1 Title:

2 High affinity F_c receptor expression indicates relative immaturity in human monocytes

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11 Short title:

12 Less mature monocytes express high affinity F_cR

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Abstract

Within monocyte heterogeneity, subsets represent discrete, well-characterised phenotypes. Although many studies have highlighted differences between subsets, there is evidence that subpopulations represent contiguous stages in a maturational series. As CD14^{hi}CD64^{hi} monocytes have higher proliferative potential than CD14^{hi}CD64^{lo} monocytes, the surface marker profile on four subsets defined by CD14 and CD64 was measured. The profiles were compared to that of subsets defined by the high affinity IgE receptor (F_cεRIα), CD16 and CD14; further differences in size, granularity and buoyancy were measured in subsets delineated by these markers. There was a positive correlation between proliferative monocyte (PM) prevalence and CD64 expression on the 'classical' monocyte subset, and also between PM prevalence and circulating F_cεRIα⁺ monocytes. The expression of CD64, the high affinity IgG receptor, on canonical human monocyte subsets was determined before and after short term culture, and in response to IL-6, IL-10, M-CSF, GM-CSF and IFNγ; the influence of these cytokines on monocyte subset transition was also measured. The loss of F_cεRIα expression preceded an increase in CD16 expression in whole blood cultures. These data indicate high affinity F_c receptors are expressed on less mature monocytes and that F_cεRIα⁺ monocytes are developmentally antecedent to the canonical classical and intermediate monocyte subsets.

1 Introduction

2 Monocytes are derived from hematopoietic bone marrow precursors. After several stages of
3 differentiation (monoblast, pro-monocyte) they enter the blood, circulate for up to three days
4 and then migrate into tissues(Kennedy and Abkowitz 1998). Monocytic cells contain
5 precursors to macrophage-lineage cells (dendritic cells(DC), osteoclasts and tissue-specific
6 subpopulations of macrophages) and may also have additional differentiation and functional
7 potential(Zhao and others 2003). Where once monocytes were identified by morphology
8 (e.g. cells with a single indented nucleus and non-specific esterase staining), light scatter
9 properties and surface antigen expression, as measured by flow cytometry, are now most
10 commonly used.

11
12 Although schemes based upon the expression of CX3CR1, CD2, CD13, CD33 or CD64 have
13 been used(Crawford and others 1999; Geissmann and others 2003; Grage-Griebenow and
14 others 1993; Thomas and others 1993), human monocytes are now classified into
15 subpopulations based upon differential expression of CD14 and CD16 (Passlick and others
16 1989; Ziegler-Heitbrock and others 2010); the majority of monocytes exhibit the classical
17 subset phenotype (CD14^{hi}CD16⁻), typically 10% have the non-classical phenotype
18 (CD14^{lo}CD16⁺⁺) leaving the intermediate subset as the least prevalent subset in the steady
19 state(Ziegler-Heitbrock and others 2010). Differences in transcriptional profiles(Ancuta and
20 others 2009) and tissue homing(Geissmann and others 2003) have confirmed functional
21 differences observed in vitro however few studies have attempted to determine the
22 developmental relationships between monocyte subsets. A proliferative monocyte (PM)
23 subpopulation has been identified which is ostensibly less mature than the bulk of the
24 monocytes, and provides evidence for differences in maturity between human monocyte
25 subsets(Cheung and Hamilton 1992; Clanchy and Hamilton 2012b; Clanchy and others
26 2006). Monocytes with relatively high expression of CD64 have higher proliferative
27 potential compared to monocytes with low expression of CD64(Clanchy and others 2006),
28 indicating that less mature, more proliferative CD14^{hi} monocytes have higher expression of
29 CD64.

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31 A comparison of surface marker profiles indicated that the expression of the high affinity F_c
32 receptor for IgE (F_cεRIα) significantly overlapped with that of monocytes with the highest
33 expression of CD64, which is also a high affinity F_c receptor. As PM have increased

1 osteoclastic potential(Clanchy and others 2006) and an as yet undetermined role in human
2 physiology, the aim of this study was to determine the relationship between CD64 expression
3 and monocyte maturity or activation on ‘canonical’ human monocyte subsets, which are
4 defined by expression of CD14 and CD16(Ziegler-Heitbrock and others 2010). Subsets
5 defined by FcεRIα, CD16 and CD14 were also characterised and the prevalence of PM within
6 measured.
7

Materials and Methods

Isolation and culture of PBMC

Human leukocytes were isolated from buffy packs as described previously (Clanchy and others 2006). After centrifugation, the PBMC layer was transferred to a new tube and washed repeatedly with PBS to deplete platelets. For cytokine stimulation experiments, PBMC were cultured at a concentration of $2.5 \times 10^6/\text{mL}$ of medium (RPMI with 10% fetal bovine serum, 1% P/S). Cytokines (PeproTech) were added to media at the following concentrations – interleukin-6 (IL-6) 50ng/mL, interleukin-10 (IL-10) 20ng/mL, macrophage colony stimulating factor (M-CSF) 20ng/mL, granulocyte/macrophage colony stimulating factor (GM-CSF) 20ng/mL or interferon- γ (IFN γ) 20ng/mL.

Whole blood culture

Medium (1% P/S, 10%FBS, α -MEM with 2mM L-glutaMaxTM (Invitrogen)) was placed in an incubator in a loosely capped tube to reach optimal temperature and pH. Into 9.5mL of medium was added 500 μ L of undiluted buffy pack ‘blood’ in addition to heparin (10U/ml, Mayne Pharma Pty Ltd, Australia). The tubes were tightly closed and placed horizontally in a 37°C shaker oscillating at ~100 os/m. PBMC were isolated using Ficoll-Paque after 20h and then stained for flow cytometry; cell surface expression of markers was compared to that of freshly isolated PBMC from the same donor.

