A Single-Cell Gene-Expression Profile Reveals Inter-Cellular Heterogeneity within Human Monocyte Subsets

Susanne T. Gren¹,², Thomas B. Rasmussen³, Sabina Janciauskiene⁴, Katarina Håkansson¹, Jens G. Gerwien⁵, Olof Grip²*

¹ Cellular Pharmacology, Novo Nordisk A/S, Måløv, Denmark, ² Department of Clinical Sciences Malmö, Lund University, Malmö, Sweden, ³ Clinical Immunology, Copenhagen University Hospital, Copenhagen, Denmark, ⁴ Department of Respiratory Medicine, Hannover Medical School, Hannover, Germany, ⁵ Global Biobanking Management, Novo Nordisk A/S, Måløv, Denmark

* olof.grip@med.lu.se

Abstract

Human monocytes are a heterogeneous cell population classified into three different subsets: Classical CD14++CD16−, intermediate CD14++CD16+, and non-classical CD14+CD16++ monocytes. These subsets are distinguished by their differential expression of CD14 and CD16, and unique gene expression profile. So far, the variation in inter-cellular gene expression within the monocyte subsets is largely unknown. In this study, the cellular variation within each human monocyte subset from a single healthy donor was described by using a novel single-cell PCR gene-expression analysis tool. We investigated 86 different genes mainly encoding cell surface markers, and proteins involved in immune regulation. Within the three human monocyte subsets, our descriptive findings show multimodal expression of key immune response genes, such as CD40, NFⱪB1, RELA, TLR4, TLR8 and TLR9. Furthermore, we discovered one subgroup of cells within the classical monocytes, which showed alterations of 22 genes e.g. IRF8, CD40, CSF1R, NFⱪB1, RELA and TNF. Additionally one subgroup within the intermediate and non-classical monocytes also displayed distinct gene signatures by altered expression of 8 and 6 genes, respectively. Hence the three monocyte subsets can be further subdivided according to activation status and differentiation, independently of the traditional classification based on cell surface markers. Demonstrating the use and the ability to discover cell heterogeneity within defined populations of human monocytes is of great importance, and can be useful in unravelling inter-cellular variation in leukocyte populations, identifying subpopulations involved in disease pathogenesis and help tailor new therapies.

Introduction

Blood monocytes are a heterogeneous population of innate immune leukocytes. They are involved in the innate immune response to pathogens by phagocytosis, the release of reactive oxygen species, cytokines and chemokines and by antigen presentation, thereby modulating and activating cells within the adaptive immune system [1]. The diversity within the human
blood monocyte subpopulations has become evident in recent years. Based on the differential expression of the co-receptor to lipopolysaccharide (LPS) CD14 and the Fcγ receptor (FcγR)-III CD16, human monocytes can be divided into different subpopulations [2]. First, two subpopulations were recognized, namely the CD14⁺CD16⁻ and the CD14⁺CD16⁺ monocytes [3], that were shown to have distinct biological functions [4] and a proportional increase of the CD14⁺CD16⁺ monocyte subset were seen in a variety of chronic and inflammatory diseases [5–8]. Thus, later it became evident that the CD16⁺ monocytes could be further divided into two subsets according to the level of CD14 expression. Three monocyte subpopulations have now been identified and characterized in humans [9], whereas two subsets are identified according to the expression of GR1 and Ly6C in mice [2]. The human monocytes have been given the following notation: Classical (CD14⁺⁺CD16⁻), Intermediate (CD14⁺⁺CD16⁺) and Non-classical (CD14⁺⁺CD16⁺⁺) monocytes [10]. Classical and intermediate monocytes are shown to be homologs to the mouse Gr⁺Ly6C⁺, whereas the non-classical monocytes resemble the mouse Gr⁻Ly6C⁻ monocytes [9]. The heterogeneity within monocytes has been unravelled by the expression of cell surface markers and by using gene expression profiling. Human classical monocytes express a diversity of genes that favours their involvement in migration, bacterial sensing, phagocytosis, immune responses and many pro-inflammatory genes, which support their role in inflammation. In contrast, intermediate monocytes display genes that account for a profile that is more prone to antigen-presenting [11] whereas genes up-regulated in non-classical monocytes are mainly involved in patrolling, sensing of nucleic acids and viruses [9].

Several studies have implied that LPS-stimulated intermediate and non-classical monocytes are the most pro-inflammatory among the three subsets [9,11]. We have previously shown that the classical monocytes are the most pro-inflammatory in regard to cytokine secretion and MMP release when stimulated with LPS and immune complexes [12]. This is in agreement with the high expression of CD14 and the FcγR I CD64 [11,12]. In relation to disease pathogenesis, the subdivision of CD16⁺ monocytes showed that in chronic and autoimmune diseases, for example Crohn’s disease (CD), the intermediate monocytes were expanded in the peripheral blood in patients with active inflammation [7,12–14], whereas the classical subset was decreased [12]. However, the role of the classical, intermediate and non-classical human monocytes in health and disease has not been fully elucidated.

