THE FATE AND LIFESPAN OF HUMAN MONOCYTE SUBSETS IN STEADY STATE AND SYSTEMIC INFLAMMATION

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ABSTRACT

In humans the monocyte pool comprises three subsets, classical, intermediate and non-classical, that circulate in dynamic equilibrium. The kinetics underlying their generation, differentiation and disappearance are critical to understanding both steady-state homeostasis and inflammatory responses. Here, using human *in vivo* deuterium-labelling we demonstrate that classical monocytes emerge first from marrow, after a post-mitotic interval of 1.6 days, and circulate for a day. Subsequent labelling of intermediate and non-classical monocytes is consistent with a model of sequential transition. Intermediate and non-classical monocytes have longer circulating lifespans, ~4 and ~7 days respectively. In a human experimental endotoxemia model a transient but profound monocytopenia was observed; restoration of circulating monocytes was achieved by the early release of classical monocytes from bone marrow. The sequence of repopulation recapitulated the order of maturation in healthy homeostasis. This developmental relationship between monocyte subsets was verified by fate mapping grafted human classical monocytes into humanized mice, which were able to differentiate sequentially into intermediate and non-classical cells.

SHORT TITLE: Human monocyte kinetics

SUMMARY: Using stable isotope labelling, Patel *et al.,* establish the lifespan of all three human monocyte subsets that circulate in dynamic equilibrium; in steady-state, classical monocytes are short-lived precursors with the potential to become intermediate and non-classical monocytes. They highlight that systemic inflammation induces an emergency release of classical monocytes into the circulation.

INTRODUCTION

The mononuclear phagocyte system (MPS) comprises three types of cell: monocytes, macrophages and dendritic cells (DC), as well as their committed bone marrow progenitors (van Furth et al., 1972; Yona and Gordon, 2015). Collectively the cells of the MPS play key functions in maintaining tissue homeostasis during steady state as well as orchestrating the genesis and resolution of the immune response (Davies et al., 2013; Ginhoux and Jung, 2014; Wynn et al., 2013).

It is now recognised that the majority tissue macrophage populations are seeded prior to birth (Ginhoux et al., 2010; Guilliams et al., 2013; Hashimoto et al., 2013; Mass et al., 2016; Schulz et al., 2012; Yona et al., 2013) and maintained via self-proliferation throughout adulthood with minimal monocyte input (Soucie et al., 2016). Conversely, DC and monocytes arise from distinct adult haematopoietic stem cell precursors in the bone marrow (Breton et al., 2015; Fogg et al., 2006; Hettinger et al., 2013; Lee et al., 2015; Liu et al., 2009; Naik et al., 2007; Onai et al., 2013; Onai et al., 2007).

Circulating monocytes represent a versatile and dynamic cell population, composed of multiple subsets which differ in phenotype, size, morphology, transcriptional profiles and are defined by their location in the blood (Cros et al., 2010; Geissmann et al., 2003; Ingersoll et al., 2010; Mildner et al., 2013a; Wong et al., 2011). These discrete monocyte subsets can be distinguished by the expression of CD14 and CD16 in humans and Ly6C, CCR2 and CX_3CR1 in mice (Ziegler-Heitbrock et al., 2010). In humans, $CD14^+$ $CD16^-$ (Classical) monocytes make up ~85% of the circulating monocyte pool, while the remaining ~15% consist of $CD14^+$ $CD16^+$ (Intermediate) and $CD14^{lo}$ $CD16^+$ (Non-classical) monocytes (Passlick et al., 1989; Wong et al., 2011). Similarly in mice, two populations of monocytes have been described: $Ly6C^{hi}$ $CCR2^+$ CX_3CR1^{int} and $Ly6C^{lo}$ $CCR2^-$ CX_3CR1^{hi}, representing classical and non-classical monocytes, respectively (Geissmann et al., 2003). Monocyte egression from the bone marrow requires expression of the chemokine receptor CCR2, which is restricted to classical monocytes (Shi and Pamer, 2011).

Classical monocytes are rapidly recruited to sites of infection (Liao et al., 2017; Serbina and Pamer, 2006) and injury (Nahrendorf et al., 2007; Zigmond et al., 2014), where they exhibit

considerable functional plastically (Arnold et al., 2007; Avraham-Davidi et al., 2013). Interestingly, classical monocytes replenish resident peripheral monocyte-derived cells under steady state conditions (Bain et al., 2014; Guilliams et al., 2014; Tamoutounour et al., 2013; Varol et al., 2009). Non-classical monocytes have been proposed to act as custodians of vasculature by patrolling endothelial cell integrity, in a LFA-1 dependent fashion (Auffray et al., 2007).

During steady state, rodent blood monocyte subsets represent stages of a developmental sequence; classical monocytes have been shown to convert into non-classical monocytes over time (Sunderkotter et al., 2004; Thomas et al., 2016; Varol et al., 2007; Yona et al., 2013; Yrlid et al., 2006). However, it remains to be shown what, if any, relationship exists between the three principal human monocyte subsets and how long these of these subsets reside in the circulation.

Although the vast majority of information concerning mononuclear phagocyte ontogeny, function and kinetics is derived from mouse studies, due to the challenging nature of performing studies in a clinical setting, some important insights into human monocyte biology have been gained from studying pathological states. Patients with a GATA2 mutation (encoding the GATA binding protein 2), have an absence of all blood monocytes; despite this, their resident dermal and lung macrophages remain unaffected, suggesting that the development of these populations is independent of blood monocytes (Bigley et al., 2011). Interestingly, patients with rheumatoid arthritis exhibit an increase in circulating intermediate monocytes (Cooper et al., 2012). Furthermore, stroke patients have been reported to increase their intermediate monocytes two days following their initial insult and this increase inversely correlated with mortality (Urra et al., 2009). These data raise the questions of whether and how circulating human monocyte subsets are related, how long each population circulates, and what impact inflammation has on this process.

