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A Csf1r-EGFP Transgene Provides a Novel Marker for Monocyte Subsets in Sheep

Clare Pridans,* Gemma M. Davis,* Kristin A. Sauter,* Zofia M. Lisowski,* Yolanda Corripio-Miyar,*§ Anna Raper,* Lucas Lefevre,* Rachel Young,* Mary E. McCulloch,* Simon Lillico,* Elspeth Milne,† Bruce Whitelaw,* and David A. Hume*

Expression of Csf1r in adults is restricted to cells of the macrophage lineage. Transgenic reporters based upon the Csf1r locus require inclusion of the highly conserved Fms-intronic regulatory element for expression. We have created Csf1r-EGFP transgenic sheep via lentiviral transgenesis of a construct containing elements of the mouse Fms-intronic regulatory element and Csf1r promoter. Committed bone marrow macrophage precursors and blood monocytes express EGFP in these animals. Sheep monocytes were divided into three populations, similar to classical, intermediate, and nonclassical monocytes in humans, based upon CD14 and CD16 expression. All expressed EGFP, with increased levels in the nonclassical subset. Because Csf1r expression coincides with the earliest commitment to the macrophage lineage, Csf1r-EGFP bone marrow provides a tool for studying the earliest events in myelopoiesis using the sheep as a model.

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ORCID: 0000-0001-9423-557X (C.P.); 0000-0001-8323-5013 (G.M.D.); 0000-0002-1344-7405 (Y.C.-M.); 0000-0002-5101-2277 (S.L.); 0000-0002-2918-1605 (B.W.).

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Address correspondence and reprint requests to Prof. David A. Hume, The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian EH25 9RG, United Kingdom. E-mail address: David.Hume@roslin.ed.ac.uk

Abbreviations used in this article: AF, Alexa Fluor; AM, alveolar macrophage; BM, bone marrow; BMDM, BM-derived macrophage; CSF1R, CSF1 receptor; FIRE, Fms-intronic regulatory element; FSC, forward light scatter; MDM, monocyte-derived macrophage; tCSF1, recombinant human CSF1; SSC, side scatter of light.

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Materials and Methods

Animals

Approval was obtained from The Roslin Institute’s and the University of Edinburgh’s Protocols and Ethics Committees. The experiments were carried out under the authority of a U.K. Home Office Project License under the...
regulations of the Animals (Scientific Procedures) Act 1986. Sheep and mice were euthanized via captive bolt or CO₂ asphyxiation, respectively.

**Con structs and lentivirus preparation**

Constructs and preparation of lentivirus (Csf1r-EGFP-FIRE) was performed as described by Pridans et al. (12). The viral titer was 2.2 \( \times 10^7 \) transducing U/ml as assayed by endpoint dilution on the D-17 cell line.

**Embry o manipulation and generation of transgenic sheep**

Zygotes were obtained via methods described by Ritchie et al. (20) using abattoir-derived oocytes and frozen sperm from a Shetland ram. The lentivirus was injected into the perivitelline space of zygotes that were developed in vitro until the blastocyst stage (days 6–7), at which point they were transplanted into the uterine horn of recipient ewes. The integration of the transgene in lambs was investigated by PCR analysis of ear biopsy DNA, amplifying EGFP using primers 5′-GCACGACTTTCAAGTCGCGCATGCCGTC-3′ (forward) and 5′-GGCGGATCTTGAATGTCACCTTGATA GCCGTC-3′ (reverse). Plasmid DNA (Csf1r-EGFP-FIRE) and genomic DNA from a wild type sheep were used as positive and negative controls, respectively.

**Isolation of PBMCs from sheep**

Bags containing citrate phosphate dextrose adenine (Infusion Concepts, Sowerby Bridge, U.K.) were used to collect blood from live animals <1 y of age. Blood was layered onto an equal volume of Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 1200 \( \times g \) for 25 min with no brake. The PBMC layer was washed in an equal volume of PBS containing 2% FCS (FACS buffer). Any contaminating RBCs were removed by resuspending the pellet in 1 ml RBC lysis buffer (BioLegend, London, U.K.) and immediately topping up to 50 ml with FACS buffer. After centrifugation (400 \( \times g \), 5 min), cells were washed once with buffer.

