


# Wnt7a induces a unique phenotype of monocyte-derived macrophages with lower phagocytic capacity and differential expression of pro- and anti-inflammatory cytokines

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## Introduction

Macrophages exist as a continuum of subtypes, each with its own unique function including phagocytosis, antigen processing and presentation, and release of tissue remodelling factors, homeostatic proteins, and pro- and anti-inflammatory cytokines. Due to their importance in health and disease, understanding the signals that regulate macrophage differentiation and phenotype is paramount to gaining a better understanding of their functions as well as revealing potential therapeutic targets. Circulating blood monocytes differentiate into specific macrophage phenotypes depending on the signals they receive.<sup>1,2</sup> Macrophage colony-stimulating factor (M-CSF) with lipopolysaccharide (LPS) and interferon- $\gamma$  (IFN- $\gamma$ ) induce monocytes to differentiate into M-1 (pro-inflammatory) like-monocyte-derived macrophage (MDMs). M-CSF with the addition of interleukin-4 (IL-4), IL-10, or

## Summary

The variation of macrophage functions suggests the involvement of multiple signalling pathways in fine tuning their differentiation. Macrophages that originate from monocytes in the blood migrate to tissue in response to homeostatic or 'danger' signals and undergo substantial morphological and functional modifications to meet the needs of the dominant signals in the microenvironment. Wnts are secreted glycoproteins that play a significant role in organ and cell differentiation, yet their impact on monocyte differentiation is not clear. In this study, we assessed the role of Wnt1 and Wnt7a on the differentiation of monocytes and the subsequent phenotype and function of monocyte-derived macrophages (MDMs). We show that Wnt7a decreased the expression of CD14, CD11b, CD163 and CD206, whereas Wnt1 had no effect. The Wnt7a effect on CD11b was also observed in the brain and spleen of Wnt7a<sup>-/-</sup> adult brain mouse tissue and in embryonic Wnt7a<sup>-/-</sup> tissue. Wnt7a reduced the phagocytic capacity of M-MDMs, decreased interleukin-10 (IL-10) and IL-12 secretion and increased IL-6 secretion. Collectively, these findings demonstrate that Wnt7a generates an MDM phenotype with both pro-inflammatory and alternative MDM cytokine profiles and reduced phagocytic capacity. As such, Wnt7a can have a significant impact on macrophage responses in health and disease.

**Keywords:** myeloid cell; Wnt; monocyte; macrophage; regulation/suppression.

transforming growth factor- $\beta$  generates an alternative/regulatory-like macrophage phenotype (M2-MDMs). M2a-MDMs, differentiated in the presence of IL-4, exhibit increased expression of scavenger receptors, CD163 and CD206, and have increased phagocytic capacity.<sup>3,4</sup> Other morphogens can skew the differentiation of monocytes, impacting their function in tissue. In particular, emerging data point to the impact of Wnts on monocyte differentiation; specifically, Wnt5a expression is associated with monocyte-derived dendritic cells, and in haematopoietic cells there are more monocyte or pro-monocyte cells in the presence of either Wnt5a or Wnt11, and the appearance of macrophages was inhibited.<sup>5,6</sup>

Wnts are ubiquitously expressed proteins that guide cell fate, development and differentiation *in utero* and a growing body of evidence points to their importance in cell differentiation and function post-development.<sup>7-15</sup> Wnts are a family of 19 small secreted glycoproteins that are

evolutionary conserved. They bind to a seven transmembrane Frizzled receptor and one of various co-receptors – culminating in either a  $\beta$ -catenin-dependent (canonical) or independent (non-canonical: i.e. Planar Cell Polarity or  $\text{Ca}^{2+}$ /Calmodulin) signalling pathway. Historically, Wnts (1, 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b, 10a, 10b, 11 and 16) were categorized on the basis of whether they induce  $\beta$ -catenin-dependent or  $\beta$ -catenin-independent signalling; however, there is an increasing recognition that this classification is no longer accurate. Wnts, depending on interaction with frizzled receptors, can mediate either pathway. For example, Wnt5a, classically, signals through the non-canonical/ $\beta$ -catenin-independent pathway.<sup>16</sup> However, by binding to Frizzled 4 and the LRP5 co-receptor, Wnt5a engages the canonical/ $\beta$ -catenin-dependent pathway.<sup>16</sup> These studies underscore an emerging paradigm of Wnt signalling, whereby the context of Wnt receptors dictates canonical versus non-canonical Wnt pathways.<sup>16,17</sup>

We focused here on evaluating the role of Wnt1 and Wnt7a in regulating monocyte differentiation, both phenotypically and functionally. Both Wnts engage the canonical  $\beta$ -catenin signalling pathway, whereby they bind to Frizzled receptors and LRP5/6 to induce a signalling cascade that inactivates glycogen synthetase kinase-3 $\beta$  to stabilize  $\beta$ -catenin.  $\beta$ -Catenin in turn translocates to the nucleus where it binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to regulate gene expression. Although Wnt1 predominantly induces canonical  $\beta$ -catenin signalling, Wnt7a can also activate the AKT/mammalian target of rapamycin (AKT/mTOR) pathway, albeit in skeletal muscles,<sup>18</sup> and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) via activation of extracellular signal-regulated kinase 5 (ERK5).<sup>19</sup> Wnt7a is expressed by stromal epithelial cells;<sup>20</sup> it is required for the development of the blood–brain barrier<sup>21–23</sup> and has also been observed to induce tumour-suppressive cellular senescence in the lung.<sup>24</sup> Notably, macrophages within tumours are known regulators of tumour progression, as signals from the tumour microenvironment regulate the macrophage phenotype, which in turn regulates the phenotype and function of infiltrating immune cells.<sup>25,26</sup> Therefore, the effect of Wnt7a on lung cancer progression may be through its influence on macrophage phenotype. As such, due to the ability of Wnt7a to activate more than one signalling pathway that potentially influences cell phenotype and function, as well as its effect on blood–brain barrier development and lung cancer pathogenesis, we hypothesize that Wnt7a may be important in the differentiation of monocytes to MDMs in the brain and other tissue. Relative to Wnt7a, the influence of Wnt1 on various cell types and disease processes has been more widely described; where in macrophages, Wnt1 induces CD36 expression<sup>27</sup> and prevents apoptosis in microglia.<sup>28</sup>

