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Chicken CSF2 and IL-4-, and CSF2-dependent bone marrow cultures differentiate into macrophages over time

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Chicken bone marrow-derived macrophages (BMM Φ) and dendritic cells (BMDC) are utilized as models to study the mononuclear phagocytic system (MPS). A widely used method to generate macrophages and DC *in vitro* is to culture bone marrow cells in the presence of colony-stimulating factor-1 (CSF1) to differentiate BMM Φ and granulocyte-macrophage-CSF (GM-CSF, CSF2) and interleukin-4 (IL-4) to differentiate BMDC, while CSF2 alone can lead to the development of granulocyte-macrophage-CSF-derived DC (GMDC). However, in chickens, the MPS cell lineages and their functions represented by these cultures are poorly understood. Here, we decipher the phenotypical, functional and transcriptional differences between chicken BMM Φ and BMDC along with examining differences in DC cultures grown in the absence of IL-4 on days 2, 4, 6 and 8 of culture. BMM Φ cultures develop into a morphologically homogenous cell population in contrast to the BMDC and GMDC cultures, which produce morphologically heterogeneous cell cultures. At a phenotypical level, all cultures contained similar cell percentages and expression levels of MHCII, CD11c and *CSF1R*-transgene, whilst *MRC1L-B* expression decreased over time in BMM Φ . All cultures were efficiently able to uptake 0.5 μ m beads, but poorly phagocytosed 1 μ m beads. Little difference was observed in the kinetics of phagosomal acidification across the cultures on each day of analysis. Temporal transcriptomic analysis indicated that all cultures expressed high levels of *CSF3R*, *MERTK*, *SEPP1*, *SPI1* and *TLR4*, genes associated with macrophages in mammals. In contrast, low levels of *FLT3*, *XCR1* and *CAMD1*, genes associated with DC, were expressed at day 2 in BMDC and GMDC after which expression levels decreased. Collectively, chicken CSF2 + IL-4- and CSF2-dependent BM cultures represent cells of the macrophage lineage rather than inducing conventional DC.

KEYWORDS

bone marrow cell cultures, chicken, dendritic cell, macrophage, transcriptome

Introduction

Cells of the mononuclear phagocyte system (MPS) differentiate from pluripotent stem cells in response to hemopoietins, such as colony-stimulating factor (CSF1), granulocyte-macrophage-CSF (GM-CSF, CSF2) or fms-like tyrosine kinase receptor 3 ligand (FLT3L). Macrophages are a heterogeneous cell population, involved in host defense against pathogens, using phagocytosis to engulf and dispose of foreign particles, a process also utilized to regulate tissue and cell damage to control tissue repair and maintain homeostasis. Macrophages acquire tissue-specific characteristics, and differ in terms of ontogeny, phenotype, and functionality despite their shared cellular origin (1). Dendritic cells (DC) are made up of distinct sub-groups; conventional DC, 1 and 2 (cDC1 & 2) and plasmacytoid DC (pDC). Conventional DC link the innate and adaptive arms of the immune response through their ability to present antigens to naïve T cells (2) while pDC have a key role in host defense against virus infection (3).

Both macrophages and DC are scattered throughout the body, it is generally difficult to isolate sufficient numbers for comprehensive studies without using enzymatic extraction steps that may detrimentally affect cell surface marker expression. Therefore, the majority of research either relies on isolating circulating monocytes to generate monocyte-derived macrophages or bone marrow (BM) cells cultured in the presence of specific cytokines to promote macrophage and DC differentiation for the production of each cell lineage on a large scale. In mouse and human, BM cells can differentiate into macrophages using CSF1 (4), while murine BMDC have been established using CSF2 (2). Soon after, the addition of interleukin 4 (IL-4) with CSF2 was shown to be required for the generation of human BM- and monocyte-derived DC (5). Based on phenotype and morphology, human BMDC (CSF2 + IL-4) represent immature DC while the addition of IL-4 to murine CSF2 treated BM cultures induces DC maturation (5). Generally in mammals, DC maturation is described based on the expression levels of major histocompatibility complex class II (MHCII), CD11c and co-stimulatory molecules, CD40 and CD86 (6). Functionally, apart from phagocytosis, DC have an additional attribute, their ability to cross-present antigens to naïve T cells (7). However, studies are increasingly showing that macrophages have the capacity to cross-present but whether this is a requirement for primary or memory T cell responses is still to be elucidated (8, 9).

Both BMM Φ and BMDC have been generated in veterinary species such as pigs, ruminants, dogs and cats, similar to the original protocol published for human and mouse (10–13). Studies have reported that classical methods for macrophage and DC generation produces a heterogeneous population of cells. For example, murine BMDC cultured with CSF2 alone generated both macrophages and neutrophils (14) while another study demonstrated the presence of macrophages and DC in CSF2 treated cells (6, 15). Similarly, when CSF2 is replaced by FLT3L in murine and porcine BM cultures, the cells resembled a bona fide cDC lineage (11, 16, 17).

Transcriptomic analysis of MPS cells derived from distinct tissues led to defined core macrophage and DC signature genes (1, 18–20). These core gene sets have been utilized to examine DC and macrophage lineages in a number of chicken tissues (21–24). Recently, novel reagents to chicken FLT3 and XCR1, cDC1 markers, demonstrated that chicken BMDC lack the expression of these proteins (25). Studies have shown macrophage development in chicken CSF1-treated BM cells (22, 26, 27). However, few studies have defined the lineage of CSF2 + IL-4 or CSF-2 treated chicken BM cells and functional analysis of CSF1 and CSF2 + IL-4 treated BM are lacking. The aim of our study was to gain a detailed understanding of the developmental kinetics and transcriptome dynamics of BM-derived cultures, based on morphology, phenotype, function and transcriptome, for validation of *in vitro* models of macrophages and DC. The present study uniquely derived BM cells from individual birds and followed their growth in the presence of both CSF2 + IL-4 (BMDC), CSF2 alone (GMDC) or CSF1 (BMM Φ) and their simultaneous characterization at days 2, 4, 6 and 8 in culture to achieve an in-depth kinetic comparison. In addition, transcriptome dynamics of BM-derived cultures gave a comprehensive characterization of cells throughout the culture period. Conclusively, chicken CSF2 + IL-4 and CSF2-dependent BM cultures represented cells of the macrophage lineage rather than inducing conventional DC.

Materials and methods

Chickens and ethical statement

CSF1R-reporter transgenic chickens and wild type Hy-Line brown chickens were used at 4–6 weeks of age and provided by the National Avian Research Facility (NARF), The Roslin Institute, Edinburgh, UK. The unvaccinated chickens were reared in floor pens and maintained under conventional conditions with water and feed *ad libitum*. Animals were housed in premises licensed under a UK Home Office Establishment License in full compliance with the requirements of the Animals (Scientific Procedures) Act 1986. Breeding of transgenic chickens was carried out under the authority of Project License PPL70/8940 with the consent of The Roslin Institute Animal Welfare and Ethical Review Board.

Bone marrow cell isolation and cell culture maintenance

Birds were humanely culled by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986, and femurs and tibias were removed and stored in PBS on ice until use. Both ends of the bones were cut and the medulla was flushed with 10 mL of PBS (pH 7.4, Ca²⁺

⁺ and MgCl²⁺ free, used throughout the study) using 21G needle and 10 mL syringe. The bone marrow was pressed through a 70 µm strainer and cells were pelleted at 400 x g for 10 min at room temperature (RT), resuspended in PBS and gradient purified (Histopaque 1.077; Sigma-Aldrich, Gillingham, UK) in 1:1 ratio for 20 min at 400 x g with no brakes. The interface and cells above were retrieved and washed twice with complete media (RPMI with 10% heat inactivated fetal bovine serum (FBS, GIBCO), L-glutamine (2 mM), penicillin (20 U/mL) and streptomycin (20 µg/mL), ThermoFisher Scientific (TFS). Live cells were counted using Trypan Blue exclusion (Corning, USA). All BM cells were cultured in complete RPMI supplemented with 10 ng/mL recombinant chicken IL-4 and CSF2 (Kingfisher Biotech Inc., USA) for BMDC, 10 ng/mL CSF2 for GMDC and 200 ng/mL of CSF1 for BMMΦ [produced in house (26)]. The optimal concentration of cytokines was determined based on the observed morphology of cells as described (26, 28). Cells were incubated at 41°C, 5% CO₂, conditions used throughout the study. For phenotypical and functional analysis, BM were isolated from six individual chickens over two independent experimental days (n=3 per experiment) and cultured independently with CSF1, CSF2 + IL-4 or CSF2 for 2, 4, 6 and 8 days of culture.

On days 3 and 6 of culture, two-thirds of the cell culture media was removed (dead and non-adherent cells) and replaced with fresh complete RPMI supplemented with the appropriate cytokines. For phenotypical analysis using flow cytometry, 10⁶ cells/mL in 3 mL were seeded in 6-well plates. Prior to harvesting, cells were gently washed with PBS and detached from the wells by the addition of 1 mL of TrypLE Express (Life Technologies, UK) and incubated at 41°C for 10 min. The cells were further dislodged from the wells by vigorous pipetting and the enzymatic reaction was quenched by the addition of complete RPMI. The cells were centrifuged at 400 x g for 5 min at RT, resuspended in complete RPMI, and counted.