**Bone marrow and alveolar macrophage isolation from sheep**

Bone marrow (BM) and alveolar macrophages (AMs) were isolated as described by Kapetanovic et al. (21), except RBC lysis buffer (BioLegend) was used and the medium was RPMI 1640 supplemented with 20% sheep serum, 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 1 mmol/l Glu-taMAX (Life Technologies, Paisley, U.K.).

**In vitro differentiation of sheep macrophages**

Freshly isolated BM and PBMCs were cultured on bacteriological plates at a density of 2 \( \times 10^5 \) and 3 \( \times 10^5 \) cells/cm², respectively. Complete medium was supplemented with 10% FBS (100 ng/ml recombinant human CSF1 (rhCSF1; a gift from Chiron, Emeryville, CA). Fresh media containing rhCSF1 was added on day 4 and cells analyzed on day 7.

**Phagocytosis assays using macrophages and whole blood from sheep**

Freshly isolated PBMCs and BM from Csf1r-EGFP sheep were cultured in two-well Lab-Tek chamber slides (Nunc) at a density of 5 \( \times 10^5 \) cells/cm² as described earlier. Zymosan A Saccharomyces cerevisiae BioParticles labeled with Alexa Fluor (AF) 594 (Thermo Fisher Scientific, Waltham, MA) were added at 8 \( \times 10^8 \) per well and incubated for 2 h at 37°C. After addition of ice-cold PBS, cells were washed four times with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were washed in PBS and viewed by confocal microscopy. For comparison of phagocytic activity between wild type and Csf1r-EGFP sheep, whole blood was collected in EDTA tubes and assays performed based on a modified protocol from Bicker et al. (22). pHoro Red Escherichia coli BioParticles (Thermo Fisher) were added to an equal volume of whole blood and centrifuged at 34 g; 37°C for 1 h. A control blood sample containing the BioParticles was left on ice during this time. Samples were incubated on ice for 10 min and then stained with mouse anti-human CD14 AF647 (clone TUK4; 1:20; AbD Serotec, Kidlington, U.K.) for 1 h on ice. Blood was then prepared for flow cytometry using Dako Ultra-lyse erythrocyte lysing solution according to instructions (Dako, Denmark).

**Microscopy**

Cells were imaged using a LSM710 confocal microscope and ZEN software (Zeiss, Cambridge, U.K.) or via standard light microscopy.

**Flow cytometry**

Whole blood or PBMCs (freshly isolated or cryopreserved) from sheep were used for analysis by flow cytometry on a FACSCalibur or Fortessa X20 (BD, Oxford, U.K.). PBMCs were washed and stained in PBS containing 2% FCS at 4°C. Propidium iodide (1 µg/ml; Sigma, Dorset, U.K.) or SYTOX Blue (Life Technologies) was used to exclude dead cells. Cells were stained with the following primary Abs: mouse anti-human CD16 (clone KDI, 1:100; LifeSpan BioSciences, Seattle, WA), mouse-anti-bovine CD14 (clone CC-G3; 1:400), anti-human CD14 (clone TUK4, 1:20), mouse-anti-bovine CD172a (clone CC149; 1:200; all from AbD Serotec), and rat anti-sheep MHC class II (clone SW73.2 [23] ascites 1:4000). Isotype controls were used at the same concentrations as primary Abs: mouse IgG2a, IgG1-RPE, IgG2b (AbD Serotec) and rat IgG2a (BioLegend). Secondary Abs were used anti-mouse IgG2a-allophycocyanin (1:200), IgG1-RPE (1:800), IgG1- allophycocyanin (1:200), IgG2b-RPE (1:400) and anti-rat IgG2a AF647 (1:4000; all from BioLegend). Blood was collected in EDTA tubes from live sheep or from mice via cardiac bleeds. Blood was prepared for flow cytometry using Dako Ultra-lyse erythrocyte lysing solution (Dako) using Zombie Violet (BioLegend) to exclude dead cells. CSF1-Fe (6) was labeled with an AF647 labeling kit (Thermo Fisher Scientific). Analysis was performed with FlowJo software (FlowJo, Ashland, OR).

