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Characterization of Conventional Dendritic Cells and Macrophages in the Spleen Using the CSF1R-Reporter Transgenic Chickens

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The spleen is a major site for the immunological responses to blood-borne antigens that is coordinated by cells of the mononuclear phagocyte system (MPS). The chicken spleen is populated with a number of different macrophage subsets in addition to the presence of conventional dendritic cells (cDC) which has been described. However, a detailed characterization of the phenotype and function of different macrophage subsets and cDC in the chicken spleen is limited. Using the CSF1R-reporter transgenic chickens (CSF1R-tg), in which cells of the MPS express a transgene under the control elements of the chicken CSF1R, we carried out an in-depth characterization of these cells in the spleen. Immunohistological analysis demonstrated differential expression of MRC1L-B by periarteriolar lymphoid sheaths (PALS)-associated CSF1R-tg+ cells. In the chicken’s equivalent of the mammalian marginal zone, the peri-ellipsoid white-pulp (PWM), we identified high expression of putative CD11c by ellipsoid-associated cells compared to ellipsoid-associated macrophages. In addition, we identified a novel ellipsoid macrophage subset that expressed MHCII, CD11c, MRC1L-B, and CSF1R but not the CSF1R-tg. In flow cytometric analysis, diverse expression of the CSF1R-tg and MHCII was observed leading to the categorization of CSF1R-tg dim MHCII int, CSF1R-tg hi MHCII int, and CSF1R-tg hi MHCII int subpopulations. Low levels of CD80, CD40, MHCI, CD44, and Ch74.2 were expressed by the CSF1R-tg hi MHCII int cells. Functionally, in vivo fluorescent bead uptake was significantly higher in the CSF1R-tg hi MHCII int MRC1L-B+ cells compared to the CSF1R-tg dim and CSF1R-tg hi MHCII int MRC1L-B+ subpopulations while LPS enhanced phagocytosis by the CSF1R-tg hi MHCII int cells. The analysis of bead localization in the spleen suggests the presence of ellipsoid-associated macrophage subsets. In addition, we demonstrated the functionality of ex vivo derived CSF1R-tg+ MRC1L-B neg cDC. Finally, RNA-seq analysis of the CSF1R-tg subpopulations demonstrated that separating the CSF1R-tg hi subpopulation into CD11c hi and CD11c dim cells enriched for cDC and macrophage lineages, respectively, while the CSF1R-tg hi MHCII int subpopulation was
INTRODUCTION

The vertebrate spleen is a secondary lymphoid organ and despite differences in the immune system among vertebrates, the spleens basic histological architecture and its role in filtering the blood for antigens and damaged, aged cells has been conserved during evolution from fish, amphibians, birds, and mammals. In the mammalian spleen, the afferent splenic artery branches into the central artery which is sheathed by the white pulp areas that include the T cell zones known as the periarteriolar lymphoid sheath (PALS), arterioles, and B cell follicles. The white pulp and red pulp are extensively populated with macrophages and dendritic cells (DC) with distinct origins and functions that contribute to the control of blood-borne pathogens and homeostatic processes (1). The venous system of the red pulp gives it the capacity to filter the blood and remove old erythrocytes by red pulp macrophages. Red pulp macrophages are defined by ADGRE1hi (ERM1, F4/80) CD11b+CD18+ or ADGRE1+ CD11b+CD18hi expression (2, 3). The murine splenic red pulp also contains reservoirs of monocytes that are transcriptionally similar to their blood counterparts, Ly-6C+ and Ly-6C++ cells (ADGRE1−/hi CD11b+ CD11c−) that egress to sites of tissue damage (4, 5).

The main difference between rodents and primates spleen is the structure of the marginal zone (MZ) that surrounds the white pulp. In contrast to mice, the human MZ has an inner and outer region that is surrounded by a large perifollicular zone [reviewed by (6, 7)]. The MZ consists of two resident macrophages subsets, metallophilic macrophages (MIM) located at the outer margins of the MZ and MZ macrophages (MzM) located in the inner regions of the MZ. Both MZ-resident macrophages express the scavenger receptor, MARCO while MZM also express SIGN-R1 (CD209b) (8). The MIM can uptake particular blood-borne antigens and present them to DC for cross-presentation to CD8+ cytotoxic T cells but they are not necessary for direct T cell activation (9).

Mammalian DC are composed of two major populations: conventional DC and plasmacytoid DC (pDC). Mammalian conventional DC are further subdivided into two major populations, cDC1 and cDC2. cDC1 develop in a BATF3 and IRF8 dependent manner and are identified by their surface expression of XCR1 and CD103, respectively (10–12). In the mammalian spleen, cDC1 are located within the T cell zones of the PALS and the outer regions of the MZ (13). The IRF4-dependent cDC2 population express CD11b, SIRPα, and DEC-205 (CD205). cDC2 are a heterogeneous cell population, the definition of these is yet to be fully resolved but they have been implicated in driving Th1, Th2, and Th17 polarization (14). The third group of DC are the IRF8−, E2−2−, and SpiB-dependent CD123+pDC which are the primary source of type I IFN production following viral infection (15, 16). In humans, CD141+ (BDCA3) and CD1c− (BDCA1), and the CD303+ (BDCA2) cells are the equivalent of murine cDC1, cDC2, and pDC subsets, respectively (17). In veterinary species where reagents are limited for the study of cDC and macrophage subsets, the identification of MPS cell ontogeny and cell type identity has been determined by specific gene expression patterns. For example, comparative genome analysis has identify homologous of cDC1 and cDC2 in sheep and pigs (18, 19), and cattle (20, 21). In the chicken, gene expression patterns have identified subsets of monocytes in the blood, resident tissue macrophages and cDC in the liver and spleen (22, 23).

The basic structure of the chicken spleen resembles its mammalian counterpart, with morphologically defined white pulp and red pulp regions. The white pulp contains the PALS and peri-ellipsoid white-pulp (PWP) which surround the penicillar capillaries. The PWP is analogous to the mammalian marginal zone. The ellipsoid, more commonly known as the capsule of Schweigger-Seidel (CSS) in mammals, is embedded within the PWP. The PWP contains three distinct cell populations, the ellipsoid-associated cells (EAC) and B cells intermingled with ellipsoid-associated macrophages (EAM). Studies have demonstrated the ability of EAC to retain antigen at their cell surface to either provide the antigen to the EAM or for transport to the PALS where they develop into FDC (24–28). Mannose receptor C1-like B (MRC1L-B, recognized by KUL01) is one of five orthologs of mammalian MRC1 (206) and a key mononuclear phagocytic cell marker in lymphoid and non-lymphoid tissues in chickens (29, 30). A previous comparative genomic analysis showed that splenic cDC lack MRC1L-B expression (23). However, there is limited research on the function and location of cDC, and the phenotype, function, and transcriptome of PALS-, PWP-, and red pulp-associated cells of the MPS in the chicken spleen.

The development, maintenance, and proliferation of cells of the MPS are dependent upon signals from CSF1 through its receptor, CSF1R (31) while DC require signals from FLT3L and its receptor, FLT3 (32). The tools to study the chicken MPS have been improved by the generation of the CSF1R-reporter transgenic chickens, in which a transgene is expressed under the control of the CSF1R-promoter and Fms-intronic regulatory element (FIRE) (33). These reporter chickens have contributed to the characterization of yolk-sac-derived macrophages, monocytes, and resident tissue mononuclear phagocytic cells (33–36). In this study, we utilize the CSF1R-reporter transgenic chickens to demonstrate the heterogeneity of the MPS in the

**Keywords:** chicken, CSF1R, dendritic cells, macrophages, mononuclear phagocyte system, RNA-seq, spleen
spleen. We show that the spleen contains at least three CSF1R-tg \(^{+}\) subpopulations that diversely express CSF1R-tg, MHCII, MRCl1-B, and CD11c along with other macrophage markers. We also identified a CSF1R-tg \(^{hi}\) macrophage population that express CSF1R protein in the PWP and demonstrate their ability to uptake beads in vivo. Furthermore, in vivo antigen uptake lead to the discovery of the diverse expression of the CSF1R-tg in EAM in the PWP. In addition, we demonstrate the phagocytic ability of cDC \(ex\) \(vivo\). Finally, using RNA-seq we demonstrate that separation of the CSF1R-tg \(^{lo}\) MHCII\(^{lo}\) subpopulation into CD11c\(^{hi}\) and CD11c\(^{dim}\) subsets, enriches for genes associated with cDC and macrophage lineages while the gene signatures characteristic of red pulp macrophages are enriched in CSF1R-tg \(^{hi}\) MHCII\(^{inter\} \) subpopulation. Future experiments will address the cDC-T cell interactions and the role of various cells of the splenic MPS in antigen-uptake and presentation.

**MATERIALS AND METHODS**

**Chickens**

CSF1R-reporter transgenic (33) and non-transgenic Hy-Line birds (Hy-Line Brown) between the ages of 4–8 weeks of age were provided by the National Avian Research Facility (NARF), Edinburgh, U.K. Unvaccinated chickens were reared in floor pens with access to water and food ad-libitum.

**Isolation of Splenocytes**

Spleens were aseptically removed and stored in PBS on ice. Spleens were homogenized using PYREX \(^{®}\) Potter-Elvejehm glass tissue grinder and passed through 70 \(\mu\)m cell strainer (Corning \(^{®}\)). Cells were overlaid on Histopaque (1.077 g, Sigma-Aldrich) and the interface and cell layer above were retrieved and washed twice with calcium and magnesium free PBS (pH 7.4). Cells were counted and stained at 1 \(\times\) 10\(^{6}\) cells for flow cytometric analysis.