CFSE-labelled cell tracking

Whole blood cultures (WBC) from buffy packs were initiated as described. PBMC from the same donor were isolated as described above. After labelling PBMC with FACS antibodies (Fc ϵ RI α -FITC and CD14-PE), monocytes were sorted. The sorted cells were resuspended at 1×10^5 cell/mL in PBS. CFSE (Invitrogen) was added to final concentration of 0.5 μ M and cells were incubated for 10 min at room temperature with frequent mixing. An equal volume of FBS was added, then cells were washed and added to the WBC for the remaining ~12 hours of the assay. PBMC from WBC were isolated using Ficoll-Paque then stained with CD16-PerCP and CD14-APC; the phenotype of the labelled monocytes was assessed by gating on CFSE⁺ cells.

Buoyancy

Percoll (Sigma Aldrich) was diluted with 10x PBS to make iso-osmotic '100% Percoll', which was diluted with PBS for lower concentrations of Percoll. For determining the relative buoyance of monocyte subsets, 3mL of 30% Percoll was layered over 3mL of 40% Percoll in a 10mL tube. PBMC ($\sim 1.5 \times 10^6$) were resuspended in 3mL of PBS and layered over the 30% Percoll. The tube was spun at 1000g for 20 min with no brake. Cells from the interfaces and soft pellet were analysed by flow cytometry. Leukocytes accumulating at Percoll interfaces were defined as low density (upper interface), high density (lower interface) and pellet. To determine the *relative subset proportion* at each interface, the percentage of a subset at each interface was expressed relative to the original subset percentage, prior to Percoll separation. To determine the *relative subset buoyancy* for each subset, the relative subset proportion of the low density interface was expressed relative to the relative subset proportion of the high density. See Supplementary Figure 1 for further details.

Flow cytometry

All antibodies were from BD Biosciences except for Fc ϵ R1 α -FITC (Abcam Inc); isotype and compensation controls were used to set gates for sorting and analysis; propidium iodide (Sigma-Aldrich) was used to identify non-viable monocytic cells (identified by FSC vs SSC) which were of similar prevalence in in vitro cultures (Supplementary Figure 2). For cell sorting, PBMC were stained with 10 μ L of antibody for 25 min on ice in the dark then washed three times with FACS buffer. Sorting was performed using a FACSVantageSE (BD Biosciences). FACS acquisition was performed with a FACSCalibur flow cytometer and subsequent analysis was performed using FlowJo (Treestar).

BrdU Assay

FACS-sorted CD14^{hi} monocytes were assessed for cell cycle entry as previously described (Clanchy and others 2006). Briefly, monocytes were cultured in 96 well U-bottom plates at a concentration of 5×10^4 cells per well in 200 μ L of media (α -MEM with 3% human serum, 1% P/S, 2 mM L-glutaMaxTM). On day 4 of culture, cells were pulsed with 10 μ L of an equimolar solution of BrdU and cytidine to give a final concentration 300 μ M. On day 8 cells were harvested, fixed and permeabilized, then stained for FACS analysis.

Statistics

Significant differences were determined by Student's t-test or ANOVA with post hoc analysis as detailed in figure legends. Significant correlations were determined by Pearson correlation coefficient. Analyses were performed with Excel (Microsoft) and Prism (Graphpad).

Results

CD64-defined subsets delineate a maturing monocyte

The monocytic expression of CD64 is highest on CD14^{hi} monocytes, particularly the CD16⁺ classical subset which accounts for the majority of monocytes (Grage-Griebenow and others 1993; Grage-Griebenow and others 2001b). As proliferative potential is higher in monocytes with the CD14^{hi}CD64^{hi} expression, compared to CD14^{hi}CD64^{lo} monocytes, the expression of several well characterised myeloid surface markers was measured on subsets of monocytes defined as CD14^{hi}CD64^{hi}, CD14^{hi}CD64^{med}, CD14^{hi}CD64^{lo} and CD14^{lo}CD64^{lo} to determine whether these subsets might represent a maturation series, as evidenced by corresponding changes in surface marker expression.

Previously, PM were determined to have a CD11b^{lo}CD33^{hi}CD13^{lo}CD64^{hi} phenotype where high and low expression were defined as the highest and lowest ~15% of expression of a particular surface marker on CD14^{hi} monocytes (Clanchy and others 2006). To further refine the description of PM, the expression of surface markers was measured on peripheral blood monocytes divided into subsets based upon expression of CD14 and CD64. High and low expression was defined as the highest and lowest ~20% of expression within CD14^{hi} monocytes for each donor and intermediate expression of CD64 was defined as the 20% of CD14^{hi} monocytes which had an expression of CD64 equidistant between the high and low gates; CD14^{lo}CD64^{lo} monocytes accounted for 5-10% of monocytic cells (Figure 1(A)).