Fifty years ago van Furth and Cohn performed a series of elegant studies examining monocyte dynamics in rodents with ^{3}H-thymidine. They concluded that monocytes transit from the bone marrow to the blood, where they circulate for around 22 hours (van Furth and Cohn, 1968). More recently, studies in mice demonstrated that classical monocytes

circulate for less than a day before converting into non-classical monocytes that circulate for around 5 days (Gamrekelashvili et al., 2016; Sunderkotter et al., 2004; Varol et al., 2007; Yona et al., 2013). Nevertheless, the fate and kinetics of human monocyte subsets under steady state and inflammation remains to be resolved. A major breakthrough in examining *in vivo* human leukocyte kinetics came with the advent of non-toxic stable isotope labeling approaches (Busch et al., 2007; Macallan et al., 1998). Specifically, the deuterium from deuterium-labeled glucose or heavy water incorporates stably into the backbone of DNA of dividing cells. The deuterium-glucose labeling approach is particularly suited to the study of rapidly-dividing cells and has been applied in humans to study the turnover of T cell populations such as regulatory T cells (Vukmanovic-Stejic et al., 2006), to memory T cell subsets in HIV infection (Zhang et al., 2013), and, more recently, to cells of the innate immune system, such as neutrophils (Lahoz-Beneytez et al., 2016).

Here we report a series of studies investigating the development and kinetics of human monocyte subpopulations. We hypothesized that the fate and kinetics of the three monocyte subsets, classical, intermediate and non-classical, were intimately linked and could be defined in kinetic terms. We first investigated the steady state kinetics in healthy human volunteers using *in vivo* deuterium-labelled glucose as a precursor. We then repeated these studies in the context of endotoxin-induced systemic inflammation, where we observed a transient depletion of almost the entire circulating monocyte pool; in this way we were able to study the early repopulation of an “empty” blood compartment. Finally we tested our sequential development hypothesis in the humanized MISTRG mouse (Rongvaux et al., 2014) in order to build a comprehensive picture of how human monocyte subsets are regulated in steady state and systemic inflammation.

RESULTS AND DISCUSSION

Characterisation of human monocyte subset kinetics under steady state

The literature has not always clearly distinguished between monocyte subsets, making interpretation confusing, we chose to follow a systematic strategy to identify the three conventional monocyte subsets of interest. Lin^- (CD3, CD19, CD20, CD56 and CD66b) $HLA\text{-}DR^+$ cells were separated into: (i) $CD14^+CD16^-$ classical monocytes, (ii) $CD14^+CD16^+$ intermediate monocytes, and (iii) $CD14^{lo}CD16^+$ non-classical monocytes (**Figure 1a**)(Ziegler-Heitbrock et al., 2010). In addition to CD14 and CD16 expression, we confirmed additional membrane marker expression between monocyte subsets (Ingersoll et al., 2010) (**Figure 1b and Supplementary Figure1a**)**.** Interestingly, these data demonstrate the discrete nature of monocyte subsets is a continuum of more than just CD14 and CD16 expression.

In order to investigate monocyte kinetics under normal physiological homeostatic conditions, we administered a short-pulse (3 hours) of deuterium-labelled glucose (6,6-$^2H_2$-glucose) to healthy human volunteers and analysed flow-sorted monocyte subsets at sequential time-points thereafter for deuterium incorporation (**Figure 1c**) (Macallan et al., 2009; Westera et al., 2013). Analysis of deuterium labelling data revealed that monocyte subsets exhibited a highly consistent pattern in all volunteers studied. Significantly, there was no deuterium labelling for the first 24 hours after administration, consistent with a post-mitotic "*maturation*" phase preceding release from bone marrow into the circulation. We then observed early integration of deuterium in classical monocytes, reaching a peak 3 days post-labelling (**Figure 1d**). At these early time points, intermediate monocytes were also labelled with deuterium but at a much lower level than classical monocytes. No label was observed in non-classical monocytes until day 7. This pattern of sequential appearance of labelling in human monocyte subsets is reminiscent of previous studies in experimental models in rodents, where classical monocytes convert into non-classical monocytes over time (Gamrekelashvili et al., 2016; Sunderkotter et al., 2004; Varol et al., 2007; Yona et al., 2013).

This chronological acquisition of deuterium by circulating monocyte subsets is most likely to be explained by a sequential ontogeny scenario in which deuterium is incorporated into

precursors that differentiate into classical monocytes in bone marrow; these classical monocytes are released into the circulation where they undergo one of two fates; they either differentiate into intermediate monocytes or disappear by death or migration. Similarly intermediate monocytes either leave the blood (by death or migration) or differentiate into non-classical monocytes. The likelihood of each onward differentiation step (classical to intermediate and then intermediate to non-classical) was denoted by the rate αr for each subpopulation, resulting in a corresponding rate of loss from the circulating pool (by death or migration) of (1-α)r. This model is summarized in **Figure 1e.** The alternative parallel ontogeny scenario was also considered; in this model the three subsets arise from separate linages, each with its own distinct post-mitotic kinetics. This model could certainly be made to fit the data mathematically, as it has so many free parameters, but was deemed unlikely on biological grounds. Firstly, it predicts the presence of intermediate and non-classical monocytes in the bone marrow, contrary to our observations where only classical monocytes were detected following bone marrow biopsy (**Figure 1f**), (blood monocyte contamination could be detected in bone marrow aspirate) and secondly, because it would be inconsistent with information from studies in rodents (Gamrekelashvili et al., 2016; Sunderkotter et al., 2004; Varol et al., 2007; Yona et al., 2013; Yrlid et al., 2006). In the sequential model used here (**Figure 1e**) proliferation is restricted to the bone marrow; we excluded models in which circulating subsets proliferate in the blood on the basis of (i) the absence of any deuterium enrichment in such cells 24 hours post-labeling (**Figure 1d**) and (ii) the absence of markers of cell cycling (**Supplementary Figure 1b**).