**In vitro Cell Cultures**

Splenocytes were cultured at 20 \(\times\) 10\(^{6}\) cells/mL in complete media [RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU of penicillin, 100 \(\mu\)g of streptomycin (Gibco), 2 mM/mL L-glutamine (Life Technologies), and 200 ng/mL of recombinant chicken CSF1-Fc (35)]. After 24 h, non-adherent cells were removed and adherent cells were treated with TrypLE express (ThermoFisher) and reseeded at 5 \(\times\) 10\(^{5}\) cells in 24-well plates (Corning) in complete media. Cells were incubated at 41\(^{°}\)C, 5% CO\(_{2}\). Twenty-four hours later, cells were stimulated with pHerD\(^{TM}\) Red labeled Zymosan or E. coli bioparticles \(^{TM}\), unlabeled or LPS labeled 1 \(\mu\)m FluoSpheres\(^{®}\) beads (ThermoFisher) (10:1 ratio of particles/beads to cells) at 41 or 4\(^{°}\)C. To inhibit actin polymerization, cells were treated with Cytochalasin D (Cayman Chemical) for 10 min prior to addition of the model antigens. Three hours post-treatment, ice-cold PBS was added to each well and cells were collected using TrypLE express (Gibco) for flow cytometric analysis. For immunostaining of \(in\) \(vivo\) cultured cells, 13 mm circular cover slips (ThermoFisher) were placed in 24-well plates and cultured as described above.

**Immunostaining of Tissue Sections**

For histological analysis, spleens were immediately flash frozen over liquid nitrogen or fixed in 4% paraformaldehyde (PFA/PBS) for 3 h and infused overnight with 30% sucrose (Sigma-Aldrich) at 4\(^{°}\)C. All samples were stored at –80\(^{°}\)C until use. Tissue sections were cut at 7 \(\mu\)m (Leica CM1900) and mounted on SuperFrost Plus\(^{TM}\) slides (ThermoFisher) and air-dried overnight at RT after which samples were stained immediately or stored at 4\(^{°}\)C until use. Sections were blocked with goat serum (Chromondex) or heat inactivated horse serum (Sigma-Aldrich) for 30 min diluted in PBS supplemented with 0.01% Triton-X and 0.5% BSA (Sigma-Aldrich). Tissue samples were stained with antibodies outlined in Table 1 for 2 h at RT followed by secondary antibodies, rabbit poly anti-GFP-Alexa Fluor 488\(^{®}\), goat anti-mouse Alexa Fluor 568\(^{®}\) IgG1, goat anti-mouse Alexa Fluor 647\(^{®}\)IgG1/IgG2a, goat anti-mouse Alexa Fluor 568\(^{®}\) IgG1 (ThermoFisher) and counterstained with 4’,6-diamidino-2-phenylindole (DAPI) or Hoechst 33258 (Sigma-Aldrich) for 1 h at RT. Slides were mounted using ProLong\(^{TM}\) Diamond Antifade mountant (ThermoFisher) and analyzed using an inverted LSM710 or 880 Zeiss confocal microscope using 405, 488, 594, and 641 nm lasers, 40X or 63X Nikon oil lenses and captured using ZEN 2012 (Black Edition) software and analyzed using ZEN 2012 (Blue Edition).

**Flow Cytometry and Flow Activated Cell Sorting**

Splenocytes were stained using antibodies described in Table 1. Briefly, cells were incubated for 20 min on ice with primary antibodies diluted in Flow Buffer (PBS supplemented with 0.5% BSA and 0.01% sodium azide Sigma-Aldrich). Cells were then incubated with secondary antibodies for 20 min on ice. For multi-color flow cytometry, antibodies were conjugated using Lightning-Link\(^{TM}\) (Novus Biologicals) with Alexa Fluor\(^{®}\)647, APC-Cy\(^{TM}\)-7, PerCP-Cy\(^{TM}\)-5.5, and PE-Cy\(^{TM}\)-7 according to the manufacturer’s instructions. All conjugated antibodies were titrated and tested for similar staining patterns observed using the unconjugated antibodies. For each experiment, live/dead cells were discriminated by the addition of SYTOX\(^{®}\) Blue Dead Cell Stain (ThermoFisher) before cell analysis. Immuno-phenotyping of cells was carried out using BD LSRFortessa\(^{TM}\) (BD Biosciences, UK) configured with 405, 488, 561, and 640 nm laser with 16 filters. Cells were sorted using BD FACS ARIA IIIu (BD Biosciences) and analyzed post-flow using FlowJo (Tree Star v10.0.2). Compensation was achieved using BD anti-mouse IgG\(\kappa\), goat anti-mouse Alexa Fluor 568\(^{®}\) IgG1, goat anti-mouse Alexa Fluor 647\(^{®}\)IgG1/IgG2a, goat anti-mouse Alexa Fluor 568\(^{®}\) IgG1 (ThermoFisher) and counterstained with 4’,6-diamidino-2-phenylindole (DAPI) or Hoechst 33258 (Sigma-Aldrich) for 1 h at RT. Slides were mounted using ProLong\(^{TM}\) Diamond Antifade mountant (ThermoFisher) and analyzed using an inverted LSM710 or 880 Zeiss confocal microscope using 405, 488, 594, and 641 nm lasers, 40X or 63X Nikon oil lenses and captured using ZEN 2012 (Black Edition) software and analyzed using ZEN 2012 (Blue Edition).

**Intravenous Bead and Liposome Administration**

One \(\mu\)m FluoSpheres\(^{®}\) beads (ThermoFisher) were labeled with E. coli LPS 055:B5 (Sigma-Aldrich) as previously described (48).
Chickens between the ages of 5–8 weeks were intravenously administered with 100 µl of beads in PBS containing 3.6 × 10^9 0.5 µm or unlabeled or LPS labeled 1 µm carboxylate FluoroSpheres® beads (ThermoFisher) or 100 µl of PBS- or clondronate-encapsulated liposomes (5 mg Liposoma, Europe) via the wing vein. Chickens were culled 3 h (beads) or 3 days (liposome) post-administration by cervical dislocation. Half of the spleen was prepared for immunohistology and the other half prepared for flow cytometric analysis. Ten thousand live, single cell events in the bead^+ gate were captured for analysis.

**RNA-Seq Sample Preparation**

Splenocytes were prepared as described above and sorted from five CSF1R-eGFP chickens at 7 weeks of age. Cells were sorted under low speed using an 80 µm diameter nozzle into 1.5 mL Eppendorf tubes at 4°C. Following cell sort, samples were pelleted at 400 g for 3 min and lysed using RLT buffer (Qiagen) supplemented with β-mercaptoethanol and stored at -20°C until use. RNA was purified using RNA Micro extraction kit according to the manufacturer’s instructions (Qiagen). RNA concentration and quality was measured using High Sensitivity RNA Screen Tape System (Agilent). All samples were used with a RIN ≥7. cDNA was transcribed using Ovation® RNA-Seq System V2 (NuGen) according to the manufacturer’s instructions. cDNA quality and quantity was analyzed using D1000 Screen Tape System (Agilent). RNA-seq was carried using TruSeq DNA Nano (350 bp insert) library preparation, sequencing using Illumina HiSeq4000 and data generation and preliminary analysis was carried out by Edinburgh Genomics, The University of Edinburgh, U.K.

**RNA-Seq Analysis**

Reads were trimmed using Cutadapt (version 1.9.dev2) (49) for quality at the 3’ end using a quality threshold of 30 and for adapter sequences of the TruSeq DNA Nano kit. Reads after trimming were required to have a minimum length of 50 bp. Reads were aligned against the Gallus gallus 5.0 genome from Ensembl using STAR (50). Raw counts for each annotated gene were obtained using the feature counts software [version 1.5.2; (51)]. Reads were normalized using the weighted trimmed mean of M-values method (52). Differential gene expression analysis was performed within the Bioconductor edgeR package [version 3.16.5; (52)]. For differential analysis the raw counts table was filtered to remove genes consisting predominantly of near-zero counts, filtering on counts per million (CPM) to avoid artifacts due to library depth. Statistical assessment of differential expression was carried out with the likelihood-ratio test. Contrasts specified were each pair of sorted cell populations. Differentially expressed genes were defined as those with a false-discovery rate (FDR) < 0.05 and log2 fold-change (FC) > 2. Heatmaps were constructed using normalized gene counts in R using the pheatmap package (v. 1.0.10; https://CRAN.R-project.org/package=pheatmap). To statistically test whether mammalian immune cell transcriptional signatures were enriched in each chicken cell population we used a pairwise gene set enrichment analysis (GSEA) approach using the BubbleGum software for statistical calculations and graphical output (53). The chicken data was compared to mouse data as more spleen specific gene sets were available than for other species. We used mouse immune cell-specific gene sets developed previously (23, 54), which are comprised of genes that show at least 1.5-fold higher expression in the cell population of interest compared to other immune cells. The gene sets used were for splenic T cells, B

| Antibody (clone) | Antigen | Product info. | References | Cellular distribution |
|------------------|---------|---------------|------------|----------------------|
| Mouse anti-chicken MHCII (2G11) IgG1 | MHCII | Southern Biotech | (37) | B cells, macrophages, DC, low levels in T cells |
| Mouse anti-chicken MHCII (TAP1) IgG2a | MHCII | DSHB4 | (38) | B cells, Macrophages, DC, low levels in T cells |
| Mouse anti-chicken MHC (F21-21) IgG1 | MHC1 | Southern Biotech | (39) | Nucleated cells |
| Mouse anti-chicken CD1.1 (CB3) IgG1 | CD1.1 | Southern Biotech | (40, 41) | B cells, cDC |
| Mouse anti-chicken CD40 (IG8) IgG2a | CD40 | In house | (42) | B cells, monocytes, macrophages, DC |
| Mouse anti-chicken CD80 (DC7) IgG2a | CD80 | In house | (42) | B cells, monocytes, macrophages, DC |
| Mouse anti-chicken KUL01 IgG1 | MRC1L-B | Southern Biotech | (29) | Monocytes and macrophages |
| Mouse anti-chicken K1 IgG2a | Unknown | Kind gift from Härtle, S. LMU. Germany | (43) | Monocytes, thrombocytes |
| Mouse anti-chicken CD11c (8F2) IgG1 | Putative CD11c | Kind gift from Härtle, S. LMU. Germany | (42) | Mononuclear phagocytes |
| Mouse anti-chicken Cvi-ChNL-68.2 IgG1 | unknown | Prions | (25) | Ellipsoid-associated cells |
| Mouse anti-chicken Cvi-ChNL-74.2 IgG1 | unknown | Prions | (24) | Subpopulation splenic macrophages |
| Mouse anti-chicken chB6 (AV20) IgG1 | Bu1 | Southern Biotech | (44) | B cells, subpopulation splenic macrophages |
| Mouse anti-chicken CD44 (AV6) IgG1 | CD44 | BioRad | (45) | Macrophages, B cells |
| Mouse anti-chicken CD8a (3-298) IgG2b | CD8a | Southern Biotech | (46) | T cells, NK cells |
| Mouse anti-chicken LEP100 (lgG1) | LAMP1 | DSHB4 | (47) | Lysosomal membrane |

[^4]: DSHB, Developmental Studies Hybridoma Bank.
cells, cDC1, pDC, cDC vs. monocytes/macrophages (Mo/Mp), Mo/Mp vs. cDC. In addition, we used blood derived mouse cMo, ncMo, and MoDC data sets. Sequences have been submitted to European Nucleotide Archive (ENA) under the accession number PRJEB40127.