Flow cytometric analysis

On days 2, 4, 6 and 8 of culture, cells were harvested as outlined above, counted and adjusted to ~10⁷ cells/mL and stained as previously described (23). For multi-color flow cytometric analysis, primary antibodies were conjugated with PerCP-Cy5.5, PE-Cy7 or APC using Lightning-Link Antibody Labelling Kit (Novus Biologicals, USA), following the manufacturer's instructions. All antibodies were titrated prior to use with details outlined in Table 1. Prior to phenotypical, phagocytosis and acidification analysis, cells were treated for 5 min with SytoxTM Blue LIVE/DEAD stain (TFS) and a minimum of 10K live, single cells were collected for each sample. Compensation was achieved using BD mouse IgGκ Compensation beads according to the manufacturer's instructions (TFS). All flow cytometry-based experiments were performed using BD LSR FortessaTM with 4

lasers and 16 filters (BD Biosciences, UK). Analysis was carried out using FlowJo (TreeStar v10). Fluorescence intensities are displayed on 'logical' scales showing negative value. Fluorescence minus one controls (FMO) and wild type, non-transgenic animals were used to apply gates.

Phagocytosis and acidification assays

The BM cell cultures were harvested on days 2, 4, 6 and 8 and reseeded at 10⁶ cells/well in two identical 96-well plates; 41°C C phagocytosis assay and 4°C controls. At 2 h post-harvest, 10⁷ (10:1 bead to cell ratio) 0.5 µm or 1.0 µm red fluorescent FluoSpheresTM Carboxylate-Modified Microspheres (TFS) were added to the cells for 2 h and incubated at 41°C or on ice to determine non-specific uptake and application of flow cytometric gates. Phagocytosis was inhibited by the addition of ice-cold PBS and cells were washed twice with ice-cold PBS before harvesting using TrypLE Express as outlined previously.

Under the same conditions, acidification of intracellular vesicles was measured using pHrodo[®] Red Zymosan BioParticles[®] (Invitrogen, UK). The bioparticles were added at a 10:1 particle to cell ratio for 2 h at 41°C. To determine non-specific bioparticle uptake and application of flow cytometric gates, cells were treated with the actin polymerization inhibitor, cytochalasin D (CytoD, 20 µM, Cayman Chemical, USA) for 15 min prior to treatment with bioparticles. Cells were washed twice with PBS and detached using TrypLE Express as outlined previously. Phagocytosis and acidification was measured using flow cytometry.

Real-time analysis of acidification across the BM cell cultures on days 2, 4, 6 and 8 of culture were measured using CLARIOstar plate reader in a controlled atmosphere, allowing for longitudinal analysis of the fluorescence emitted by the acidified pHrodo Red Zymosan BioParticles. Cells were harvested and seeded at 10⁵ cells per well in black 96-well clear bottom plates (Greiner Bio-One Inc., USA) and incubated for a further 4 h at 41°C, 5% CO₂ before addition of the BioParticles at 10:1 particle to cell ratio for 18 h. Control cells were treated with CytoD as outlined above. The intensity was measured at 15 min post-treatment followed by every hour for 18 h. The raw data were normalized to control cells treated with CytoD.

Measurement of nitric oxide (NO) production

Nitrite (NO₂) concentration, as an index of nitric oxide (NO) production, was measured at days 2, 4, 6 and 8 of culture. The BM cells cultures were reseeded at 10⁶ cells/well in duplicate wells on 12-well plates and treated with 10 ng/mL lipopolysaccharide (LPS) *E. coli* O55:B5 (Sigma-Aldrich) for 48 h after which the supernatant was collected and stored at

TABLE 1 Primary antibodies for the phenotypical analysis of BM-derived cultures.

Antibody	Isotype	Clone	Antigen	Supplier	Working concentration
Mouse Anti-chicken Monocyte/Macrophage-PE	IgG1	KUL01	MRC1L-B	SB ¹	0.125 µg/ml
Mouse Anti-chicken CD45-SPRD	IgM	LT40	CD45	SB	0.5 µg/ml
Mouse anti chicken MHC II	IgG1	2G11	MHC II β chain	SB	1 µg/ml
Mouse anti-chicken CD40	IgG2a	IG8	CD40	RI ²	2 µg/ml
Mouse anti-chicken CD11c	IgG1	8F2	Putative CD11c	LMU ³	1 µg/ml

¹Southern Biotech; ²Immunological Toolbox, The Roslin Institute; ³gift from Dr. S. Härtle, LMU, Germany.

-20°C until use. Griess Reagent Kit was used to measure NO production in 96-well flat bottom plate following the manufacturer's instructions (Invitrogen, UK). To calculate NO production, dilutions of sodium nitrite, ranging from 0.78 to 100 µM, were measured to generate a standard curve. Absorbance was measured at 570 nm using a SpectraMax plate reader.

Total RNA extraction and RNA-sequencing

Bone marrow cultures derived from three individual chickens were independently cultured with CSF1, CSF2 + IL-4 or CSF2 on 6-well plates. On days 2, 4, 6 and 8 of culture, floating cells were discarded and cells were gently washed with PBS to prevent dislodging of the semi-adherent cells. Cells were lysed with RLT buffer (QIAGEN, UK) supplemented with β-mercaptoethanol (10 µM, TFS). Total RNA was isolated using the RNEasy mini kit (QIAGEN) following the manufacturer's instructions. RNA quantity and quality were assessed using Agilent RNA ScreenTape using TapeStation 2200 (Agilent, UK). All samples had an RNA integrity index > 9.5. Illumina TruSeq stranded mRNA-seq libraries were generated for the thirty-six samples and sequenced on NovaSeq by Edinburgh Genomics UK yielding at least 26-123.9M mapped read pairs per sample.

RNA-seq analysis

RNA-seq reads were trimmed for quality at the 3' end using a quality threshold of 30 and for adapter sequences of the TruSeq stranded mRNA kit (AGATCGGAAGAGC) with a minimum length of 50 bp using Cutadapt1 (version cutadapt-1.9.dev2). Transcripts were mapped to the Gallus_gallus-5.0 genome using STAR2 (version 2.5.2b). Raw counts for each annotated gene were obtained using the featureCounts3 software [version 1.5.1). Principal Components Analysis (PCA) was analyzed using normalized and filtered data to explore observed patterns with respect to experimental factors. Statistical assessment of differential expression analyzed using quasi-likelihood (QL) F-test using a false-discovery rate (FDR) < 0.05 and log2 fold-

change (FC) > 2. The heat maps of core signature genes expression in the cultures were created in GraphPad based on the fragment per kilobase per million reads mapped (FPKM) values and presented as log10 FPKM with addition of 1 to avoid visual bias from genes with FPKM values <1.

Network gene analysis

Gene-to-gene network analysis was performed in Graphia [<https://graphia.app/>] (29). Pairwise Pearson correlations ($r > 0.95$) were calculated between all the cultures from day 4 to day 8 to create a matrix of correlations for each pair of genes. Network graphs were created by connecting nodes (genes) with edges (connectivity based on correlation above the defined threshold) and its local structure defined by applying the Louvain clustering algorithm at an inflation value (cluster granularity) of 0.650. Over-representation of gene ontology (GO) terms were derived from PANTHER (version 15.0) (<http://pantherdb.org/tools/> Gallus gallus as a reference organism), statistical overrepresentation test using Fisher's Exact with FDR multiple test correction, DAVID Bioinformatics Resources (version 6.8, <https://david.ncifcrf.gov/>) and gProfiler (<https://biit.cs.ut.ee/gprofiler/gost>, Homo sapiens as a reference organism).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.00 (GraphPad, San Diego, USA). All data was analyzed for normality. All data was analyzed by two way Anova test adjusted for *post-hoc* analysis. The probability level for significance was taken as $p \leq 0.05$.

Results

CSF1 induces a morphologically homogenous cell culture in contrast to CSF2 + IL-4 treated cultures

BM cells from 4- to 6-week-old chickens were cultured for up to 8 days in the presence of different cytokines; CSF1 to

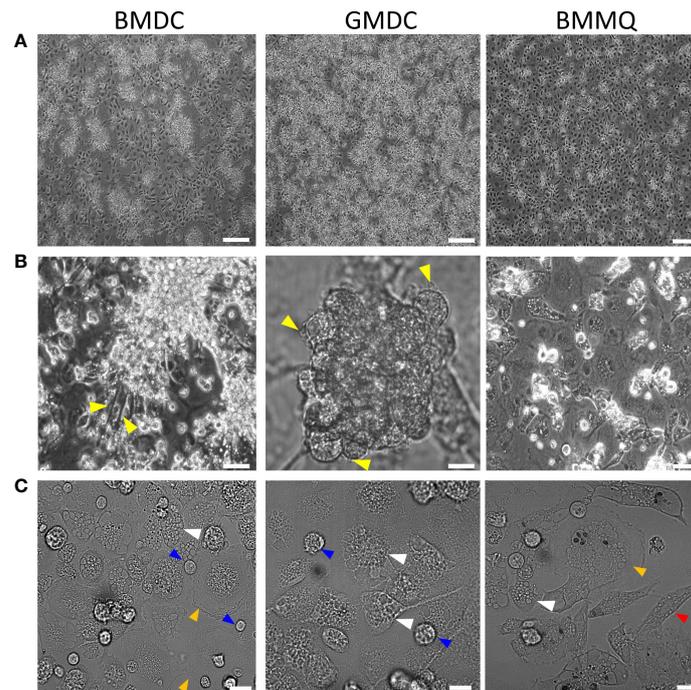


FIGURE 1

CSF1 induces a morphologically homogenous BM cell cultures in contrast to BM treated with CSF2 + IL-4 or CSF2 alone. **(A)** Representative DIC images of chicken BM cells treated with CSF2 + IL-4 (BMDC), CSF2 (GMDC) or CSF1 (BMMΦ) on day 4 of culture. **(B)** Yellow triangles indicate veiled cells **(C)** white triangles indicate cells with numerous intracellular vacuoles, blue triangles shows small round cells and orange triangles show large cells and red triangles show elongated cells. Images of three independent BM cultures derived from 5-week-old chickens. Scale bar represents 100 μm in A and 50 μm in B and C.