Results from fitting the model to the experimental data are shown in **Table 1** and **Supplementary Figure 2**. We found that classical monocytes have a very short circulating lifespan (mean 1.0 ± 0.26 days). Most cells leave the circulation or die, while the remaining cells transition to intermediate monocytes. Intermediate monocytes have a longer lifespan (mean 4.3 ± 0.36 days) and all transition to non-classical monocytes. Non-classical monocytes in turn have the longest lifespan in blood (mean 7.4 ± 0.53 days), before either leaving the circulation or dying, as summarized graphically in **Figure 1g**.

Other studies have found evidence for a delay between intermediate monocytes and non-classical monocytes (Tak et al., 2016). We therefore investigated the consequences of including such a delay (Δ_3) in our model. The goodness of the fits (ssr) were very similar with

or without a delay. However, in three of four subjects, the model without Δ_3 outperformed the model with Δ_3 in terms of the corrected Akaike Information Criterion (AIC) (**Supplementary Table 1**). The estimates of monocyte lifetime were very similar for models with or without Δ_3 (**Supplementary Table 2**).

Our data are consistent with earlier murine studies, which provided evidence that the lifespan of each monocyte subpopulation varies, classical Ly6C^{hi} monocytes having shorter circulating half-lives (20 hours) compared to non-classical Ly6C^{lo} monocytes (5 days) (Yona et al., 2013). The difference in circulating half-life between monocyte subsets is likely to correlate with their functional attributes. Classical monocytes replenish the large resident macrophage pool of the gut (Bain et al., 2014), skin (Tamoutounour et al., 2013) and are poised to migrate to sites of inflammation where they display a pro- or anti-inflammatory phenotype depending on microenvironmental cues (Mildner et al., 2013b). More recently, these cells have been shown to enter tissues under steady-state and transport antigen to lymph nodes without differentiating (Jakubzick et al., 2013). Less is known regarding the fate of non-classical monocytes, but it is well documented that mouse and human non-classical monocytes patrol the endothelium (Auffray et al., 2007; Cros et al., 2010) and represent a more terminally-differentiated blood resident monocyte-derived cell.

Human endotoxemia provokes the early release of bone marrow monocytes

We next investigated the response of monocytes to major systemic inflammation using the human experimental endotoxemia model (**Figure 2a**) (Fullerton et al., 2016). A single injection of endotoxin induced a profound acute monocytopenia, following which, population numbers in blood recovered rapidly **(Figure 2b and 2c)**. Corresponding *in vitro* studies have reported functional differences in the response to LPS between monocyte subpopulations - classical and intermediate monocytes exhibit pro-inflammatory responses, whereas non-classical monocytes are described as anti-inflammatory in this setting (Cros et al., 2010).

Volunteers challenged with 2 ng/kg endotoxin (**Figure 2a**) experienced a complete loss of circulating Lin$^-$ HLA-DR$^+$ cells within the first 2 hours after receiving endotoxin **(Figure 2b)**.

Strikingly, repopulation of the blood monocyte pool began very rapidly. Classical monocytes were the first subset to repopulate the circulation and appeared as early as 4 hours post-endotoxin; intermediate and non-classical monocytes remained absent from the circulation until 24 and 48 hours, respectively but they then were also restored (**Figure 2b**). By day 7 monocyte numbers had returned to steady state values (**Figure 2c**).

These data are consistent with previous studies in rodents, in which there is a surge in circulating classical Ly6C^{hi} monocytes following both peripheral and systemic inflammation (Griseri et al., 2012; Heidt et al., 2014; Shi et al., 2011). Interestingly, the recovery of monocyte subsets following systemic inflammation recapitulates the order in which deuterium labelling appeared in monocyte subsets in healthy homeostasis (**Figure 1d**).

We set out to address whether classical monocytes marginate and then return to the circulation, or whether their reappearance is due to an early "emergency" release from the bone marrow monocyte pool? To address this question, volunteers were pulsed with deuterium-labelled glucose 20 hours prior to endotoxin challenge. We deliberately chose this time-point pre-endotoxin as we knew from the healthy labelling data that at this time-point post-labelling, no circulating monocytes would normally be labelled **(Figure 1d)**, whereas cells in the post-mitotic phase within the bone marrow pool could be expected to be highly labelled. Hence unlabelled cells reappearing from margination could be readily distinguished from highly-labelled cells released early from bone marrow.

We observed very high levels of deuterium labelling in classical monocytes at 8 hours following endotoxin challenge (**Figure 2d**), demonstrating that these cells must have been recently released from the bone marrow. Although it cannot be confirmed that all classical monocytes were released from the bone marrow, due to the limitations of human experimentation, the fraction labelled were very similar to those seen 72 hours post-labelling in healthy homeostasis and are consistent with the proposal that most, if not all, circulating monocytes in the early recovery phase are bone marrow derived, rather than monocytes returning from a marginated pool. Certainly it is clear that the transition time from bone marrow to the circulation is reduced dramatically in comparison to steady state as a result of the emergency release of classical monocytes.