We report here that activated monocytes down-regulate Wnt7a expression and that Wnt7a, but not Wnt1, significantly inhibited the expression of CD14, CD11b, CD163 and CD206 on MDMs. The aforementioned proteins are classical markers associated with myeloid cell phenotype and function, specifically; CD14 is a pathogen recognition receptor (PRR) found on human monocytes and macrophages.<sup>29</sup> CD11b is the  $\alpha$  subunit of the integrin heterodimer CR3 or Mac-1; known functions include cellular migration,<sup>30</sup> phagocytosis,<sup>31</sup> and cellular activation.<sup>32</sup> CD11b is primarily expressed on myeloid cells and neutrophils and is essential for their defining functions; hence, assessing the expression of this protein is important when defining monocyte and macrophage subtypes. CD163 and CD206 are scavenger receptors and mannose receptors responsible for clearing low-density lipoproteins and glycoproteins, respectively.<sup>33,34</sup>

*In vitro* observations of the Wnt7a effect on CD11b were also made in an *in vivo* Wnt7a<sup>-/-</sup> mouse model, where absence of Wnt7a resulted in higher CD11b expression relative to the wild-type mice. Wnt7a also inhibited the phagocytic capacity of MDMs, inhibited the secretion of IL-10 from alternative MDM controls, and inhibited the secretion of IL-12 from M1-like MDMs. Conversely, IL-6 secretion was significantly increased relative to all MDMs, including MDMs differentiated in the presence of Wnt1. Overall, these data demonstrate that Wnt7a induces monocytes to differentiate towards a unique phenotype with both M1-like and alternative MDM properties. As such, Wnt7a can potentially be used as a tool to influence the MDM phenotype for therapeutic intervention for example, where anti-inflammatory macrophages dominate and inhibit appropriate immune responses.

## Materials and methods

### Ethics statement

Research involving human subjects was conducted in accordance with institutional (IRBL06080703) and U.S. Government guidelines on human research.

### Animal care

Animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by respective institutional Animal Care and Use (IACCU) committees. Adult animals were housed in the Biologic Resources Laboratory (BRL), UIC under protocol (15-264). Wnt7a<sup>-/-</sup> embryos (e13.5) (Parr and McMahon, 1995) (JAX 004715) were from the Frederick National Laboratory in Frederick, MD, under IACUC protocol (ASP 14-078).

*Wnt7a<sup>-/-</sup> mice*

*Wnt7a<sup>-/-</sup>* mice (Parr and McMahon, 1995) were used as frozen embryos (e13.5) and 11- to 12-week-old adults. *Wnt7a<sup>-/-</sup>* adult mice and their wild-type (WT) littermates are inbred strains on a B6/129S1 genetic background (The Jackson Laboratory, Bar Harbor, ME; Stock number 004715). *Wnt7a<sup>-/-</sup>* mice were bred until congenic in C57BL/6J strain. As the homozygous *Wnt7a<sup>-/-</sup>* mice were sterile, we made use of heterozygotes for colony maintenance. The genetic background of the mice was determined using PCR of DNA from tail biopsies. For embryos; after removing a limb for genotyping, the embryos were briefly fixed in 2% paraformaldehyde in PBS before being embedded in optimal cutting temperature compound (OCT) and snap frozen. Adult mice were killed using CO<sub>2</sub> and perfused with PBS, before removal of brain and spleen. Half of the adult brain was fixed overnight in 2% paraformaldehyde followed by 30% sucrose and fixed in OCT blocks before cryosectioning. Mouse spleens were crushed and incubated with red blood cell lysis buffer (Clontech Laboratories, Mountain View, CA) before cell culture experiments.

*Generation of monocyte-derived macrophages*

Monocyte populations were enriched directly from healthy human whole blood by negative immune-selection (STEMCELL Technologies, Vancouver, BC, Canada). Approximately  $0.5 \times 10^6$  cells/ml were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) for 7 days in the presence of 50 ng/ml M-CSF (R&D Systems, Minneapolis, MN). To obtain M1-like and M2-like MDM cells, 50 ng/ml IFN- $\gamma$  (R&D Systems) and 1  $\mu$ g/ml LPS (Sigma-Aldrich, St Louis, MO, USA) or 10 ng/ml IL-4 (R&D Systems), respectively, were added. Experimental groups were treated with 50 ng/ml M-CSF in addition to 300 ng/ml of either Wnt1 or Wnt7a (Abcam, Cambridge, UK) active full-length human recombinant proteins. Growth factors, cytokines and Wnt proteins were added every other day, whereas IL-4 was added during the last 48 hr of the experiment, IFN- $\gamma$  was added 48 hr before, followed by stimulation with LPS 24 hr before ending the experiment. Mouse MDMs were obtained by culturing splenocytes overnight in complete macrophage medium. Briefly, complete macrophage medium consisted of Dulbecco's modified Eagle's medium with high glucose and pyridoxine hydrochloride (Gibco, Waltham, MA), heat-inactivated fetal calf serum, modified Eagle's medium essential amino acids (Gibco), L-glutamine (Gibco), HEPES buffer (Gibco), penicillin and streptomycin, and 50 mM 2-mercaptoethanol. After overnight activation and adherence of monocytes, suspension cells were removed and adherent cells were washed twice with Dulbecco's PBS (Corning, Manassas, VA) before replacing complete macrophage medium. Cells were cultured for 7 days followed by

overnight incubation with Wnt7a and subsequent flow cytometric analysis.

*Multiplex assay*

Secretion of IL-6, IL-1 $\beta$ , IL-10, IL-12p70 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) from differentiated MDMs were measured with a human cytokine magnetic 5-Plex Panel (EMD Millipore, St Louis, MO) according to the manufacturer's protocol.

*Phagocytosis assay*

Phagocytosis was assessed on 7-day differentiated MDMs using pHrodo *Staphylococcus aureus* bioparticles (Life Technologies, Carlsbad, CA) following the manufacturer's protocol scaled to a 12-well format. Briefly, differentiated macrophages were cultured with pHrodo *S. aureus* bioparticles for 90 min, washed with PBS and fluorescence signal was assessed by microscopy and by flow cytometry to calculate median fluorescence intensity to identify phagocytic cells.