Statistical Analysis
All data was analyzed for normality using Shapiro-Wilk test and non-parametric analysis was performed using GraphPad Prism 8 (GraphPad, USA). Differences between the groups were statistically evaluated by the non-parametric Mann-Whitney or Kruskal-Wallis test adjusted for post-hoc analysis. For all statistical analysis, \( P < 0.05 \) were considered significant.

RESULTS
Splenic PALS-Associated CSF1R-tg\(^+\) Cells Do Not Uniformly Express MRC1L-B
Whole mount microscopic analysis of the CSF1R-tg chicken spleen demonstrates the high density of CSF1R-tg\(^+\) cell sheaths within the splenic PALS and the PWP (Figure 1A). A microscopic overview of CSF1R-tg and CD11c expression, using the putative anti-chicken CD11c monoclonal, 8F2, demonstrates low expression levels of CD11c in the PALS and PWP regions and high levels of expression in cells of the red pulp (Figure 1B and Supplementary Figure 1). At the origin of the PALS, CSF1R-tg\(^+\) cells that surround the central artery express the chicken monocyte/macrophage markers, Ch74.2, which binds to an unknown epitope, MHCII and mannose receptor CI-like B [MRC1L-B formally known as KUL01, Figures 1C,D; (30)]. CSF1R-tg\(^+\) interdigitating cells are scattered throughout the PALS and express Ch74.2 and MHCII, while they differentially express MRC1L-B (Figures 1C,D). A lack of MRC1L-B expression is a hallmark for cDC in the chicken spleen (21). At the bifurcation of the central artery resides the GCs that contain CSF1R-tg\(^+\) follicular dendritic cells (FDC) that lack Ch74.2, MHCII, and MRC1L-B expression (Figures 1C,D) and bind IgY (Figure 1E). ChB6\(^+\) B cells are located within the GC and in the PWP where they form a ring around the ellipsoid (Figure 1F). PWP-associated chB6\(^+\) B cells are intermingled with ellipsoid-associated macrophages (EAM) that express the CSF1R, Ch74.2, MHCII, and MRC1L-B (Figures 1G,H). Closer analysis of the red pulp demonstrated that not all red pulp-associated CSF1R-tg\(^+\) cells express Ch74.2 (Figure 1G image insert) but they strongly express MRC1L-B (Figure 1H). Within the inner regions of the PWP are EAC that express the CSF1R-tg (33) and can also be identified using the monoclonal Ch68.2 which binds to an unknown epitope [Figure II; (25)]. The CSF1R-tg\(^+\) expression demonstrates that cells of the splenic spleen differentially express MRC1L-B and help to distinguish cDC in the chicken spleen.

A CSF1R-tg\(^{neg}\) Subpopulation Is Located in the Ellipsoid and Express CSF1R Protein

The PWP is structurally defined as the region from the discontinuous basement membrane of the penicilliform capillary that harbors supportive cells surrounded by the capsule of the Schweigger-Seidel sheath [Figure 2A; (28)]. The ellipsoid, embedded within the PWP, is the filtering apparatus of the spleen and represents a key component of chicken splenic lymphoid system. Using high-resolution confocal analysis, the CSF1R-tg\(^+\) EAC were observed anchored to the ellipsoid, positioned to be the primary cells to encounter blood-borne antigens (Figure 2B). Double staining of spleen sections with Ch68.2 and MHCII, lead to the identification of a ring of Ch68.2\(^{neg}\) MHCII\(^+\) cells within the ellipsoid that were found to be CSF1R-tg\(^{neg}\) (Figure 2C). Although these cells lack transgene expression, they were found to express the CSF1R protein (Figure 2D) and also expressed MRC1L-B and CD11c (Figures 2E,F). To distinguish these cells from EAM we denotate them as ellipsoid macrophages (EM). The expression of MHCII by the EAC was not easily identifiable. In some instances, intracellular MHCII staining was observed in the EAC whereas surface expression was difficult to demonstrate which may suggest a lack of MHCII surface expression or expression levels being undetectable via confocal microscopy (Figures 2C–E and Supplementary Figure 2). We also observed lower levels of CD11c expression by the EAM compared to the EAC (Figure 2D). LAMP1 expression was observed throughout the PWP and its expression was found within the EAM, EAC, and the EM cell populations (Figure 2G). Overall, our analysis show that a network of EM reside within the inner margins of the ellipsoid that express a number of chicken macrophage markers and the CSF1R protein but not the CSF1R-tg.

The Chicken Spleen Contains Cells of the MPS That Diversely Express the CSF1R-tg, MHCII MRC1L-B, and CD11c

We next analyzed the phenotype of splenic cells of MPS from the CSF1R-tg chickens using flow cytometry. After gating the cells based on size/granularity and the removal of cell debris, doublets, and dead cells (Supplementary Figure 3), typically five subpopulations were observed based on their CSF1R-tg and MHCII expression patterns (Figure 3A). Firstly, the CSF1R-tg\(^{neg}\) population was gated into MHCII\(^{dim}\) (Gate 1) and MHCII\(^{hi}\) subpopulations (Gate 2). The CSF1R-tg\(^+\) subpopulation made up ~5% of the total live cell population in which three distinct subpopulations were observed based on their CSF1R-tg\(^+\) and MHCII expression levels (Gates 4–6). Histogram overlays of the CSF1R-tg\(^+\) subpopulations demonstrated diversity of CSF1R-tg and MHCII expression (Figure 3B). The median fluorescent intensity (MFI) of CSF1R-tg and MHCII expression levels indicated that cells within Gate 4 had significantly lower levels of CSF1R-tg expression compared to cells in Gates 5 and 6. This subpopulation expressed intermediate to high levels of MHCII (Figure 3C) and were named CSF1R-tg\(^{dim}\) MHCII\(^{int}\)–hi subpopulation. Cells within Gates 5–6 expressed high levels of the CSF1R-tg and differ in their MHCII expression levels. Cells in Gate 5 expressed significantly higher levels of MHCII compared to cells in Gate 6 and hence named the CSF1R-tg\(^{hi}\) MHCII\(^{int}\) (Gate 5) and CSF1R-tg\(^{hi}\) MHCII\(^{hi}\) (Gate 6) subpopulations, respectively. In terms of size and granularity, the
FIGURE 1 | CSF1R-tg+ mononuclear phagocytes differentially express MRC1L-B and CD11c in the spleen. The white pulp of the chicken spleen consists of two morphologically distinct areas, the peri-arteriolar lymphoid sheaths (PALS) that surround the central artery (CA) and the peri-ellipsoid white pulp (PWP) surrounding the periciliary capillaries. (A) Whole mount microscopic analysis of the spleen demonstrates the abundance of CSF1R-tg+ cells in the PALS and PWP; (B) CD11c is differentially expressed across the PALS and PWP and high concentrations of CD11c hi cell sheaths were observed in the red pulp (RP); (C) the chicken macrophage marker, Ch74.2, is expressed by a majority of the CSF1R-tg+ MHCII+ PALS-associated mononuclear phagocytic cells in contrast to the FDC and the EAC in the PWP; (D) CSF1R-tg+ cells surrounding the CA express MRC1L-B while some scattered CSF1R-tg+ PALS-associated mononuclear phagocytic cells lack MRC1L-B expression which is also observed in FDC; (E) FDC and plasma cells express IgY; (F) GC are packed with chB6+ B cells and chB6+ B cells surround the ellipsoid in the PWP; (G) in the PWP, Ch74.2+ EAM reside in the outer regions of the PWP and in the red pulp, not all CSF1R-tg+ cells express this chicken macrophage marker [insert image of Ch74.2+ (yellow arrow) and Ch74.2- (white arrow) staining in red pulp macrophages]; (H) EAM express MHCII and MRC1L-B in contrast to the EAC; (I) CSF1R-tg is expressed by the Ch68.2+ EAC. Yellow dashed circles indicate GC, white dashed lines indicates the PWP. Data is representative of five CSF1R-eGFP transgenic chickens at 5–6 weeks of age. Images (B-D-F-G-I) are 2X2 tiled images with 100 µm scale bars and 50 µm scale bars for images (C,E).

CSF1R-tg hi MHCII inter subpopulation was more homogenous than the CSF1R-tg dim MHCII inter− hi and CSF1R-tg hi MHCII hi subpopulations (Figure 3D).