Previous gene-expression profiling has distinguished the three monocyte subsets and addressed mechanisms of transcriptional regulation and differential functional genes [11,15,16]. However, an increasing body of evidence indicates that cell subpopulations can be comprised of cells with distinct gene expression profiles, though this is masked when using techniques such as micro-array analysis. To the best of our knowledge, no studies have performed single-cell gene expression analysis of the three monocyte subsets, a technique that can elucidate the inter-cellular heterogeneity. Here, we investigated the three monocyte subsets using the single-cell gene analysis technique from Fluidigm. We investigated 86 selected genes, involved mainly in adhesion, migration, phagocytosis, tissue remodelling and immune functionality, in order to obtain a greater knowledge of the heterogeneity within each monocyte subset. Our results showed a hitherto unknown inter-cellular variation within the three human monocyte subsets, which other techniques such as micro-array analysis and flow cytometry are unable to identify.

Results

Gene expression profile

Live, single cells within the three human monocyte subsets were single-cell-sorted according to forward and side scatter, and the expression of the cellular surface markers CD14 and CD16 (Fig 1A). Cellular lysates from 94 classical monocytes, 92 intermediate monocytes and 90 non-
classical monocytes were converted to cDNA and amplified with 85 target genes (Table 1). The 85 genes analysed were chosen based on differential expression shown in earlier micro-array results [11,15], and by their relation to cell function, as well as biological or immune regulated processes. The genes were plotted into a PCA score plot showing that the single-sorted cells were clustered according to their subset, classical, intermediate or non-classical, verifying that the cells were sorted correctly (Fig 1B). The expression of each gene was analysed using the SINGuLAR R package. No signal in the Fluidigm qPCR were interpreted as expression below detection limit and referred to as non-expression. We found a differential expression of 80 genes within the 3 subgroups of monocytes (Fig 1C and Table 2). Five of the genes, CCR7, MMP12, MRC1, ULBP1 and ULBP2 were not expressed by any of the subsets. Four genes, CCR2, CD163, CLEC4E, and SERPINB2 were exclusively not expressed by the non-classical subset. Expression of IL6 was not detectable in the classical subset and very low expression was observed in the intermediate and non-classical subset. MMP9 was weakly expressed by the classical and non-classical monocytes and no expression was found in the intermediate subset. TLR3 was not expressed by the classical and intermediate, but weakly by the non-classical monocytes, whereas TREM2 was weakly expressed by the classical subset but not by the intermediate and non-classical subsets. Expression of CCR5 and RAET1G was found only in the intermediate monocytes. These data show that there is great diversity within the three monocyte subsets and that the subsets have a differential gene expression profile with regard to the genes investigated in this study.

Comparison of gene expression investigated by single-cell PCR gene expression analysis versus micro-array

Previous microarray data have shown that genes within diverse cellular and immunologic processes are differentially expressed within the three subsets. We therefore wanted to validate our gene expression data generated by Fluidigm (Table 2) with existing array data. We compared our single cell gene-expression data to current expression profiles published by Wong et al. by looking at the general expression pattern since data have not been derived using the same methods. In agreement with the study conducted by Wong et al., the classical monocytes showed the highest expression of CCR1, CCR2, CD14, CD163, CD36, SELL, and SERPINB2 whereas the intermediate showed highest expression of CD40, CD74, HLA-DRA, MARCO and TIMP, and the non-classical showed the highest expression of C1QA, ITGAL, and SIGLEC10. However, our data deviated from Wong et al., by showing the highest expression of CTSL and HMOX1 by the intermediate monocytes and CX3CR1 by the classical monocytes, which were found by Wong et al. to be most highly expressed by the non-classical subset.

Functional characteristics of the three subsets

To investigate unique characteristics of each monocyte subset, the genes examined were grouped into categories based on functionality (Table 3). The classical monocytes were shown
Table 1. Genes of interest investigated in the three monocyte subsets.

