directly assess the role of iMo in disease outcome, a subject that has been studied by others. Satoskar and colleagues have previously reported that iMo were associated with enhanced parasite loads in the spleen of B6 mice and that these iMo showed an activation profile biased at the transcriptional level towards arginase production. In contrast, iMo ingress to the spleen and liver using a CCR2 antagonist led to improved host resistance in both tissues, suggesting that phenotypic characteristics measured in vitro may not always reflect in vivo function. In contrast, another study using CCR2-deficient mice failed to demonstrate a role for iMo in host resistance and hepatic granuloma formation (45). We have not directly measured arginase in BM iMo in this study, but iNOS production was clearly elevated throughout the course of infection through an IFNγ-dependent pathway, most likely involving STAT1 signaling (44). Whilst this study also showed that iMo in the peritoneal cavity could efficiently phagocytose *L. donovani* amastigotes, they did not show that this directly occurred at the major sites of infection. Abidin et al. (15) demonstrated that alterations in myelopoiesis led to the generation of regulatory iMO that suppress protective immunity and these monocytes were identified to be parasitized in the BM. Phenotypic changes in MHCIi and Ly6C expression suggested exposure to IFNγ, in keeping with our direct demonstration of a role for IFNγR signalling, but also up-regulation of galectin-3, associated with alternate activation (46). Further studies will be necessary to identify if changes in monocyte activation affects anti-leishmanial immunity in other sites of infection such as the spleen and the liver.

Our data also provide an in vivo example of the intimate relationship between immune activation and parasite load. Although IL-10 deficiency leads to a rapid acceleration and augmentation of monocyte activation, this is transient and by d56 post infection, monocyte activation in B6.IL10−/− mice has returned to a homeostatic baseline. One explanation for this apparently paradoxical observation is that maintenance of monocyte activation and local IFNγ production is dependent upon the presence of parasites in the BM environment, a suggestion borne out by experimentation. Thus, reduction of parasite load by drug treatment rapidly reduced expression of MHCIi on BM monocytes whereas reinfestation of previously cured B6.IL10−/− mice with *L. donovani* led to a rapid increase in BM monocyte MHCIi expression. Similarly, adoptive transfer of CD4+ T cells from infected mice into naive recipient hosts does not lead to monocyte activation, indicating a need for parasites to trigger this response in BM monocytes. The lack of monocyte activation in the BM of infected B6.Rag2−/− mice suggests that parasites alone are not sufficient for such activation. Rather, IFNγ-producing CD4+ T cells and *Leishmania* are both required to induce this response. Currently, we cannot distinguish between a model in which parasite antigens contribute to CD4+ T cell activation through cognate pathways of antigen presentation or one in which parasites induce bystander CD4+ T cell activation through innate (e.g. TLR) signaling pathways. Alternatively, parasites and or their products may directly stimulate monocytes through similar engagement of pattern recognition receptors, though this itself is insufficient to drive MHCIi and iNOS expression in the absence of T cell-derived IFNγ. Nevertheless, our data clearly demonstrate the importance of parasite load as a parameter influencing local immunoregulatory pathways.

Whilst the kinetics of induction of MHCIi expression following T cell activation was expected based on the known role of IFNγ in the transcriptional regulation of MHCIi expression on myeloid cells (47), the rapid change seen after pathogen clearance with AmBisome® in terms of both the frequency of MHCIi+ BM monocytes and the abundance of MHCIi was surprising. One explanation for a reduced frequency of MHCIi+ monocytes is that MHCIi+ iMo are released from the BM as a consequence of a change in the balance of retention/egress signals (48) and that newly produced monocytes fail to become activated. CXCL12, the ligand for the main retention receptor CXCR4, is produced locally by BM and splenic stromal cells but expression is reduced at the mRNA and protein level in these tissues as well as in the BM following *L. donovani* infection (17, 49–51). In contrast, the monocyte attracting chemokines CCL2, CCL7 and CCL8 remain highly expressed in spleen (51) and splenic CCL2 mRNA accumulation remains elevated even after AmBisome® treatment (52). Alternatively, the more marked reduction in MFI for MHCIi on BM monocytes is suggestive of cell intrinsic changes in protein expression. E3 ubiquitin ligases of the MARCH family play a role in regulating cell surface expression of a variety of membrane proteins (53). Of note, MARCH1 plays a central role in IL-10-mediated post translational regulation of surface MHCIi expression in human monocytes and other myeloid cells (54, 55), in addition to regulating monocyte differentiation (56). Additional studies would be needed to further dissect this novel aspect of monocyte MHCIi expression following parasite clearance.

In summary, we have demonstrated that BM monocyte activation is a prominent feature of *L. donovani* infection in mice and reflects the outcome of an intricate balance between IFNγ, IL-10 and parasite load. Little is currently known about the role of BM monocytes in the progression of human VL and during the response of patients to therapy. The studies outlined here, together with the relative ease and safety with which bone marrow aspirates can be obtained from VL patients, provide a foundation to explore in more detail this aspect of myeloid cell function during human VL.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.
ETHICS STATEMENT

The animal studies were carried out in accordance with the Animals and Scientific Procedures Act 1986, under UK Home Office Licence (project licence number PPL 60/4377 approved by the University of York Animal Welfare and Ethics Review Board).

AUTHOR CONTRIBUTIONS

Conceptualization: AR and PMK. Data curation: AR and PMK. Formal analysis: AR and JD. Funding acquisition: PMK. Investigation: GA, NB, HA, JD, JH, MO, ND, GR, and TF. Methodology: AR and PMK. Project administration: PMK. Resources: PMK. Supervision: PMK. Visualization: AR. Writing, original draft: AR and PMK. Writing, review and editing: AR and PMK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Monocyte Activation in Visceral Leishmaniasis

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