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## Characterisation of endogenous Galectin-1 and -9 expression in monocyte and macrophage subsets under resting and inflammatory conditions



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### ABSTRACT

Macrophages are key cells in both acute and chronic inflammatory settings. Their activation and function highly depends on the cytokines, chemokines and adhesion molecules that direct monocytes to infiltrate tissues, differentiate into macrophages, and finally lead to the clearance of such inflammatory signals. Galectins,  $\beta$ -galactoside-binding lectins, are differentially expressed by various immune cells, and some members of this family have been identified as regulators of leukocyte recruitment and activation. Galectin-1 (Gal-1) and galectin-9 (Gal-9) expression has been described in immune cells, but the specific molecular mechanisms by which they modulate the inflammatory response in macrophages/monocytes are not completely understood. In this study we sought to comprehensively characterise the expression profile of endogenous Gal-1 and Gal-9 in different murine and human monocyte/macrophage populations in response to different inflammatory stimuli. All subsets of murine and human macrophages expressed significant levels of Gal-1 and -9. Interestingly, murine bone marrow derived macrophages stimulated with M2 (pro-resolution) polarising agents preferentially upregulated Gal-1, while Gal-9 expression was upregulated by M1/pro-inflammatory stimulation. However, we observed differing results in human monocyte derived macrophages. Collectively, our findings report a differential expression pattern of endogenous Gal-1 and -9 in macrophage and monocyte subsets in response to a range of inflammatory stimuli. Future studies will endeavour to elucidate whether the galectins make attractive therapeutic targets or agents for regulating the inflammatory response.

### 1. Introduction

The inflammatory response is a physiological process that protects host tissues from injury and infection. A controlled series of events culminate in the trafficking of leukocytes from the bloodstream into tissue; a process fundamental for successful cell-mediated immunity [1].

Once the initiating stimulus is cleared, removal of leukocyte infiltrates and a return to tissue homeostasis is required for termination of the acute inflammatory response. This active process is known as resolution. It is driven by macrophages and includes the removal of harmful materials produced by neutrophils, clearance of apoptotic

inflammatory cells, and delay of apoptosis [2]. Failure of resolution triggers an uncontrolled inflammatory response and a persistent infiltration of leukocytes into tissue which in turn unleashes a chronic and pathological inflammation [3]. Numerous important human pathologies including arthritis, asthma and atherosclerosis are a result of a chronic inflammatory response. Although several inflammatory mediators (adhesion molecules, cytokines and chemokines) involved in the recruitment of leukocytes into tissues have been identified, there is still much to be discovered. In particular, key processes involved in the persistence of leukocyte infiltrates, widely observed in chronic inflammation, have not been elucidated [4,5]. In this context, members of the galectin (Gal) family have recently been identified as

**Abbreviations:** CL, Classical monocyte; CRD, Carbohydrate recognition domain; Gal, Galectin; ITM, Intermediate monocyte; NC, Non-classical monocyte

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immunoregulatory proteins whose actions include both positive and negative modulation of leukocyte recruitment [6,7].

Galectins are a family of soluble  $\beta$ -galactoside-binding lectins that display different functions depending on their tissue-specific and sub-cellular location. In vertebrates, 15 galectins have been identified to date, and can be found either intracellularly, in the nucleus, cytoplasm and organelles, or extracellularly [8]. All members of the family share close sequence homology in their carbohydrate recognition domains (CRD, of approximately 130 amino acids), but exhibit distinct affinities for different saccharide ligands. Based on their biochemical structure they are classified into proto-type (containing one CRD in a monomeric form such as Gal-5, -7, -10, or in a dimeric form such as Gal-1, -2, -11, -13 and -14), chimera-type (one CRD and an additional non-lectin domain involved in the protein oligomerization as is the case of Gal-3), and tandem-repeat-type (composed of two CRDs connected by a linker peptide such as Gal-4, -6, -8, -9 and -12) [9,10]. Once synthesized, galectins may remain within the intracellular compartment and participate in protein-protein interactions to regulate intracellular events [11,12]. However, most galectins are released through an unconventional route to the extracellular compartment [13]. Once secreted, galectins bind to  $\beta$ -galactoside sugars and crosslink cells-surface N-acetyllactosamine-enriched glycoconjugates. This leads to the activation of different signal transduction responses through aggregation of specific cell-surface glycoreceptors. Each galectin may exert different and contrasting functions depending on whether it acts extracellularly or intracellularly [9]. Compelling evidence highlights major roles of galectins modulating inflammation and immune responses [14,15]. Indeed, expression of galectin proteins has been described in infiltrating inflammatory cells displaying actions at key stages of the inflammatory response including mast cells degranulation, platelet activation, cell adhesion, chemotaxis and T-cell apoptosis induction [14]. Importantly, they are also known to modulate both leukocyte trafficking into tissue and also clearance of the resulting inflammatory infiltrate; both processes being crucial for the initiation and resolution of inflammation [9]. Among all the family members, Gal-1, -3 and -9 are highly expressed in immune cells. Gal-3 has been widely studied as an immunoregulatory protein in macrophage biology, however, the expression pattern and function of endogenous Gal-1 and -9 has not been completely characterized. Gal-1 is composed by two subunits of 14.5 kDa and it is known to weaken acute inflammatory responses by controlling neutrophil adhesion, function and turnover, and modulating monocyte and macrophage activation [16,17]. Furthermore, Gal-1 controls T-cell viability, mitigates Th1- and Th17- mediated responses and leans the balance toward a Th2 cytokine profile [18,19]. Its essential role in the regulation of the inflammatory response has been shown in experimental models of autoimmunity, allergy and cancer [20–22]. The majority of published studies relating to Gal-9 focuses on its role in T cell biology, and largely supports an anti-inflammatory and protective role for the protein. This is thought to be a result of its ability to induce apoptosis in Th1 and Th17 cells via T-cell immunoglobulin mucin domain (Tim)-3 [23]. Published studies have identified a role for Gal-1 and -9 in regulating immune responses in human and murine models of disease. However, how these regulations differ with macrophage populations under different inflammatory stimuli has not been explored.

The aim of this study was to characterise the expression profile of endogenous Gal-1 and -9 in different murine and human macrophage and monocyte populations under both steady state and inflammatory conditions, in order to determine their suitability as potential therapeutic targets in inflammatory diseases.

## 2. Materials and methods

All buffers, cell culture media and other laboratory chemicals were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise specified. Cytokines and chemokines were purchased from Peprotech

(London, UK) and R&D Systems (Abingdon, UK).  $\beta$ -actin and  $\alpha$ -tubulin antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA), polyclonal Gal-1 (clone AF1125) and Gal-9 (clone AF2045) antibodies were purchased from R&D Systems. Polyclonal inducible nitric-oxide synthase (iNOS; clone ab3523) and polyclonal anti-arginase 1 (clone ab60176) were purchased from Abcam. HRP-conjugated secondary antibodies were obtained from BioRad Laboratories (Hemel Hempstead, UK) and Cell Signaling.