Using the gating strategy outlined in Figure 3A, we next analyzed the expression of MRC1L-B and CD11c across each subpopulation (Gates 1–6, Figure 3E). We found no MRC1L-B+ cells within the CSF1R-tg neg MHCII dim subpopulation and they were largely CD11c+ (Figure 3E). Within the CSF1R-tg neg MHCII hi subpopulation a small percentage of MRC1L-B hi CD11c hi cells were observed (Figure 3E), which may represent the CSF1R-tg neg EM observed in the ellipsoid via immunohistolgy. The CSF1R-tg dim MHCII inter− hi and CSF1R-tg hi MHCII hi subpopulations were very heterogeneous, consisting of MRC1L-B hi CD11c hi, MRC1L-B inter CD11c inter− hi cells while a third subset expressed low level or no MRC1L-B and high levels of CD11c, making up around 30–35% of the cell population in each of these subpopulations, respectively (Figure 3E and Supplementary Figure 4). The CSF1R-tg hi MHCII inter subpopulation were homogenous in their MRC1L-B and CD11c (88%) expression and lacked the MRC1L-B inter cells observed in the CSF1R-tg dim MHCII inter− hi and CSF1R-tg hi MHCII hi subpopulations (Figure 3E).
Figure 2 | The ellipsoid contains CSF1R-tg negative cells that co-express chicken macrophage related markers. (A) Schematic overview of the chicken PWP, ellipsoid and associated cells, EC; ellipsoid macrophages, EAC; ellipsoid-associated cells, EAM; ellipsoid-associated macrophages; (B) confocal analysis of the PWP demonstrates the dendrites of the CSF1R-tg EAC cells protruding into the ellipsoid (white arrow); (C) MHCI and Ch68.2 staining demonstrates the presence of CSF1R-tg\textsuperscript{neg} MHCI\textsuperscript{hi} Ch68.2\textsuperscript{neg} cell population named ellipsoid macrophages (EM) that are located within the ellipsoid; (D) CSF1R is expressed by the CSF1R-tg\textsuperscript{neg} EM; (E) in contrast to EM and EAM, EAC lack MRC1L-B expression; (F) EAC and EM express high levels of CD11c in contrast to the EAM; (G) LAMP1 is expressed by all CSF1R-tg\textsuperscript{+} and CSF1R-tg\textsuperscript{neg} cells in the PWP. Yellow lines outline the location of the EM. Data is representative of five CSF1R-eGFP transgenic chickens at 5–6 weeks of age. Scale bars for images (B,E–G) are 100\,\mu m and images (C,D) are 50\,\mu m.

Overlays of MRC1L-B and CD11c expression in each CSF1R-tg subpopulations shows the uniform level of expression of these markers in the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} subpopulation compared to the CSF1R-tg\textsuperscript{dim} MHCI\textsuperscript{inter–hi} and CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} subpopulations (Supplementary Figure 4). Although numerous extraction approaches were carried out, we did not observed a CSF1R-tg\textsuperscript{+} MHCI\textsuperscript{neg/low} MRC1L-B\textsuperscript{neg} CD11c\textsuperscript{+} phenotype that would represent the EAC and FDC subpopulations in the chicken spleen. This may reflect the low survival rates of these cells post-extraction or they may express low levels of MHCI and are represented in the CSF1R-tg\textsuperscript{+} subpopulations. Even with these limitations, our complex multicolor flow cytometric analysis demonstrates the existence of at least three CSF1R-tg\textsuperscript{+} subpopulations in the chicken spleen that diversely express the CSF1R-tg, MHCI, MRC1L-B, and CD11c.

The CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} Subpopulation Expresses Low Levels of CD80, CD40 and MHCI, CD44, and Ch74.2

We next analyzed the expression of co-stimulatory molecules, CD80, CD40, and MHCI and CD44, across the three CSF1R-tg\textsuperscript{+} MHCI\textsuperscript{+} subpopulations by flow cytometry (Figure 4A). Histogram overlays indicated that the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter}...
FIGURE 3 | CSF1R-tg and MHCII are diversely expressed by CSF1R-tg+ cells. Splenic live, single cells could be separated based on their diverse CSF1R-tg and MHCII expression by flow cytometry. (A) The CSF1R-tg negative populations were gated into MHCII[hi] (Gate G1) and MHCII[lo] (G2) subpopulations. By applying a gate to the CSF1R-tg+ population (G3), the differential expression of the transgene and MHCII could be observed. Three CSF1R-tg+ subpopulations were separated into CSF1R-tg[lo] MHCII[lo]−[hi] (G4) and two CSF1R-tg[hi] cell subpopulations; CSF1R-tg[hi] MHCII[lo] (G5) and CSF1R-tg[hi] MHCII[inter] (G6) subpopulations; (B) Representative histograms of CSF1R-tg and MHCII expression levels in the CSF1R-tg+ subpopulations (G4-6); (C) The median fluorescent intensity (MFI) analysis across 20 CSF1R-eGFP chickens shows the consistent difference of CSF1R-tg and MHCII expression. Error bars indicate 95% confidence intervals, Asterisks indicate statistical significance ($P < 0.05$); (D) The CSF1R-tg[hi] MHCII[inter] subpopulation has more uniformed cell size and granularity pattern compared to the CSF1R-tg[lo] MHCII[inter]−[hi] (G4) and CSF1R-tg[hi] MHCII[lo] (G5) subpopulations; (E) CSF1R-tg[hi] MHCII[inter] (G6) subpopulation has more uniformed expression of MRCL1-B and CD11c compared to the CSF1R-tg[lo] MHCII[inter]−[hi] (G4) and CSF1R-tg[hi] MHCII[hi] (G5) subpopulations. Analysis were applied to cells gated based on size/granularity, live, single cells, CSF1R-tg+ MHCII+ gates using FMO-1 controls. Data is representative of 5 or more independent experiments from 4-8 week old CSF1R-eGFP transgenic chickens.
Figure 4. The CSF1R-tg<sup>+</sup> MHCII<sub>inter</sub>-hi subpopulation express low levels of CD80, CD40, MHCI, CD44, and Ch74.2. The three CSF1R-tg<sup>+</sup> subpopulations were gated as outlined in Figure 3A and analyzed for a number of chicken mononuclear phagocytic cell markers by flow cytometry. (A) Representative histograms and MFI expression levels of CD80, CD40, MHCI, and CD44, across the three CSF1R-tg<sup>+</sup> subpopulations; (B) representative histograms of CD1.1, K1, and Ch74.2 expression across the three CSF1R-tg<sup>+</sup> subpopulations; (C) representative flow plots of chB6, CD3, and CD8α expression across the CSF1R-tg<sup>+</sup> subpopulations indicates that the CSF1R-tg<sup>+</sup> MHCII<sub>inter</sub>-hi subpopulation express B cell and T cell markers along with macrophage markers. Gates were applied to size/granularity, live, single cells, CSF1R-tg<sup>+</sup> MHCII<sup>+</sup> gates using FMO controls. Data is representative of 3–6 independent experiments from 4 to 6 week old chickens. Error bars indicate 95% confidence intervals. Asterisks indicate statistical significance (P < 0.05).
subpopulation. The CSF1R-tg^hi MHCII^inter cells contained Ch74.2^hi and Ch74.2^neg cell populations whereas the CSF1R-tg^dim MHCII^inter–^hi and CSF1R-tg^hi MHCII^inter cells had a more uniformed expression pattern of Ch74.2 (Figure 4B). The chicken B cell marker, chB6, binds to a subset of splenic macrophages (55). We therefore analyzed the expression of chicken T/NK cells and B cell markers within the subpopulations and found that ChB6 label cells within the CSF1R-tg^dim subpopulation while a small population expressed CD3 and CD8α, possibly indicating the presence of T cells and NK cells along with macrophages (Figure 4C). The CSF1R-tg^neg MHCII^hi MRC1L-B^+ subpopulation expressed higher levels of CD80, CD40, and K1 (Supplementary Figure 5A). We also verified the lack of CD41/61 expression in CSF1R-tg^+ cells indicating that thrombocyte contamination was not altering the expression levels of the cell markers (Supplementary Figure 5B).

Overall, our multicolor flow cytometry analysis indicates that the CSF1R-tg^hi MHCII^inter subpopulation expressed lower levels of CD80, CD40, MHCII, CD44, CD1.1, and Ch74.2 compared to the CSF1R-tg^dim MHCII^inter–^hi and CSF1R-tg^hi MHCII^hi subpopulations.

The Phagocytosis Abilities of the CSF1R-tg^hi MHCII^inter Cells Are Enhanced by LPS

To determine the function of the CSF1R-tg^+ subpopulations, 5–6 week old CSF1R-eGFP transgenic chickens were intravenously administered 0.5 µm or unlabeled and LPS labeled 1 µm fluorescent beads. Three hours post-IV administration, the phenotype of the splenic phagocytic cells was analyzed by applying the gating strategy outlined in Figure 3A on the bead^neg cell population which was subsequently applied to the bead^+ cell population (Figure 5A). Due to low number of bead^+ events in the CSF1R-tg^neg MHCII^inter subpopulation, these cells were analyzed with the CSF1R-tg^neg MHCII^hi subpopulation. In the 0.5 µm bead^+ gate, the cell percentages of the CSF1R-tg^neg, CSF1R-tg^dim MHCII^inter–^hi, and CSF1R-tg^hi MHCII^hi subpopulations were significantly higher compared to the CSF1R-tg^hi MHCII^inter subpopulation. When the MFI of the 0.5 µm beads was analyzed in each subpopulation, all CSF1R-tg subpopulations had statistically significant higher bead uptake compared to the CSF1R-tg^neg subpopulation (Figure 5B).

Next, the ability of the CSF1R-tg cells to phagocyte 1 µm beads was analyzed. After 3 h, the percentage of CSF1R-tg^neg cells within the 1 µm bead^+ gate was significantly higher compared to the CSF1R-tg^dim MHCII^inter–^hi and CSF1R-tg^hi subpopulations (Figure 5C). Between the CSF1R-tg^+ subpopulations, the percentage of the CSF1R-tg^hi MHCII^hi subpopulation was significantly higher compared to the CSF1R-tg^dim and CSF1R-tg^hi MHCII^inter subpopulations. The MFI analysis of the 1 µm beads show that although the CSF1R-tg^dim subpopulation was significantly underrepresented in the bead^+ gate, they had significantly higher bead uptake compared to the CSF1R-tg^hi MHCII^inter subpopulation. In addition, the CSF1R-tg^neg and CSF1R-tg^hi MHCII^hi subpopulations phagocytosed significantly higher number of beads compared to the CSF1R-tg^hi MHCII^inter subpopulation (Figure 5C).