generate BMMΦ, CSF2 to generate GMDC, and a combination of CSF2 + IL-4 to generate BMDC. Firstly, we examined the effect of cytokines on cellular morphology at day 4 of culture (Figure 1). Semi-adherent clusters with underlying adherent cells, with a “fried egg” morphology, were observed in both BMDC and GMDC cultures (Figure 1A). In contrast, clusters of cells were rarely observed in the CSF1 treated cultures. Upon closer analysis, BMMΦ cultures were more homogenous and consisted predominantly of cells with a “fried egg” morphology (Figures 1A, B). Clusters in the BMDC and GMDC cell cultures consisted of numerous small round cells with short protrusions (Figure 1B). Irrespective of treatment, adherent cells presented with numerous intracellular vacuoles and both large round, elongated cells were observed in each culture. There was little difference in the cellular morphology from day 4 onwards (data not shown). Without the addition of cytokines, the presence of few floating cells and very little cell adherence was observed by day 4 (Figure S1). As few cells survived after 4 days of culture, these non-treated cells were omitted from further downstream studies.

BMDC, GMDC and BMMΦ develop similar MHCII⁺ and CD11c⁺ subpopulations

BMDC, GMDC and BMMΦ cultures were primarily analyzed based on flow cytometric side and forward scatter profiles (Figure S2). Side scatter analysis did not show any differences in granularity between cultures and the forward scatter patterns indicated that BMMΦ were slightly larger in size at day 2 of culture compared to BMDC and GMDC cultures. From day 4 of culture, all cell cultures were characterized by the same pattern, based on size and granularity (Figure S2).

To investigate phenotypic differences between cultures, expression of chicken MPS cell surface markers was analyzed over time using flow cytometry. Due to possible differences in individual culture reactivity, we conducted two independent experiments with each consisting of three individual chickens for all phenotypical and functional analysis (Figure 2, open & closed circles). Phenotypical analysis was carried out by applying a live, single cell gates to each culture and analyzing the

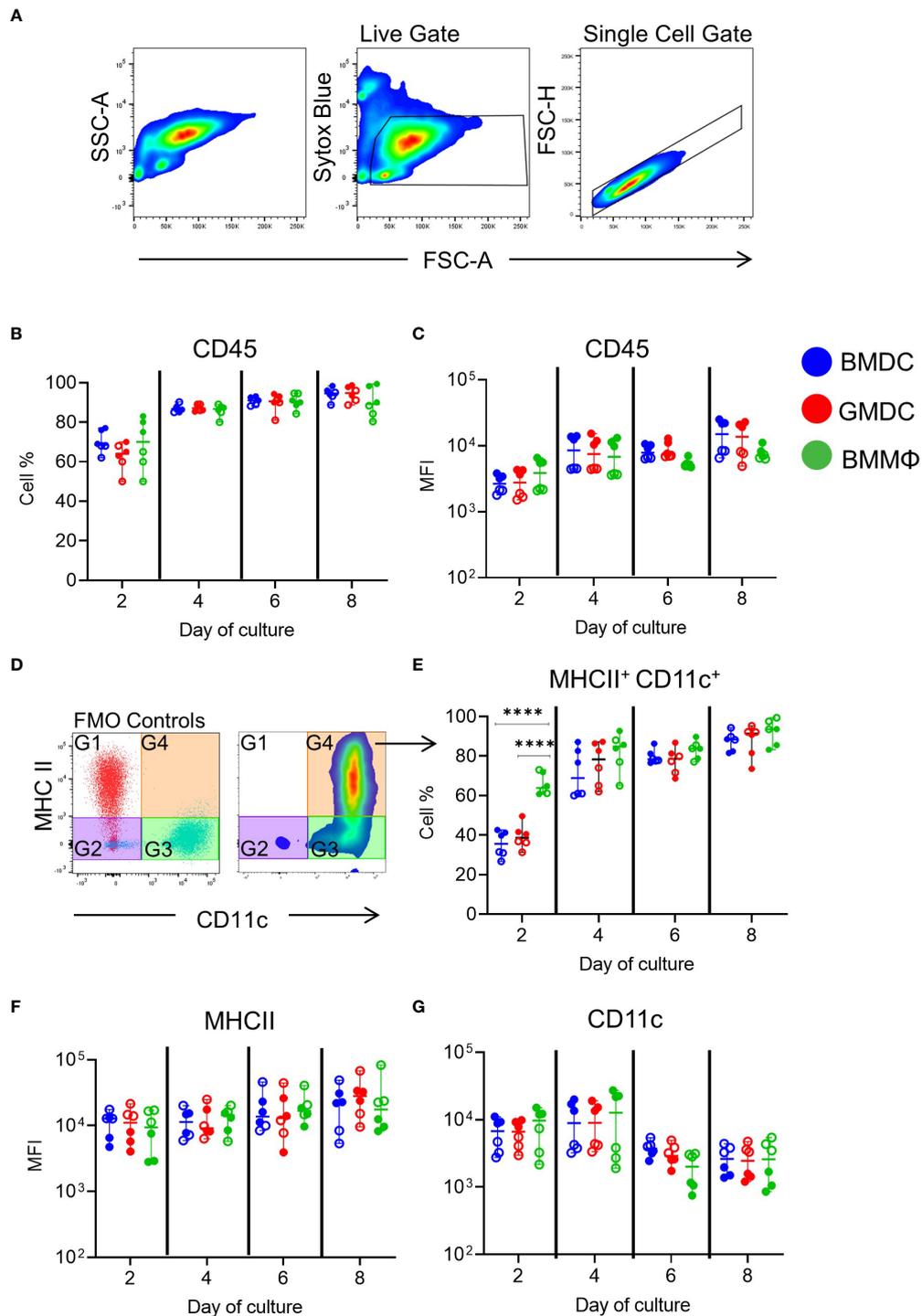


FIGURE 2

Phenotypic analysis of chicken BMDC, GMDC and BMMΦ on days 2, 4, 6 and 8 of culture. **(A)** Representative flow plots of SSC-A and FSC-A and live and single cell gating strategy applied to all cultures at each day of analysis. **(B)** The percentage of CD45⁺ cells and **(C)** median fluorescence intensity (MFI) levels of CD45 expression live, single cells in BMDC (blue), GMDC (red) and BMMΦ (green) on days 2, 4, 6 and 8. **(D)** Dot plots representing FMO-1 control gating strategy on live, single cells for the analysis of MHCII⁺ CD11c⁺ (G3) cell percentages and **(E)** MFI of **(F)** MHCII and **(G)** CD11c expression levels in G4 across each culture on days 2, 4, 6 and 8. Data represents the median (± range). From each bird (n=6), BMDC, GMDC and BMMΦ were generated. Data represents two independent experiments (n=3 per experiment, open & closed circles). Statistical significant differences between cultures on day of analysis are indicated by ****p<0.001.

expression of specific markers (Figure 2A). Firstly the percentage of CD45⁺ cells in the live, single gate was analyzed (Figure 2B) and its median fluorescence intensity (MFI; Figure 2C), showing an increase over time but did not differ significantly between the cultures.

Next, cells expressing both MHC class II (MHCII) and CD11c, using the putative anti-chicken CD11c monoclonal, 8F2, were analyzed across each culture over time to resemble the approach used in mammals. Using FMO controls, four gates

were applied to the live, single cells in each culture (Figure 2D). The percentage of MHCII⁺ CD11c⁺ cells within each culture increased over time and reached ~80-90% by day 8 (Figure 2E; Figure S3A). At day 2, a significantly higher percentage of MHCII⁺ CD11c⁺ cells were observed in BMMΦ compared to BMDC and GMDC cultures, while from day 4 onwards all cultures had similar percentages of MHCII⁺ CD11c⁺ cells (Figure 2E; Figure S3). The MFI of MHCII and CD11c did not