As shown in Fig. 1(B), the expression of CD16 was low on CD14^{hi}CD64^{hi/int} monocytes, slightly higher on CD14^{hi}CD64^{lo} monocytes and highest on the CD14^{lo}CD64^{lo} subset, as has previously been described (Grage-Griebenow and others 2000; Grage-Griebenow and others 1993). The expression of CD2, CD11b, CD33, CD38, CD62L and CD163 was highest on CD14^{hi}CD64^{hi} monocytes and is reduced at a uniform rate across CD14^{hi}CD64^{med},

CD14^{hi}CD64^{lo} and CD14^{lo}CD64^{lo} monocytes. CD14^{hi} monocytes with low expression of CD13 were previously found to have higher proliferative potential compared to CD14^{hi}CD13^{hi} monocytes(Clanchy and others 2006) and CD14^{hi}CD64^{hi} monocytes have lower expression of CD13 compared to CD14^{hi}CD64^{lo} monocytes. HLA-DR was expressed in a bi-modal fashion, with highest expression on the CD14^{hi}CD64^{hi} and CD14^{lo}CD64^{lo} monocytes but lower on the other subsets. The expression of CD4 was similar in all subsets.

FcεRIα, CD16 and CD14 define five monocyte subsets

The expression of CD2 was higher on CD64^{hi} monocytes. Monocytes which express higher levels of CD2 have been described as a putative DC precursor since, in the presence of IL-4 and GM-CSF, they differentiate rapidly into CD83⁺ cells with higher antigen presentation potential compared to that in CD2⁻ monocytes(Crawford and others 1999; Di Pucchio and others 2003). These monocytes bear some resemblance to CD14^{lo}CD16⁻CD33^{hi} monocytes which have been characterised as myeloid DC precursors as they display high antigen presentation capacity, DC morphology and DC FACS profile after culture(Fagnoni and others 2001; Thomas and others 1993); the high expression of CD2 on monocytes from patients with myelo-proliferative disorders further indicates a lack of monocyte maturity associated with this marker(Shoda and others 2005). The CD2^{hi} monocyte subset has been reported to express the high affinity receptor for IgE (F_εRIα), the expression of which on monocytes correlates with the amount of IgE present in the blood in allergic conditions(Cheng and others 2006).

The expression of CD14, CD16 and F_εRIα was measured on freshly isolated cells and is shown in Figure 2(A-C), demonstrating the mutually exclusive expression of CD16 and F_εRIα on both CD14^{hi} and CD14^{lo} monocytes. When defined by the expression of CD14, CD16 and F_εRIα, monocytic cells can be divided into five subsets – CD14^{lo}F_εRIα⁺, CD14^{hi}F_εRIα⁺, CD14^{hi}(F_εRIα/CD16)⁻, CD14^{hi}CD16⁺ (the intermediate subset) and CD14^{lo}CD16⁺⁺ (the non-classical subset); CD14^{hi}F_εRIα⁺ and CD14^{hi}(F_εRIα/CD16)⁻ comprise the classical subset. The expression of common myeloid cell surface markers was measured on each subset (Figure 2(D)). Whilst CD34 (not shown) was not significantly expressed, generally the pattern of expression across the five subsets falls into three categories. CD33 and CD38 had a similar profile with highest expression on the CD14^{hi}F_εRIα⁺ subset and much lower expression on the non-classical CD14^{lo}CD16⁺⁺ subset.

CD2, CD62L and CD163 were expressed at higher levels on FcεRIα⁺ monocytes but reduced at varying rates in a stepwise fashion on the other populations. CD11b and CD13 have high expression on the most prevalent CD14^{hi}(FcεRIα/CD16)⁻ subset and lowest expression on both CD14^{lo} subsets; this pattern is inverted for HLA-DR. Of particular interest was the CD11b^{lo}CD13^{lo}CD33^{hi}CD64^{hi} phenotype of CD14^{hi}FcεRIα⁺ monocytes, as cells expressing this combination of markers have previously been shown to have higher proliferative potential, and are thus potentially less mature (Clanchy and others 2006). The prevalence of each subset varied from donor to donor though FcεRIα⁺ monocytes were less abundant than CD16⁺ monocytes which were in turn far less abundant than CD14^{hi}(FcεRIα/CD16)⁺ monocytes (Figure 3(A)).

Size, granularity and buoyancy

Early techniques for human monocyte separation, based on size and density (reviewed in (Grage-Griebenow and others 2001a)), identified two subsets broadly defined as ‘large’ and ‘small’, which appear to correlate with CD14^{hi} and CD14^{lo}CD16⁺⁺ monocytes, respectively (Ziegler-Heitbrock and others 1991). To determine differences in size and granularity (approximated by forward and side scatter, respectively) between the five subsets defined by the expression of CD14, CD16 and FcεRIα, the flow cytometric scatter profiles of antibody-labelled PBMC were ascertained. Figure 3(B) illustrates the relative differences in mean forward and side scatter between the five subsets. The size and granularity of the CD14^{lo}CD16⁺⁺ and CD14^{hi}FcεRIα⁺ subsets were similar, as were those of the CD14^{hi}(FcεRIα/CD16)⁻ and CD14^{hi}CD16⁺ subsets; CD14^{lo}FcεRIα⁺ cells have the lowest size and granularity.