### 2.1. Animals

All animal studies (isolation of different cell populations) were conducted with ethical approval from the Sir William Dunn School of Pathology, University of Oxford Local Ethical Review Committee and in accordance with the UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). Male (7–10 weeks) C57BL/6 mice and neonatal C57BL/6 pups were obtained from the Biomedical Services Unit (Oxford, UK). All mice were housed in a 12 h light/12 h dark cycle unit with free access to food and water. Adult animals were euthanised via asphyxiation with a rising concentration of CO<sub>2</sub>, whereas neonatal mice were sacrificed by decapitation.

#### 2.1.1. Cell culture

**2.1.1.1. Murine bone marrow-derived macrophages (BMDMs).** BMDMs were generated as previously described [24]. Briefly, fresh bone marrow cells from tibiae and femurs of male C57BL/6 mice were isolated and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 10 % L929 cell-conditioned media as a source of macrophage colony-stimulating factor [25], and 1 % penicillin/streptomycin for 6–7 days. Bone marrow cells were seeded into 8 mL of medium in 100 mm non-tissue culture treated Petri dishes (ThermoFisher Scientific, UK). On day prior the experiment, BMDMs were lifted off dish surface by gently scrapping and were counted and resuspended in FBS free media at the desired cell concentration.

**2.1.1.2. Resident peritoneal macrophages.** male C57BL/6 mice were sacrificed, and peritoneal cavities were lavaged with 10 mL ice-cold PBS supplemented with 2 mM EDTA. Cells were pelleted by centrifugation at 300 g for 5 min, resuspended in DMEM 10 % FBS and plated for 2–4 h to allow macrophages to attach to the plate.

**2.1.1.3. Murine microglia.** C57BL/6 pups were sacrificed by decapitation at 1–3 postnatal days, and heads were placed into a Petri dish for dissection as previously described (10.21769/BioProtoc.1989). Briefly, meninges were carefully removed from brains, minced into small pieces, and incubated with trypsin 0.25 % and DNase I. After tissue digestion, the suspension was filtered and resuspended in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin and seeded in culture flasks. After 10–12 days in culture, detached microglia were collected and plated on 6-well culture dishes [26].

### 2.2. Human blood samples

Blood was collected from healthy donors with written and verbal informed consent and approval from the University of Birmingham Local Ethical Review Committee (ERN\_18–0382). An equal proportion of male and female donors were used with an age range between 22–70.

#### 2.2.1. Cell culture

**2.2.1.1. Human monocyte-derived macrophages (hMDMs).** Whole blood was separated, using density gradients Histopaque 1119 and 1077 (Sigma-Aldrich, U.K.), to obtain the peripheral blood mononuclear cell (PBMC) fraction. Mixed monocytes were isolated from PBMC, using PBS

without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  supplemented with 0.5 % BSA and 2 mM EDTA at 4 °C, by positive selection for CD14 using anti-CD14 microbeads and MACS separation columns (Miltenyi Biotec, Germany). The purity of mixed monocytes was measured and was consistently  $\approx 95$  %. Purified monocytes were cultured at 37 °C in 5 %  $\text{CO}_2$  in M199 media (Life Technologies, Paisley, U.K.) containing 10 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich, Poole, UK) and 10 % autologous human serum.

### 2.3. Compounds and treatments

BMDMs, resident peritoneal macrophages and murine microglia were seeded onto tissue culture plastic in FBS free media and allowed to adhere overnight at 37 °C, 5 %  $\text{CO}_2$ . To induce “pro-inflammatory” (M1) or “pro-resolution” (M2) phenotypes, previously established treatment protocols were followed (26,699,615). Briefly, M1 and M2 phenotypes were generated by treating BMDMs with LPS (100 ng/mL) and  $\text{IFN}\gamma$  (20 ng/mL) or IL-4 (20 ng/mL) respectively. BMDMs were additionally treated with compounds at the following concentrations: Flagellin (500 ng/mL), IL-13 (20 ng/mL), Poly I:C (10  $\mu\text{g}/\text{mL}$ ) and Zymosan (10  $\mu\text{g}/\text{mL}$ ). Resident peritoneal macrophages and murine microglia were treated with either LPS (100 ng/mL) and  $\text{IFN}\gamma$  (20 ng/mL) or IL-4 (20 ng/mL).

hMDMs were supplemented with M199 media containing 10 ng/mL EGF and 10 % human autologous serum every two days for six days. Following this, cells were cultured in M199 media containing 10 ng/mL EGF and 1 % autologous human serum and three culture conditions were established; i) untreated ii) treatment with LPS (100 ng/mL) and  $\text{IFN}\gamma$  (20 ng/mL) or iii) treatment with IL-4 (20 ng/mL) [27]. Cells were stimulated for 16 h before lysis and storage at  $-80$  °C until gene and protein analysis.

Viability assays (CellTiter Glo Viability Reagent and morphological analysis) for human monocyte derived and murine macrophages were performed and no increased cell death was observed in any of the polarisation conditions (data not shown).

### 2.4. mRNA expression analysis

Cultured cells were extracted with TRIzol reagent (ThermoFisher Scientific) and total RNA concentration and purity was determined with a ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, USA). cDNA was synthesized from 700 ng human RNA or 1000 ng murine RNA using the QuantiTect Reverse Transcription kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions. Real-time quantitative PCR was performed using either Taqman or Sybr Select gene expression master mix (Life Technologies) in the StepOnePlus™ thermal cycler (Applied Biosystems). Primers were purchased from Thermo Fisher Scientific (Hs00175478\_m1 (CD80); Hs00267207\_m1 (MRC1, coding for CD206); Hs00355202\_m1 (LGALS1); Hs04190742\_mH (LGALS9); Hs01567026\_m1 (CD86); Hs03003631\_g1 (18S)) or Qiagen (NM\_008495 (Lgals1, coding for murine Gal-1); NM\_001159301 (Lgals9, coding for murine Gal-9)). Cycle threshold values were determined by the StepOne software and target gene expression was normalised to housekeeping gene (*18 s*). Relative expression results were plotted as mRNA expression divided by actin expression, and normalised to basal samples when convenient [28].