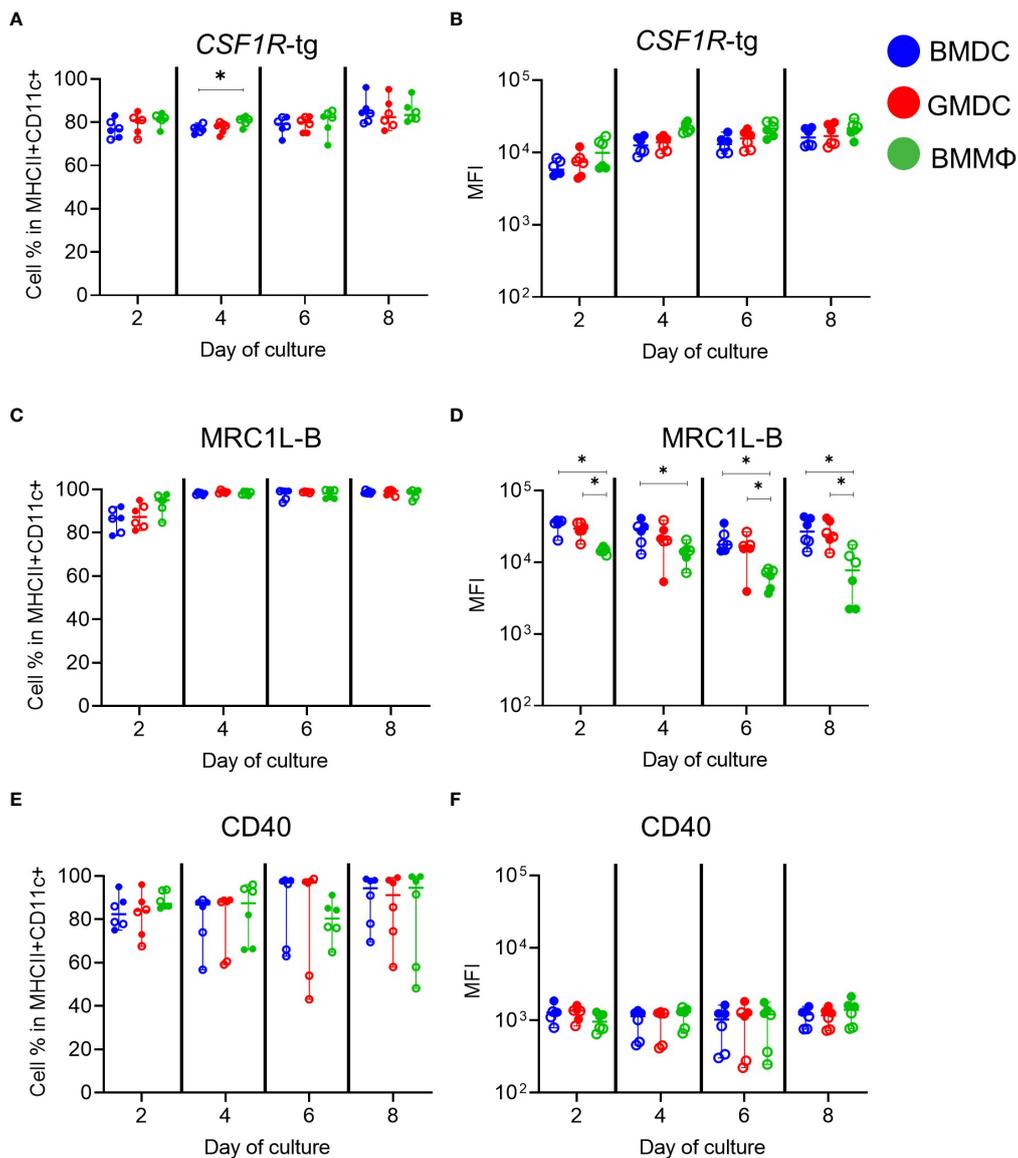


FIGURE 3

Phenotypic analysis of MHCII⁺ CD11c⁺ subpopulations in chicken BMDC, GMDC and BMMΦ cultures on days 2, 4, 6 and 8. The cell percentages and MFI of (A, B) CSF1R-tg, (C, D) MRC1L-B and (E, F) CD40 in the MHCII⁺ CD11c⁺ subpopulations in BMDC (blue), GMDC (red) and BMMΦ (green) on days 2, 4, 6 and 8. Data represents the median (\pm range). From each bird ($n=6$), BMDC, GMDC and BMMΦ were generated. Data represents two independent experiments ($n=3$ per experiment, open & closed circles). Statistical significant differences analyzed between cultures on day of analysis are indicated by * $p < 0.05$.

significantly differ between the cultures (Figures 2F, G). Each culture contained a major MHCII⁺ CD11c⁺ subpopulation.

All MHCII⁺ and CD11c⁺ cells express similar levels of chicken macrophage markers

Next, further phenotypical analysis focused on the MHCII⁺ CD11c⁺ subpopulation in each culture (Figure 3). We used *CSF1R*-reporter transgenic chickens, which express a transgene under control of the *CSF1R* promoter and FIRE enhancer, essentially labelling most cells of the MPS (30). Approximately 70–80% of the MHCII⁺ CD11c⁺ subpopulation expressed the *CSF1R*-transgene (*CSF1R*-tg; Figure 3A). There was no significant difference observed in the MFI of *CSF1R*-tg expression between the cells across the different days of culture (Figure 3B). Expression of MRC1L-B (recognized by the MoAb KUL01) in the MHCII⁺ CD11c⁺ subpopulations increased from day 2 to day 4 of culture reaching 95–100%, and plateaued for the duration of the culture period (Figure 3C). This also demonstrates that all cells express MRC1L-B but ~10–20% lack *CSF1R*-tg expression (Figure S3B). The MFI of MRC1L-B expression was significantly lower on the BMMΦ on all days compared to BMDC (Figure 3D). The percentage of MHCII⁺ CD11c⁺ cells expressing the co-stimulatory molecule CD40 varied between cultures. From days 4–8, two birds consistently contained lower percentage of CD40⁺ cells, regardless of culture conditions (Figure 3E) and correlated with the MFI of CD40 expression, with the same two birds having the lowest expression levels (Figure 3F). Throughout the culture period, BMMΦ, BMDC and GMDC contained small subpopulations of cells lacking both MHCII and CD11c expression (Figure S3A). The MHCII[−] CD11c[−] subpopulations were correspondingly low/negative for CD40, MRC1L-B and expressed low levels of the *CSF1R*-tg in all culture conditions (Figure S3B). Overall, multi-color flow cytometric analysis indicates that BMDC, GMDC and BMMΦ cultures derived from post-hatch chickens all contained a major cell population expressing MHCII, CD11c and MRC1L-B, but the BMMΦ consistently expressed lower levels of MRC1L-B in comparison to both BMDC and GMDC. Furthermore, addition of IL-4 in BMDC cultures induced no difference in cellular phenotype or expression levels of MPS markers compared to GMDC cultures.

BMMΦ, BMDC and GMDC have limited phagocytosis abilities

To determine any functional differences between the cultures, uptake of small (0.5 μm) and large (1 μm) fluorescent beads was analyzed by flow cytometry 2 h post-treatment. To control for non-specific bead adherence, cells were incubated on

ice and the percentage of bead⁺ cells and the number of beads per cell were determined (Figure 4A). Although only significant at day 2, the BMMΦ had a higher percentage of 0.5 μm bead⁺ cells (Figure 4B) compared to the corresponding BMDC and GMDC. Analysis of bead uptake in two independent experiments consisting of three individual chickens, demonstrates large bird-to-bird variation within the cultures (Figure 4, filled vs empty circles). In contrast to the percentage of 0.5 μm bead⁺ cells, the percentage of 1 μm bead⁺ cells was much lower across all cell cultures and time points, reaching only a maximum of ~20% and no statistical difference was observed between cultures (Figure 4C).

To analyze whether there was a difference across the cultures in the number of beads per cell (beads/cell), the MFI of all bead⁺ cells was divided (Figure 4A, gate G1) by the MFI of cells containing a single bead (Figure 4A, gate G2). At day 2, BMMΦ contained significantly more 0.5 μm beads/cell (average of 3 beads) compared to the BMDC and GMDC cultures (average of 2 beads). However, from day 4 onwards, the number of 0.5 μm beads/cell did not differ between cultures, and this coincided with the greater variation between two independent experiments. Cultures derived from the same experiment resulted in similar values (percentage bead positive and number beads/cell) suggesting that despite experimental variation there was no functional difference in bead uptake between the three cultures. No significant difference was observed in the number of 1 μm beads/cell between the cultures on each day of analysis and the low percentage of bead⁺ cells corresponded with a lower number of 1 μm beads/cell (average of 1–2 beads; Figure 4E). All BM cultures were more proficient at uptake of 0.5 μm beads compared to 1 μm and BMMΦ were more efficient at uptake on day 2 compared to BMDC and GMDC.

Phagosomal acidification is highest in BMMΦ cultures on day 2

Cell cultures were analyzed for their ability to uptake and acidify pH sensitive pHrodo labelled zymosan bioparticles. To control for non-specific zymosan uptake, cells were treated with an actin-polymerization inhibitor, CytoD, for 30 min prior to a 2 h incubation with zymosan (Figure 5A). All cells were capable of acidifying zymosan bioparticles from day 2 of culture and there was no significant differences in the MFI across the days or between the cultures (Figure 5B).

To determine if the kinetics of phagosomal acidification may be associated with functional differences between the cell cultures, the levels and increase in fluorescence were measured every hour for 18 h on days 2, 4, 6 and 8 (Figures 5C–F). On day 2 of culture, there was a rapid increase in fluorescence in all cultures, with the fluorescent intensity in BMMΦ higher than in