To determine the differences in buoyancy in the five human monocyte subsets defined by expression of CD14, CD16 and FcεRIα, PBMC were layered over a two-step discontinuous Percoll gradient and, after centrifugation, cells at each interface and the pellet were analysed by flow cytometry (Materials and Methods, Figure 3(C)). As seen in Figure 3 (D) the low and high density interfaces both had ~43% CD14^{hi} monocytes whilst denser cells formed the pellet and averaged ~8% CD14^{hi} monocytes. The proportion of monocytes from each subset at both the high or low density interface was expressed relative to the respective proportion of each subset in unseparated PBMC for each donor, with no change being equal to 1 (Figure 3 (E)). The proportion of CD14^{hi}(FcεRIα/CD16)⁻ monocytes in the high and low density

fraction was equal to that in unseparated PBMC. In the high density fraction, the relative proportion of each subset appears to increase as the expression of FcεRIα decreased and CD16 increased. This pattern is inverted in the low density fraction, with the exception of the CD14^{lo}CD16⁺⁺ subset which has a higher prevalence. To compare the differences in buoyancy between subsets, the high density interface values were expressed relative to low density interface values for each subset (Figure 3(E), see Supplementary Figure 1 for further particulars). CD14^{hi}CD16⁺ and CD14^{hi}(FcεRIα/CD16)⁻ monocytes are significantly more buoyant than the other subsets using this analysis. There was a strong correlation between forward scatter and relative buoyancy for the five subsets (Figure 3(F)), suggesting that each monocyte subset may represent a stage in development from FcεRIα⁺ monocytes to monocytes that lack FcεRIα/CD16 and then to CD16⁺ monocytes, although the non-classical subset does not appear to be an immediate continuation of the intermediate subset based on this analysis of physical properties.

PM prevalence is associated with high affinity Fcε receptor expression

CD64 expression on canonical monocyte subsets or the prevalence of a monocyte subset, as defined by FcεRIα, CD16 and CD14, was measured to determine the association between these parameters and circulating PM. A proliferation assay was performed using CD14^{hi} monocytes (Figure 4(A)) and the relative expression of CD64 on each canonical subset at day 0 was correlated with the proportion of BrdU⁺ cells (Figure 4(B)). There was an association between CD64 expression and PM prevalence for the classical subset and a weak trend for the intermediate subset; there appeared no correlation between PM prevalence and CD64 expression for non-classical monocytes.

As CD14^{hi}FcεRIα⁺ monocytes share the same surface marker phenotype as PM (CD14^{hi}CD11b^{lo}CD13^{lo}CD33^{hi}CD64^{hi})(Clanchy and others 2006), it is unsurprising that proliferation, as measured by BrdU assay, was also significantly higher in cultures containing sorted CD14^{hi}FcεRIα⁺ monocytes, compared to sorted CD14^{hi}FcεRIα⁻ monocytes (Figure 4(C, D)). As shown in Figure 4(E), there was a significant positive correlation between the proportions of day 0 CD14^{hi/lo}FcεRIα⁺ monocyte subsets and circulating PM, as determined by BrdU assay performed using the relatively abundant CD14^{hi} monocytes sorted from each donor.

Monocyte subset CD64 expression is uniformly modulated by cytokine stimulation

A measurement of CD64 expression in different culture conditions was undertaken to determine whether the expression of CD64 on monocytes was upregulated due to surface marker modulation or an alteration in the composition of monocyte subsets. Previous studies of monocyte differentiation found a reduction in CD64 expression during culture (Chapuis and others 1997; Li and others 2009) however, the expression of CD64 on cells defined as canonical monocyte subsets has not been measured. In addition, the effects of cytokines that can modulate the expression of CD64 (Chapuis and others 1997; Li and others 2009) and PM activity (Cheung and Hamilton 1992; Clanchy and Hamilton 2012a), which have been investigated using total monocytes, were investigated within monocyte subsets over the same period.

The surface expression of CD64 was measured on canonical monocyte subsets before and after cytokine stimulation (Figure 5). For each subset the effect of individual cytokines was broadly similar after culture. IL-6 modestly increased CD64 expression, whereas M-CSF stimulation induced no change and GM-CSF slightly decreased expression. In all subsets, the presence of IL-10 or IFN γ significantly increased CD64 expression, compared to medium alone. The increase in CD64 expression in the presence of IFN γ was most pronounced in the monocytes with an intermediate or non-classical phenotype after culture, compared to the expression on these monocyte subsets prior to culture (Figure 5(D-F)).

Cytokine stimulation modulates monocyte subset transition

The proportion of monocytes with the phenotype of a canonical monocyte subset was determined before and after cytokine stimulation (Figure 6). Prior to culture, the classical monocytes were most prevalent and after culture the intermediate monocytes predominated; monocytes with the non-classical phenotype were slightly reduced in proportion after culture, in the absence of cytokine stimulation. The presence of GM-CSF or IFN γ significantly attenuated the reduction in classical monocytes and consequent increase in the proportion of intermediate monocytes. The presence of M-CSF, GM-CSF or IFN γ modestly elevated the proportion of non-classical monocytes compared to medium alone, to a level similar to pre-cultured cells. The apparent transition from classical to intermediate subset without a

significant change in the non-classical subset (Figure 6(E-G)) was also observed in whole blood cultures stimulated with or without LPS (see Materials and Methods). Based on these data, changes in the composition of monocyte subset phenotypes in the absence of cytokine stimulation indicate an increase in the proportion of the intermediate monocyte phenotype at the expense of classical monocytes.

Loss of FcεRIα precedes expression of CD16

To determine whether the three CD14^{hi} subsets defined by expression of CD16 and FcεRIα represent incremental steps in development, whole blood cultures were used to mimic maturation that occurs in the circulation in vivo (Skinner and others 2005). A comparison of freshly isolated PBMC and PBMC isolated from whole blood cultures shaken for 20h indicates that on CD14^{hi} monocytes (Figure 7 (A, B)) the expression of CD16 is upregulated (Figure 7 (C)); for the donors tested, the increase in fluorescent intensity was six-fold higher (Figure 7 (D)). As indicated by Figure 7 (E) and (F) there is a decrease in the proportion of CD14^{hi}FcεRIα⁺ monocytes and a concurrent increase in the proportion of CD14^{hi}CD16⁺ monocytes between 0 and 20 h; of note is the increase in background staining and autofluorescence at 20 h (Figure 7 (G)), which was also observed in Figure 7(C). As detailed in Figure 7 (H), the proportions of CD14^{hi} monocytes in each quadrant (labelled α, β, γ, δ in Figure 7 (E)) indicate that the decrease in CD14^{hi}FcεRIα⁺ (α) and CD14^{hi}(FcεRIα/CD16)⁻ (β) monocytes and increase in CD14^{hi}CD16⁺ monocytes (γ) occurs without a transitional phase where FcεRIα and CD16 are co-expressed, as, in this in vitro model, the maturing cells do not 'transit' through the δ quadrant. This aspect of the in vitro model reflects what is seen in freshly isolated monocytes, which also do not co-express FcεRIα and CD16.