Classical human monocytes have the potential to give rise to intermediate and non-classical monocytes

Given the sequential maturation of monocyte subsets during healthy homeostasis and, reappearance of monocytes following endotoxin challenge, we investigated the developmental relationship between human monocytes subsets in a humanised animal model. To this end we analysed the fate of classical human monocytes isolated from healthy volunteers and grafted into MISTRG mice (**Figure 3**). The MISTRG mouse is a novel humanised mouse containing human versions of four genes encoding the cytokines thrombopoietin, IL-3, CSF2 (GM-CSF), SIRPα and CSF1 (M-CSF) that help maintain human mononuclear phagocyte development (Deng et al., 2015; Rongvaux et al., 2014). Recipient mice were sacrificed at various time points following transfer and peripheral blood was subjected to flow cytometry analysis. Ten minutes after transfer engraftment, all (human) $CD45^{+}$ cells detected in recipient blood displayed a classical monocyte phenotype; by 24 hours, the grafted cells had transitioned to intermediate monocytes, and by 96 hours all grafted cells were non-classical monocytes (**Figure 3c**). Collectively, this establishes for the first time that classical monocytes have the potential to become intermediate monocytes before finally differentiating into non-classical monocytes *in vivo*. These studies are reminiscent of previous rodent experiments, where classical $Ly6C^{hi}$ monocytes were shown to convert into non-classical cells over time (Gamrekelashvili et al., 2016; Varol et al., 2007; Yona et al., 2013), although the conversion times differed from those seen in the *in vivo* deuterium labelling studies, this is most likely due to grafted cells already being mature classical monocytes. Recent murine studies have demonstrated Notch2 signalling is required for classical $Ly6C^{+}$ monocytes to convert to non-classical monocytes (Gamrekelashvili et al., 2016). Due to the challenging nature of *ex vivo* monocyte culture, this has not been demonstrated in human cells, but hopefully future advances in cell culture will enable us to fully comprehend the mechanisms involved in human monocyte conversion.

Collectively, these data suggest that monocyte precursors first differentiate into classical monocytes that are retained in marrow for a post-mitotic “maturation” phase of about 38 hours. As a result of this delay, a reserve population of newly-generated classical monocytes is retained in bone marrow. Following acute systemic inflammation, this reserve population

is rapidly released to replace lost circulating cells. Once in the circulation, both *in vivo* modelling and humanised animal experiments are most consistent with a model in which most classical monocytes leave the circulation after a circulating lifespan of around a day. A small proportion of classical monocytes further mature into intermediate monocytes in the circulation; most of these cells finally convert to non-classical monocytes before leaving the circulation. Clearly this is a very tightly controlled process, with remarkably consistent results between individuals. Establishing the regulatory mechanisms that control these processes will be the next step in exploring human monocyte biology regulation. Understanding the fundamental regulation of monocyte subsets generation, differentiation and function, will dictate future therapeutic avenues - depleting them when they are detrimental and boosting them when they are beneficial.

MATERIALS AND METHODS

Subjects and Ethics

Subjects were healthy volunteers (20M: 5F). All volunteers gave written informed consent and all studies were conducted according to the principles of the declaration of Helsinki after approval by the relevant institutional review boards: for deuterium and steady state experiments NRES Committee West London (10/H0803/102), University College London Research Ethic Committee (8081/001) for endotoxaemia study (5060/001), Human bone marrow samples were obtained from haematopoietic stem cell donors, or femoral heads following total hip replacement. Newcastle and North Tyneside Research Ethics Committee for bone marrow biopsy (REC 14/NE/113) hip (REC 14/NE/1212).

Flow cytometry and cell sorting

Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll-Paque Plus (GE Healthcare) by density centrifugation (1000 x *g.* low acceleration no brake), then resuspended in PBS containing 2% FCS and 2mM EDTA. Isolated PBMCs were incubated with Human Trustain FcX (Biolegend) before antibody labelling with (from Biolegend unless otherwise stated): CD3 (HIT3a); CD11b (ICRF44); CD11c (B-ly6) BD Biosciences; CD14 (M5E2); CD16 (3G8); CD19 (HIB19); CD20 (2H7); CD33 (WM53); CD36 (5-271); hCD45 (H130); mCD45 (30F11); CD56 (MEM-188); CD62L (DREG-56); CD64 (10.1); CD66b (G10F5); HLA-DR (G46-6) BD Biosciences; CX_3CR1 (2A9-1); CCR2 (KO36C2) and SLAN (MDC-8) Miltenyi Biotec. DAPI staining was performed in specified experiments following surface staining, cells were fixed and permeabilised in Fixation Buffer and Intracellular Staining Permeabilisation Wash Buffer (Biolegend) according to manufactures instructions prior to incubation with 0.05 ng/ml DAPI. For a positive control, the human monocyte cell line Mono Mac 6 were used (Ziegler-Heitbrock et al., 1988). For bone marrow isolation cells from hip arthroplasty specimens, bone fragments were excavated from femoral heads, the cavity and fragments washed with PBS and filtered through a 50 µm filter. Mononuclear cells were prepared from the resulting cell suspension, or bone marrow aspirate from HSC healthy donors, by density centrifugation as described above. Cells were stained for (from BD Biosciences unless otherwise stated): CD3 (SK7-Leu9); CD19 (HIB19); CD20 (L27); CD7 (4H9); CD14 (M5E2) Biolegend; CD16 (3G8); HLA-DR (G46-6) and DAPI (Sysmex) for dead cell

exclusion. Flow cytometry was performed with LSR Fortessa X20 (BD Biosciences), cell sorting by FACS Aria II (BD Biosciences) and data analysed offline with FlowJo (Tree Star, Inc.) and Cytobank (Cytobank, Inc.).