**Cell sorting and cytospins**

Freshly isolated BM and PBMCs were used for sorting EGFP+ populations on a FACSaria III cell sorter (BD). SYTOX Blue (Life Technologies) was used to exclude dead cells. Cells were stained with Leishman’s stain and mounted with DPX mounting media.

**cDNA synthesis and PCR**

Total RNA from sheep BM-derived macrophages (BMDMs) was isolated using TRIzol (Invitrogen), and the aqueous phase was purified with an RNasey Mini kit (Qiagen). cDNA was prepared as in Pridans et al. (12). For standard PCR, EGFP and ovine Csf1r were amplified with Invitrogen Taq polymerase using the following oligonucleotides: EGFP, 5′-CCCAATTT GGTCAAGGTGTCGTC-3′ and 5′-CTTTGACACGCTGCCATGC-3′; Csf1r, 5′-AGTCCGACCCTAAAACTCG-3′ and 5′-GTTGAGGTGTTGGTGTTGTA TAT-3′. For quantitative PCR, cDNA was amplified with Power SYBR Green PCR Master Mix using the 7500 fast Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific). The oligonucleotides used were: Hprt 5′-GACACTGGAGAAGGAACTGCA-3′ and 5′-GTTCTTTTCCAC GCAACTC-3′; Csf1r 5′-TGTTGAGTCCTCCATGACTC-3′ and 5′-CCT- TGATGTGCGCACCAGTTC-3′. Primer efficiency was validated with a standard curve of four serial dilution points (efficiency ranging between −3.28 and −3.38), and tests for nonamplification of genomic DNA were carried out systematically. Data were normalized according to the Acq model (24).

**Western blot**

Radioimmunoprecipitation assay lysates (50 mM of Tris pH 7.0, 150 mM of NaCl, 0.1% SDS, 1% Igepal CA-630, 1.27 mM of sodium deoxycholate) were prepared from CSF1-starved BMDMs (1 ml per 5 \( \times 10^5 \) cells) and EGFP RAW264.7 cells (12). Samples were mixed with 4X loading dye (0.25 M Tris-HCl pH 6.8, 8% SDS, 3% glycerol, 0.02% bromophenol blue, 10% 2-ME), heated for 5 min at 95°C, run on a 4–12% gradient SDS-PAGE gel, and transferred onto a polyvinylidene difluoride membrane as per Bio-Rad instructions. The membrane was blocked using a GPF Tag mAb (Life Technologies) and β-actin Ab (C4; Santa Cruz Biotechnology). Secondary Abs were anti-rabbit and -mouse IgG HRP (Cell Signaling Technology).

**Results**

**Production and screening of Csf1r-EGFP transgenic sheep**

Our recent in vitro study of a lentiviral vector containing control elements of murine Csf1r revealed macrophage-specific gene reporter expression in multiple species including sheep (12). Zygotes were injected with lentivirus (Csf1r-EGFP-FIRE [12]) and developed to blastocysts in vitro to create germline transgenic Csf1r-EGFP sheep. Eight recipient ewes were transplanted with either two or three blastocysts, which resulted in three pregnancies. Four founder lambs (two male and two female) were born and all were positive for the transgene (EGFP) via confocal microscopy and PCR (Fig. 1A). The LPS receptor CD14 is a monocyte marker (25) and was used to initially screen the founders by flow cytometry. Two reporter gene expression patterns were observed: all CD14+ EGFP+ monocytes expressed EGFP in two of the lambs (Fig. 1Bi), whereas the other two lambs had a population of CD14+ EGFP+ monocytes...
(Fig. 1Bi). There was also a small percentage of EGFP<sup>hi</sup> cells that did not express CD14, which is discussed later (Fig. 1B). The two founder males (as shown in Fig. 1B) were bred to wild type Blackface ewes. Seven lambs were born to the CD14<sup>+</sup>EGFP<sup>+</sup> founder (Fig. 1Bi), and six were positive for the transgene via PCR. Five of these expressed detectable EGFP in blood in a preliminary screen by flow cytometry. There was a lower percentage of offspring with EGFP<sup>+</sup> monocytes born to the CD14<sup>−/−</sup>-EGFP<sup>+</sup> founder (Fig. 1Bi). Of the 13 lambs born, 10 were EGFP<sup>+</sup> via PCR, yet only three expressed EGFP via flow cytometry. Although this founder ram had a population of CD14<sup>+</sup>EGFP<sup>+</sup> monocytes (Fig. 1Bi), analysis of the offspring’s blood revealed all CD14<sup>+</sup> monocytes expressed EGFP (data not shown). At the time of this article’s publication, lambs were born from the F1 generation (Csf1r-EGFP male bred with wild type ewe) and had the same phenotype as the founders in Fig. 1Bi. All EGFP<sup>+</sup> offspring were used in subsequent experiments.