### 2.5. Protein expression analysis

#### 2.5.1. Western blot

cells were lysed by adding RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich) followed by manual disruption. BCA protein assay kit (ThermoFisher Scientific) was used to determine protein concentration. Total cell protein (20–30 mg) was added to 4x Laemmli buffer (250 mM

Tris–HCl, pH 6.8, 8 % SDS, 40 % glycerol, 0.004 % bromophenol blue, 20 % b-mercaptoethanol) and heated at 95 °C for 5–10 min. Samples were then resolved on SDS-PAGE gels and transferred onto Hybond ECL nitrocellulose/PVDF membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked with 5 % milk in TBS-T (Tris-buffered saline, 0.1 % Tween-20, pH 7.6) for 1 h at room temperature and then incubated with either polyclonal goat anti-Gal-1, goat anti-Gal-9 (1:1000; R&D Systems), rabbit anti-iNOS, goat anti-arginase 1 (1:2000; Abcam), rabbit anti- $\beta$ -actin or rabbit anti-tubulin (both 1:1000, Cell Signaling Technologies) in 5 % BSA/TBS-T overnight at 4 °C. Next, membranes were incubated with a HRP-conjugated anti-Goat IgG secondary antibody for 1 h at room temperature. Protein bands were visualised by incubating the membranes with Amersham ECL prime and subsequent exposure to X-ray film over a range of exposure times. For successive antibody incubations using the same membrane bound antibodies were removed with stripping buffer (ThermoFisher Scientific).

#### 2.5.2. ELISA

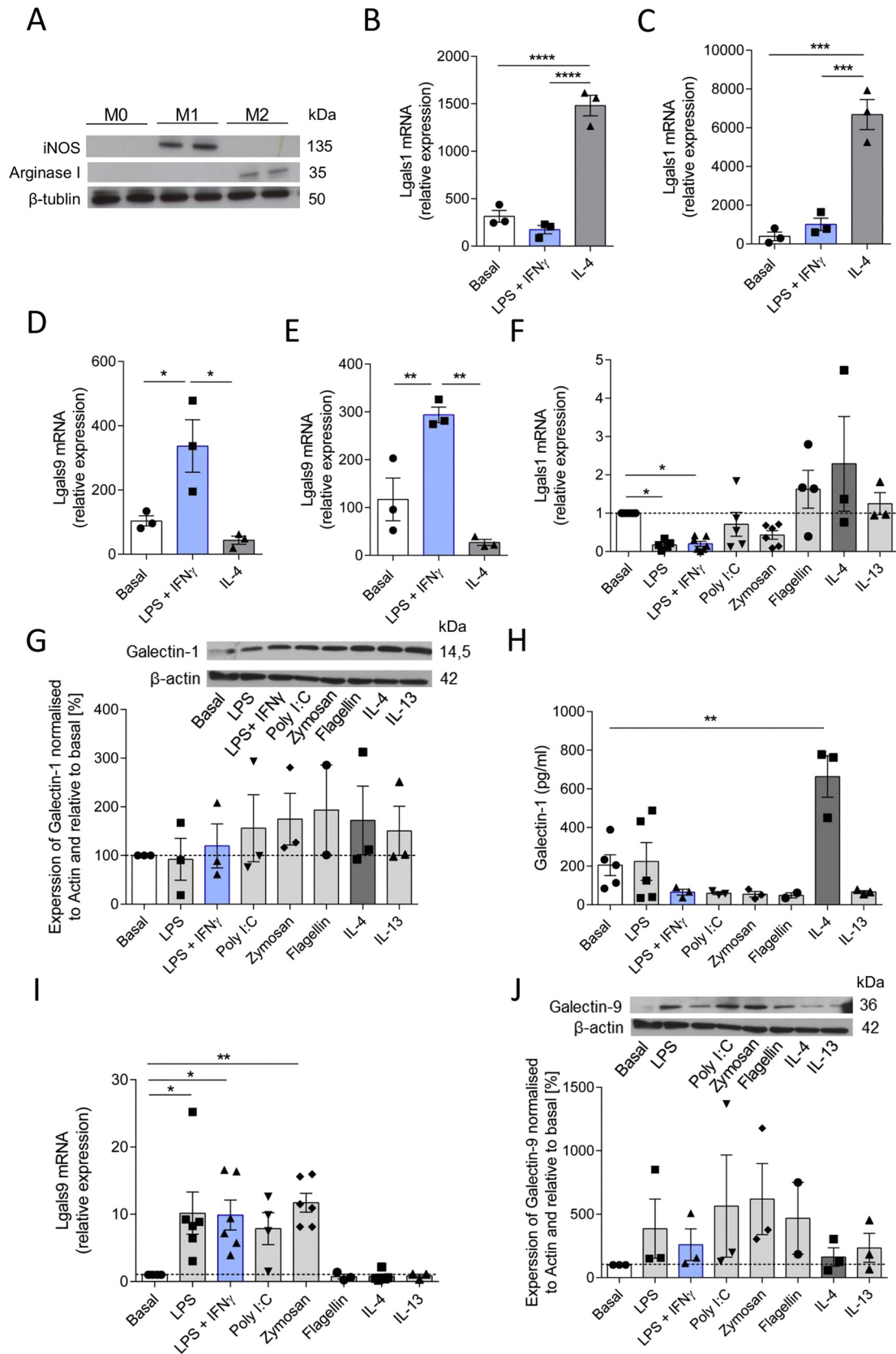
Measurement of Gal-1 and Gal-9 secreted protein levels in cell supernatants was performed by ELISA assay (R&D Systems) according to manufacturer’s instructions.

#### 2.5.3. Flow cytometry

PBMCs were isolated from whole blood, using density gradients as described above. Cells were either processed directly or washed with PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , PBS containing 25 mM lactose or PBS containing 25 mM sucrose for 20 min at room temperature with occasional mixing. Cells were incubated with FcR blocking agents (Miltenyi) in PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  containing lactose or sucrose in the appropriate experiment before staining cells with antibodies against monocyte subset markers CD14 (TexasRed, clone TuK4, Thermo Fisher) and CD16 (BV421, clone 3G8, Biolegend, San Diego, USA) as well as against surface Gal-1 (PE, polyclonal antimouse, R&D Systems) and -9 (BV421, clone 9M1-3, eBioscience, San Diego, USA). Cells were fixed using 2 % PFA. Intracellular staining was performed using eBioscience Foxp3/ Transcription Factor Staining Buffer set (ThermoFisher Scientific) according to the manufacturer’s instructions. Briefly, cells were stained extracellularly with monocyte subset markers and fixed as described above. The cells were permeabilised using 1x Permeabilization Buffer before incubating cells with Gal-1 and Gal-9 antibodies (details above). Unbound antibodies were removed by washing in 1x Permeabilization Buffer. Protein expression was analysed by flow cytometry on a Dako CyAn (Beckman Coulter, High Wycombe, U.K.), and data were analysed using Summit software (Dako). The un-specific binding of Ab was quantified by using corresponding isotype controls.