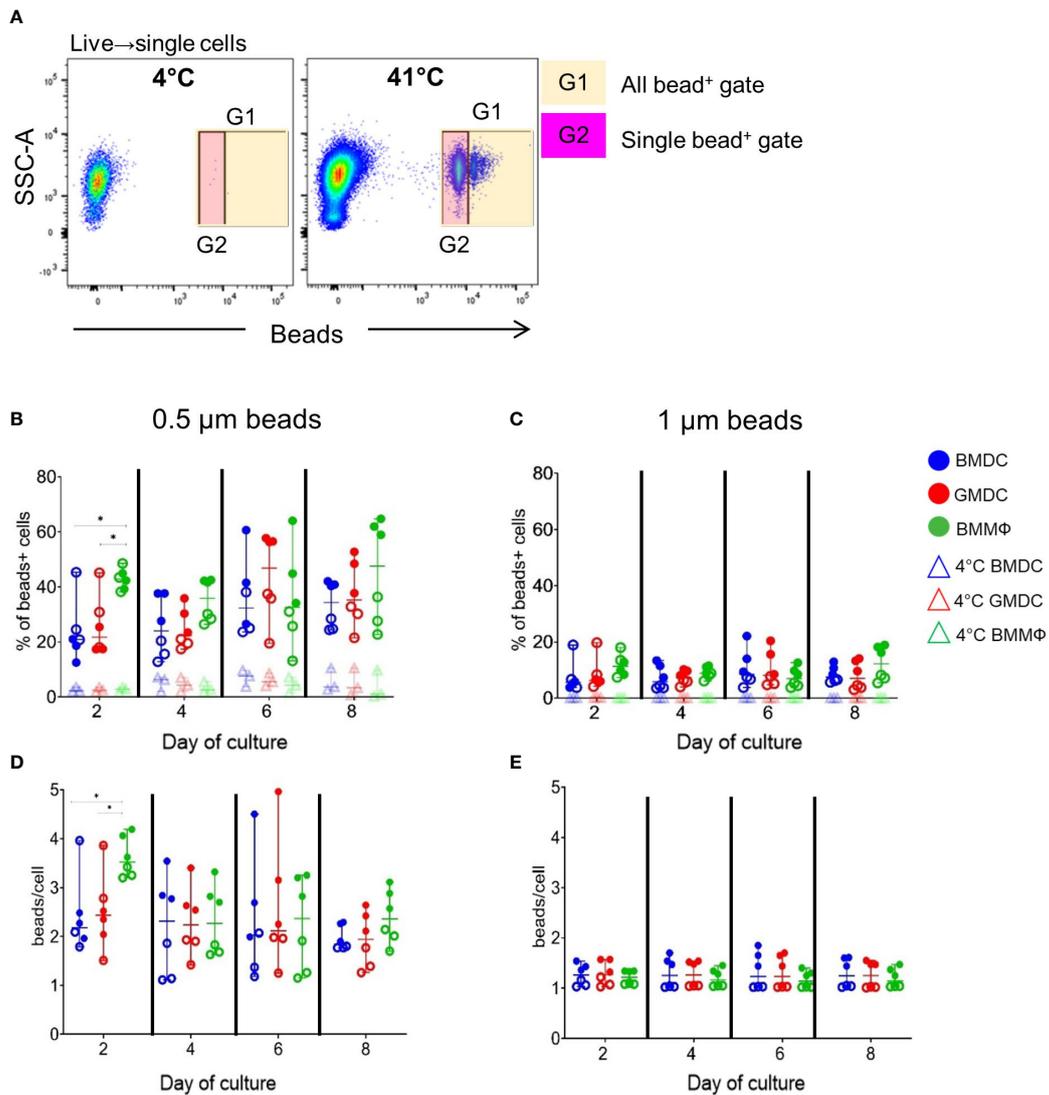
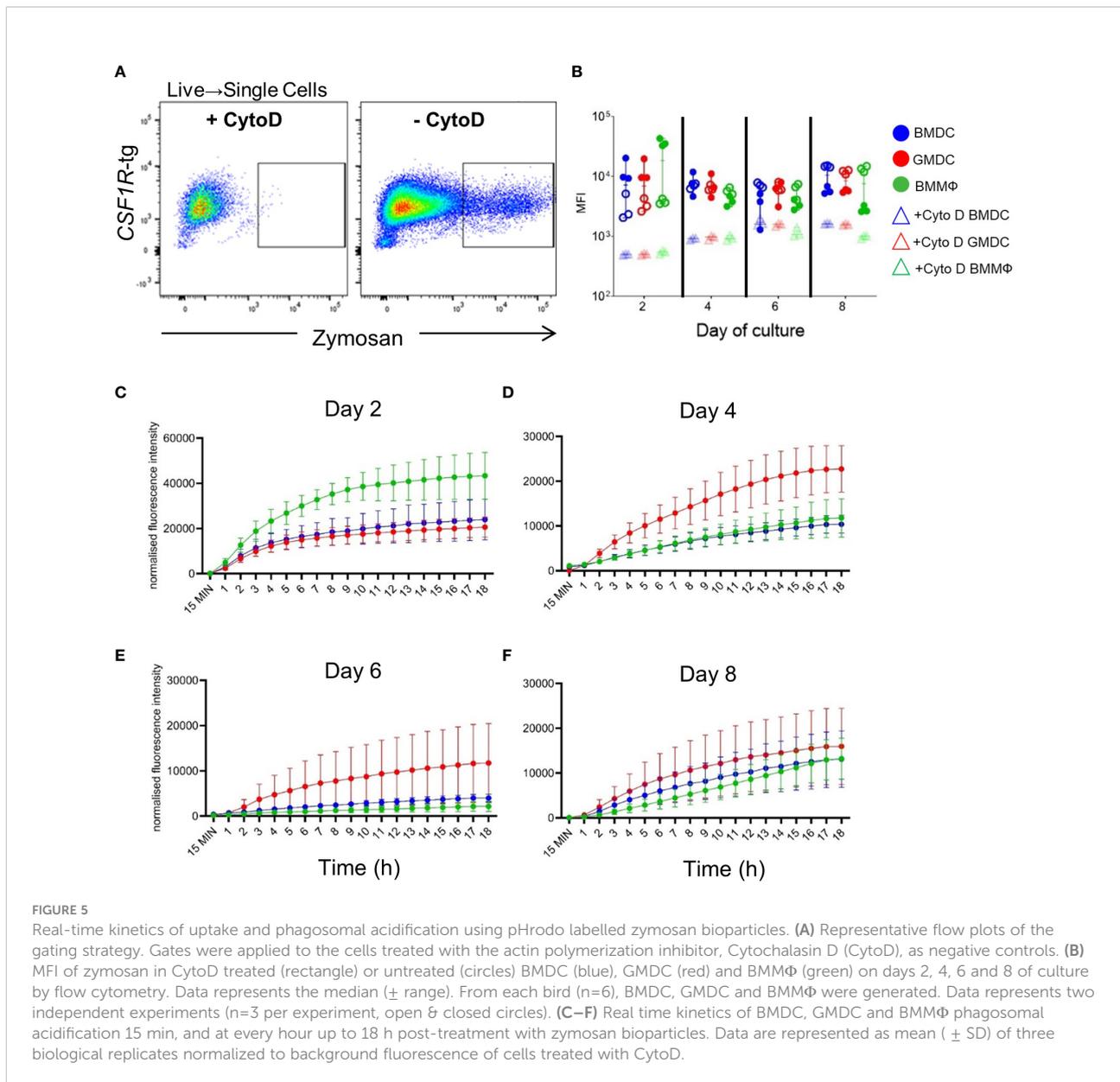


FIGURE 4
 Endocytic activity of BMDC, GMDC and BMMΦ cultures. BMDC (blue), GMDC (red) and BMMΦ (green) were incubated with 0.5 μm or 1 μm fluorescent beads at 41°C (circles) or 4°C (triangles) for 2 h on days 2, 4, 6 and 8 of culture and phagocytosis was analyzed by flow cytometry. (A) Representative flow plots of the gating strategy to determine specific bead uptake using the cells treated at 4°C and applied to the cells treated at 41°C. An all bead+ gate (G1) and a gate for cells with a single bead (G2) were applied. (B) The percentage of 0.5 μm bead+ cells; (C) and 1 μm bead+ cells; (D) the number of 0.5 μm beads per cell; (E) the number of 1 μm beads per cell; The number of beads per cell was calculated by dividing MFI of cells in G1 by cells in G2. Data represents the median (± range). From each bird (n=6), BMDC, GMDC and BMMΦ were generated. Data represents two independent experiments (n=3 per experiment, open & closed circles). Significant differences are indicated by *p < 0.05.

BMDC and GMDC (Figure 5C). On days 4 and 6, a higher increase in acidification was observed in the GMDC compared to the BMDC and BMMΦ (Figures 5D, E). On days 6 and 8 of culture, the fluorescence intensities were much lower compared to day 2 (Figures 5E, F), while in day eight cultures there was no differences observed between the cultures. Overall, no difference in the level and speed of acidification was observed across the cultures.

NO production decreases over time in BMMΦ

NO production by the BMDC, GMDC and BMMΦ was analyzed after LPS stimulation on days 2, 4, 6 and 8 of culture. Forty-eight hours post-LPS stimulation all cell cultures produced NO compared to unstimulated cells (Figure 6), but no significant difference in NO production between the



cell cultures was observed on day 2 and 4. However, on day 6, the GMDC produced significantly higher levels of NO compared to BMDC. There was significant variation in NO production by BMMΦ and the variation observed within the cultures did not correlate with any specific individual bird/culture i.e high responders on particular time point were not correlated with high responders at the following time point. At day 8, NO production was highly consistent across the individual birds within each cell culture and both the BMDC and GMDC produced significantly higher levels of NO compared to BMMΦ (Figure 6).