To determine the phagocytosis abilities of cells exposed to bacterial antigens, 1 µm beads were labeled with LPS and phagocytosis was analyzed 3 h post-IV administration. The percentages of both CSF1R-tg^neg and CSF1R-tg^dim MHCII^inter–^hi subpopulations in the bead^+ gate were significantly higher compared to the CSF1R-tg^hi MHCII^hi and CSF1R-tg^hi MHCII^inter subpopulations (Figure 5D). The MFI levels of the LPS labeled 1 µm beads were significantly higher in the CSF1R-tg^hi MHCII^hi and CSF1R-tg^hi MHCII^inter subpopulations compared to the CSF1R-tg^dim MHCII^inter–^hi subpopulation. All CSF1R-tg^+ subpopulations had significantly higher bead uptake compared to the CSF1R-tg^neg subpopulation (Figure 5D). In vivo functional analysis indicates that exposure to LPS enhances the phagocytosis of 1 µm beads by the CSF1R-tg^hi MHCII^inter subpopulation.

In vivo Antigen Uptake Demonstrates Diversity in the EAM in the PWP

Flow cytometric analysis demonstrated the differential expression of MRC1L-B across each of the CSF1R-tg^+ subpopulations and the existence of the CSF1R-tg^neg MRC1L-B^+ subpopulation (Figure 3D). We analyzed the percentage of MRC1L-B expressing cells in the bead^+ CSF1R-tg^+ and CSF1R-tg^neg subpopulations and used immunohistochemistry to analyze the location of the beads in the spleen 3 h post-administration (Figure 6). Firstly, we found a majority of 0.5 µm bead^+ CSF1R-tg^+ cells consisted of MRC1L-B expressing cells (~95%) which was significantly higher compared to the percentage of MRC1L-B^+ in both the 0.5 µm bead^+ CSF1R-tg^dim MHCII^inter–^hi and CSF1R-tg^hi MHCII^inter subpopulations (~75–70%). In the 0.5 µm bead^+ CSF1R-tg^neg subpopulation, we found that ~10% of cells expressed MRC1L-B. In the unlabeled 1 µm bead^+ CSF1R-tg^hi MHCII^hi subpopulation (~98%), the percentage of MRC1L-B expressing cells was significantly higher compared to the CSF1R-tg^dim MHCII^inter–^hi and CSF1R-tg^hi MHCII^inter MRC1L-B subpopulations (~90–85%) while no significance difference between the CSF1R-tg^+ cells was observed after phagocytosis of LPS labeled 1 µm beads. In the CSF1R-tg^neg unlabeled and LPS labeled 1 µm bead^+ subpopulation, MRC1L-B expressing cells made up ~50% of the cell population demonstrating the phagocytosis abilities of the EM (Figure 6A).

We next analyzed the location of the bead^+ cells in the spleen 3 h post-administration. Whole mount microscopic analysis shows that irrespective of bead size or LPS coating, beads were localized to the PWP (Supplementary Figure 6). Additional in depth analysis found that the 0.5 µm beads were located within the CSF1R-tg^+ Ch68.2^+ cells but high concentrations of beads were observed in the CSF1R-tg^– Ch68.2^neg cells, which represent the EAM (Figure 6B). Uptake of 1 µm beads was observed in the CSF1R-tg^+ Ch68.2^+ EAC but a majority of beads were associated with the CSF1R-tg^– Ch68.2^neg EAM (Figure 6Bii). Beads were also found closely associated with chB6^+ cells in the ellipsoid representing the CSF1R-tg^neg MRC1L-B^neg bead^+ cell phenotype observed in flow cytometry (Supplementary Figure 6).
FIGURE 5 | The phagocytosis abilities of the CSF1R-tg\(^{hi}\) MHCII\(^{int}\) cells is enhanced by LPS. (A) Representative flow plots and histograms of the gating strategy for the analysis of the cell phenotype based on CSF1R-tg and MHCII expression levels and phagocytosis of beads 3 h post-IV bead administration by flow cytometry. The percentage of cells in each subpopulations within the bead\(^{+}\) gate and MFI of the beads within each subpopulation; (B) 0.5 \(\mu\)m; (C) 1 \(\mu\)m and; (D) LPS labeled 1 \(\mu\)m carboxylate fluorescent beads. Data is shown as the median of 5 or 4 individual chickens and error bars indicate 95% confidence intervals. Asterisks indicate statistical significance (\(P < 0.05\)).

deposits of LPS labeled 1 \(\mu\)m beads were also located in the CSF1R-tg\(^{+}\) MRC1L-B\(^{+}\) EAM compared to the CSF1R-tg\(^{+}\) MRC1L-B\(^{neg}\) EAC (Figure 6Biii). Due to their location in the PWP the uptake of beads by the different MRC1L-B\(^{+}\) CSF1R-tg\(^{+}\) subpopulations indicates the variable expression of the CSF1R-tg by EAM. Thus, in vivo antigen uptake demonstrates the existence of EAM subsets that differ in their CSF1R-tg expression and antigen uptake but not in their MRC1L-B expression.

The CSF1R-tg\(^{+}\) MRC1L-B\(^{neg}\) cDC Can Phagocytose Model Antigens

We observed a vast majority of CSF1R-tg\(^{+}\) MRC1L-B\(^{neg}\) cells in the splenic PALS and very little in the PWP. These cells were found to be a minor population in CSF1R-tg\(^{+}\) bead\(^{+}\) cells in the in vivo uptake analysis by flow cytometry while immunohistolological analysis demonstrated the lack of beads in the PALS 3 h post-administration (Supplementary Figure 6B).
In vivo functional analysis indicates diversity in the EAM. (A) The percentage of MRC1L-B$^+$ cells in the 0.5 µm$^+$, unlabeled and LPS labeled 1 µm bead$^+$ CSF1R-tg$^+$ and CSF1R-tg$^-$ subpopulations 3h post-IV bead administration analyzed by flow cytometry. Data is shown as the median of 5 or 4 individual animals and error bars indicate 95% confidence intervals. Asterisks indicate statistical significance ($P < 0.05$); (B) histological analysis of the location of the beads in the PWP; (i) few 0.5 µm beads were located in CSF1R-tg$^+$ Ch68.2$^+$ EAC cells (yellow arrow) whereas CSF1R-tg$^+$ Ch68.2$^-$ EAM contain numerous beads (yellow arrow); (ii) the 1 µm beads co-localize with the CSF1R-tg$^+$ Ch68.2$^+$ EAC (white arrow) but numerous beads were found in the CSF1R-tg$^+$ Ch68.2$^-$ EAM (insert image); (iii) LPS labeled 1 µm beads were located throughout the PWP in CSF1R-tg$^+$ MRC1L-B$^-$ EAM (white arrow) while high concentrations of beads were located in the CSF1R-tg$^+$ MRC1L-B$^+$ EAM (yellow arrows). Data is representative of analysis of three individual chickens.

As previously mentioned, cells with the MHCII, MRC1L-B$^-$, CD11c$^+$ phenotype consist of cDC while MRC1L-B expression is associated with cells of the macrophage lineage (23). Therefore, we used in vitro splenic cultures to determine if the low levels of phagocytosis observed in the CSF1R-tg$^+$ MRC1L-B$^-$ cells was due to their location in the spleen rather than a functional aspect of these cells. Firstly, we analyzed the MRC1L-B phenotype of splenocytes grown in the presence of CSF1 by flow cytometry (Figure 7A). After 48 h, CSF1R-tg$^+$ MRC1L-B$^+$ cells made up around 75% of the total cell population while the CSF1R-tg$^-$ MRC1L-B$^-$ subpopulation made up $\sim$4% (Figure 7A and gating strategy Supplementary Figure 7A). We did observe the presence of the CSF1R-tg$^-$ MRC1L-B$^+$ subpopulation but not the CSF1R-tg$^-$ MRC1L-B$^+$ subpopulation in the cultures. As we were interested in analyzing the difference between the MRC1L-B$^+$/MRC1L-B$^-$ subpopulations, we focused our analysis on the CSF1R-tg$^+$ MRC1L-B$^+$ and MRC1L-B$^-$ subpopulations. Firstly, cells were treated with unlabeled and LPS-labeled 1 µm beads at 41 or 4°C for 3 h and the phenotype of the bead$^+$ cells followed by the MFI of beads within the subpopulations were analyzed (Supplementary Figure 7B). After 3 h, the CSF1R-tg$^+$ MRC1L-B$^+$ subpopulation phagocytosed significantly more beads than the CSF1R-tg$^+$ MRC1L-B$^-$ subpopulation, a similar pattern observed in vivo (Figure 7A). Confocal analysis demonstrated the adherence of both MRC1L-B$^+$ and MRC1L-B$^-$ subpopulations in the cell cultures (Figure 7Bi) and the high phagocytic abilities of the MRC1L-B$^+$ cells (Figure 7Bii). We also observed the co-localization of MRC1L-B expression with internalized beads indicting its internalization with beads from the cell surface to intracellular vesicles. In addition we observed the co-localization of LAMP1 expression with the phagocytosed beads (Figure 7Biii).