#### 2.5.4. Immunohistochemistry

CD14<sup>+</sup> monocytes were isolated from PBMCs as described above.  $3 \times 10^5$  cells were cultured per well in an ibidi  $\mu$ -slide 8 well dish (ibidi, Germany) for 16 h as described above. For extracellular staining the cells were fixed in 2 % PFA, followed by repeated washing in PBS or PBS containing 25 mM lactose. Cells for intracellular staining were fixed with ice cold methanol and repeatedly washed in PBS afterwards. The cells were blocked using 1 % BSA and 10 % donkey serum in PBS before incubating the cells with the primary antibody for 1 h at room temperature. Antibodies used were polyclonal anti-Gal-1 and -Gal9 antibodies (both R&D). Cells were washed repeatedly in PBS before the Alexa Fluor 488 donkey anti-Goat IgG (H + L) antibody (Invitrogen) was applied for 1 h at room temperature in the dark. The cells were washed repeatedly after the incubation. DAPI staining was applied just before imaging the cells using the Zeiss LSM780 confocal microscope (Zeiss, Germany). All buffers for the intracellular staining contained 1 % Tween-20 (Sigma-Aldrich). Monocytes, both permeabilized and non-permeabilized, were also stained with the secondary antibody in the absence of the primary to measure the extent of non-specific binding.



(caption on next page)



**Fig. 1. Gal-1 and -9 mRNA and protein levels in murine BMDM, microglia and peritoneal macrophages.** Macrophages were polarised towards M1 or M2 phenotypes treating BMDM using LPS + IFN $\gamma$  or IL-4 respectively. **A** Representative Western Blot analysis of arginase I and inducible nitric oxide-synthase in either untreated, M1 or M2 polarised BMDMs. **B-E** Murine microglia (**B, D**) and peritoneal macrophages (**C, E**) were harvested from C57BL/6 mice. **B, C** IL-4 treatment increases Lgals1 mRNA levels compared to basal levels in microglia and peritoneal macrophages. **D, E** LPS and IFN $\gamma$  upregulate Lgals9 mRNA levels compared to basal levels in microglia and peritoneal macrophages. Data acquired via qRT-PCR (**F, I**), Western blot (**G, J**) and ELISA (**H**) using BMDM treated with various stimulations over 24 h. **F** qRT-PCR shows varying Lgals1 mRNA expression in BMDM after different treatments for 24 h. **G** Relative expression of Gal-1 protein in BMDM after treatment with various stimulations for 24 h alongside a representative Western Blot. **H** Gal-1 in supernatant of stimulated BMDMs. **I** qRT-PCR shows Lgals9 mRNA expression in BMDM is increased after treatment with LPS with and without IFN $\gamma$  and Zymosan for 24 h. **J** Relative expression levels of Gal-9 protein in BMDM after treatment with various stimulations for 24 h alongside a representative Western Blot. Data are expressed as mean  $\pm$  SEM (n = 2-6). Statistical analysis was conducted using One Way ANOVA/Kruskal-Wallis test\*: p = 0.05, \*\*: p = 0.01, \*\*\*: p = 0.005, \*\*\*\*: p = 0.001.

## 2.6. Statistical analysis

All quantitative data are expressed as mean  $\pm$  SEM of n independent biological replicates. Normally distributed data was analysed using ANOVA followed by multiple comparison using Bonferroni post-test. Skewed data was analysed using Kruskal-Wallis and Dunn's Multiple Comparison tests (Prism 7 GraphPad Software, San Diego, CA, USA). A P value of < 0.05 was taken to be statistically significant.

### 3.1. Primary murine M1 and M2 macrophages differentially express Gal-1 and -9

To confirm the expression of Gal-1 and -9 in different murine macrophage populations, we began by polarising macrophages into either an "M1-like" pro-inflammatory using LPS + IFN $\gamma$  or "M2-like" pro-resolution phenotype using IL-4. In order to validate polarisation we measured the expression of inducible nitric oxide-synthase (iNOS; M1 marker) and Arginase 1 (Arg1; M2 marker) by western blot (Fig. 1 A).

Gal-1 mRNA remained unchanged following challenge by LPS and IFN $\gamma$  in both microglia and resident peritoneal macrophage (Fig. 1 B, C). However, Lgals1 mRNA expression was upregulated in both, microglia and peritoneal macrophages following IL-4 stimulation (Fig. 1 B, C). Lgals9 mRNA expression in resident peritoneal macrophages and microglia was unchanged following IL-4 treatment but significantly increased with LPS and IFN $\gamma$  stimulation when compared to basal conditions (Fig. 1 D and E). To further characterise macrophage galectin expression, BMDMs were challenged with a range of inflammatory stimuli including several pathogen-associated molecular patterns (PAMPs) and selected cytokines. As previously noted, addition of IL-4 trended towards increased expression of Lgals1 mRNA. However, LPS with or without IFN $\gamma$  caused a significant reduction in Lgals1 mRNA expression (Fig. 1 F). Western blot analysis showed that protein levels remained unaffected across all treatment groups (Fig. 1 G). Interestingly, conditioned culture medium from BMDM supernatants contained significantly increased levels of Gal-1 upon IL-4 stimulation (Fig. 1 H). Conversely, LPS with or without IFN $\gamma$ , Poly I:C and zymosan were all potent inducers of both Lgals9 mRNA and protein expression compared to non-stimulated (basal) conditions (Fig. 1 I, J).

These results suggest that Gal-1 is upregulated in a M2 macrophages whilst upregulation of Gal-9 is associated with an M1 Phenotype.

### 3.2. All human peripheral monocyte subsets have large intracellular pools of Gal-1 and 9

Peripheral blood mononuclear cells were isolated from whole blood. CD14 and CD16 were used to identify classical (CL), intermediate (ITM) and non-classical (NC) monocytes (Fig. 2 A). The flow cytometric analysis showed that all subsets express Gal-1 and Gal-9 on the surface (Fig. 2 B-E). A trend for the enrichment of Gal-1 on the surface of CL and ITM monocytes and Gal-9 on the surface of ITM and NC monocytes was shown (Fig. 2 D, E). However, a substantial difference in endogenous expression of Gal-1 and -9 was observed between donors (Fig. 2 F, G).

Intracellular pools were detected in both freshly isolated and

cultured monocytes, as seen by flow cytometry and confocal microscopy imaging (Fig. 2 D, E, H).