To confirm that the monocytes are losing FcεRIα and gaining CD16, FACS-sorted monocytes (CD14^{hi} or CD14^{hi}FcεRIα⁺) were labelled with CFSE and added to whole blood cultures. After short term culture, the PBMC were then isolated as before. As expected, after gating on CFSE⁺ monocytes from the cultured blood, the sorted CD14^{hi} monocytes expressed CD16 on most cells after culture however, a large proportion of sorted monocytes that were originally CD14^{hi}FcεRIα⁺ also expressed CD16 after culture (Figure 7 (J) and (K) respectively). Figure 7 (L) details the proportions of CFSE⁺ CD16⁻ and CD16⁺ monocytes (corresponding to quadrant labels α/β and γ in Figure 7 (J)).

Discussion

Surface markers delineated by the high affinity F_c receptors CD64 or $F_c\epsilon R1\alpha$

Several markers were differentially expressed on CD14^{hi}CD64^{hi} monocytes, though none were found solely or inordinately on this subset. The differences between surface profiles of monocyte subsets defined by high affinity F_c receptor expression indicated a gradual change in monocyte phenotype from the classical to intermediate subset phenotype. Expression of CD16, along with CD14, is currently the main differentiator of human monocyte subsets and, as previously observed, CD64 expression was lowest on the CD14^{lo}CD16⁺⁺ non-classical subset (Grage-Griebenow and others 1993; Grage-Griebenow and others 2001b). Within CD14^{hi} monocytes, the pattern of expression for several markers (CD2, CD13, CD62L, CD38, and CD163) was suggestive of a maturational series, as there is a steady change in expression of these markers between the highly proliferative CD14^{hi}CD64^{hi} and the less proliferative CD14^{hi}CD64^{lo} (Figure 1).

The correlation between CD64 expression and CD2 expression lead to the delineation of monocyte subsets based upon $F_c\epsilon R1\alpha$, which is expressed on CD2⁺ monocytes (Cheng and others 2006). BDCA-1 (CD1c) was previously found to be expressed at low levels on CD14^{lo/hi} $F_c\epsilon R1\alpha$ ⁺ monocytes whereas BDCA-2 and BDCA-3 was expressed only on CD14⁻ cells (Cheng and others 2006). A small proportion of classical monocytes express $F_c\epsilon R1\alpha$ compared to CD64, which is expressed by the majority of classical monocytes. In contrast, the intermediate monocyte subset expressed no $F_c\epsilon R1\alpha$ and much lower levels of CD64. Based on the expression of CD14, CD16 and $F_c\epsilon R1\alpha$, five monocyte subsets were defined. The following additional features of these subsets were analysed: expression of common myeloid markers, size, granularity and density.

Differences in cell surface markers demonstrated a particular profile for each subset and, on CD14^{hi} monocytes, the cells expressing $F_c\epsilon R1\alpha$ or CD16 had the same changes in expression of CD2, CD13, CD62L, CD38 and CD163 as was observed for CD64 defined subsets. Of interest was the surface profile of CD14^{hi} $F_c\epsilon R1\alpha$ ⁺ monocyte subset as, relative to all of the CD14^{hi} monocytes, this subset expressed the lowest amount of CD11b and CD13 and somewhat higher levels of CD33 and CD64; it has previously been shown that circulating PM have this cell surface phenotype – CD14^{hi}CD11b^{lo}CD13^{lo}CD33^{hi}CD64^{hi} (Clanchy and others 2006).

The expression and function of CD62L is necessary for the sequestration of monocytes within the circulation(Xu and others 2008), and murine and human monocyte subsets that are most strongly associated with the endothelium have the lowest expression(Kamei and Carman 2010). The reduction in CD62L expression, concurrent with a reduction in CD64 expression, is consistent with a process of maturation prior to egress from the circulation. Likewise, Figure 1(B) indicates a stepwise reduction in CD163 concordant with a reduction in CD64 expression. CD163 is present on cells of the monocyte/macrophage lineage, from progenitors through to terminally differentiated macrophages(Buechler and others 2000). Whilst in the pig(Fairbairn and others 2013) and mouse(Bain and others 2013) higher CD163 expression is found on more mature monocytes, expression of CD163 on human monocytes is lowest on the intermediate and non-classical subsets and comparatively higher on FcεRIα⁺ monocytes.