Temporal transcriptomic analysis suggest BMDC, GMDC and BMMΦ differentiate to the macrophage cell lineage

To define the enriched MPS cell lineage and their dynamics in the chicken bone marrow cultures grown under the influence of different cytokines, RNA-seq analysis was performed on the day 2, 4, 6 and 8 cultures. Samples for RNA-seq were preferential towards the adherent and semi-adherent cells in each culture as floating cells were discarded during sample preparation. Firstly, PCA identified six clusters, grouping day 2 BMDC and GMDC

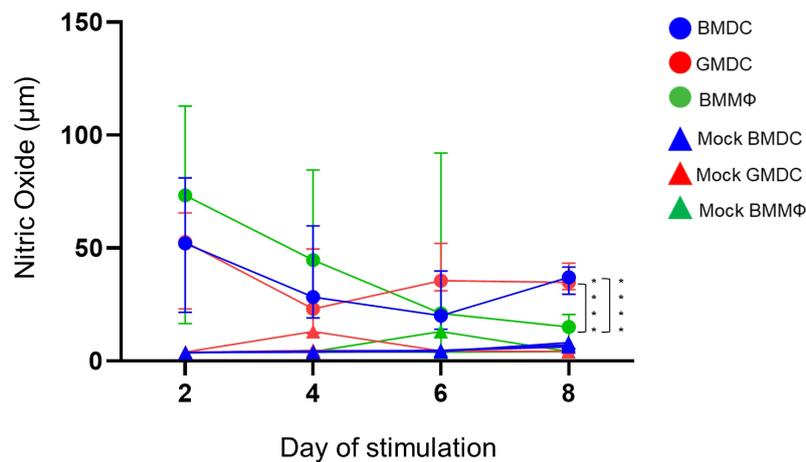


FIGURE 6

BMDC and GMDC produce high NO levels post-LPS treatment. BM cultures were stimulated on days 2, 4, 6 and 8 with LPS from *E. coli* (circles) or untreated (Mock, rectangles). NO levels in cell supernatant were analyzed 48 h post-treatment using Griess reaction. Data represents the median (\pm range). From each bird ($n=6$), BMDC, GMDC and BMM Φ were generated. Data represents two independent experiments ($n=3$ per experiment, open & closed circles). Significant differences between the cultures on the day of stimulation are indicated by * $p<0.05$ and **** $p<0.001$.

together and separated from day 2 BMM Φ (Figure 7A). Day 4 to 8 BMM Φ clustered together whereas the BMDC and GMDC cultures were clustered based on the specific culture day.

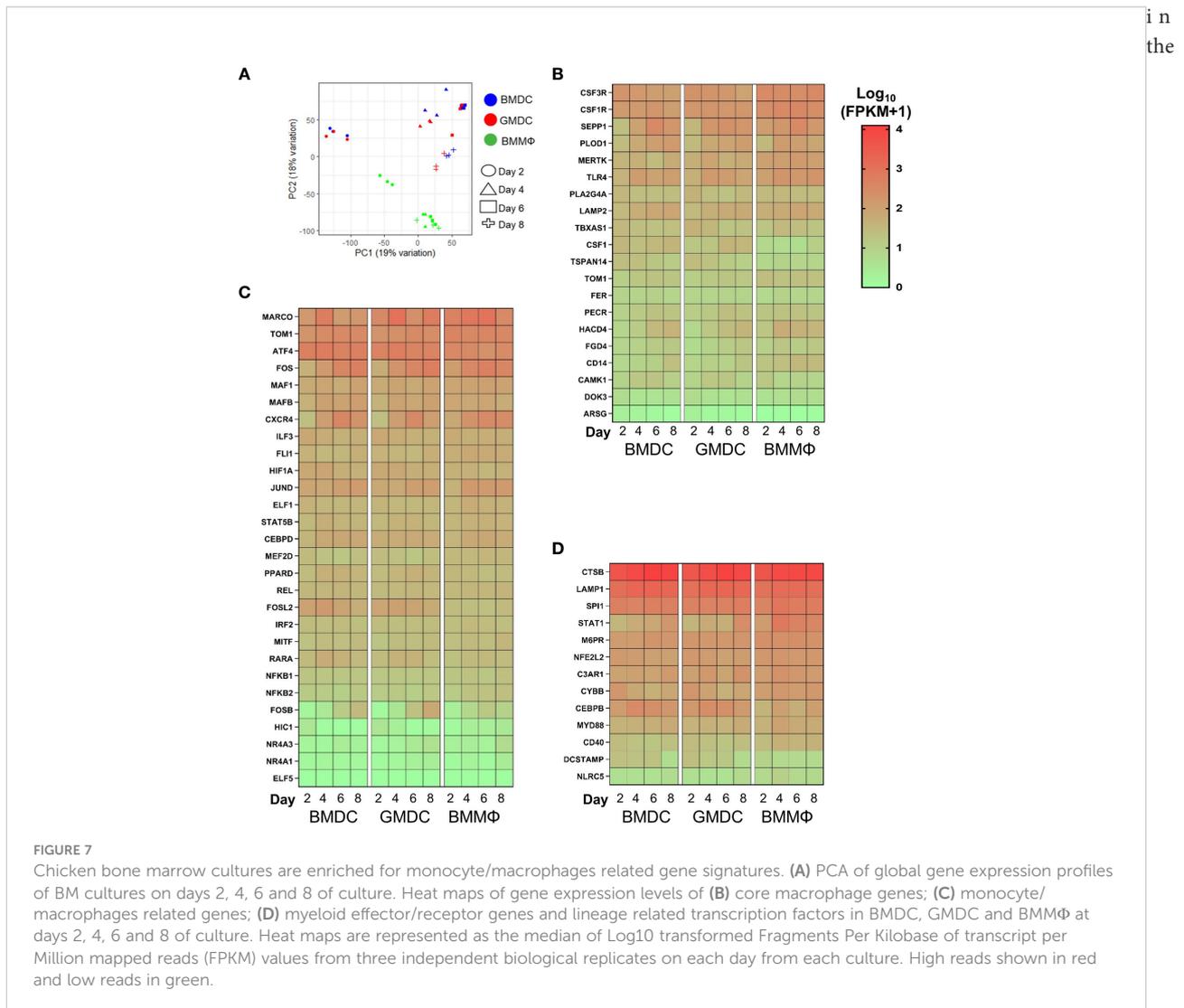
Next, each culture was analyzed over time for the enrichment of monocyte/macrophage and DC cell lineage genes, related transcription factors, myeloid receptors and lysosomal components previously identified in mammals (19, 31, 32) and in chicken (21, 22, 33, 34) (Figures 7B–D). Firstly, the enrichment of core macrophage related genes showed little differences in expression between the cell cultures (Figure 7B). *CSF3R*, *MERTK*, *SEPP1* and *TLR4* were expressed at modestly higher levels in the BMM Φ compared to the BMDC and GMDC cultures. *CD14*, *FGD4*, *HACD4*, *PECR* and *TOM1* were expressed in a temporal fashion, with expression levels increasing with time in BMDC and GMDC cultures (Figure 7B). The enrichment of monocyte/macrophage related genes further demonstrated no major differences in expression levels across the cultures (Figure 7C). The most notable differences were observed for FOS gene family, with FOS mRNA expression levels increasing with time in BMDC and GMDC in comparison to BMM Φ cultures. *FOSL2* was expressed at higher levels in the DC cultures compared to the BMM Φ cultures irrespective of time point, an expression trend also observed for *RARA*. *FOSB* mRNA expression levels were also higher in the BMDC and GMDC cultures compared to BMM Φ on days 6 and 8 (Figure 7C). The macrophage-associated transcription factors *SPI1* and *STAT1* were expressed slightly higher in BMM Φ , contrastingly *CEBPB* expression levels were elevated in BMDC and GMDC (Figure 7D). To further distinguish transcriptomic differences between the BM

cultures, a number of genes related to myeloid effector and receptor functions were analyzed. Similarly, little differences in expression levels of these genes were observed between and over time in the cultures (Figure 7D).

We next analyzed the expression levels of DC related genes across the cultures over time. Interestingly, no enrichment of a core set of DC related genes were observed in any of the cultures over time (Figure 8A). Analysis of cDC related genes demonstrated the high level of expression of *CTSC* and *LGDN* across all cultures. However, there was little difference in the expression of key cDC related transcription factors such as *ID2* or *IRF8*, cell markers such as *CADMI* and *XCRI* and the majority of cDC related genes were expressed at low levels in all cultures over time. Of note, *BAFT3* expression decreased over time in the BMDC and GMDC cultures (Figure 8B). In addition, pDC related gene signatures were not enriched in any of the cultures over time (Figure 8C). Overall, chicken bone marrow cells grown under lineage determining cytokines are biased towards macrophage lineage development with no DC gene signature enrichment observed in any of the cultures.

BMDC and GMDC developmental pathway towards macrophages is slower compared to BMM Φ

In order to compare global gene expression profiles, relationships between the cultures over time and examination of differences observed in the PCA, pairwise Pearson correlations (r) were calculated between all the transcripts



using Graphia (Figure 9). The day 2 cultures sample-to-sample analysis revealed a time effect relative to all other samples, and therefore the data were excluded in further analysis. A network graph was constructed using a correlation threshold $r=0.95$ producing three clusters (A-C). Cluster A was enriched for the BMDC and GMDC cultures while cluster C was enriched for the BMMΦ cultures. Using Louvain clustering algorithm co-expression clusters were compiled and analyzed using gene ontology databases. The enrichment profiles of each cluster are provided in File S1. Genes in clusters 3, 7, 8 and 10 were highly expressed in BMDC and GMDC whereas they were lowly expressed in BMMΦ. These clusters consisted of genes involved in protein modification processes (Cluster 3; *AK9*, *DHTKD1*, *ENO2*, *INPP5K*, *MRPL13*, *PKM2*, *PGMA1*), cellular metabolic processes, such as glucose metabolism (*HK1*, *HK2*, *H6PD*, *GPI*, *LDHA*), amino acid metabolism (Cluster 10; *ASNS*, *GPD2*, *SARS*, *SCL7A1*, *SCL7A11*, *YARS*) and repressor of *KLF4* expression (*ZNF706*). Cluster 7 also consisted of genes involved

positive regulation of leukocyte activation (*CD274*, *ENO1*, *HES1*, *KARS*, *PRELID1*) and autophagy (*ARMC8*, *ATG4B*, *EIF3E*, *EXOG*, *TPI1*, *Ufd1l*). Genes within cluster 2 were expressed at low levels in BMDC and GMDC and even further downregulated in BMMΦ. This cluster consisted of genes involved in the regulation of cell adhesion (*CLDN5*, *HRSA*, *ITGA6*, *LGAL53*, *VEGFA*, *VEGFC*).