### 3.3. Gal-1 is eluted from the cell surface using lactose

Surface expression of Gal-1 and -9 on monocyte subsets was further investigated using competitive binding assays with  $\beta$ -lactose. Surface Gal-1 was eluted following  $\beta$ -lactose treatment; however, total Gal-1 levels remained unaffected (Fig. 3 A-C). Irrespective of the presence of  $\beta$ -lactose, total and surface Gal-9 levels remained unchanged (Fig. 3 D-F). Confocal imaging confirmed that despite  $\beta$ -lactose treatment, levels of surface Gal-9 remained unchanged while Gal-1 was efficiently eluted (Fig. 3 G).

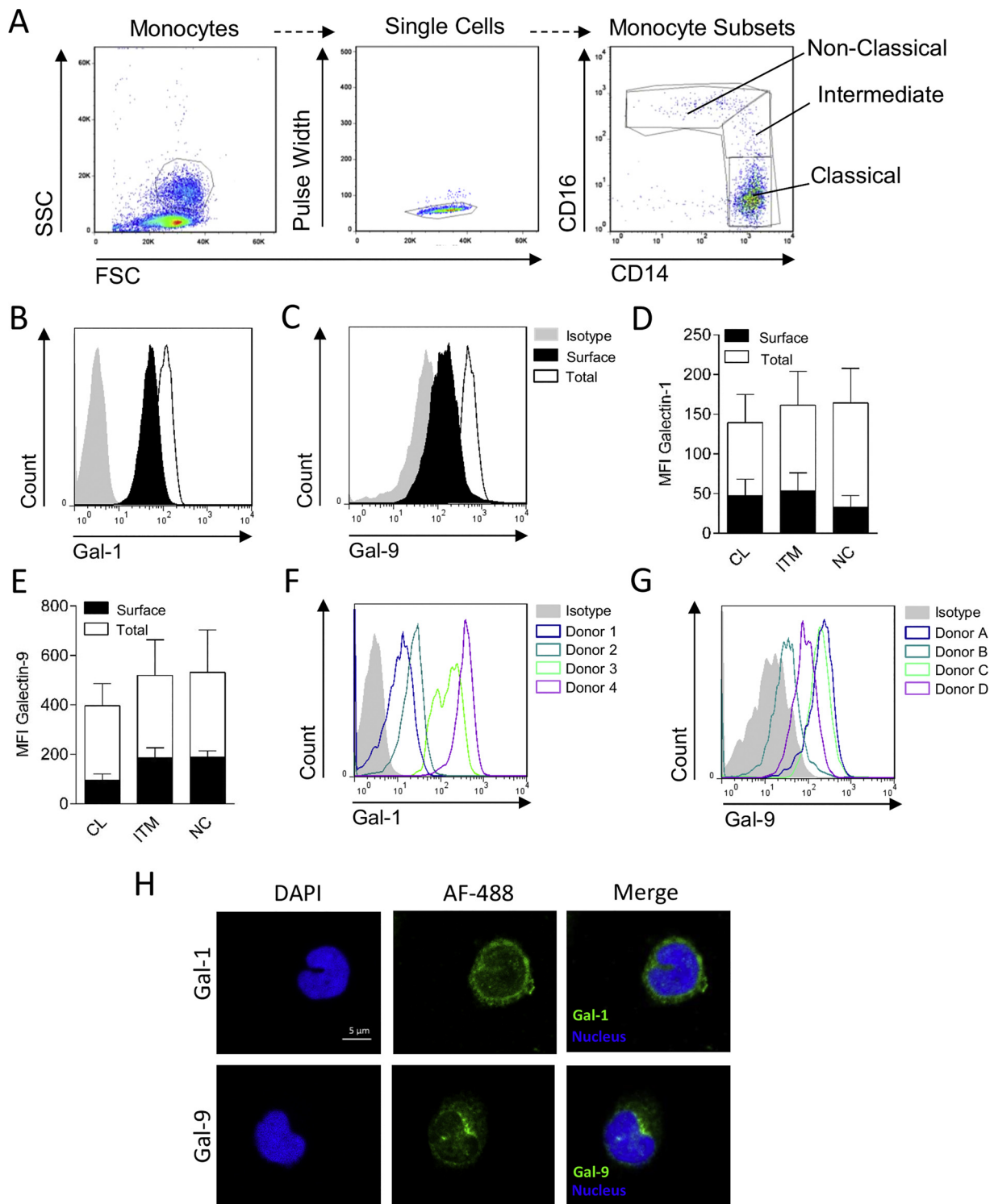
### 3.4. Endogenous Gal-1 and -9 expression in human macrophage subsets remains stable in response to stimulation

To investigate Gal-1 and -9 profiles in M1- or M2-like phenotypes, hMDMs were polarised using LPS and IFN $\gamma$  or IL-4, respectively. Successful M1 polarisation was confirmed by increased CD80 and CD86 and reduced CD206 mRNA after treatment with LPS and IFN $\gamma$ . Conversely, M2 polarisation was corroborated by increased CD206 mRNA levels and reduced CD80 and CD86 expression following treatment with IL-4 (Fig. 4 A-C). Gal-1 expression trended to increase upon differentiation into M1 but not M2 macrophages at the mRNA (Fig. 4 D) and protein (Fig. 4 E) level. However, unlike in murine macrophages, we observed no increase in Gal-1 release by both subsets of macrophages (Fig. 4 F). The pattern of Gal-9 expression in human M1 and M2-like macrophages at mRNA (Fig. 4 G), protein (Fig. 4 H) and secreted (Fig. 4 I) levels were similar to that of Gal-1.

## 4. Discussion

Gal-1 and -9 are two members of the galectin family which have been previously reported to be expressed by immune cells [29]. However, potential roles for Gal-1 and -9 in modulating the inflammatory response mediated by macrophages have not been fully explored. In this study, we demonstrated that Gal-1 is up-regulated in murine M2-like macrophages whereas Gal-9 increased in M1-like macrophages. Changes in expression levels of Gal-1 and -9 in M1 vs M2 macrophages held true at both, mRNA and protein level. Interestingly, we also report that the pattern of expression for Gal-1 and -9 differs between mice and humans; stimulation of human monocyte derived macrophages (hMDM) yielded no significant difference in the expression of either Gal-1 or -9.