*Flow cytometry*

Differentiated macrophages were assessed by flow cytometry for extracellular markers: lineage marker CD3/CD19/CD20/CD56 (allophycocyanin; Biolegend, San Diego, CA), CD14 (BV786; Biolegend), CD16 (Peridinin chlorophyll protein-Cy7; BD Biosciences, San Jose, CA), CD11b (allophycocyanin-Cy7; BD Biosciences), HLA-DR (Peridinin chlorophyll protein; BD Biosciences), CD163 (PeTexas Red, BD Biosciences) and CD206 (phycoerythrin, FITC; BD Biosciences). Data were collected on the BD Fortessa Flow Cytometer with BD FACS DIVA software (BD Biosciences) and analysed using FLOW JO FLOW Analysis Software (Treestar Inc., Ashland, OR).

*Immunofluorescence*

Mouse embryos and adult brain cryosections (20  $\mu$ m) from *Wnt7a<sup>-/-</sup>* and WT mice were stained with F4/80 (1 : 100, anti-rabbit Cy3; Abcam), CD11b (1 : 100, anti-rat AF647; Abcam), isolectin b4 (1 : 100, AF488; Life Technologies), and DAPI (Molecular Probes, Eugene, OR) and visualized using an LSM 710 or LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

*Quantitative real-time RT-PCR*

Total RNA was extracted using the RNeasy mini prep kit (Qiagen, Hilden, Germany), followed by incubation with DNase (Sigma-Aldrich) and retrotranscription (cDNA qScript; Quantabio, Beverly, MA). Quantitative real-time RT-PCR was performed using Ssofast Evagreen Supermix

with a Low Rox kit (Bio-Rad, Hercules, CA) in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using 7500 software v2.0.1. Melting curve analysis was performed to ensure the amplification of a single product. The change in binding was calculated by relative quantification using the comparative threshold cycle (CT) method, with results reported as fold change relative to control ( $\Delta CT = CT_{\text{Target}} - CT_{\text{control}}$ ;  $\Delta\Delta Ct = \Delta CT_{\text{Target}} - \Delta CT_{\text{experimental control}}$ ; fold change relative to control =  $2^{-\Delta\Delta Ct}$ ). Primers were designed using IDTDNA (San Diego, CA) PRIMERQUEST design software. They are: Wnt1 Forward GAA CGC TCT CTT CCA GTT CTC, Wnt1 Reverse GGA GAG ATG GAT CGC TAT GAA C; Wnt2 Forward 5'-TAC TGT ATC AGG GAC CGA GAG, Wnt2 Reverse GCC TCT CCC ACA ACA CAT AA; Wnt2b Forward 5'-GAT GGA TTT AGG AGG CTG AGT G-3', Wnt2b Reverse GAG GAT GAG CCT TGA CTT AGT G; Wnt3 Forward CTG GAC CAC ATG CAC CTA AA, Wnt3 Reverse CGT ACT TGT CCT TGA GGA AGT C; Wnt3a Forward GGC AGC TGT GAA GTG AAG A, Wnt3a Reverse GGT GTT TCT CTA CCA CCA TCT C; Wnt4 Forward CAG TGG AGA ACT GGA GAA GTG, Wnt4 Reverse TCC ACA AAG GAC TGT GAG AAG; Wnt5a Forward GCT AGA GAA AGG GAA CGA ATC C, Wnt5a Reverse CCA GAC ACT CCA TGA CAC TTA C; Wnt5b Forward CGA GAG CGT GAG AAG AAC TTT, Wnt5b Reverse GGC GAC ATC AGC CAT CTT AT; Wnt6 Forward TCA GTT CCA GTT CCG TTT CC, Wnt6 Reverse GAA AGC TGT CTC TCG GAT GTC; Wnt7a Forward GGT GCG AGC ATC ATC TGT AA, Wnt7a Reverse CAT TTG GGA GCC TTC TCC TAT G; Wnt7b Forward GGC AGT GTG GAT GGA TGT T, Wnt7b Reverse GGG TGT CCT CAA ATA GGG TTA G; Wnt8a Forward GTG TCA TGG CAT CTC AGG AA, Wnt8a Reverse GCG GTC ATA CTT GGC CTT TA; Wnt8b Forward CTA ACC GGG AGA CAG CAT TT, Wnt8b Reverse GAG TCA TCA CAG CCA CAG TT; Wnt9a Forward CAG TAC CAG TTC CGC TTT GA, Wnt9a Reverse GAA GAG ATG GCG TAG AGG AAA G; Wnt9b Forward GAA GCA GTG TGA CCT ACT GAA G, Wnt9b Reverse GCC TGA ACT GGA ACT GAC AT; Wnt10a Forward ACT CCG ACC TGG TCT ACT TT; Wnt10b Forward GAA TGC GGA TCC ACA ACA AC; Wnt11 Forward GGG CTT CAA AGG AAA CTG ATA GG, Wnt11 Reverse ATC CCT GCC CTG ACT TCT T; Wnt16 Forward GTC CTG CTA GCC ATG AAT CTA C, Wnt16 Reverse GGC AGT CCA CAG ACA TTA ACT; GAPDH Forward TGACTTCAACAGCGACACCCACT, GAPDH Reverse ACCACCCTGTTGCTGTAGCCAAAT.

### Statistical analyses

When the data were distributed normally, analysis of variance and post-hoc tests were used. When the data were not normally distributed, non-parametric analysis was performed. All tests assumed a two-sided significance

level of 0.05 using GRAPHPAD INSTAT 3 Software (San Diego, CA) for data analysis.

## Results

### Wnt7a mRNA expression decreases after monocyte activation

We evaluated mRNA expression of the 19 Wnts 24 hr after monocytes were cultured in the presence of M-CSF (Table 1). At 24 hr, both Wnt2 and Wnt7a were significantly decreased, whereas the expression of Wnt1 and Wnt5a was significantly increased in comparison to freshly isolated monocytes. These data suggest that Wnts are differentially expressed upon monocyte activation at the early stages of differentiation.