We next analyzed whether there was a difference in the ability of the subpopulations to uptake model antigen for transport to intracellular vesicles, such as lysosomes and phagosomes, using E. coli and Zymosan bioparticles labeled with the pH-sensitive pHrodo red. As a control, cells were treated with the actin-polymerization inhibitor, Cytochalasin D, 10 min prior to exposure to the bioparticles and the data was normalize by subtracting background fluorescence (Supplementary Figure 7). After 3 h, the CSF1R-tg$^+$ MRC1L-B$^+$ subpopulation engulfed and transported significantly more bioparticles compared to the CSF1R-tg$^+$ MRC1L-B$^-$.
**The CSF1R-tg^hi MHCII^hi CD11c^hi and CD11c^dim Subpopulations Are Enriched With Cells of the cDC and Macrophage Lineages**

We carried out RNA-seq analysis on FACS sorted splenic subpopulations as shown in Figure 8A. To determine if the level of putative CD11c expression was specific to a cell subset within the CSF1R-tg^+ subpopulation, we further split the CSF1R-tg^hi MHCII^hi subpopulation into CD11c^hi and CD11c^dim subpopulations due to insufficient cell numbers for their analysis via RNA-seq and therefore these cells were collected with the CSF1R-tg^neg MHCII^hi subpopulation. Pairwise comparisons of all subpopulations are shown in Supplementary Table 1. Using pairwise GSEA, we analyzed whether the transcriptome signatures of murine immune cells were enriched in the sorted subpopulations (23, 54). The CSF1R-tg^neg MHCII^dim and CSF1R-tg^neg MHCII^subpopulations were significantly enriched for T cell and B cell gene fingerprints compared to the CSF1R-tg^dim MHCII^inter−^hi subpopulation (Figure 8A). The separation of the CSF1R-tg^neg MRC1L-B^+ subpopulation from the CSF1R-tg^neg MRC1L-B^neg cells lead to insufficient cell numbers for their analysis via RNA-seq and therefore these cells were collected with the CSF1R-tg^neg MHCII^hi subpopulation. Pairwise comparisons of all subpopulations are shown in Supplementary Table 1.

**FIGURE 7** | In vitro functional analysis demonstrates the ability of the CSF1R-tg^+ MRC1L-B^neg cDC to phagocytose model antigens. Splenocytes were cultured for 48 h in the presence of CSF1 and phagocytosis was analyzed 3 h post-treatment via flow cytometry. (A) After 48 h in culture, CSF1R-tg^+ MRC1L-B^+ are the dominant subpopulation (75%) compared to the CSF1R-tg^neg MRC1L-B^neg (12%), CSF1R-tg^dim MRC1L-B^neg (4%), and CSF1R-tg^neg MRC1L-B^neg subpopulations (17.5%). MFI of unlabeled and LPS labeled 1 µm beads was significantly higher in the CSF1R-tg^+ MRC1L-B^+ subpopulation compared to the CSF1R-tg^neg MRC1L-B^neg cell population 3 h post-bead treatment; (B) confocal analysis demonstrates the (i) adherence of the MRC1L-B^+ and MRC1L-B^neg cells after 48 h in culture; (ii) MRC1L-B^+ cells phagocytose numerous beads and its expression is localized with internalized beads (white arrows); (iii) intracellular LAMP1 expression co-localizes with MRC1L-B+ (Continued)
Mo/Mp vs. cDC gene signatures. cDC, pDC, Mo/Mp vs. cDC, ncMo, and MoDC gene signatures were enriched in the CSF1R-tg<sup>hi</sup> MHCII<sup>inter</sup> subpopulation (Figure 8B). The mammalian cDC2 transcriptomic fingerprint could not be established due to the low number of genes to generate reliable statistical analysis. GSEA analysis indicate that the CSF1R-tg<sup>neg</sup> MHCII<sup>dim</sup> subpopulation consist of macrophages and cDC.

![Image](https://via.placeholder.com/150)

**Figure 8** The CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> and CSF1R-tg<sup>hi</sup> MHCII<sup>inter</sup> subpopulations Express Gene Signatures Characteristic of cDC, Macrophages, and Red Pulp Macrophages

We next analyzed the enrichment of conserved mRNA transcripts characteristic of different cell lineages across the sorted subpopulations. Firstly, core T/NK cell and B cell related genes signatures were analyzed and as expected the CSF1R-tg<sup>neg</sup> MHCII<sup>dim</sup> subpopulation were enriched for T/NK cells, significantly expressing CD3E, CD3D, CD28, CD5, RUNX3, and FR2 transcripts whereas the CSF1R-tg<sup>neg</sup> MHCII<sup>hi</sup> subpopulation were enriched for B cells, significantly expressing key transcripts such as CD79B, CXCR5, and PAX5 (Supplementary Figure 8). The CSF1R-tg<sup>dim</sup> MHCII<sup>inter</sup>−hi subpopulation also strongly expressed CD79B and PAX5 (Supplementary Figure 8).

Next, the enrichment of cell-fate determining transcriptional regulators involved in macrophage and cDC lineages as reported in the ImmGen Consortium (56) and the mammalian literature were analyzed in the CSF1R-tg<sup>hi</sup> subpopulations (Figure 8B). The CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> subpopulation was enriched for cells expressing cDC lineage related transcriptional regulators or genes positively associated with cDC differentiation [EGR1, ID2, IRF8 (56, 57), NRB4A3 (58), and ZBTB46 (59, 60)] while the CSF1R-tg<sup>hi</sup> MHCII<sup>bi</sup> CD11c<sup>dim</sup> subpopulation was enriched for macrophage-related transcription factors [MAFB (61, 62), MITF (63), SPI1 (64), TFEC (65), and ZEB2 (66); Figure 8B]. The red pulp macrophage-lineage related transcription factor, SpiC (67) was expressed at the highest level in the CSF1R-tg<sup>hi</sup> MHCII<sup>inter</sup> subpopulation but also strongly expressed in the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> and CSF1R-tg<sup>dim</sup> MHCII<sup>inter</sup>−hi subpopulations.

The CSF1R-tg<sup>dim</sup> MHCII<sup>inter</sup>−hi subpopulation expressed higher levels of genes encoding transcripts characteristic of cDC and macrophages (ETS1, ID2, IRF4, IRF8, SpiC, and SLA2). This observation, along with the expression of B cell and T cell related transcripts, indicates that this subpopulation is very heterogeneous. Overall the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> and the CSF1R-tg<sup>hi</sup> MHCII<sup>bi</sup> CD11c<sup>dim</sup> subpopulations express higher levels of genes characteristic of cDC and macrophage lineages across warm blooded vertebrate species (18, 19, 54). Thus, it can be deduced from this data that the CSF1R-tg<sup>hi</sup> CD11c<sup>hi</sup> and the CSF1R-tg<sup>hi</sup> CD11c<sup>dim</sup> subpopulations must primarily encompass cDC and macrophages, respectively.

We next analyzed the enrichment of cell-lineage related surface receptors involved in a number of cellular processes, such as T cell co-stimulation, antigen presentation and migration. cDC related markers FLT3, CADM1, and XCR1 (10, 13), were significantly enriched in the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> subpopulation whereas macrophage-associated genes, CSF1R, CD14, CX3CR1, CCR2, CXCR4, and TNFSF1A were enriched in the CSF1R-tg<sup>hi</sup> MHCII<sup>bi</sup> CD11c<sup>dim</sup> subpopulation (Figure 9A). The CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>dim</sup> subpopulation were enriched for migratory and adhesion related transcripts such as, CCR7, CDH2, C3AR1, and CDH2 (Supplementary Figure 9).

The integrin ITGAD was enriched in the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> and CSF1R-tg<sup>hi</sup> MHCII<sup>inter</sup> subpopulations whereas...
ITGAV transcript was enriched in CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>dim</sup> subpopulation. Co-stimulatory molecules, CD80 and CD86 transcripts were significantly expressed by cells within the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> and CD11c<sup>dim</sup> subpopulations. DPP4 (CD26) is highly expressed in the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>dim</sup> subpopulation (not CD11c<sup>hi</sup>), which is not consistent with DPP4 being expressed to high levels selectively on cDC1 in humans, mice, and sheep (19). We next analyzed the expression of Toll-like receptors (TLR) transcripts across the CSF1R-tg<sup>−</sup> subpopulations and found that the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>dim</sup> subpopulation was enriched for cells expressing TLR4, TLR2A and co-receptor, CD14. The CSF1R-tg<sup>−</sup> MHCII<sub>inter</sub> subpopulation expressed high levels of C-type lectin receptors, FCER2 (CLEC17AL-A), and CLEC17A (CLEC17AL-B) (68). The apoptotic cell recognition receptor, TIMD4 (69) transcript expression was enriched in both the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> and CD11c<sup>dim</sup> subpopulations (Figure 9A).

The CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> subpopulation significantly expressed genes involved in cross-presentation and MHCII-associated genes [ACVRL1 (70), CD74, LAMP5 (71), and WDFY4 (72)]. Key macrophage related genes, MERTK, VCAM1, and genes related to red pulp and alternatively activated macrophages, C1QA, C1QB, and PPARG (73), and suppressor of cytokine signaling molecules (SOCS2, SOCS4, and SOCS6) were highly expressed in the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>dim</sup> subpopulation compared to the CD11c<sup>hi</sup> subpopulation. An array of transcripts involved in antigen processing [CD74, CTSS, LGMN, LAMP1, LAMP3, LAMP5, MERTK, and PSAP (71)] were also strongly expressed by the CSF1R-tg<sup>hi</sup> MHCII<sub>inter</sub> CD11c<sup>dim</sup> subpopulation (Figure 9B). The CSF1R-tg<sup>hi</sup> MHCII<sub>inter</sub> subpopulation expressed the highest levels of genes characteristic of the red pulp macrophage lineage, MARCO, C3, and LRG1 (73). Chicken heterophils express the CSF1R-tg at low levels (33, 34). Therefore, we analyzed the expression of core heterophil gene transcripts across the CSF1R-tg<sup>−</sup> subpopulations (74) and found no significant enrichment of heterophil related gene transcripts in any of the CSF1R-tg<sup>−</sup> subpopulations (Supplementary Table 2). The CSF1R-tg<sup>dim</sup> MHCII<sub>inter−hi</sub> subpopulation were enriched for cathepsins family members (CTSC, CTSH, and CTSS) but no clear definition of what enriched macrophage cell type this subpopulation represented was identified. To analyze whether the CSF1R-tg<sup>dim</sup> subpopulation were true phagocytic cells and not an artifact of transgene expression or cells undergoing physiological stress, we carried out an in vivo liposome depletion study, where phagocytic cells were targeted for depletion by apoptosis induction after the uptake of clondronate liposomes (75). Three days after a single IV-administration of liposomes, we observed a severe depletion of the CSF1R-tg<sup>dim</sup> MRC1L-B<sup>B+</sup> subpopulation in chickens treated with the clondronate liposomes compared to those treated with PBS liposomes indicating that this subpopulation contains phagocytic cells belonging to the MPS (Supplementary Figure 10). Although our sorting approach would not have led to pure cell populations, we were able to determine the enrichment of cDC in the CSF1R-tg<sup>hi</sup> MHCII<sup>hi</sup> CD11c<sup>hi</sup> subpopulations as previously described (23). Furthermore, we demonstrated the existence of at least
three subsets of resident tissue macrophages in the chicken spleen. Based on the RNA-seq and immunohistological analysis, the CSF1R-tg^{hi} MHCI^{hi} CD11c^{lo} subpopulation are enriched with macrophages from the PWP and PALS while the CSF1R-tg^{dim} MHCI^{inter} cells are enriched with red pulp macrophages. Although we cannot demonstrate which splenic cell they represent, the CSF1R-tg^{dim} MHCI^{inter–hi} MRC1L-B^{+} are a subset of the chicken splenic MPS.