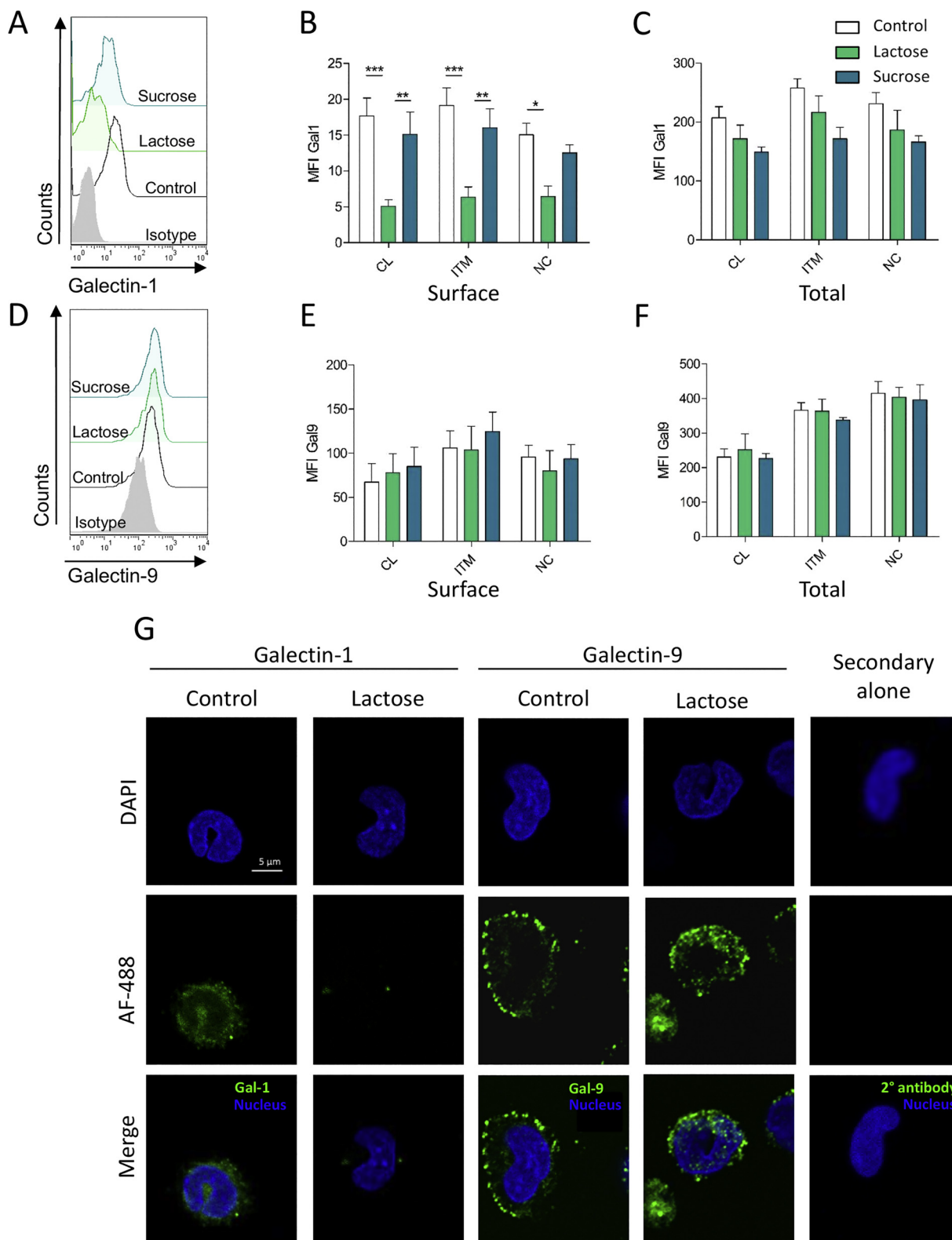
Our findings that Gal-1 is increased in murine pro-resolving, M2, macrophages are in line with current reports which characterise Gal-1 as having broadly pro-resolving properties. It is known that the pro-resolving effects of Gal-1 include the induction of IL-10 production in activated T-cells and promotion of T-cell anergy in the tumour micro-environment [30]. Additionally, Gal-1 has been observed to induce IL-10 production in CD40 stimulated B-cells [31]. It may be possible that the polarisation of macrophages into a M2 phenotype during resolution is a means of initiating anti-inflammatory processes via Gal-1 expression. In contrast however, short-term stimulation of BMDM with LPS



**Fig. 2. Total and surface staining for Gal-1 and -9 on human primary monocyte subsets.** The monocyte subsets were distinguished by using CD14 and CD16 surface markers. Classical (CL), intermediate (ITM) and non-classical (NC) monocytes were characterised as CD14<sup>++</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>-</sup>CD16<sup>++</sup> cells respectively. **A** Representative dot-plots of the gating strategy employed in order to distinguish between the CL, ITM and NC monocyte subsets displaying CD16<sup>+</sup> events along the Y-axis and CD14<sup>+</sup> events along the X-axis. **B, C** Representative histograms of fluorescence intensities of fluorophore-conjugated antibodies against Gal-1 and Gal-9 respectively on mixed monocytes. **D, E** Bar graph of MFIs of total and extracellular Gal-1 and -9 respectively on monocyte subsets (n = 8). **F, G** Representative histograms of fluorescent intensities of Gal-1 and Gal-9 on mixed monocytes of individual donors. **H** Representative images of fluorescently labelled, intracellular Gal-1 and -9 of cultured CD14<sup>+</sup> monocytes imaged using confocal microscopy. Data are expressed as mean ± SEM (n = 8).