Deuterium labelling

Deuterium labelling followed a shortened version of published protocols (Macallan et al., 2009; Westera et al., 2013). Subjects received 20g deuterium-labelled glucose (6,6-$^{2}H_2$-glucose, Cambridge Isotopes, Cambridge, MA) as an oral solution in half-hourly aliquots over 3 hours, following a priming dose equivalent to 1.8 hours dosing at time zero. Blood glucose enrichment was monitored at baseline, during and after labelling. At selected time points after labelling, mononuclear phagocytes subsets were stained and sorted by FACS Aria II (BD Biosciences), DNA extracted, and deuterium enrichment measured by gas chromatography mass spectrometry (GC/MS), as previously described (Busch et al., 2007; Macallan et al., 2009).

Modelling of data

A schematic of the model is shown in **Figure 1e**. We denote N as the number of monocytes in the bone marrow, B_1 the number of classical monocytes in the blood, B_2 the number of intermediate monocytes in the blood and B_3 the number of non-classical monocytes in the blood. The dynamics between these four compartments can then described by the following equations:

$$\frac{dN}{dt} = pN - r_1 N$$

$$\frac{dB_1}{dt} = r_1 N(t - \Delta_1) - r_2 B_1$$

$$\frac{dB_2}{dt} = \alpha_2 r_2 B_1 - r_3 B_2$$

$$\frac{dB_3}{dt} = \alpha_3 r_3 B_2(t - \Delta_3) - r_4 B_3$$

We assume that all the compartments are in steady state. The relative sizes of B_1, B_2 and B_3 were taken from flow cytometry data for each individual (**Table 1**). From these equations, we derive the dynamics of the fraction of labelled cells in each compartment - F_N for the bone marrow, F_x for blood compartments B_x:

$$\frac{dF_N}{dt} = pU(t)b - r_1 F_N$$

$$\frac{dF_1}{dt} = r_1 \frac{N}{B_1} F_N(t - \Delta_1) - r_2 F_1$$

$$\frac{dF_2}{dt} = \alpha_2 r_2 \frac{B_1}{B_2} F_1 - r_3 F_2$$

$$\frac{dF_3}{dt} = \alpha_3 r_3 \frac{B_2}{B_3} F_2(t - \Delta_3) - r_4 F_3$$

Here U(t) is the precursor enrichment (plasma glucose) at time t, described empirically as a plateau function with exponential decay.

We used the R packages modFit and dede to fit the model to the observed values of deuterium enrichment (F_x in the equations above). The fitting algorithm sought to minimize the sum of squared residuals (ssr) between the modelled curves and observed values. This ssr was translated into an AICc (Akaike Information Criterion, corrected for small sample sizes) allowing us to compare the models with and without Δ_3 (since a model with more parameters will trivially result in an equal or better fit, but comes with a risk of overfitting).

Intravenous administration of Endotoxin. 2 ng/kg Endotoxin (E.coli 0:113; NIH Clinical Center, Bethesda) was administered i.v. to ten healthy male volunteers as described (Fullerton et al., 2016). At selected time points blood samples were taken and analysed by flow cytometry. Three subjects received deuterium-labelled glucose 20 hours prior to endotoxin administration and monocyte labelling kinetics analysed as above (**Figure 2a**).

Mice. 10 week-old MISTRG mice (Deng et al., 2015; Rongvaux et al., 2014) were used for adoptive transfer experiments described below. Mice were maintained under specific

pathogen-free conditions and handled under protocols approved by the Yale Institutional Animal Care and Use Committee protocols.

Mouse adoptive transfer. Blood was collected from healthy volunteers and mononuclear phagocytes enriched using RosetteSep™ Human Monocyte Enrichment Cocktail (Stemcell Technologies) following the manufacturer's instructions. Enriched cells were labeled with CD3, CD19, CD20, CD56, CD66b, HLA-DR, CD14 and CD16 antibodies prior to sorting classical monocytes by FACS AriaII (BD Biosciences). Sorted classical monocytes were adoptively transferred intravenously into MISTRG mice (Deng et al., 2015; Rongvaux et al., 2014). Peripheral blood was collected by cardiac puncture under terminal anaesthesia; erythrocytes were lysed by ACK (Lonza). The leukocyte fraction was stained and analysed by flow cytometry. The fate of the classical monocytes were analysed at selected time points post transfer identified as human $mCD45^{-}hCD45^{+}HLA\text{-}DR^{+}CD33^{+}Lin^{-}$ by flow cytometry.

ONLINE SUPPLEMENTAL INFORMATION: Fig. S1 quantifies human blood monocyte subset membrane marker expression and cell cycle analysis. Fig. S2 shows modelling curves generated with and without a delay between intermediate and non-classical monocytes. Table S1 shows model data fit with and without a delay. Table S2 Lifespans, proliferation rates and delays for the model with a delay.

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Author contributions: SY, DM & DG conceived the project. SY designed and performed all experiments and analysis except where otherwise specified and wrote the manuscript with DM. AAP performed all the human studies except where otherwise stated. YZ processed and isolated DNA from sorted monocyte populations and performed GC/MS analysis. LB, BA, DM and AAP wrote and performed the mathematical modeling. VB performed the comparison of bone marrow vs. blood monocytes. JNF, SY & AAM performed the human endotoxin study. AR & RAF provided the MISTRG mice and assistance in the adoptive transfers of human monocytes and hosted SY.