**DISCUSSION**

Mononuclear phagocytes reside within distinct niches of the spleen where they partake in the number of cellular processes from homeostasis, removal of aged, or damaged erythrocytes to killing of pathogens and induction of adaptive immune responses. The development, proliferation, and survival of monocytes, macrophages, and cDC are controlled by CSF1R [reviewed by (76)]. Lineage specific expression of CSF1R has allowed for the detailed study of the MPS using the CSF1R-reporter transgenic chickens. Since their generation, transcriptome analysis of bursal macrophages, liver macrophages, and to some extent cDC, along with subsets of blood monocytes have been described (22). Hu et al., observed the lack of CSF1R-tg expression in chicken Kupffer cells that express similar CSF1R mRNA levels as tissue macrophages. In certain cell types in mice, CSF1R expression is under controlled by a 150 bp distal promoter not included in the generation of the CSF1R-reporter chickens and thus is inferred as the reason for the lack of CSF1R-tg expression in chicken Kupffer cells (22, 77). The splenic MPS cells has been described based on their MHCII, MRC1L-B, Ch74.2 expression, and acid-phosphatase staining and to a certain extent using the CSF1R-reporter transgenic chickens (24, 25, 29, 33, 78–80). However, the separation of PALS and PWP macrophages has not been described while comparative gene profiling has identified the existence of the XCR1 cDC in the chicken spleen but not their location (23). Using the CSF1R-reporter transgenic chickens we carried out a comprehensive analysis of the splenic MPS.

In the chicken spleen, interdigitating cells (also known as interdigitating DC) and PALS-associated macrophages express MHCI and MRC1L-B while MHCI^{hi} CD11c^{+} MRC1L-B^{neg} cells are enriched for cDC (28, 29). As the CD11c, 8F2 antibody is not commercially available, progress in the field of chicken DC research has been limited. Using the CSF1R-reporter transgenic chickens, we observed a CSF1R-tg^{+} subpopulation lacking MRC1L-B expression in the PALS (Figure 1) demonstrating that these chicken models can be used to study cDC without requiring specific cDC antibodies such as FLT3. In the PWP, the equivalent of the mammalian MZ, two subpopulations have previously been shown to express the CSF1R-tg (22, 33). On the outer margins of the PWP, intermingled with B cells are the CSF1R-tg^{+} EAM while the CSF1R-tg^{+} EAC are anchored into the ellipsoid (81). In contrast to EAM, EAC lack MRC1L-B and MHCI expression and specifically stain for Ch68.2, a chicken monoclonal which binds to an unknown epitope (25, 28). In this study, we show that EAC express higher levels of CD11c compared to the EAM [Figure 1; (23, 42)]. Due to their location in the PWP, the EAM resemble the mammalian MZM which function to trap and display antibody-coated antigens from the blood for transfer to the MZ B cells which is subsequently deposit to FDC in the follicles (82). However, in chickens, surface antigen display has been observed in EAC but not EAM (28, 83). The high level of CD11c expression by the EAC may correlate with their ability to detach from the ellipsoid upon antigen exposure and their subsequent migration to the PALS to develop into FDC (28).

We found intracellular punctate expression of MHCI by EAC but could not fully determine whether these cells express surface MHCI (Figure 2). These cells were found to express LAMP1, possibly demonstrating their ability to digest substances delivered by endocytosis and phagocytosis (84). In the red pulp, we found that the CSF1R-tg^{+} cells express high levels of MRC1L-B but not all stained for the chicken macrophage marker, Ch74.2 as previously described (24). A previous study has described the presence of CD45^{+} Ch68.2^{neg} cells within the inner regions of the ellipsoid (28). We observed cells lacking CSF1R-tg expression in a similar location in the ellipsoid and found they express high levels of MHCII, MRC1L-B, CD11c, LAMP1, and CSF1R (22). We have called this cell population “ellipsoid macrophages” (EM). The EM may represent incoming monocytes or ellipsoid resident macrophages and requires further study in the future. Through flow cytometry we show that CSF1R-tg^{neg} MRC1L-B^{+} cells express high levels of CD80, CD40, and the chicken monocyte/thrombocyte marker, K1 (Supplementary Figure 4).

When analyzing the phenotype of cells via flow cytometry we found that splenic MPS diversely express the CSF1R-tg and MHCI. One subpopulation could be distinguished based on their CSF1R-tg^{dim} MHCI^{inter–hi} phenotype while two subpopulations expressed similar levels of the CSF1R-tg and were separated based on MHCI^{hi} and MHCI^{inter} levels (Figure 3). We found that the CSF1R-tg^{dim} and CSF1R-tg^{hi} MHCI^{hi} subpopulations were very heterogenic in their MRC1L-B and CD11c expression levels while the CSF1R-tg^{hi} MHCI^{inter} subpopulation expressed these markers more uniformly (Figure 3). We did not identify a cell phenotype resembling EAC or FDC which have the CSF1R-tg^{+} MHCI^{neg/low} MRC1L-B^{neg} CD11c^{+} phenotype (28, 33). This may be due to their lack of survival during cell isolation or they express low or dynamic levels of MHCI. Although there is an EAC-specific surface marker, Ch68.2, this antibody fails to provide consistent staining in flow cytometry (data not shown). Further phenotypical analysis demonstrated low expression levels of CD80, CD40, MHCI, and CD44 in the CSF1R-tg^{hi} MHCI^{inter} subpopulation compared to the CSF1R-tg^{dim} and CSF1R-tg^{hi} MHCI^{hi} subpopulations (Figure 4). The CSF1R-tg^{hi} MHCI^{inter} subpopulation express low levels of Ch74.2 which was also observed for red pulp macrophages via immunohistology (Figure 4). Mammalian red pulp macrophages express low level of co-stimulatory molecules and MHCI due to their reduced role in T cell antigen presentation (2). These observations would suggest that the CSF1R-tg^{hi} MHCI^{inter} subpopulation may consist of red pulp macrophages.

In vivo functional analysis demonstrated that the CSF1R-tg^{hi} MHCI^{inter} subpopulation phagocytosed significantly more...
beads compared to the CSF1R-tg\textsuperscript{dim} and CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} subpopulations while labeling beads with LPS increased the phagocytosis of the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} subpopulation (Figure 5). LPS has been shown to enhance antigen uptake by chicken red pulp macrophages (24). In a recent study, ex vivo isolated splenic MRC1L-B\textsuperscript{hi} MHCI\textsuperscript{lo} cells showed higher phagocytosis of dead cells than MRC1L-B\textsuperscript{lo} MHCI\textsuperscript{hi} cells with the latter cell population suggested to represent red pulp macrophages. Another study has also demonstrated the expression of the apoptotic cell receptor, Tim4, by EAM which we show express high levels of MRC1L-B [Figure 1; (22, 85)]. Therefore, the previous characterized, MRC1L-B\textsuperscript{hi} MHCI\textsuperscript{lo} cells may represent a heterogeneous population of red pulp macrophages and EAM. In vivo functional analysis indicated that the majority of the bead\textsuperscript{+} CSF1R-tg\textsuperscript{+} subpopulations expressed MRC1L-B. When we analyzed location of the beads in the spleen we observed large deposits in the EAM while few beads were observed associated with EAC (Figure 6). Both flow cytometric and immunohistological analysis suggests that a subset of CSF1R-tg\textsuperscript{dim} MHCI\textsuperscript{inter−}\textsuperscript{hi} MRC1L-B\textsuperscript{+} and CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} MRC1L-B\textsuperscript{+} subpopulations are located in the PWP demonstrating that EAM differ in their CSF1R-tg expression levels but not MRC1L-B. For the bead\textsuperscript{+} CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} subpopulation, we speculate that these cells are either a subset of EAM or red pulp macrophages residing in close proximity to the EAM, hence their ability to capture antigens.

We observed very little in vivo bead uptake by the CSF1R-tg\textsuperscript{+} MHCI\textsuperscript{+} MRC1L-B\textsuperscript{neg} cells which may be due to their higher numbers in the PALS and not the PWP where antigen capture primarily takes place. In vitro functional analysis demonstrated that the CSF1R-tg\textsuperscript{+} MRC1L-B\textsuperscript{neg} subpopulation were capable of phagocytosis (Figure 7). Mammalian macrophages express large intracellular pools of MRC1 within their early endosomes, which undergo continual rapid recycling to the cell surface to endocytose bound antigens (86). Due the high recycling nature of MRC1 in mammals and the fixture of adherent cells with PFA in this study, we only observed intracellular punctate expression of MRC1L-B (Figure 7). However, we found the co-localization of MRC1L-B and LAMP1 expression with the internalized beads in vitro which for the first time demonstrates the ability of MRC1L-B to traffic with antigens to lysosomes and endosomes similar to mammalian MRC1 (86). Our in vivo functional analysis provided the first demonstration of antigen uptake by the CSF1R-tg\textsuperscript{+} MRC1L-B\textsuperscript{neg} cDC subpopulation in the spleen further demonstrating the ability to study cDC ex vivo using the CSF1R-transgenic chickens. In addition, future studies using the CSF1R-transgenic chickens and pathogens would allow for the in vivo visualization of cDC-T cell interactions in the chicken spleen.