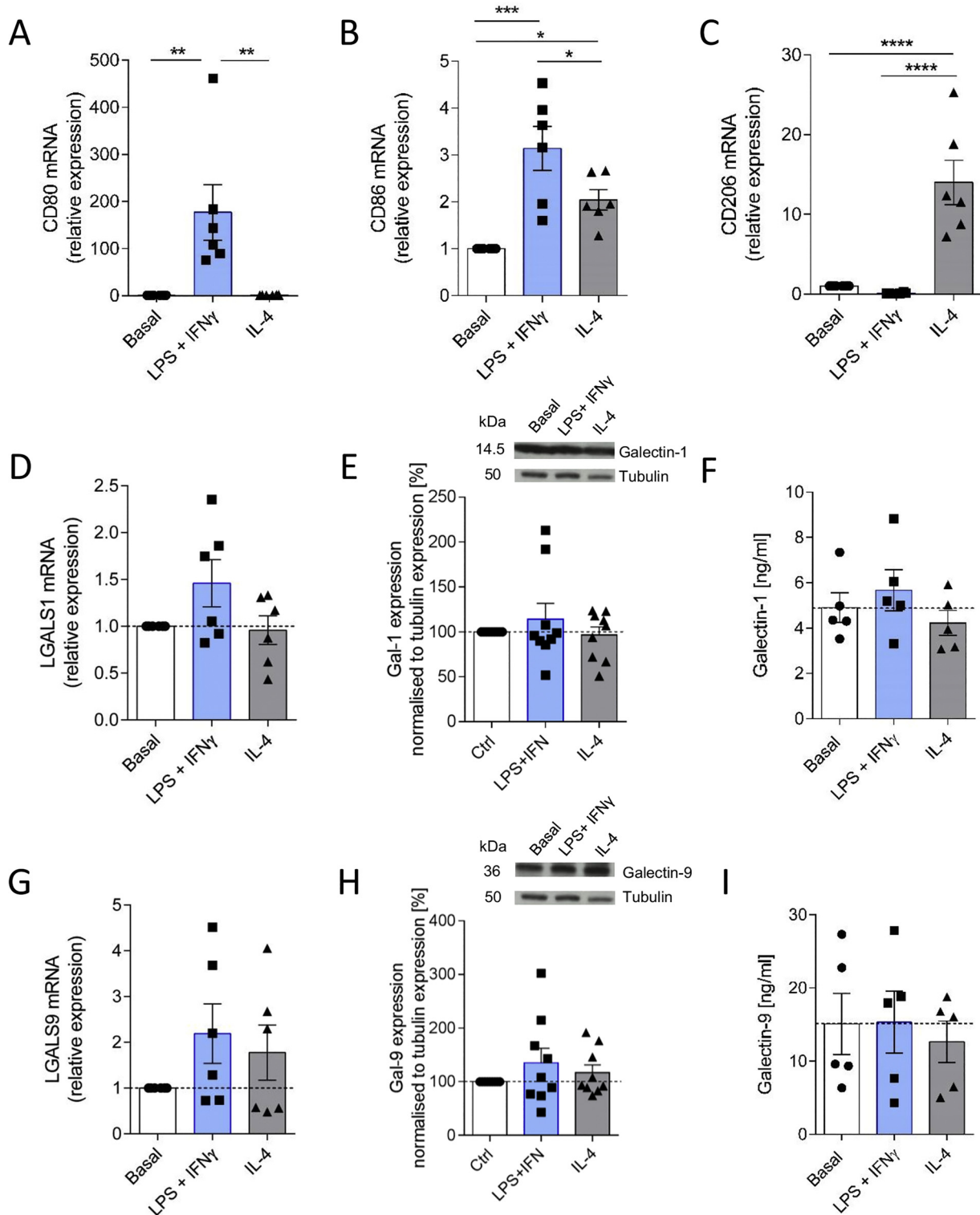
has previously been reported to induce increased expression of Gal-9 in a TIM-3 dependent manner [32]. Similarly, we have shown that Gal-9 expression increased in murine pro-inflammatory, M1, macrophages in responses to pro-inflammatory stimuli (Fig.1 D, E). These findings

corroborate reports whereby addition of exogenous Gal-9 promote inflammation. For example, the injection of exogenous Gal-9 into mouse knee joint has been reported to drive arthritogenicity by the increased infiltration of monocytes into the joint [33]. Mice deficient in



**Fig. 3. Total and surface staining for Gal-1 and -9 on human primary monocytes washed with  $\beta$ -lactose.** **A** Representative histograms of fluorescent intensities of fluorophore-conjugated antibodies against extracellular Gal-1 on mixed monocytes. **B, C** MFI of surface and total Gal-1 on monocyte subsets respectively after elution with lactose and controls. **D** Representative histograms of fluorescent intensities of fluorophore-conjugated antibodies against extracellular Gal-9 on mixed monocytes. **E, F** Bar graphs of MFI of surface and total Gal-9 respectively of untreated monocyte subsets and monocyte subsets washed with lactose or sucrose. **G** Cultured CD14<sup>+</sup> monocytes were washed with lactose and stained with Gal-1 and -9 antibodies before imaging with a confocal microscope. The nuclei were stained with DAPI. Data are expressed as  $\pm$  mean (n = 8). Statistical analysis was conducted using 2-Way ANOVA with Bonferroni post-test. \*: p = 0.05, \*\*: p = 0.01, \*\*\*: p = 0.005.





**Fig. 4. Human macrophages increase Gal-1 and Gal-9 expression after treatment with LPS and IFN $\gamma$ .** Macrophages were derived from primary human CD14<sup>+</sup> monocytes and stimulated with LPS and IFN $\gamma$  or IL-4 over 16 h. A-C Macrophage phenotype markers were detected after the stimulation with LPS and IFN $\gamma$  or IL-4 to confirm successful polarisation. qRT-PCR was used to detect CD80 and CD86 levels to confirm the polarisation towards M1 macrophages. CD206 mRNA levels were investigated to confirm polarisation towards M2 macrophages. D, G LGALS1 and LGALS9 mRNA levels of macrophages were quantified using qRT-PCR after 16 h of treatment with LPS and IFN $\gamma$  or IL-4. E, H Western blot was used to analyse total Gal-1 and -9 levels of macrophages treated with LPS and IFN $\gamma$  or IL-4. F, I Gal-1 and -9 released by stimulated macrophages was detected using ELISA. Data are expressed as mean  $\pm$  SEM (n = 5-9). Statistical analysis was conducted using Kruskal-Wallis test \* p = 0.05 \*\*p = 0.01 \*\*\* p = 0.005 \*\*\*\* p = 0.001.

endogenous Gal-9 have also been shown to have attenuated pathology in models of lung inflammation and addition of exogenous Gal-9 to wildtype mice with endotoxic shock has been reported to increase the

incidence of mortality [34]. However, contrasting findings have been reported on the roles of both Gal-1 and -9 in the context of immune regulation. Both can be described as pro- or anti-inflammatory,

ultimately this depends on the primary cell types, cellular niche or environment. For example, the addition of exogenous Gal-1 to osteoarthritic chondrocytes has been shown to upregulate a pro-inflammatory gene profile [35]. However, the lack of endogenous Gal-1, in Gal-1 deficient mice, was clinically beneficial in models of experimental arthritis [36]. Therefore, further study is required to understand the kinetics of Gal-1 and -9 expression in macrophage subsets during inflammation and resolution and how this may direct the outcomes of pre-clinical models of inflammation.

In this study, we found that unstimulated human monocytes, isolated from healthy whole blood, express both Gal-1 and -9. Despite surface expression of both proteins on all three major monocyte subsets, CL, ITM and NC, each subset also had relatively large intracellular pools of both Gal-1 and -9 (Fig. 2 B–E). This observation was expected, as similar patterns of expression have been previously reported in other cell types, such as endothelial cells and T-cells [37,38]. The importance of surface versus intracellular expression has been well documented as the different cellular localizations of galectins can have different, and sometimes opposing, functions [39]. Galectins are reported to shuttle between the cytoplasm and the nucleus, and specifically Gal-1 has been shown to be involved in pre-mRNA splicing events. The addition of exogenous Gal-9 into cultures of human synovial fibroblasts was shown to have pro-apoptotic effects; in contrast, endogenous Gal-9 within these synovial fibroblasts suppressed apoptosis [40]. In the context of human monocytes specifically, Matsuura et al. showed that intracellular Gal-9 is responsible for the activation of pro-inflammatory genes in THP-1 cells; a human monocyte cell-line [41]. Interestingly, overexpression of intracellular Gal-9 induced the expression of pro-inflammatory genes, whereas, the addition of exogenous Gal-9 had no impact on pro-inflammatory genes [41]. Similarly, Ma et al. showed that the association of intracellular Gal-9 with TIM-3 induced a pro-inflammatory phenotype in the presence of toll-like receptor (TLR) stimulation, in THP-1 monocytes/macrophages. It was observed that intracellular ligation of Gal-9 with TIM-3 prompted the secretion of IL-12/IL-23 in a STAT-3 dependent manner [42]. Further characterisation of intracellular versus surface galectins and their relative functions in polarised primary macrophages still requires thorough investigation. Interestingly, we have seen that the surface of CL and ITM monocytes trend towards an increase in Gal-1 levels when compared to NC monocytes (Fig. 2 D). In contrast, NC and ITM monocytes trend towards a greater enrichment of surface Gal-9 compared to CL monocytes (Fig. 2 E). Further studies are needed in order to determine whether the differential expression of galectins amongst monocyte subsets impacts their function.