Genes within clusters 1, 4, 5 and 6 were expressed at higher levels in the BMMΦ with little difference in expression levels across the time-points analyzed (Figure 9B). In contrast, the genes within these clusters were expressed in a temporal fashion, increasing with time in BMDC and GMDC and consisted of genes related to leukocyte activation and immune system processes (Cluster 1; *B2M*, *CARD11*, *CCR5*, *CD40*, *FL11*, *IKZF1*, *IRF7*, *IRF8*, *LY86*, *NFATC2*, *NFKB2*, *NKBKB2*, *PLCG2*, *TAP1*, *TAP2*, *TGFB1*, *TNFSF10*), anti-viral immune responses (Cluster 5; *IFI35*, *IFIH1*, *EIF2AK2*, *MyD88*, *STAT1*, *STAT2*, *TRIM25*). The clusters also consisted of genes involved in the

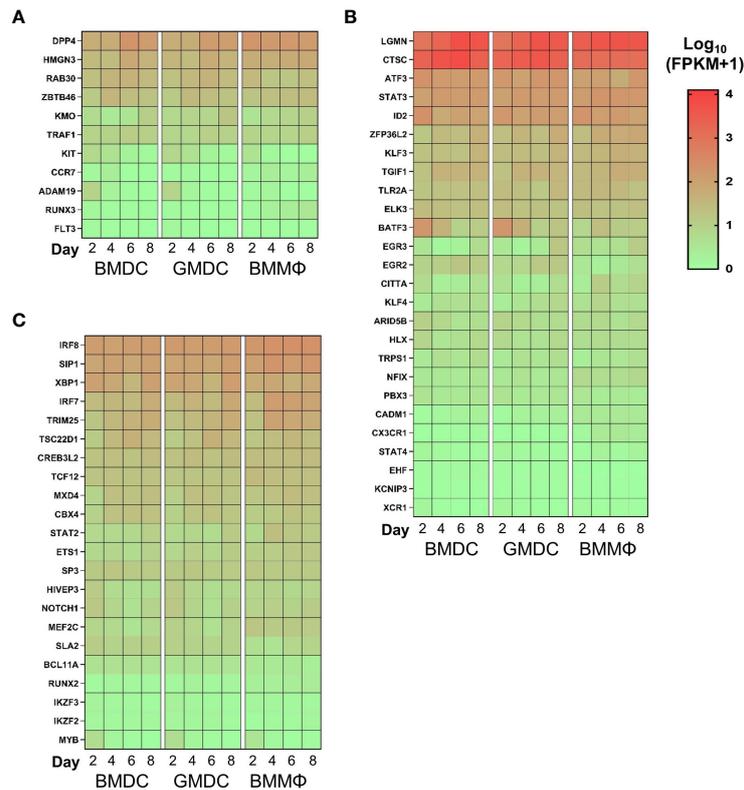


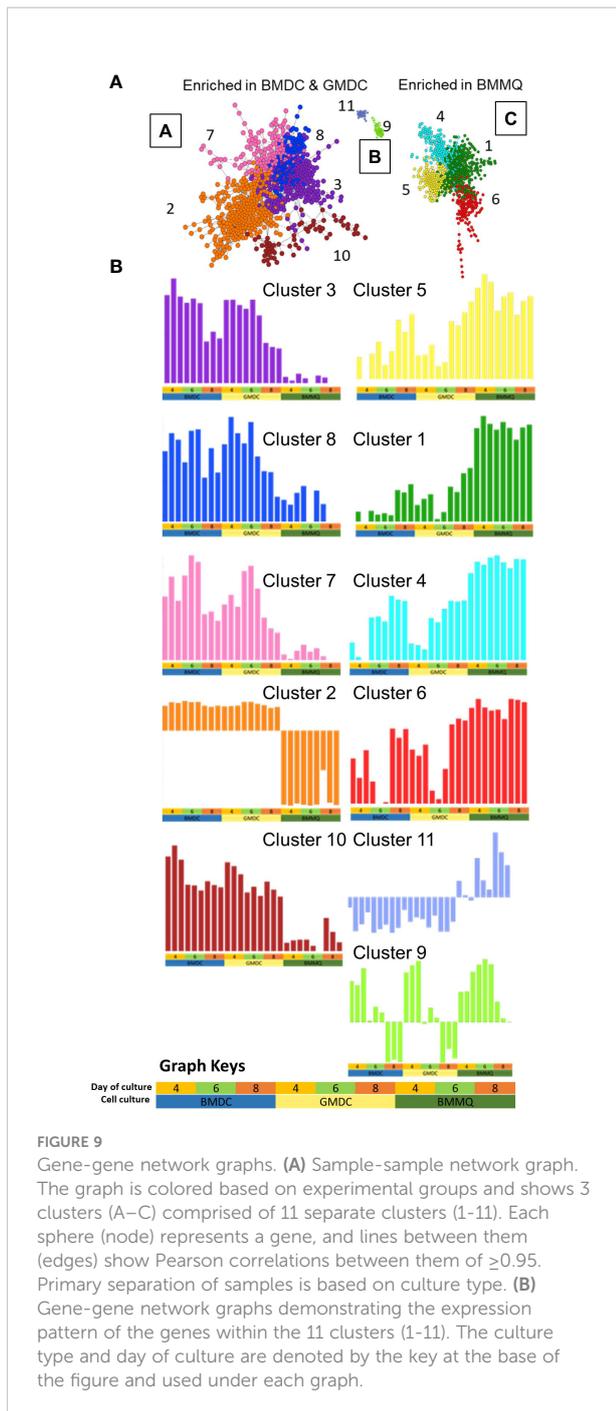
FIGURE 8
 Chicken BM cultures are not enriched for DC related transcripts. Heat maps of gene expression levels of (A) pDC; (B) cDC and (C) core DC markers. Heat maps are represented as the median of Log₁₀ transformed Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values from three independent biological replicates on each day from each culture. High reads shown in red and low reads in green.

complement cascade (*CIQA*, *CIQB*, *CIQC*), lysosomal function (Cluster 4; *ACP5*, *ASAHI*, *CTSA*, *CTSH*, *CYB561A3*, *GLA*, *NEU3*, *SNX14*, *VPS11*) and mRNA stability (Cluster 6; *DHX36*, *HNRNPM*, *HNRNPR*, *TAF15*, *ZC3H14*).

Cluster 11 represented a profile that was downregulated in BMDC and GMDC and increased over time in BMMΦ. It comprised of genes involved in endothelial cell proliferation (*BMPER*, *LOXL2*) and regulation of endodermal cell differentiation (*COL5A1/6A/6A1/6A3/12A1*). Genes within cluster 9 had a pronounced time-dependent co-expression relationship, decreasing overtime in BMDC and GMDC while increasing from day 4-6 in BMMΦ and decreasing by day 8 (Figure 9). Genes within this cluster are related to the cell cycle and DNA replication processes (*CENPF/I/K/L/N/P/W*, *KIF11/14/18A/20A23/2C* and *MCM5/6/10*). Network gene analysis demonstrated key differences in the temporal expression of genes involved in cellular functions between BMMΦ and BMDC, GMDC cultures. It further demonstrated that the addition of IL-4 to BMDC induced no significant global transcriptional differentiation from GMDC.

Discussion

Chicken MPS cells derived from the BM permit investigation of key cellular functions including scavenging of dying cells, pathogens, and molecules through phagocytosis, endocytosis and T cell activation, making these models vital for understanding their contribution to immunity and inflammatory responses to pathogenic microorganisms. To obtain large numbers of macrophages or cDC, BM progenitor cells are cultured in the presence of hematopoietic cytokines CSF1, CSF2 + IL-4, to drive macrophage or DC development, respectively (26, 28, 35–37). Recent studies have shown that the addition of FLT3L to BM cultures can promote the growth of cDC (11, 16). However, biologically active chicken FLT3L is currently unavailable and therefore its role in cDC development in chicken BM cells was not analyzed in this study. To this end, a thorough investigation of chicken BM cultures treated with the traditional CSF2 + IL-4 or with CSF2 or CSF1 alone, was carried out to understand the heterogeneity across the cultures, their function and cell lineage investigated by temporal transcriptomic analysis.