**Analysis of BM from Csf1r-EGFP sheep**

BM contains hematopoietic stem cells that create monocytes via a series of progenitor cells (26). To determine whether EGFP was expressed in the progenitors of monocytes within the BM of Csf1r-EGFP sheep, we isolated cells from ribs and analyzed them by flow cytometry. To exclude autofluorescent eosinophils (27) and other granulocytes, we gated the narrow population on lymphocytes/monocytes based on forward light scatter (FSC) and side scatter of light (SSC) profiles (Fig. 2A). The small mononuclear population had an average of 28.5% EGFP<sup>+</sup> cells (n = 5) and could be divided into EGFP<sup>hi</sup> and EGFP<sup>lo</sup> populations. There was an average of 1.7% EGFP<sup>hi</sup> cells that were CD16<sup>−/−</sup>CD172a<sup>+</sup> and expressed an increasing level of CD14. The EGFP<sup>lo</sup> cells were more heterogeneous. There was a wider range of CD14 expression than the EGFP<sup>hi</sup> cells, and only a small percentage (<10%) also expressed CD16 and CD172a (Fig. 2B). Leishman staining of sorted EGFP<sup>+</sup> cells revealed that the majority of cells were monocyteoid. The large blast cells were characteristic of myeloblasts and monoblasts (28) (Fig. 2C).

**Analysis of PBMCs from Csf1r-EGFP sheep**

Peripheral blood monocytes in mice, humans, and pigs can be divided into functional subsets based upon expression of surface markers including CD14, CD16, CX3CR1, and CD163 (29–31). Differentiation of these subsets appears to be controlled by the macrophage growth factor, CSF1 (1, 2). Monocytes in MacGreen differentiate monocytes (33) whereas the EGFP<sup>hi</sup> cells displayed the nonclassical monocyte phenotype (CD14<sup>+</sup> CD16<sup>+</sup>). Both populations expressed CD172a (Fig. 3B), and FSC could be used to separate the EGFP<sup>hi</sup> cells into two distinct populations. Nonclassical monocytes are known to be smaller than classical monocytes (34), and Leishman staining on sorted cells revealed that both subsets presented monocyte morphology (Fig. 3C). CD16<sup>+</sup> human monocytes express higher levels of Csf1r mRNA than CD16<sup>−</sup> monocytes (35), and this could be reflected in the increase in EGFP expression in CD16<sup>+</sup> monocytes from Csf1r-EGFP sheep.

**Downregulation of the EGFP transgene in macrophages of sheep**

Treatment of BM cells or blood monocytes from other species with CSF1 can promote maturation into macrophages in vitro (4, 21, 36). To analyze EGFP expression in macrophages from Csf1r-EGFP sheep, we isolated AMs via lung lavage and PBMCs/BM were differentiated in vitro in the presence of rhCSF1. The resulting cell populations were analyzed for surface expression of CD14, CD16, and CD172a. All three cell populations retained high levels of surface CD14, but they differed in expression of CD16 and CD172a. AMs were the only cell type to express CD16, and BMDMs expressed very low levels of CD172a (Fig. 4). In all of these populations of cells, detectable expression of the EGFP transgene was extinguished. Hence the transgene provides a novel marker for blood monocytes that is lost from mature macrophages.