Transcriptomic analysis has shown the conservation of MPS lineage specific gene signatures throughout distantly related mammals (18, 19, 23, 54). A previous comparative analysis of chicken splenic cells demonstrated the existence of cDC which expressed the MHCI\textsuperscript{+} MRC1L-B\textsuperscript{neg} CD11c\textsuperscript{+} phenotype by their enrichment for XCR1, FLT3, and key cDC lineage gene transcripts. The authors also described the enrichment of macrophage related genes in MRC1L-B\textsuperscript{−} cells (23). We expanded on this study by separating the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} subpopulation into CD11c\textsuperscript{hi} and CD11c\textsuperscript{dim} subsets and analyzing two additional subsets of splenic MPS via RNA-seq analysis (Figure 8). It should be noted that MRC1L-B\textsuperscript{+} cells were not excluded from the CSF1R-tg\textsuperscript{+} subpopulations as performed in the Vu Manh et al., study (23). Primarily we analyzed the presence of murine splenic immune cell gene signatures using GSEA across all the cell subpopulations (Figure 8). We initially found an enrichment of the cDCI gene signatures in the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} CD11c\textsuperscript{hi} subpopulation and macrophage gene signatures in the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{lo} CD11c\textsuperscript{dim} subpopulation. We found that the CSF1R-tg\textsuperscript{dim} MHCI\textsuperscript{inter−} hi subpopulations contained cells expressing B cell, T cell, cDC, and macrophage gene signatures which correlates with the immuno-phenotyping analysis (Figures 3, 4). Upon closer analysis of the enrichment of genes associated with cell lineage development and function, we found that the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} CD11c\textsuperscript{hi} subpopulation were enriched for genes characteristic of the cDC lineage (IRF8, ID2, ZEB2, FLT3, XCR1, and CAMD1) although they contain ~30% of MRC1L-B\textsuperscript{neg−hi} cells which may suggest that a significant fraction of chicken cDC express MRC1L-B contrary what was previously reported or this population contains non-cDCs that express MRC1L-B (23). This subpopulation contained cells enriched for genes also involved in cross-presentation and MHCII processing [WDFY4, ACVRL1, CD74, and LAMPS; (72)]. However, in contrast to human, mouse, and sheep cDC, we found strong expression of DPP4 in chicken cells corresponding to macrophages, CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} CD11c\textsuperscript{dim} cells, discussed later, and not chicken cDC (19, 87). This suggests that chicken macrophages may have a role in T cell-stimulation (85). The hallmark of cDC is their ability to present to and activate T cells, however, numerous attempts to demonstrate DC-T cell interaction through MLR were to no avail, with numerous cells of the MPS engulfing/phagocytosing labeled CD4\textsuperscript{+} T cells (data not shown).

The CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} CD11c\textsuperscript{dim} subpopulation were enriched for genes characteristic of macrophages [CCR2, CD14, CD36, CSF1R, CXCR4, CX3CR1, HIF1A, MAFB, MITF, STAT1, SPI1, TLR4, and TNFSF1A; (22, 76)]. This subpopulation was also enriched for M2 anti-inflammatory cell phenotype and red pulp macrophage related gene transcripts (CIQA, CIQB, and PPARG) which are associated with tissue repair and homeostasis (89). Based on their CD11c\textsuperscript{dim} phenotype these macrophages likely represent the EAM and PAL-associated macrophages in the spleen. Distinguishing the difference between the EAM and PAL-associated macrophages requires further investigation. The CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} subpopulation was enriched for genes associated with red pulp macrophages [HEPHL1, LRPI, SpiC, and TT; (67)]. Similar to the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{hi} subpopulation, we cannot rule out the possibility of the CSF1R-tg\textsuperscript{hi} MHCI\textsuperscript{inter} subpopulation containing more than one macrophage cell lineage, such as PALS-associated macrophages. The CSF1R-tg\textsuperscript{dim} subpopulation were phenotypically heterogeneous and may represent cells undergoing different levels of development or activation in the spleen and require further analysis. In the murine spleen, pDC make up around 0.5% of cells (90). In our analysis, we found low levels of pDC gene signatures in the CSF1R-tg\textsuperscript{neg} MHCI\textsuperscript{hi} subpopulation which may be
TABLE 2 | An overview of the phenotype and phagocytosis abilities of MPS in the chicken spleen as determined by immunohistology and flow cytometry.

| Phenotype | In vivo phagocytosis |
|-----------|---------------------|
|           | CSF1R-tg | MHCII | MRC1L-B | CD11c | CD80 | CD40 | MHCI | ChB6 | Ch74.2 | MRC1L-B positive | MRC1L-B negative |
| Negative  | Hi       | Hi     | Hi      | Hi    | N/D  | –     | N/D  |   +   | +       |          |                      |
| Dim       | Inter    | Neg/Low| Hi      | Hi    |     |     |     |   +/– | +       |          |                      |
|           |          |        |         | Hi    |     |     |     |   +   | + +     |          |                      |
|           |          |        |         | Hi    |     |     |     |   +   | + +     |          |                      |
| High      | Hi       | Neg/Low| Hi      | Hi    |     |     |     |   –   | + + +    |          |                      |
|           |          |        |         | Hi    |     |     |     |   +   | + +     |          |                      |
| High      | Inter    | Neg/Low| Hi      | Low   | Low | Low | Low |   –   | + +     |          |                      |
|           |          |        |         | Hi    |     |     |     |   +   | + +     |          |                      |

The level of surface marker expression in the CSF1R-tg+ and CSF1R-tg– cells is indicated as negative to low (Neg), intermediate (Inter), and high (Hi). N/D, not determined. The + symbols indicate the level of phagocytosis in the CSF1R-tg+ MRC1L-B+ or MRC1L-B– cells.

related to the MRC1L-B+ cells present in this subpopulation (Figure 3E). However, more analysis is required to confirm the existence of this cell lineage in chickens. As of yet, no definitive study has identified different chicken cDC subsets as observed in mammals possible indicating the existence of just one cDC subset in chickens. We did observe the cDC2 related transcription factor, IRF4, in the CSF1R-tg dim subpopulation but this may be due to the mixture of B cell, cDC, and macrophage lineages in this subset (91–93). Future single cell RNA-seq analysis of splenocytes has the potential to further contribute to delineating the cells of the chicken splenic MPS.

In conclusion, we have shown that the CSF1R-reporter transgenic chickens can be used to distinguish different cell subsets of the MPS in the spleen. We have demonstrated that the PALS-associated CSF1R-tg+ cells do not uniformly express MRC1L-B helping to differentiate PALS-associated cDC (MRC1L-B+ cells) from macrophages (MRC1L-B–) in the chicken spleen. In the PWP, a novel CSF1R-tg– cell population, called ellipsoid macrophages were also characterized. We demonstrated that there are at least three distinctive cell subpopulations expressing diverse levels of CSF1R-tg and MHCII in the chicken spleen. In addition, we demonstrated that the CSF1R-tg+MHCII– subpopulation, phenotypically, and functionally resembles red pulp macrophages and identified functional diversity in EAM. For the first time we demonstrated the functionality of CSF1R-tg+MRC1L-B+ cDC in vitro. The phenotype and function of chicken splenic MPS are provided as an overview in Table 2. This is the first description of the function and transcriptome of a number of MPS cell lineages in chicken spleen that will contribute to future studies to dissect their roles in antigen presentation, immune responses, and resolve the EAC and FDC cell lineages and functions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Materials and Methods.

ETHICS STATEMENT

The animal study was conducted under Home Office project license PE263A4FA according to the requirements of the Animal (Scientific Procedures) Act 1986, with the approval of the Roslin Institute’s ethical review committees. Birds were humanely culled in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986.

AUTHOR CONTRIBUTIONS

KS, LV, and AB conceptualized the study. KS, DB, and LV performed or assisted with the bird experiments. KM performed the transcriptomic data analysis. KS performed all experiments and analysis. LV, PK, and HS secured the funding. KS wrote the manuscript. All authors contributed to read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.636436/full#supplementary-material

**Supplementary Figure 1** The dynamic expression of CD11c and MHCII in the chicken spleen.

**Supplementary Figure 2** Multicolor confocal analysis of the PWP.

**Supplementary Figure 3** Gating strategy for flow cytometric analysis and FACS.

**Supplementary Figure 4** Immuno-phenotyping of the CSF1R-tg+ and CSF1R-tg- cells in the chicken spleen.

**Supplementary Figure 5** Phenotypical analysis of the CSF1R-tg+ MFC1L-B+ cells in the chicken spleen and thrombocyte cell analysis.

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**Supplementary Figure 6** Analysis of the location of fluorescent bead particles 3 h post-IV administration in chicken spleen.

**Supplementary Figure 7** Flow cytometric analysis of adherent primary chicken splenocytes cultured for 48 h and in vitro phagocytosis controls at 4 °C and Cytochalasin D treatment.

**Supplementary Figure 8** qRT-PCR analysis of the chicken splenic subpopulations indicate enrichment for core T cell, NK cell, and B cell in the CSF1R-tg+ subpopulations.

**Supplementary Figure 9** qRT-PCR analysis of the chicken CSF1R-tg+ subpopulations indicate enrichment of migratory and adhesion related genes in the CSF1R-tg+ MHCII+ CD11c+tg+ subpopulations.

**Supplementary Figure 10** qRT-PCR analysis of the chicken CSF1R-tg+ subpopulations are depleted 3 days post-clondronate liposome treatment.

**Supplementary Table 1** Differentially expressed genes comparison in splenic CSF1R-tg+ and CSF1R-tg- subpopulations (FDR <0.5).

**Supplementary Table 2** Differentially expressed chicken heterophil related genes comparison in splenic CSF1R-tg+ subpopulations (FDR <0.5).
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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