### Wnt7a inhibits the expression of CD14, CD11b, CD163 and CD206

Isolation of monocytes, flow cytometry gating strategy/culture purity, generation of MDM, M1-MDMs and M2a-MDMs, and the treatment paradigm with Wnt1 or

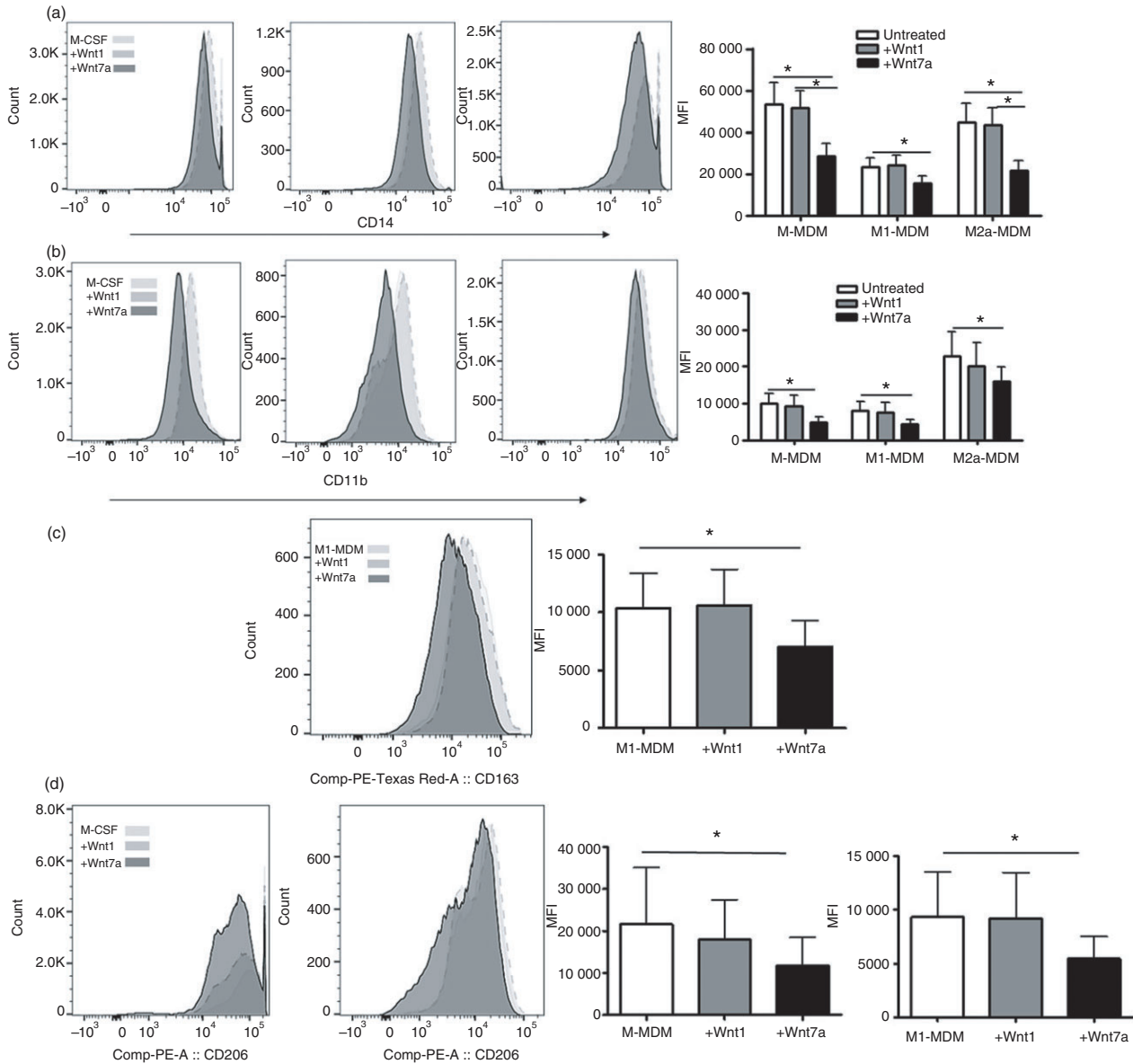
**Table 1.** Differential expression of Wnts mRNAs after monocyte activation.

Wnt	Fold change rel. to T = 0 hr
Wnt1	49.035*
Wnt2	0.194*
Wnt2b	2.757
Wnt3	7.117
Wnt3a	22.651
Wnt4	5.267
Wnt5a	56.482*
Wnt5b	18.389
Wnt6	1.560
Wnt7a	0.118*
Wnt7b	8.641
Wnt8a	17.467
Wnt8b	35.426
Wnt9a	2.552
Wnt9b	19.885
Wnt10a	2.716
Wnt10b	5.179
Wnt11	4.366
Wnt16	2.516

mRNA expression of 19 Wnts measured from freshly isolated whole blood human monocytes and at 24 hr post M-CSF stimulation. RNA was measured by RT-qPCR using 19 custom designed Wnt primer pairs.  $C_t$  values were normalized to the  $C_t$  for GAPDH, followed by calculations of  $\Delta C_t$  and  $\Delta\Delta C_t$  and fold change calculated relative to  $T = 0$ . Data are representative of three donors

\* $P < 0.05$ .





**Figure 1.** Wnt7a reduces CD14, CD11b, CD163, and CD206 expression on monocyte-derived macrophages *in vitro*. Representative histogram for median fluorescence intensity (MFI), and cumulative data of CD14 (a), CD11b (b), CD163 (c), and CD206 (d) expression on human M-MDM, M1-MDM, M2a-MDM with or without Wnt1 and Wnt7a treatments as described in the Supplementary material (Fig. S1) ( $n = 6$  or  $n = 7$  using four different donors,  $*P < 0.05$  in comparison with monocyte-derived macrophages (MDMs) or between M1-MDM versus M1-MDM+7a, as indicated).