FIGURE LEGENDS

Figure 1. In vivo labelling and a methodological approach of modelling human monocyte subset kinetics at steady state

(a) Polychromatic flow cytometry gating strategy for blood monocyte subsets. Peripheral blood mononuclear phagocyte cells were identified as Lin^- (CD3, CD19, CD20, CD56, CD66b) HLA-DR^+ cells. This population comprises classical monocytes ($CD14^+$ $CD16^-$:black gated population), intermediate monocytes ($CD14^+$ $CD16^+$:grey gated population) and non-classical monocytes ($CD14^{lo}$ $CD16^+$:red gated population) representative of >ten subjects. Below, representative cytospin images from 10 healthy volunteers stained with haematoxylin and eosin, scale bar 25 μm.

(b) Flow cytometry viSNE analysis of monocyte subsets illustrating membrane expression for CCR2, CD62L, CD36, CD64, CD11b, CD11c, HLA-DR, SLAN and CX_3CR1, representative of eight healthy volunteers.

(c) Schematic of protocol for labelling newly-divided cells. Healthy volunteers received 20 g deuterium-labelled glucose over 3 hours. Monocytes subsets were then sorted from whole blood over a 30-day period, DNA was extracted to quantify the deuterium enrichment in each monocyte subset by gas chromatography mass spectrometry (GC/MS).

(d) Percentage of deuterium label in peripheral blood classical (black), intermediate (grey) and non-classical (red) monocytes following oral admission of deuterated glucose in four healthy volunteers; values shown are mean ± SEM.

(e) Model of circulating monocyte kinetics. Cartoon depicts the sequential model for the fate of circulating monocyte subsets. Monocytes mature in the bone marrow, where their precursors proliferate at a rate p. Classical monocytes leave the bone marrow at rate r_1, after a delay of Δ_1 days between the last proliferation and release into the circulation. In the blood, classical monocytes either mature into intermediate monocytes at rate α_2 r_2, where α = proportion of the subset, or they disappear from the blood (either by death or by moving to other organs) at rate $(1–\alpha_2)r_2$. The total disappearance

rate is thus r_2. Likewise, a proportion α_3 of the intermediate monocyte subset develop into non-classical monocytes, the remainder disappearing from blood. A parameter Δ_3 has been included to allow for a potential delay in the differentiation of intermediate monocyte to non-classical monocytes.

(f) Polychromatic flow cytometry comparing bone marrow (BM) with circulating monocyte subsets. Human BM was initially gated as $Lin^-HLA\text{-}DR^+$. Human BM obtained as either an aspirate or femoral head excavated biopsy were examined by flow cytometry to identify resident monocyte subsets. Only classical monocytes could be detected in the biopsy. These data are representative of three donors for each procedure.

(g) Summary of the steady state kinetics for monocyte subsets. Figures in black bold text denote lifespans in each compartment figures in italics denote the relative probability of each cell undergoing the respective fate (death/disappearance versus phenotype transition). Progenitor cells in the bone marrow proliferate at rate of 0.42/day (blue), where the post-mitotic cells remain within the bone marrow for 1.6 days before being released into the circulation as classical monocytes. Classical monocytes contribute 87% to the total monocyte pool while intermediate and non-classical monocytes make-up 5% and 8%, respectively. 99% of classical monocytes leave the circulation and 1% go onto become intermediate monocytes. 100% of intermediate monocytes mature in the circulation to become non-classical monocytes under steady state.

Figure 2. Sequential reappearance of monocytes subsets following endotoxin challenge

(a) Schematic protocol for administrating deuterium-labelled glucose 20 hours prior to i.v endotoxin 2 ng/kg in healthy volunteers. Classical monocytes were then sorted from whole blood, DNA extracted and deuterium enrichment quantified by GC/MS over the ensuing 10 days.

(b) Flow cytometry analysis of human monocyte subsets at 0, 2, 8, 24, 48, 72 hours and 7 days following intravenous administration of endotoxin, representative of 10 individuals.

(c) Time course of absolute monocyte numbers at selected time-points following endotoxin challenge for classical, intermediate, and non-classical monocytes, mean ± SEM x 10^9/L of three individual subjects; note the different scale for each subset.

(d) Comparison of deuterium labelled classical monocyte egression from the bone marrow under normal physiological conditions (triangles, dashed line, four subjects) and following endotoxin challenge (circles, solid line, three subjects). Values represent mean ± SEM.

Figure 3. Development of intermediate and non-classical human monocytes from classical monocytes

(a) Classical human monocytes LIN^- $HLA\text{-}DR^+$ $CD14^+$ $CD16^-$ cells were sorted from healthy blood by FACS.

(b) 1.5 x10^6 sorted classical monocytes, were grafted intravenously into the humanised MISTRG mouse. Grafted cells could be readily identified by expression of the human isoform of CD45, compared to recipient leukocytes expressing mouse CD45.

(c) Flow cytometry analysis identified human $CD45^+$ circulating monocytes from MISTRG recipients following adoptive transfer of human $CD14^+CD16^-$ classical monocytes at 10 minutes, 24, 72 and 96 hours post-infusion. Results are representative of three analysed mice per time point.

ONLINE SUPPLEMENTARY FIGURES

Supplementary Figure 1. Heterogeneity of membrane markers within monocyte subsets

(a) Polychromatic flow cytometry analysis of circulating monocyte subsets membrane expression of CCR2, CD62L, CD64, CX_3CR1, HLA-DR, CD11c, CD11b, CD36 and SLAN, from eight healthy volunteers. Bar represents mean MFI.

(b) Cell cycle analysis of circulating blood monocytes. DAPI was used to quantify DNA levels to determine whether circulating monocytes are in various phases of the cell cycle. The Mono mac 6 (MM6) monocyte cell line was used as a positive control to show various stages of the cell cycle.