To determine when EGFP expression was lost during differentiation, we cultured BM cells in rhCSF1 and analyzed EGFP expression via flow cytometry (Fig. 5A). After 24 h in culture with rhCSF1, the EGFP<sup>+</sup> population was no longer visible and 50% of the cells expressed low levels of EGFP. This pattern of expression continued until day 4, when there was a slight increase in the granularity of the cells. After 7 d in culture, once the BM had differentiated into macrophages, EGFP expression was no longer detected. Expression of Csf1r mRNA is downregulated by CSF1 (37), and macrophages express lower levels of Csf1r compared...
with monocytes (38). Because the levels of EGFP in Csf1r-EGFP sheep are very low, downregulation of Csf1r during differentiation would likely result in loss of detectable EGFP. Indeed, comparison of EGFP in PBMCs from Csf1r-EGFP sheep and mice demonstrates the sheep express 1 log-fold less EGFP (Fig. 5B) and still express EGFP mRNA in BMDMs (Fig. 5C). Mature macrophages in Csf1r-EGFP mice are easily detected via FACS and microscopy (4). Notwithstanding the lower expression, EGFP could still be detected in the sheep macrophages via Western blot (Fig. 5D). As we have previously shown that Csf1r-EGFP lentivirus is capable

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** BM of Csf1r-EGFP sheep contains a heterogeneous EGFP+ population of cells. BM from Csf1r-EGFP sheep was isolated from ribs and analyzed via flow cytometry. (A) Gating strategy. Dead cells were excluded with propidium iodide staining and granulocytes excluded via FSC/SSC. EGFPlo and EGFPhi were selected once quadrants were set with negative control BM. (B) Analysis of CD14, CD16, and CD172a expression in GFPlo and GFPhi cells. Quadrants were set using isotype controls for each population. (C) Leishman-stained EGFP+ cells (combined EGFPlo and EGFPhi). Scale bar, 10 μm. Dot plots and Leishman staining of EGFP+ cells representative of five and two sheep, respectively. Leishman staining was representative of 15 images per animal.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Monocytes are EGFP+ in Csf1r-EGFP sheep. Peripheral blood monocytes were isolated from the blood of live animals and analyzed via flow cytometry. (A) EGFP+ gating strategy. (B) Expression of CD14, CD16, and CD172a in EGFPlo and EGFPhi PBMCs. Quadrants were set using isotype controls for each population. (C) Leishman-stained EGFP+ cells. Scale bars, 10 μm. Dot plots and Leishman staining of EGFP+ cells are representative of nine and three sheep, respectively. Leishman staining was representative of 10 images per animal.
of transducing primary sheep macrophages in vitro to generate an EGFP+ population (12), producing Csf1r-EGFP sheep with a higher titer of lentivirus may result in higher levels of EGFP+ in macrophages.

Functional analyses of monocytes and macrophages from Csf1r-EGFP sheep

MacGreen (Csf1r-EGFP) mice express the transgene in the same locations as the endogenous gene (4). To verify whether expression of the transgene in sheep was restricted to cells expressing Csf1r, we used fluorescently labeled CSF1-Fc (6) to detect Csf1r in whole blood via flow cytometry. Both EGFPhi and EGFPlo PBMCs bound the labeled CSF1-Fc, whereas the EGFPlo PBMCs did not (Fig. 6A). Surprisingly, the granulocyte population showed low levels of CSF1-Fc binding. Murine granulocytes from the MacGreen mouse express Csf1r mRNA and are EGFP+, yet they do not express Csf1r on the cell surface (39). There is evidence that human granulocytes express functional CSF1R, albeit at lower levels than CD14+ monocytes (40). We examined EGFP expression in sheep granulocytes. Consistent with the level of CSF1-Fc binding, and identical to the levels of CSF1R in humans, this population expressed low levels of EGFP (Fig. 6A). Expression of endogenous Csf1r was not affected by the transgene: the level of mRNA detected by quantitative PCR in BMDMs from wild type and transgenic sheep was not significantly different (Fig. 6B).

BM and PBMCs from Csf1r-EGFP sheep differentiated into macrophages in the presence of rhCSF1 (Figs. 4, 5). Phagocytosis assays were performed to further investigate whether the transgene had any effect on monocyte and macrophage function in Csf1r-EGFP sheep. Both monocyte-derived macrophages (MDMs) and BMDMs phagocytosed Zymosan A S. cerevisiae BioParticles within 2 h (Fig. 6C), highlighting that they were indeed functional macrophages. To compare the function of monocytes from wild type and Csf1r-EGFP sheep, we performed phagocytosis assays on whole blood. There was no difference in the capability of CD14+ monocytes to phagocytose E. coli (Fig. 6D), suggesting that the transgene also had no effect on the function of monocytes.