Wnt7a are described in the Supplementary material (Fig. S1). MDM, M1-MDMs or M2a-MDMs were treated with either Wnt1 or Wnt7a and expression of CD14, CD11b, CD163 and CD206 was evaluated by flow cytometry. CD14 and CD11b expression distinguish macrophages from other cell types. These proteins are involved in response to microbial products, adhesion and migration, and are therefore important for macrophage function.<sup>35,36</sup> CD163 and CD206 are scavenger and mannose receptors, respectively, often associated with alternative

macrophage phenotypes and phagocytic function.<sup>37</sup> Wnt7a significantly reduced CD14 expression on M-MDM, M1-MDM and M2a-MDM in comparison with untreated cultures and in comparison with Wnt1-treated cultures (Fig. 1a). Wnt7a also significantly inhibited the expression of CD11b on M-MDM, M1-MDM and M2a-MDM (Fig. 1b) and reduced CD163 expression on M1-MDM ( $P < 0.05$ ), but not on M-MDMs or M2a-MDMs (Fig. 1c). Wnt7a reduced CD206 expression on both M-MDM and M1-MDM cells ( $P < 0.05$ ) but not on M2a-

MDMs (Fig. 1d). The M2a-MDM control expressed higher levels of CD163 and CD206 relative to the M-MDM and M1-MDM controls and Wnt7a addition to M2a-MDMs did not decrease CD163 or CD206 expression (data not shown). Wnt5a, which was enhanced at the transcriptional level after monocyte activation (Table 1), did not show significant effects on these surface markers and as such was excluded from further analysis (data not shown). These data demonstrate that Wnt7a inhibits the expression of classical markers of macrophage differentiation and polarization in MDMs differentiated *in vitro*.

### Wnt7a<sup>-/-</sup> mice express higher levels of CD11b

To assess whether these *in vitro* observations translate to *in vivo* conditions, we assessed CD11b and F4/80 (a mouse macrophage marker) expression on Wnt7a<sup>-/-</sup> e13.5 mouse embryos and 11- to 12-week-old mice by immunofluorescence. Wnt7a<sup>-/-</sup> embryos had higher levels of F4/80 and CD11b expression than WT controls (Fig. 2a). To assess whether the difference observed in embryos also occurs in adult Wnt7a<sup>-/-</sup> mice, we obtained whole brain tissue from WT and Wnt7a<sup>-/-</sup> mice for immunofluorescence and flow cytometry analysis of the macrophage population. Adult brain tissue from Wnt7a<sup>-/-</sup> mice had a significantly higher frequency of CD11b<sup>+</sup> staining relative to WT controls (Fig. 2b) as well as significantly higher median fluorescence intensity (Fig. 2c). Finally, to determine if exposure of macrophages from Wnt7a<sup>-/-</sup> mice to recombinant Wnt7a would further reduce CD11b expression, we differentiated monocytes from mouse splenocytes into macrophages in macrophage-conditioned media then incubated the differentiated cells for 48 hr with Wnt7a protein. Macrophages differentiated from Wnt7a<sup>-/-</sup> animals showed significant reduction in CD11b expression after Wnt7a treatment (Fig. 2d). These data demonstrate that Wnt7a down-regulates CD11b expression on monocytes both *in vitro* and *in vivo*.

### Wnt7a inhibits phagocytosis

Given that Wnt7a reduced the expression of two molecules associated with macrophage phagocytic capability, CD11b and CD206, we assessed the impact of Wnt7a on

MDM phagocytic function. MDMs, after *in vitro* differentiation, were incubated with pHrodo *S. aureus* bioparticles for 90 min. M-MDMs differentiated in the presence of Wnt7a showed a significantly reduced ability to take up foreign particles (Fig. 3a,b). Wnt1 or Wnt7a had no significant effect on the phagocytic capacity of M1-MDMs or M2a-MDMs. These data demonstrate that Wnt7a reduces the phagocytic potential of M-MDM.

### Wnt7a influences the cytokine profile of MDMs

To assess the impact of Wnt7a on cytokine secretion of MDMs, we collected cell culture supernatant of fully differentiated cells for analysis with a human cytokine magnetic 5-Plex Panel for the Luminex<sup>TM</sup> for IL-1 $\beta$ , TNF- $\alpha$ , IL-10, IL-6 and IL-12p70. In the presence of Wnt7a, there was a decrease in IL-10 relative to the M-MDM and M2a-MDM controls ( $p < 0.05$ ), but not relative to the M1-MDM control (Fig. 4a). There was also a reduction in IL-12p70 in the presence of Wnt7a, but not Wnt1, in the M1-MDM control, and IL-12p70 was undetectable in M-MDM and M2a-MDMs (Fig. 4b). Interleukin-6 secretion was significantly increased in the presence of Wnt7a relative to all MDM controls (Fig. 4c). Neither IL-1 $\beta$  nor TNF- $\alpha$  levels were significantly altered by Wnt7a (Fig. 4d, e). These data demonstrate that Wnt7a influences cytokine secretion from MDMs, particularly in a manner that does not promote classical polarization of cytokine responses associated with M1 or M2 phenotypes.