Supplementary Figure 2. Curve fits to experimental data from modelling with and without a delay between intermediate and non-classical monocytes

Dots represent experimental data expressed as percentage DNA enrichment with deuterium in the three sorted blood monocyte subsets. Lines represent the best model fit to the data classical monocytes (black line); intermediate monocytes (grey line); non-classical monocytes (red line). Each row represents an individual subject. Columns represent alternative models for Δ_3; left hand figures, constrained to zero; right-hand panels, as a free parameter.

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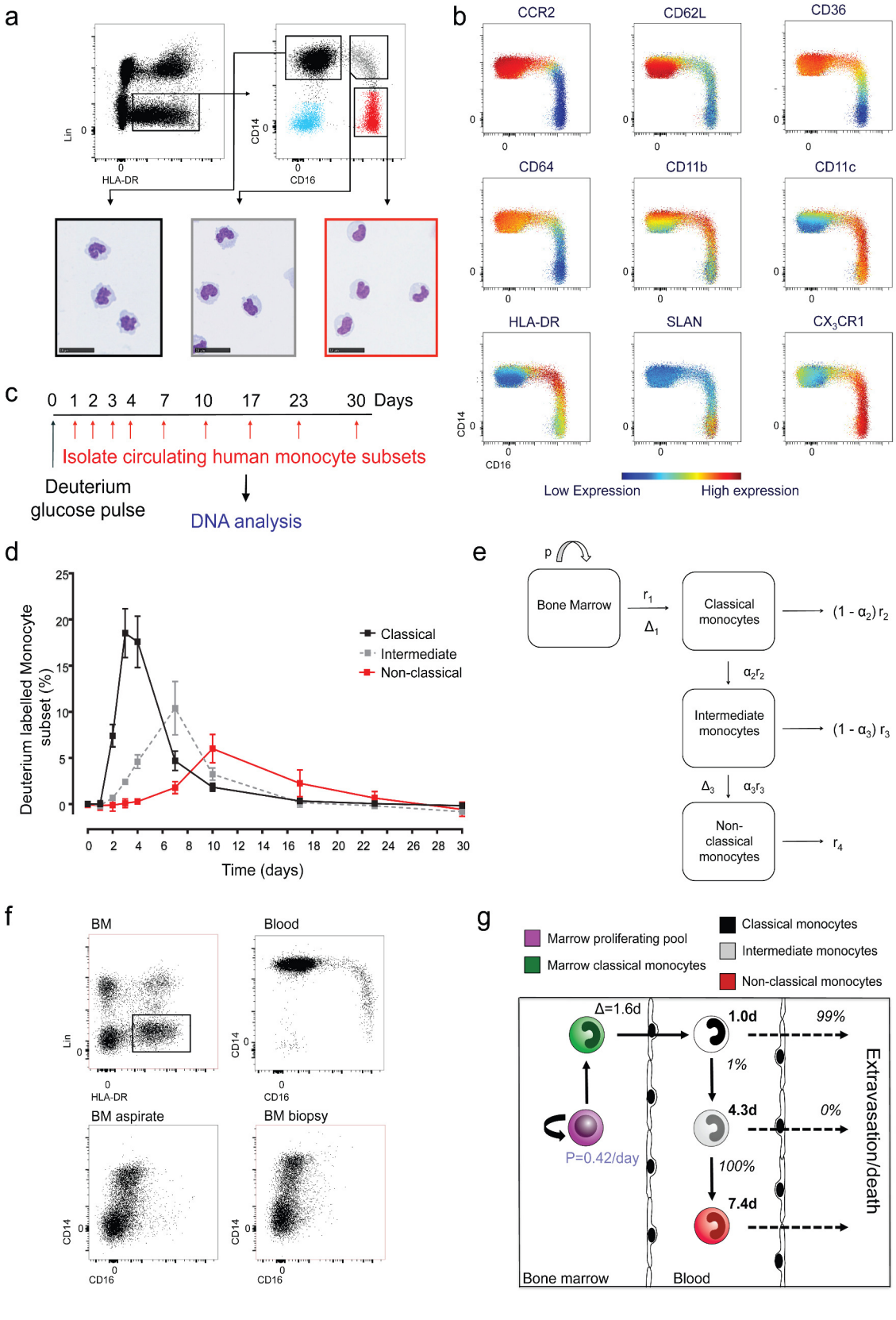
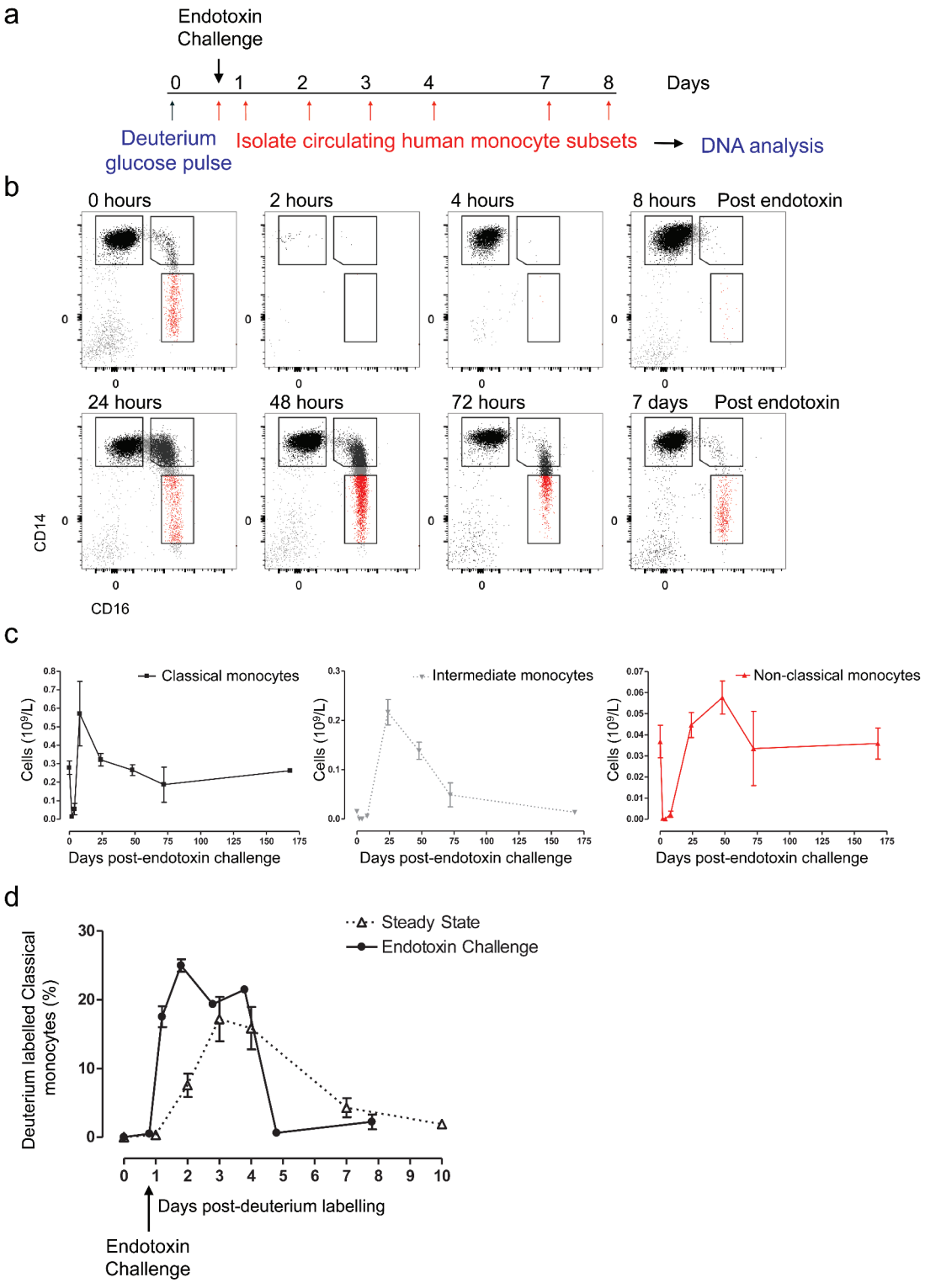
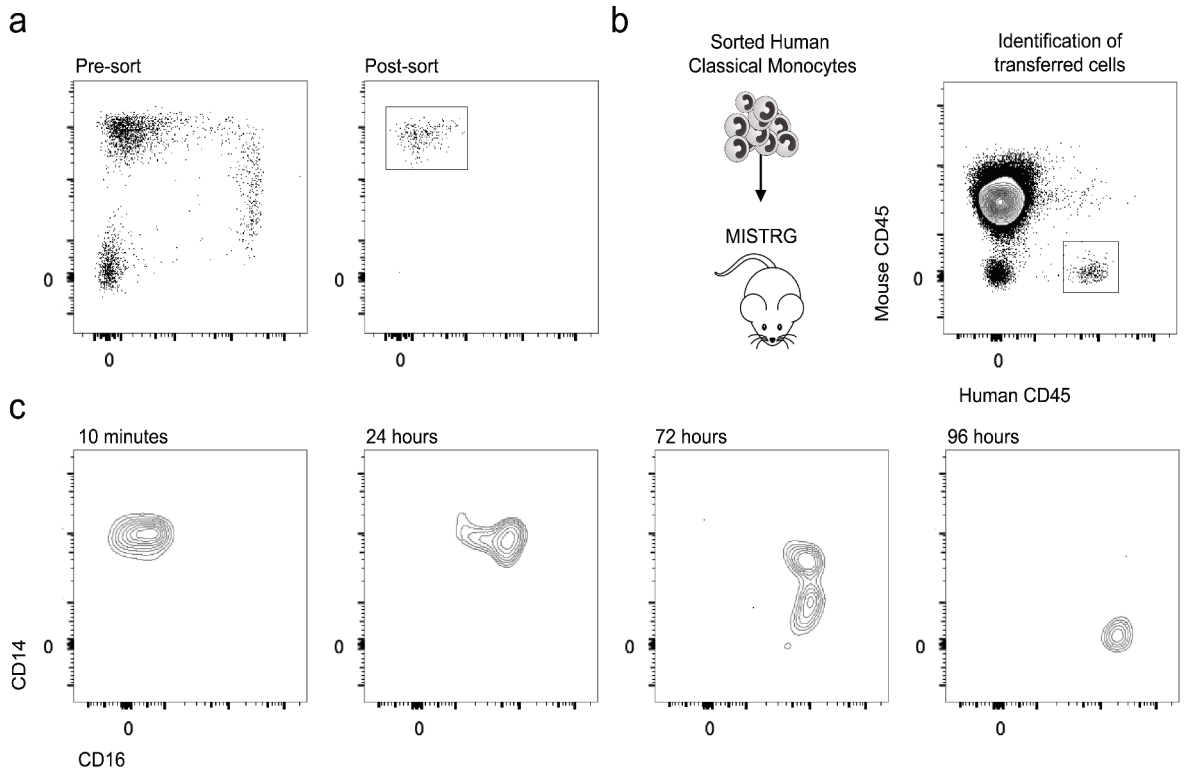


Patel et al., Figure 1



Patel et al., Figure 2



Patel et al., Figure 3