HLA-DR was expressed in a bi-modal fashion, with the lowest expression on monocytes defined as CD14^{hi}CD64^{med} or CD14^{hi}(FcεRIα/CD16)⁻ but higher on the other subsets. An increase in CD74 expression in the intermediate subset(Zawada and others 2011), compared to the classical subset, may account for the increased surface expression of HLA-DR, due to greater trafficking to the cell surface via this chaperone(Borghese and Clanchy 2011). Monocytes which have increased potential to acquire a DC phenotype(Crawford and others 1999) are found in the subpopulation of monocytes that express CD2 at low to intermediate levels. Although CD2⁺ cells have been described as DC precursors based upon this differentiation potential, another interpretation of the data supporting this is that they are merely less mature monocytes with greater differentiation potential than CD2⁻ monocytes. CD16⁻ monocytes, which include CD2⁺ monocytes, have higher osteoclastic potential than CD16⁺ monocytes(Komano and others 2006), which uniformly lack CD2(Fagnoni and others 2001), which suggests CD2⁺ monocytes have higher differentiation potential rather than DC-specific potential.

When comparing subsets defined by CD64/CD14 or FcεRIα/CD14/CD16, it is evident from the surface marker profiles that some CD14^{lo}FcεRIα⁺ cells would be present in the CD14^{lo}CD64^{lo} gate, indicating that the delineation of monocyte subsets by the expression of FcεRIα, CD14 and CD16 achieves comparatively superior deconvolution of monocyte heterogeneity. The incremental changes in surface markers were paralleled by changes in

physical properties. A putative transition of CD14^{lo/hi}FcεRIα⁺ monocytes to CD14^{hi}CD16⁺ monocytes appears concurrent with an increase in size and granularity; the differences in buoyancy between subsets were similar and may influence the phenotype of monocytes isolated by elutriation or aphaeresis. A substantial overlap between one or more subsets in buoyancy, size and granularity would strengthen the hypothesis that there is more than one monocyte 'lineage' (potentially derived from different precursors(Hanna and others 2011)) maturing in the circulation. The non-classical subset did not uniformly follow the trends of the other subsets in surface marker profile or physical properties, nor did it increase in proportion during short term culture, indicating that it may not be immediately contiguous with the intermediate subset; other researchers have identified features that support a developmental link between these subsets(Wong and others 2011). By comparison, the physical properties and surface profile of the CD14^{lo}FcεRIα⁺ monocyte subset appeared more congruous with a monocytic developmental continuum.

PM activity and high affinity Fc defined subsets

The correlation of circulating PM prevalence and classical monocytes with the highest CD64 expression confirmed a previous finding of this subset's increased proliferative potential(Clanchy and others 2006), and provides further evidence that CD64 expression is indicative of monocyte maturity on CD14^{hi} monocytes. The proportion of CD14^{hi}FcεRIα⁺ monocytes was more strongly associated with circulating PM prevalence. This is a refinement to the means by which circulating PM could be estimated, as the measurement of CD14^{hi}FcεRIα⁺ monocytes offers more precision than the relative expression of CD64 on the classical monocyte subset, compared to an isotype control. The use of a FACS-based metric for circulating PM also offers practical advantages over an eight day proliferation assay that requires monocyte isolation, although further testing in the steady state and disease would be necessary to confirm this association.

Monocyte subset transition in vitro

The expression of CD64, the high affinity receptor for IgG, has been used to classify monocytes with increased anti-viral and antigen presentation potential(Grage-Griebenow and others 1993; Grage-Griebenow and others 2001b); monocyte subsets defined by CD64 also have differences in phagocytosis and the generation of reactive oxygen species(Grage-

Griebenow and others 2000). An increase in CD64 expression on monocytes has been measured in autoimmune disease(Li and others 2009), and has also been suggested as indicative of enhanced innate immunity during pregnancy(Neuteboom and others 2011). The increase in CD64 expression during disease may be due in part to increased concentrations of circulating IFN γ , as this cytokine is known to up-regulate CD64 expression(Li and others 2009). Increased circulating levels of IFN γ have been associated with increased CD64 expression in AIDS(Capsoni and others 1994) and the effects of IFN γ , IL6 and IL-10 on total monocyte expression of CD64 have been evaluated in vitro(Chapuis and others 1997; Li and others 2009), with broadly similar effects to those described here.

In this study, two short term culture systems were used to measure monocyte subset transition from the classical to the intermediate subset. An increase in non-classical monocytes has been reported in whole blood cultures (Skinner and others 2005) however this was not observed in the current study, despite the presence of a TLR ligand (Fig. 6(E-G)). The presence of GM-CSF and IFN γ appeared to attenuate the transition from classical to intermediate subset compared to cells cultured in medium alone. Although the changes in monocyte phenotype in response to IFN γ and other mediators have been reported(Buckle and others 1990; Firestein and Zvaifler 1987; Jayaram and others 1989) changes in phenotype with regard to monocyte subsets have not been adequately detailed.

CD64 expression on each canonical subset differed after culture according to the presence of each cytokine. It was determined that GM-CSF and IFN γ caused an attenuation of monocyte subset transition although IFN γ (and IL-10, to lesser extent) appeared to maintain or increase CD64 expression on monocytes with a classical subset phenotype after culture, whereas GM-CSF did not. After culture, monocytes with the intermediate and non-classical subset phenotype had increased expression of CD64 in the presence of IFN γ and IL-10. Stimulation with IFN γ (and to a lesser extent IL-10) permitted supra-physiological CD64 expression on the monocytes with a CD16⁺ intermediate and non-classical subset phenotype after culture.