We also observed high variations in Gal-1 and -9 expression in human monocytes and macrophages between donors which did not coincide with gender or age (Fig. 2 F, G). Further studies are required to determine the possible causes for these variations, such as lifestyle. Furthermore, culturing hMDMs in autologous serum may also give rise to variations due to differences in M-CSF levels in serum amongst donors [43].

Previous studies, using mast cells for example, have shown that treatment with exogenous Gal-9 can be competitively inhibited with  $\beta$ -lactose [44]. The elution of endogenous galectin from immune cells has not been widely published. Here we showed that surface levels of Gal-1 were bound to monocytes in a glycan dependent manner as the protein was eluted following treatment with  $\beta$ -lactose (Fig. 3 A, B). However, changes in surface Gal-1 following  $\beta$ -lactose elution were diminutive in the context of the total cellular pool of Gal-1 (Fig. 3 C). Symons et al. have previously reported a rapid glycan-dependent dissociation of Gal-1, after approximately 10 h, from lymphocytic CD45 [45]. A possible explanation for presence of glycan bound Gal-1 on the surface of monocytes is its potential role in homeostatic regulation of monocyte physiology. Barrionuevo et al. have demonstrated that the binding of exogenous Gal-1 to monocytes is essential for the regulation of constitutive MHC II and Fc $\gamma$ RI expression [46]. Additionally, sugar

dependent binding of Gal-1 to its ligands has been previously shown to be regulated dynamically [10]. Alterations to the redox state of Gal-1 has been proposed to disable its sugar binding capabilities and the presence of glycan-bound Gal-1 on unstimulated monocytes may represent a mechanism for the rapid release of Gal-1 under inflammatory and oxidative conditions [47]. However, further experiments need to be conducted in order to determine the true origin of the Gal-1 on the surface of the primary monocytes.

Interestingly, we observed that Gal-9 was not eluted from the surface of monocytes in the presence of  $\beta$ -lactose and therefore the protein is presented on the cell surface in a glycan-independent manner (Fig. 3 D, E). Earlier studies have suggested both, glycan-dependent and -independent binding of Gal-9 to its binding partners on the cell surface. PDI, a Gal-9 ligand, has been previously reported to primarily bind the protein in a glycan dependent manner and less so through protein-protein interactions [48]. The dependency of Gal-9-glycan interactions holds true for a number of Gal-9 ligands, such as CD44 and CD45 [49]. However, Chabot et al. demonstrated the release of Gal-9 from Jurkat T-cells was partly dependent on the activity of matrix metalloproteinase and protein kinase C activity despite Gal-9 itself lacking a signalling peptide [50,51]. It has been postulated that the release of Gal-9 is through association with a carrier protein and the inability to elute surface Gal-9 from monocytes under basal conditions in the current study may provide greater evidence to this end. Furthermore, Dai et al. showed that lactose failed to inhibit Gal-9-dependent upregulation of CD83 and other proteins during dendritic cell maturation [52]. TIM-3 has been shown to be released in association with Gal-9 from PMA activated THP-1 cells. Even though these previous studies confirm glycan-independent binding of Gal-9 to cells, further investigation is required to delineate the mechanistic basis of this binding.

To our surprise, no significant differences in expression of either LGALS1 and LGALS9 mRNA or Gal-1 and -9 protein were observed, in hMDM in either LPS + IFN $\gamma$  or IL-4 treated conditions (Fig. 4 D–I). The potential difference between mouse and human galectins has been previously observed in the case of Gal-9. Leitner et al. reported the potential of the Gal-9 receptor, TIM-3, as a therapeutic target in HIV associated T-cell exhaustion. In earlier mouse studies, blockade of TIM-3 was sufficient for restoring exhausted T-cells back to a functional state. However, they reported that exhausted human T-cells did not upregulate TIM-3, as murine T-cells do, and the presence of Gal-9 during human T-cell activation did not induce T-cell exhaustion [53]. Similarly, the data presented here may represent genuine variation between murine and human patterns of expression of various galectin family members and highlights the importance of caution when making such interpretations between species.

We have observed no difference in levels of Gal-9 released from hMDM between treatment groups (Fig. 4 I). However, the current commercially available ELISA kit for Gal-9 has been reported to measure degraded Gal-9 in human serum samples and may explain our observations, especially if the stability of Gal-9 released by differentially stimulated human macrophages may or may not be altered, like Gal-3 [54]. This is especially pertinent since Gal-9 has been shown to have altered angiogenic properties and have either pro-apoptotic or anti-apoptotic effects on T<sub>H</sub>1 cells when at relatively low or high concentrations, respectively [33,55,56].

In summary, our findings support the view that macrophages are important galectin-expressing cells. However, the results of the current study highlight that there are differential patterns of expression of Gal-1 and -9 in mice and human. These differences between species must be taken into consideration when planning experiments, otherwise this may lead to incorrect conclusions being made. We have observed that under basal conditions surface levels of Gal-1 are glycan-dependent while Gal-9 remains glycan independent. However, the surface expression of both Gal-1 and -9, and any changes in them, were in total overshadowed by large intracellular pools of both proteins. Further investigation is required to determine the exact binding and expression

mechanisms of Gal-1 and -9 on monocytes and how macrophage culture conditions and polarisation from either CD14+ or CD14- monocytes influences the relative expression of galectins. Nevertheless, the future of galectins in the context of macrophage biology looks to be 'sweet'.

#### Author contributions

CR, FK, MTH, AJI, DRL, MC, FM performed experiments; CR, FK, MTH, AJI analysed results and made the figures; CR, AJI designed the research. CR, FK, MTH, AJI wrote the paper. All authors provided critical revision of the manuscript.

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#### Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.110595>.

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