The phenotypical characterization of mammalian DC and macrophages and their distinction from each other are associated with expression of several marker genes. Higher expression of MHCII and CD11c are historically associated with the DC phenotype, related to their antigen-presenting abilities (6). However, such phenotypical differences were not present in our chicken BM cultures, irrespective of cytokines used. In a recent study, GMDC-derived from ED18 chick embryos, consisted of MHCII^{low} and MHCII^{high} subpopulations (36). This study found

that both the species origin and concentration of serum used can alter the phenotype of the BM cells grown with CSF2, as the MHCII^{low} subpopulation was absent in the 10% FBS treated cultures compared to the 5% chicken serum treated cultures (36). In our study, 10% FBS was utilized, potentially lacking adult chicken-derived serum components, that may drive development of the MHCII^{low} subpopulation. Phenotypical analysis found the percentage of MHCII⁺ CD11c⁺ subpopulation was significantly higher in BMMΦ on day 2 compared to BMDC and GMDC. Interestingly, the mannose receptor, MRC1L-B, expression levels were significantly lower on the BMMΦ at each day of analysis compared to both BMDC and GMDC. In the chicken spleen, MRC1L-B is highly expressed by macrophages and either lowly expressed or lacking in cDC (21, 23, 25). However, murine and human BM or monocyte derived-DC express the mannose receptor (MR, also known as CD206) (38). It has been reported that IL-4 increases MR expression on peritoneal macrophages, which is normally down-regulated by IFN- γ treatment (39). Our previous study showed a reduction in MRCL1-B expression in chicken BMMΦ post-LPS treatment, hence its level of expression may indicate maturation status in chicken BMMΦ (40). Murine BM cultured with CSF2 can include macrophages, in addition to DC and neutrophils (2). A small population of MHCII⁻ CD11c⁻ CSF1R-tg^{lo} cells was evident in each culture indicating the presence of granulocytes in the chicken BM cell cultures (30). We also detected CSF1R-tg⁻ MRCL1-B⁺ cells in all cultures, a subpopulation previously observed in the chicken spleen (23) and more recently in BMDC (25).

Both phagocytic and acidification assays across the cultures indicated no differences in the ability of the cells to uptake and undertake luminal acidification of phagosomes. Low levels of 1 μ m bead uptake by the BM cultures in our study coincides with previously observed receptor-mediated uptake of IgY-coated 1 μ m beads in GMDC (36). Similar levels of zymosan bioparticle uptake and acidification was observed across all cultures. It may be plausible that for more efficient phagocytosis of larger particles TLR activation in chicken BM cultures is required, similarly to mammals (41). Targeting C-type lectins, such as dectin-1, has been showed to increase the phagocytosis capacity of BMDC (37). Recently, exposure of chicken splenic cDCs (FLT3^{Hi}) and macrophages (FLT3^{low-neg}) to non-invasive *Salmonella* Typhimurium also resulted in equally efficient phagocytosis by both cell types (25), suggesting that both cell populations have the capacity to uptake larger particles. The response of cultures to LPS, measured *via* NO production, shown no significant difference between the cultures and levels decrease with time. This coincides with a previous study in which little difference in pro-inflammatory cytokine mRNA expression levels was observed between LPS treated BMMΦ and BMDC (40). Although attempts were made to compare the antigen-presenting abilities of the BMDC, GMDC and BMMΦ using syngenic and allogenic T cells, T cell proliferation was only achieved after stimulation with ConA (data not shown).

Reproducible T cell proliferation assays in the chicken using antigen-presenting cells still requires further development.

Comparative transcriptomic analysis of immune cells between mammals and chickens provides a framework to support model systems in the context of MPS biology (1, 18, 20, 42). Chicken blood monocytes, tissue-resident macrophages and cDC have been described based on their expression of conserved mammalian cell lineage related genes (21–24). RNA-seq analysis of CSF1-treated chicken BM cells has demonstrated their macrophage lineage by day 7 of culture (22, 30). The transcriptome of CSF1-treated chicken BM cells were further analyzed in this study on days 2, 4, 6 and 8 and used to examine the enriched cell lineages along with BM treated with CSF2 + IL-4 or CSF2 alone. Firstly, the BM cell culture transcriptome were analyzed for core macrophage and DC related gene signatures on days 2, 4, 6 and 8 of culture. Interestingly, a core macrophage-lineage related gene signature (*CEBPB*, *CSF1R*, *NFE2L2*, *MAFB*, *STAT1*, *TLR4*) (22, 31, 43–45) along with lysosomal components (*CTSB*, *LAMP1/2*, *M6PR*) (46, 47) were found to be highly expressed across all culture conditions, a co-expression that suggests a core macrophage transcriptional program is being driven in all cultures. In addition, sustained levels of *SPI1* (*Pu.1*) expression was observed across all time-points and cultures, a transcription factor that is required at high levels to induce and maintain macrophage differentiation (48). When analyzing cDC related gene enrichment in all cultures, (*FLT3*, *BATF3*, *CADM1*, *XCR1*, *Zbtb46*) (49–54), the highest expressions levels were observed in BMDC and GMDC on day 2. This may indicate that without FLT3L, chicken BM-derived DC do not differentiate or have longevity in culture. Since this study, Wu et al. (25) confirmed the absence of FLT3 and XCR1 protein expression on day 7 BMDC. FOS genes (*FOS*, *FOSB*, *FOSL2*) are involved in osteoclast formation and their high expression indicates the presence of these cell lineages in the cultures (55, 56). Colony-stimulating factors are known to “prime” or “activate” macrophages as well as induce their differentiation (57). Our data further demonstrates that BM cells treated with CSF2 + IL-4 or CSF2 alone induce a core macrophage transcriptional program similar to CSF1 treated cells.

Network gene analysis demonstrated key differences in the temporal expression of genes with related functions across the cultures. At all time-points analyzed, BMM Φ expressed high levels of MHCII related genes (*B2M*, *TAP1*, *TAP2*) (22), genes involved in immune regulation, such as transcriptional control (*IFI35*, *IKZF1*, *IRF7*, *IRF8*, *FLI*, *NFATC2*, *NFKB2*, *NKBKB2*, *STAT2*) and immune function (*CARD11*, *CCR5*, *CD40*, *EIF2AK2*, *IFIH1*, *IL1R2*, *IL31RA*, *LY86*, *MyD88*, *PLCG2*, *TGFB1*, *TNFAIP8L2*, *TNFSF10*, *TRIM25*) (58). In contrast, the expression levels of these genes increased with time in BMDC and GMDC cultures, with the highest levels observed at day 8, demonstrating their developmental progress towards a similar transcriptome as BMM Φ .

Macrophage metabolic requirements change with development and functional polarization (59). Genes involved in cellular metabolic processes, including glucose transport (*HK1* and *HK2*), amino acid metabolism and solute transport, (*ENO1*, *SCL7A1*, *SCL7A11*, *SLC16A3*, *SLC16A4*, *SLC25A17*) (60, 61) were expressed at higher levels in BMDC and GMDC compared to BMM Φ throughout the developmental period analyzed. This may indicate that the BMDC and GMDC are more metabolically active compared with BMM Φ , potentially linked to their ongoing differentiation to a macrophage, or reflect the heterogeneity of cells in the culture (59). Macrophages are sub-categorized into either pro-inflammatory M1 macrophages, polarized by LPS or IFN- γ , or anti-inflammatory M2 macrophages, polarized by IL-4 or IL-13 (62–64). KLF4 can differentially affect a repertoire of genes that characterize the M1 and M2 phenotype. With respect to M2 polarization, following IL-4 stimulation, KLF4 and STAT6 synergistically induce M2 gene targets, such as Arg-1 (65). The high expression of *ZNF706*, an inhibitor of KLF4 transcription, in BMDC and GMDC, indicates that these cultures may not represent M2 macrophages. Interestingly, we observed an enrichment of collagen related genes (*COL5A1/6A/6A1/6A3/12A1*) and low expression of cell-adhesion related genes (*CLDN5*, *HRSA*, *ITGA6*, *LGAL53*, *VEGFA*, *VEGFC*) in all cultures. While macrophages can produce proteases to degrade and destabilize the extracellular matrix, they can also produce collagen to maintain tissue integrity, which may encourage adherence along with macrophage-leukocyte communication (66). It should be noted that BM-derived culture protocols not only differ in the concentration of differentiation factors and duration of growth, but also the inclusion of loosely and non-adherent cells. In rodents BM cultures, non-adherent cells consisted of mature DC, whereas adherent cells consisted of tolerogenic DC and firmly adherent cells were considered macrophages (67). In our study, the adherent and loosely attached cells were included in characterization of the cultures. Therefore, future studies should consider whether the morphological heterogeneous nature of the BMDC and GMDC cultures could account for different cell lineages. Overall, RNA-seq transcriptomic and network gene analysis indicates that chicken BM cultures treated with CSF1, CSF2 + IL-4 or CSF2 alone activate a core macrophage transcriptional program. Whilst BMM Φ have a more “primed” macrophage within 2 days of culture, the developmental pathway towards a macrophage is somewhat delayed in BMDC and GMDC. In addition, subtle differences between the transcriptomes of BMDC and GMDC suggests they follow a similar development pathway irrespective of the presence of IL-4.

FLT3L-derived porcine BM-derived cell cultures supported the development of three distinct cell populations based on expression of *CADM1*, *CD14*, *MHCII* and *CD172a* defining putative cDC1, cDC2 and a novel CD14⁺ cell population (11). FLT3L treated bone marrow cultures in chicken have not been developed, but may lead to the *in vitro* generation of bona fide

chicken cDC. Simplified *in vitro* BM-derived cell models can be meaningful; however, these cell sources to study tissue-derived MPS biology should be met with caution as tissue-specific factors alter the function of MPS cells in mammals (32). With the advent of unbiased single cell sequencing technology, the ability to identify more heterogeneity within the MPS in healthy and diseased animals is now becoming more achievable.

Data availability statement

The datasets generated and analyzed for this study are included in the published article (and its additional files) or in the following data repository. The RNA-seq data for this study was deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB56177.

Ethics statement

The animal study was reviewed and approved by The Roslin Institute, University of Edinburgh, Ethical Review Committee.

Author contributions

DB, KS, and LV conceptualized the study. DB, KS, and LV performed or assisted with the experiments. DB performed the data analysis. SS contributed to the transcriptome analysis. DB, KS and LV wrote the manuscript. LV secured the funding. All authors contributed to, read and approved the final manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1064084/full#supplementary-material>

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