Monocytes with a classical subset phenotype after culture in medium alone express CD64 at a level that is equivalent to circulating intermediate monocytes (Figure 5(E)) whereas intermediate monocyte CD64 expression was unchanged by culture in medium alone. The transition from classical to intermediate monocyte phenotype was also observed in whole

blood cultures without co-expression of FcεRIα and CD16; a definitive change was also observed using sorted CD14^{hi}FcεRIα⁺ monocytes which became CD16⁺. Although monocyte subsets in culture appear to progress from the classical subset to the intermediate subset, further analyses will be required to confirm freshly isolated intermediate subset monocytes are phenotypically similar with those derived in vitro, as has been demonstrated by other studies (Calzada-Wack and others 1996; Randolph and others 2002). Nevertheless, these data suggest, for classical monocytes, that the reduction in the expression of the high affinity Fcε receptors CD64 and FcεRIα is a condition precedent to subset transition in the steady state.

Conclusion

It was determined that several key myeloid surface markers are co-expressed at similar levels depending on CD64 expression. Five subsets defined by the expression of CD14, FcεRIα and CD16 were characterised and found to differ in surface phenotype and physical properties (size, granularity and buoyancy). The expression of CD64 on the classical subset and the prevalence of the CD14^{hi}FcεRIα⁺ monocyte subset correlated with the prevalence of circulating PM. These findings indicate that the decline in the expression of high affinity Fcε receptors on monocytes is accompanied by changes in cell surface expression and a degree of maturation which is evidenced by a reduction in proliferative potential. Further characterisation of FcεRIα⁺ monocytes may reveal a mechanism for increased proliferative potential. Although these data indicate FcεRIα⁺ monocytes are antecedent to the canonical classical and intermediate monocyte subsets, further functional analyses are required to determine whether these “pre-classical” monocytes require separate classification within the current nomenclature.

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Figure legends

Figure 1. Relative CD64 expression is indicative of a maturing monocyte surface profile

(A) Gating on monocytic cells within PBMC, four subsets were defined by the expression of CD14 and CD64 – (a) CD14^{hi}CD64^{hi}, (b) CD14^{hi}CD64^{med}, (c) CD14^{hi}CD64^{lo} and (d) CD14^{lo}CD64^{lo}. (B) Cell surface phenotype of monocyte subsets, as defined in (A); mean±SEM, n=8 donors. Statistically significant differences in expression between subsets are indicated in the matrices below each column (* p<0.05, ANOVA with Newman-Keuls Multiple Comparison post hoc analysis).

Figure 2. Five human monocyte subsets defined by the expression of CD14, FcεRIα and CD16

Within monocytic cells (based on FSC vs SSC gating of PBMC), subsets were defined by the expression of (A) CD14 then (B, C) FcεRIα^{+/++} and CD16^{+/++} monocytes are divided based on CD14 expression; CD14^{hi}(FcεRIα/CD16)⁻ monocytes were defined by high expression of CD14 and lack FcεRIα and CD16. (D) Cell surface phenotype of five human monocyte subsets, as defined in (A-C). Statistically significant differences in surface marker expression between subsets are indicated in the matrices below (mean±SEM, p<0.05, ANOVA with Newman-Keuls Multiple Comparison post hoc analysis, n=8 donors).

Figure 3. Prevalence, size, granularity and buoyancy of monocyte subsets

(A) The proportion of each subset within monocytic cells was determined; n=17 donors. (B) The size and granularity of 5 human monocyte subsets; axis labels a-e in (A) correspond to those in the magnified inset. Data are means±SEM of 8 donors, with each donor the mean of ≥ 10 replicates; statistically significant differences between subsets are indicated in the matrices (ANOVA with Newman-Keuls Multiple Comparison post hoc analysis, p<0.05). (C) Schematic illustration of density separation result. 'High' and 'Low' indicate interstices of differing isosmotic Percoll/PBS solutions; see Supplementary Figure 1 for further details. (D) Proportions of CD14^{hi} monocytes in the pellet and low or high density Percoll interstices, compared to unseparated PBMC (Unsep); Data are means±SEM. (E) Prevalence of each subset in the low density and high density interstices expressed relative to unseparated cells from the same donor. Data are means±SEM for five donors; *, p<0.05, high vs low density, Student's t-test. Relative buoyancy was defined as the high density values for each subset

expressed relative to the low density values; statistically significant differences between subsets are indicated in the matrices (ANOVA with Newman-Keuls Multiple Comparison post hoc analysis, $p < 0.05$). (F) For the five subsets, the relative subset buoyancy values (for the subsets in Figure 5(E)) correlate with the forward scatter values (an approximation of cell size) from Figure 5(B); Pearson correlation $R = 0.954$, $p = 0.0119$.

Figure 4. Circulating PM prevalence is associated with high affinity FcR expression

(A) For each donor, the prevalence of PM was determined using sorted $CD14^{hi}$ monocytes in a BrdU incorporation assay. (B) The expression of CD64 on each monocyte subset at day 0 was correlated with PM prevalence as measured by BrdU assay using sorted cells; R^2 values are correlations derived from the 2-tailed Pearson correlation coefficient ($n = 10$ donors). (C) Sorted $CD14^{hi}Fc\epsilon RI\alpha^+$, $CD14^{hi}Fc\epsilon RI\alpha^-$ and $CD14^{hi}$ monocytes were assayed for proliferation using the BrdU assay; mean $\pm$ SEM, * $p < 0.05$, Student's t-test, $n = 5$ donors. (D) Proportion of $BrdU^+$ cells from each subset, relative to $CD14^{hi}$ monocytes for each donor; mean $\pm$ SEM, * $p < 0.05$, Student's t-test, $n = 5$ donors. (E) The prevalence of PM within $CD14^{hi}$ monocytes, as measured by BrdU assay using sorted cells, correlated with the proportion of cells in each subset at day 0; R values are correlations derived from the 2-tailed Pearson correlation coefficient ($n = 11$ donors).