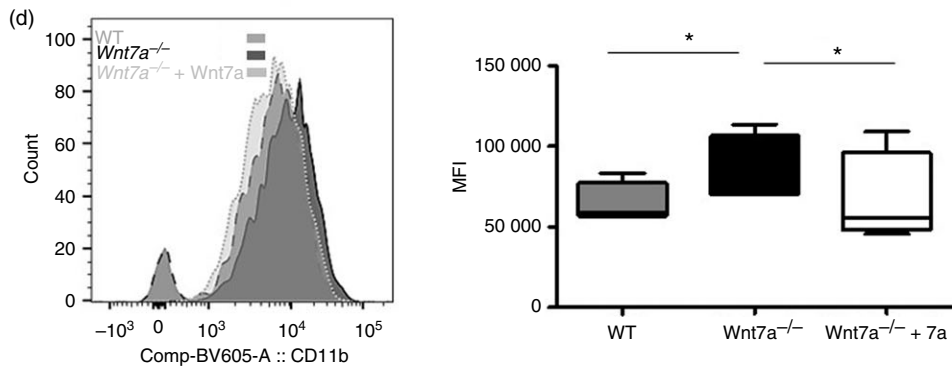
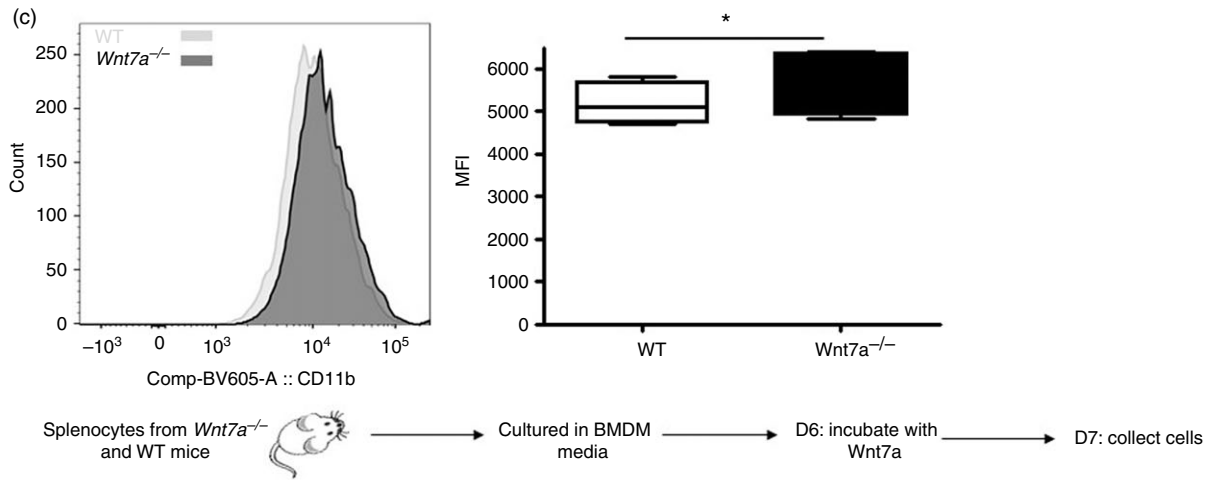
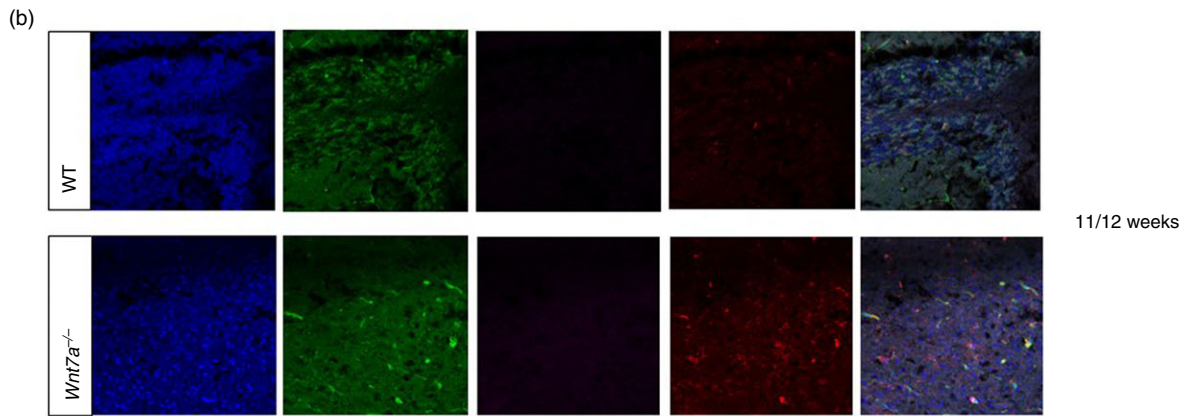
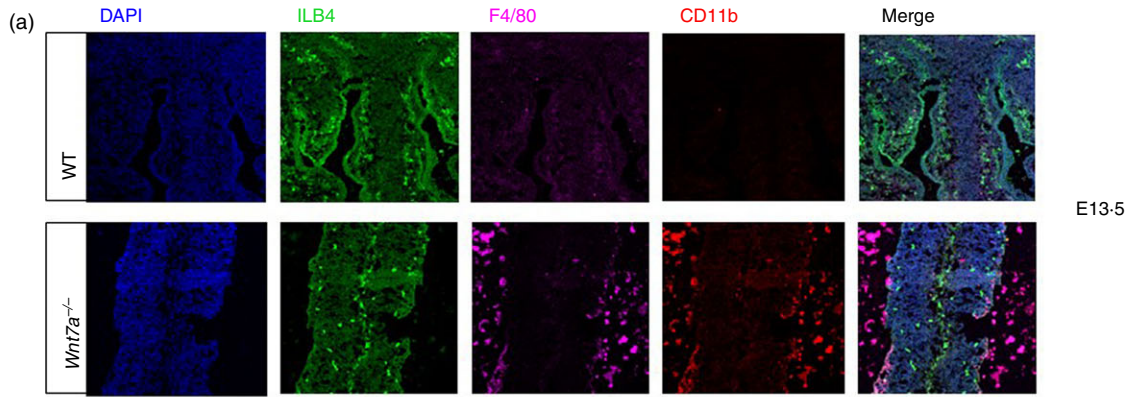
### Discussion

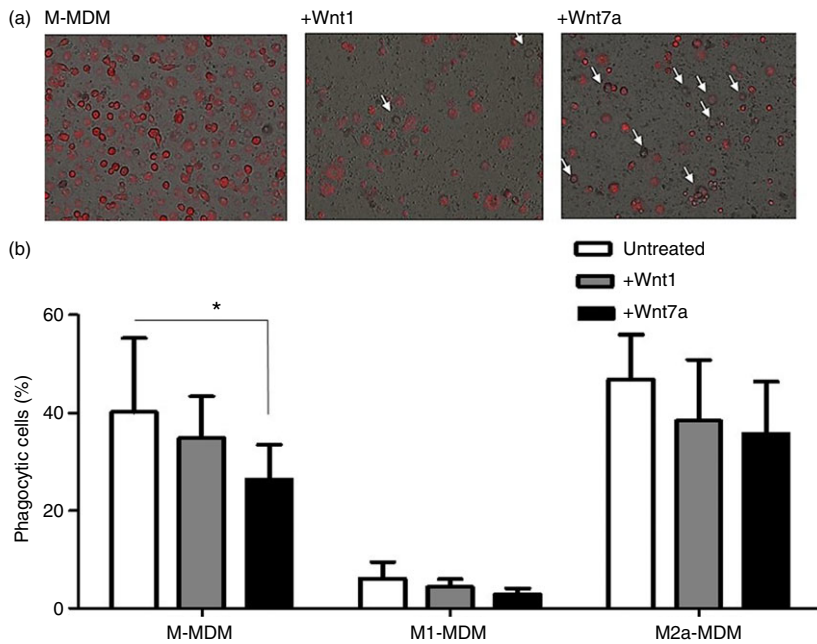
Monocyte-derived macrophages are exceptionally plastic, a trait that allows them to alter their function to meet the requirements of their microenvironment. Therefore, it is important to define physiologically relevant factors that fine-tune MDM phenotype and function, including their rate of development, the proteins they secrete, and the receptors they express. While skewing of monocytes to M-1 or M2a-MDM phenotypes is well characterized in response to classical stimulation (e.g. M-CSF/LPS/IFN- $\gamma$  or M-CSF+IL-4/IL-10/or transforming growth factor  $\beta$ , respectively), little is known about the role of Wnts in skewing monocyte differentiation.