The development of mature APCs relies on the expression of surface MHC class II molecules. We assessed the expression in wild type and transgenic monocytes via flow cytometry and found...
that both groups had CD14+ monocytes that expressed MHC class II (Fig. 6E). In humans, the highest expression levels of MHC class II are found on CD16+ monocytes (30). As in humans, all monocytes express high levels of MHC class II and it is unaffected in the Csf1r-EGFP sheep. Again, in common with humans, the CD16+ monocytes expressed higher levels than the CD14++ subset.

Discussion

CSF1 controls the proliferation, differentiation, and survival of monocytes and macrophages and their BM progenitors (41), and the receptor, CSF1R, provides a marker for cells of the mononuclear phagocyte lineage. MacGreen (Csf1r-EGFP) mice express the transgene in the same locations as the endogenous gene, and expression is dependent on FIRE (4). FIRE acts as an antisense promoter in macrophages (42) and is remarkably conserved from humans to reptiles (43). It is even more highly conserved than the proximal promoter. We have recently described a lentivirus containing control elements of murine Csf1r capable of driving transgene expression in macrophages from multiple species, including sheep, in vitro (12). This lentivirus was used in the generation of Csf1r-EGFP transgenic sheep. Alignment of the Ensemble predicted sheep Csf1r gene with the murine promoter, and FIRE reveals 50 and 80% identity, respectively (data not shown). All of the myeloid-expressed transcription factor binding sites contained within FIRE (44) are conserved between mouse and sheep. Our data show that the apparent conservation reflects function in that the mouse elements were sufficient to drive EGFP expression in monocytes from all four Csf1r-EGFP founder sheep.

The Csf1r promoter region in mice contains two separate promoters to drive expression in macrophages and placental trophoblast cells (45). In humans, the trophoblast promoter is located 26 kb upstream of CSF1R (46). When the human trophoblast promoter sequence is aligned against the sheep genome (Oar_v3.1/oviAri3) using BLAST-like alignment tool, the highest scoring match is located at the 3' end of Pdgrfb. This suggests the ovine trophoblast promoter, as in humans, is located at least 20 kb upstream of Csf1r. Deletion of the trophoblast promoter in the MacBlue mice abolishes transgene expression in the majority of tissue macrophages (10). Hence the lack of EGFP expression in AMs from the transgenic sheep may be caused by the lack of the trophoblast promoter in the construct used to generate these animals. We have previously shown that sheep macrophages are EGFP+ after incubation with Csf1r-EGFP lentivirus, which used polybrene to increase transduction efficiency (12). The low levels of EGFP expression in Csf1r-EGFP sheep could likely be increased by the use of a higher titer lentivirus (and hence more lentiviral insertion events) or by use of the ovine instead of murine Csf1r in the lentiviral construct. However, it is also possible that the reduced expression level in sheep versus mouse is associated with either species- or lentiviral-specific methylation of the transgene. A study of EGFP+ sheep generated by lentiviral injection of zygotes using a ubiquitous promoter indicated increased methylation patterns correlated with lower EGFP intensity (47).