Figure 5. Monocyte subset CD64 expression is modulated uniformly after cytokine stimulation

Histograms detailing CD64 expression on monocytes in the (A) $CD14^{hi}CD16^-$, (B) $CD14^{hi}CD16^+$ and (C) $CD14^{lo}CD16^{++}$ monocyte subsets before and after 20 h of cytokine stimulation in PBMC cultures; filled and unfilled histograms are isotype control and CD64 staining, respectively, for each subset. The relative expression of CD64 was measured on (D) $CD14^{hi}CD16^-$, (E) $CD14^{hi}CD16^+$ and (F) $CD14^{lo}CD16^{++}$ monocyte subsets before and after stimulation with medium alone (Nil), IL-6, IL-10, M-CSF, GM-CSF and interferon- γ (IFN); statistical differences compared to Nil treatment were determined (* $p < 0.05$ vs Nil treatment, ANOVA with Dunnett's post hoc analysis, $n = 7$ donors).

Figure 6. Monocyte subset transition is modulated by cytokine stimulation

(A) The proportion of each monocyte subset was determined for (B) CD14^{hi}CD16⁻, (C) CD14^{hi}CD16⁺ and (D) CD14^{lo}CD16⁺⁺ monocytes before and after 20 h stimulation with medium alone (Nil), IL-6, IL-10, M-CSF, GM-CSF and interferon- γ (IFN); statistical differences compared to Nil treatment were determined (* $p < 0.05$ vs Nil treatment, ANOVA with Dunnett's post hoc analysis, $n = 7$ donors). The proportion of monocytes that were (E) CD14^{hi}CD16⁻, (F) CD14^{hi}CD16⁺ and (G) CD14^{lo}CD16⁺⁺ was measured before and after 20 h whole blood cultures $\pm$ LPS; statistical differences compared to Nil treatment were determined (* $p < 0.05$ vs Nil treatment, ANOVA with Dunnett's post hoc analysis, $n = 8$ donors).

Figure 7. Loss of Fc ϵ R1 α precedes CD16 expression in WBC

(A, B) CD14^{hi} monocytes increase expression of CD16 (C) after 20 hours of WBC; dashed lines on the histograms indicate isotype controls. (D) For 7 donors the 6.2-fold mean increase in expression was statistically significant (Student's t-test, $p = 0.021$). Gating on CD14^{hi} monocytes, the expression of Fc ϵ R1 α and CD16 was assessed on (E) freshly isolated PBMC and (F) PBMC isolated from 20 hour WBC; (G) the differences in isotype control staining should be noted. (H) The percentage of CD14^{hi} monocytes expressing Fc ϵ R1 α , CD16 or neither was determined for five donors; X-axis labels (α , β , γ , δ) in (H) correspond to the quadrant markers in (E); p values for changes in α , β , γ quadrants are 0.115, 0.040 and 0.008 respectively (Student's t-test, $n = 5$ donors). Quadrant markers in (E, F) correspond to equivalent subsets in (J, K). Sorted monocytes labelled with CFSE were added to WBC, the PBMC from which were isolated after culture and the phenotype examined. (I) Gating on CFSE-labelled cells, the expression of CD14 and CD16 was determined on sorted (J) CD14^{hi} and (K) CD14^{hi}Fc ϵ R1 α ⁺ monocytes that had been cultured for 12 hours in WBC; quadrant markers were set according to isotype control staining. (L) The proportion of CD16⁻ (α/β) and CD16⁺ (γ) monocytes was determined for monocytes that were originally CD14^{hi} ((α , β and γ) $n = 4$ donors) or CD14^{hi}Fc ϵ R1 α ⁺ ((α) $n = 7$ donors).

Authorship and disclosures

FILC conceived, designed and performed research, analysed data and wrote the paper. There are no conflicts of interest to declare.

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4

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□ a: CD14^{hi}CD64^{hi}
 ■ b: CD14^{hi}CD64^{med}
 ■ c: CD14^{hi}CD64^{lo}
 ■ d: CD14^{lo}CD64^{lo}

B

$$A_{low} = \frac{Y_{\%subset}}{X_{\%subset}} \quad B_{high} = \frac{Z_{\%subset}}{X_{\%subset}}$$

C

$$Relative\ subset\ bouyancy_{subset} = \frac{A_{low}}{B_{high}}$$

Supplementary Figure 1

Supplementary Figure 1. Determination of the relative density of human monocyte subsets

(A) PBMC suspended in PBS were layered over a discontinuous Percoll gradient and centrifuged as described in Methods and Materials. The proportion of each subset within total monocytes was determined before centrifugation (X%). After centrifugation the proportion of subsets was determined at the Low density interface (Y%) and the High density interface (Z%). (B) To determine relative subset proportion, the prevalence of each subset was determined at both interfaces (Y or Z) and expressed relative to the starting subset proportion (X). (C) Relative subset buoyancy was calculated by dividing the relative subset proportion of the Low density interface by the High density relative subset proportion.

Supplementary Figure 2

Supplementary Figure 2. Determination of the relative effects of cytokine-supplemented medium on monocyte viability. After 20 hours of (A) PBMC cultures or (B) whole blood cultures (see Materials and Methods and Figures 5, 6 and 7), the viability of single-cell events gated on (i) monocytic cells was measured by (ii) propidium iodide; representative FACS plots. (C) Viability of (i) CD14^{+/++} monocytic cells in (ii) PBMC cultures as determined by propidium iodide. There were no significant differences between day 0 for any condition (ANOVA with Dunnett's post hoc test) although variation was greater in cultures containing GM-CSF and IFN γ .