To date, the role of Wnt3a and Wnt5a has been evaluated in the context of macrophage functions. Wnt5a

**Figure 2.** Wnt7a<sup>-/-</sup> mice have a higher expression of CD11b. Anti-CD11b and mouse F4/80 antibodies were used to stain for mouse macrophages/microglia in E13.5 mouse embryo telencephalon (a) and in 11- to 12-week-old mouse brain cortex (b) for both wild-type (WT) and Wnt7a<sup>-/-</sup> mice. Green: Isolectin B4, blue: DAPI, red: CD11b, magenta: F4/80. (c) Flow cytometric analysis of CD11b expression on macrophages/microglia isolated from the brain of Wnt7a WT and Wnt7a<sup>-/-</sup> mice represented as median fluorescence intensity (MFI). Data are representative of four mice in each group,  $P < 0.05$ . (d) Splenocytes isolated from Wnt7a<sup>-/-</sup> and WT mice were used to culture macrophages as indicated in the experimental paradigm then cells were stained for CD11b expression ( $n = 4$  for each experimental group; \* $P < 0.05$ ; WT versus Wnt7a<sup>-/-</sup> and Wnt7a<sup>-/-</sup> versus Wnt7a<sup>-/-</sup> +Wnt7a).

Effect of Wnts on monocyte differentiation





**Figure 3.** Wnt7a reduces phagocytic ability of monocyte-derived macrophages (MDMs). (a) Phagocytic potential of human M-MDM with or without Wnt1 or Wnt7a treatment was assessed using immune fluorescence (a) of pHrodo® Red *Staphylococcus aureus* Bioparticles®, white arrows indicate non-phagocytic cells ( $n = 3$ ) and by flow cytometry (b) for PE (excitation 585 nm) MFI of experimental group in comparison with control MDMs ( $n = 3$ ,  $*P < 0.05$  for M-MDM versus M-MDM + Wnt7a).

through CamMK11 signalling induced inflammatory cytokines of macrophages<sup>38</sup> and it was also reported to induce immunosuppressive macrophages.<sup>39</sup> These rather dichotomous reports may be context dependent. Further, Wnt5a was reported to stimulate phagocytosis, although this was based on mouse macrophage studies rather than human macrophages.<sup>40</sup> Wnt3a also induced pro-inflammatory cytokines by mouse macrophages, but led to sensitization of macrophage responses to subsequent Toll-like receptor 2 and 9 stimulation.<sup>41</sup> We show here that Wnt7a induces monocytes to differentiate towards a unique MDM phenotype with both M1-like and alternative-like MDM properties. As such, levels of Wnt7a in various tissues can significantly alter monocyte differentiation, regulating the phenotype and function of MDMs.

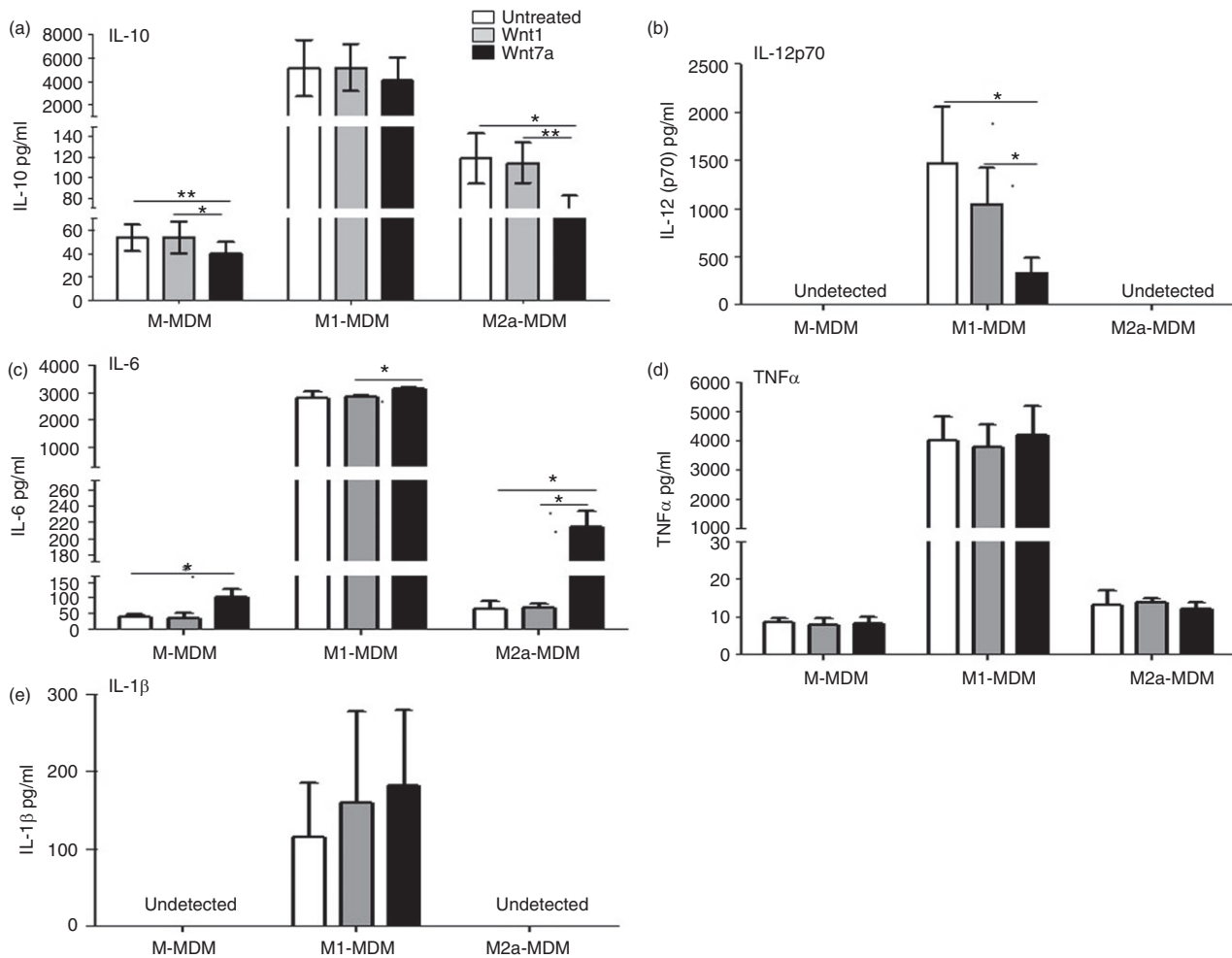
Specifically, we show that activated monocytes down-regulate Wnt7a expression and that Wnt7a but not Wnt1 significantly inhibited the expression of CD14, CD11b, CD163 and CD206 on MDMs. For CD11b, this inhibition was also observed *in vivo* in a Wnt7a<sup>-/-</sup> mouse model. Wnt7a also inhibited phagocytic capacity of MDMs, which is probably due to Wnt7a-mediated down-regulation of CD206 and CD11b, two molecules associated with phagocytosis.<sup>37,42</sup>