Monocytes are a population of leukocytes that can be functionally characterized by their ability to phagocytose, produce cytokines, and present Ag. They make up 5–10% of the PBMCs in humans and show both antigenic and morphological (size, granularity, and nuclear morphology) heterogeneity. Their initial identification was based on expression of CD14; however, variation in surface expression of Ags has led to the description of various subsets (reviewed in Ref. 48). Human and bovine monocytes can be divided into subpopulations based on surface expression of
CD14/CD16, whereas porcine and murine monocytes can be identified by CD14/CD163 and Ly6C/CX3CR1, respectively (reviewed in Ref. 19). The majority of knowledge about monocyte development in the BM stems from work performed in mice (reviewed in Ref. 49). Monocytes develop from hematopoietic stem cells in the BM via a series of progenitors such as the common myeloid progenitor, monocyte-macrophage dendritic cell progenitor, and the common monocyte progenitor, which all express Csf1r (50–53). These progenitors can be identified as Lin− and by differential expression of CD117 (c−kit), CX3CR1, Ly6C, and CD31. In humans, the common myeloid progenitor can be distinguished from other progenitors based on expression of CD38, CD45RA, FLT3, CD7, and CD10 (54). In-depth studies of monocyte development in larger animals have been hampered by the lack of species-specific Abs. In pigs, the Swine Workshop Cluster molecules were originally used to identify committed monocyte precursors in the BM. Swine Workshop Cluster 3 was identified as the earliest marker of myeloid cell development (55) and was later identified as CD172a (56). In cows and sheep, CD34+ progenitor cells have been identified (57, 58), yet specific analysis of monocyte progenitors has not been performed.

As noted earlier, the expression of the EGFP transgene in the sheep is downregulated by CSF1 in vitro and in tissue macrophages in vivo. Tissue macrophages depend upon CSF1 for their differentiation, and they are rapidly depleted after treatment of mice with anti-CSF1R Ab (5). The downregulation of the reporter gene by CSF1 probably reflects direct actions on FIRE. FIRE enhancer activity is acutely regulated by transcription factor Runx1, which is expressed at high levels in progenitors and acutely downregulated by CSF1 (59). There is an emerging view that most tissue macrophages are replaced by self-renewal, rather than replacement from the blood monocyte pool (60, 61). Like the MacBlue transgene in mice, which is effectively monocyte specific (62, 63), the Csf1r-EGFP lentiviral transgene could provide a useful marker to monitor monocyte extravasation and trafficking in tissues, and might be applied to other species.

Mice and humans differ immunologically, and the mouse has limitations as a model (64–66). Macrophages from the pig more closely resemble those of humans in terms of their response to bacterial LPS (21) or sheep in terms of pattern recognition receptor expression (67). Hence larger animal models are likely to represent a better model of human macrophage development. Phenotypic analysis of macrophage development in sheep has not been described. Instead, studies have focused on the response of monocytes and macrophages to infection. We have shown that monocytes from Csf1r-EGFP sheep are functional and comparable with their wild type counterparts, and that the transgene had no effect on expression of the endogenous gene. Hence, as expression of Csf1r is one of the earliest markers of macrophage lineage commitment (51), BM from Csf1r-EGFP sheep could be a valuable resource to study the earliest events in myelopoiesis in an underused species.

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Disclosures
The authors have no financial conflicts of interest.

FIGURE 6. (A) Freshly isolated blood from wild type and Csf1r-EGFP sheep was analyzed via flow cytometry for binding of CSF1-Fc and EGFP expression. PBMCs and granulocytes were gated via FSC/SSC profiles and dead cells excluded with Zombie Violet. Quadrants were set with wild type sheep blood. Dot plots are representative of three Csf1r-EGFP sheep and two repeat experiments. (B) Quantitative PCR was used to determine the fold change of Csf1r expression between wild type and Csf1r-EGFP sheep BMDMs (n = 4 per group). (C) PBMCs from Csf1r-EGFP sheep were differentiated into macrophages with rhCSF1. Phagocytosis assays performed with Zymosan A BioParticles and viewed by confocal microscopy. Images are representative of two MDMs and two BMDMs. Scale bars, 20 μm. (D) Phagocytosis assays were performed on freshly drawn blood from Csf1r-EGFP sheep and wild type controls using pHrodo Red E.coli BioParticles. CD14+ monocytes were gated in the PBMC fraction by flow cytometry. Results are representative of four wild type and six Csf1r-EGFP sheep. The control contained BioParticles and was incubated on ice. (E) Representative histograms of MHC class II expression in CD14+ PBMCs from wild type (n = 5) and Csf1r-EGFP (n = 7) sheep, analyzed by flow cytometry. (F) Representative histograms of MHC class II expression in CD14+ and CD16+ (GFP+) monocytes from Csf1r-EGFP sheep (n = 5), analyzed by flow cytometry.

The authors have no financial conflicts of interest.
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