Wnt7a inhibited the secretion of IL-10 from alternative MDM controls and inhibited the secretion of IL-12 from M1-like MDM controls. Conversely, IL-6 secretion was significantly increased relative to all MDM controls as well as MDMs differentiated in the presence of Wnt1. IL-6 has been shown to skew MDMs toward an alternative phenotype;<sup>43,44</sup> however, IL-6 is notoriously pleiotropic and needs to be assessed in combination with other factors to determine its role in each context. In this case, cells primed with IFN- $\gamma$  and stimulated with LPS secreted

significantly higher amounts of IL-6 than cells that were differentiated in just M-CSF or M-CSF with IL-4. The cells that secreted more IL-6 also down-regulated CD206 and CD163 expression and had significantly higher expression of IL-12p70, TNF- $\alpha$  and IL-1 $\beta$  relative to the M-CSF and alternative controls. The significant decrease in IL-12p70 expression as well as the limited effect on IL-1 $\beta$  and TNF- $\alpha$  suggests that the Wnt7a MDMs may not be completely pro-inflammatory. Further, Wnt7a-mediated down-regulation of IL-12p70 and IL-10 simultaneously underscores the heterogeneity of MDMs *in vivo* to display both pro- and anti-inflammatory characteristics simultaneously.<sup>45,46</sup>

The finding that Wnt7a skews monocyte differentiation is particularly relevant to HIV invasion in the central nervous system. HIV invades the central nervous system within weeks of infection through both trafficking of T cells and also monocytes that harbour HIV DNA, yet are not productively infected unless they differentiate into macrophages.<sup>47</sup> Studies from our laboratory demonstrated that robust  $\beta$ -catenin is expressed in monocytes and blocks active HIV transcription, yet as these monocytes differentiate into macrophages they support productive infection. As such, Wnt7a secreted by endothelial cells and neurons<sup>48-51</sup> can potentially regulate monocyte differentiation in the brain, as the data suggest, in a way that may inhibit their differentiation, and subsequent susceptibility to HIV infection. Further, Wnt7a-responsive macrophages are likely to be less phagocytic and as such may not contribute significantly to controlling neuroinflammation by way of clearing debris and dying cells that can perpetuate inflammation. However, because microglia and astrocytes have both demonstrated phagocytic capacity,<sup>52-54</sup> it remains to be seen whether Wnt7a-mediated





**Figure 4.** Wnt7a regulates cytokine expression in monocyte-derived macrophages (MDMs). Interleukin-10 (IL-10) (a), IL-12p70 (b), IL-1 $\beta$  (c), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (d) and IL-6 (e) secreted protein levels of untreated human MDMs, and Wnt1- or Wnt7a-treated MDMs was measured by Luminex assay ( $n = 4$ , \* $P < 0.05$ , \*\* $P < 0.01$ ).

reduction in phagocytic capacity has a significant impact on neuroinflammation. These findings underscore the complexity of monocyte/macrophage differentiation in response to a variety of morphogens and highlight the complexity of these responses as context dependent and not necessarily as just M-1 and M-2 like macrophages. Indeed, Wnt7a, and similar physiological signals, may serve to regulate inflammatory responses, resulting in reduced cell damage that can occur from chronic inflammation. Simultaneous reduction in IL-10 also suggests that Wnt7a-MDM may not inhibit the response of other immune cells, but rather serves as a regulator that modulates excessive inflammatory or anti-inflammatory responses. An intermediate MDM phenotype may be favourable in disease and homeostasis; in neuroAIDS inflammatory M1 macrophages perpetuate neuroinflammation,<sup>55</sup> whereas alternative macrophage phenotypes are a marker of a worsened prognosis due to the inability of macrophages to promote anti-viral CD8<sup>+</sup> T-cell

responses.<sup>56</sup> Additionally, macrophages inhibit would healing,<sup>57,58</sup> while anti-inflammatory macrophages are notorious allies in tumour development through their ability to inhibit anti-tumour responses from other immune cells.<sup>59</sup> Future experiments should assess the relevance of Wnt7a on MDM function in neuroAIDS by gain and loss of function studies using *in vitro* and *in vivo* Wnt7a knockout and over-expression models. Ultimately, understanding how monocyte/macrophages respond to signals in their microenvironment, particularly from proteins such as Wnts that demonstrated ubiquitous expression and ability to influence cell fate and function, can serve as novel approaches to regulate immune responses in particular diseases and/or tissues.

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## Disclosure

The authors declare no competing interests.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Gating strategy for monocytes, generation of monocyte-derived macrophages and treatment paradigm.