| Gene symbol | Gene title                                      | Protein function |
|-------------|-------------------------------------------------|------------------|
| ACTB        | Actin beta                                      | Conserved motif, ubiquitously expressed in all eukaryotic cells |
| ADAM17      | ADAM Metallopeptidase Domain 17                | Cleave membrane-bound precursor TNF-α |
| BAX         | BCL2-associated X protein                       | Apoptotic activator |
| BCL2        | B-cell lymphoma 2                               | Apoptotic suppressor |
| BCL2L1      | BCL2-like1                                      | Inhibits activation of caspases |
| C1QA        | Complement component, q subcomponent, A chain  | First component of complement system |
| CCR1        | Chemokine (C-C motif) receptor 1               | Recruitment of effector cells to sites of inflammation |
| CCR2        | Chemokine (C-C motif) receptor 2               | Mediates monocyte chemotaxis |
| CCR5        | Chemokine (C-C motif) receptor 5               | Receptor for a number of inflammatory CC-chemokines |
| CCR7        | Chemokine (C-C motif) receptor 7               | Migration of memory T cells and stimulate DC maturation |
| CCR9        | Chemokine (C-C motif) receptor 9               | thymocyte recruitment and development, localization to the gastrointestinal tract |
| CD14        | CD14 molecule                                   | Innate immune responses to bacterial lipopolysaccharide |
| CD163       | CD163 molecule                                  | Phagocytosis of hemoglobin/haptoglobin complexes |
| CD209       | CD209 molecule                                  | C-type lectin, pathogen recognition receptor and cell adhesion |
| CD33        | CD33 molecule                                   | Sialic acid adhesion molecule |
| CD36        | CD36 molecule                                   | Receptor for thrombospondin, function in cell adhesion |
| CD40        | CD40 molecule                                   | Receptor for CD40L, co-stimulatory molecule, T and B cell activation |
| CD68        | CD68 molecule                                   | Intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interaction |
| CD74        | CD74 molecule                                   | Invariant chain, regulates antigen presentation |
| CD93        | CD93 molecule                                   | Intercellular adhesion and clearance of apoptotic cells |
| CLEC4E      | C-type lectin domain family 4, member E         | Recognise pathogenic fungi |
| CSF1R       | Colony stimulating factor 1 receptor           | Regulates survival, proliferation and differentiation of hematopoietic precursor cells, promote release of inflammatory cytokines in response to CSF1 |
| CST3        | Cystatin C                                      | Inhibitor of cysteine proteases |
| CTSB        | Cathepsin B                                     | Intracellular degradation and turnover of proteins |
| CTSK        | Cathepsin K                                     | Bone remodelling, endoprotease activity against fibrinogen |
| CTSN        | Cathepsin L                                     | Degradation of proteins in lysosomes |
| CTSS        | Cathepsin S                                     | Degradation and turnover of proteins, maturation MHC II complex |
| CX3CR1      | Chemokine (C-X3-C motif) receptor 1             | Receptor for fraktalkine, mediates adhesion and migration |
| FCGR3A      | Fc gamma receptor IIIA                          | Receptor for Fc region of immunoglobulin G |
| HLA-DRA     | Major histocompatibility complex, class II, DR alpha | Alpha chain of HLA-DR class II complex, antigen presentation |
| HLA-DRB1    | Major histocompatibility complex, class II, DR beta | Beta chain of HLA-DR class II complex, antigen presentation |
| HMOX1       | Heme oxygenase (Decycling) 1                   | Cleave the heme ring, to form biliverdin |
| IL10        | Interleukin 10                                  | Immune regulation, inhibits number of cytokines, enhances cell survival and proliferation |
| IL12A       | Interleukin 12A                                 | Act as growth factor for T cells and NK cells, stimulate production of IFN-gamma by T cells |
| IL15        | Interleukin 15                                  | Stimulate proliferation of T cells |
| IL18        | Interleukin 18                                  | Stimulate IFN-gamma production by T cells, augments NK cell activity |
| IL1B        | Interleukin 1beta                               | Inflammatory mediator, cell proliferation, differentiation, and apoptosis |
| IL23A       | Interleukin 23A                                 | Stimulate memory T cells, stimulates production of IFN-gamma active response to infection |
| IL6         | Interleukin 6                                   | Induce acute phase response. Leukocyte differentiation |
| IFN4        | Interferon regulatory factor 4                  | Lymphocyte specific transcriptional activator that regulates TLR signalling |
| IFR5        | Interferon regulatory factor 5                  | Transcriptional activator that regulates TLR7 and TLR9 signalling |
| IFR8        | Interferon regulatory factor 8                  | Binds to the upstream region of type I IFN and IFN-inducible MHC class I genes |

(Continued)
| Gene symbol | Gene title               | Protein function                                                                 |
|-------------|--------------------------|-----------------------------------------------------------------------------------|
| ITGAE       | Integrin Alpha E (CD103 molecule) | Intergrin alpha E/beta-7 is a receptor for E-cadherin                             |
| ITGAL       | Integrin Alpha L (Antigen CD11A) | Intercellular adhesion through interaction with ICAM1-3, leukocyte-endothelial cell interaction, antibody dependent killing by monocytes and granulocytes |
| ITGAM       | Integrin Alpha M (CD11B)     | Adherence to stimulated endothelium, receptor for IC3B fragment and mediates uptake of complement-coated particles |
| ITGAX       | Integrin Alpha X (CD11C)     | Receptor for fibrinogen, mediates cell-cell interaction, monocyte adhesion and chemotaxis |
| LTB         | Lymphotoxin beta           | Type II membrane protein of TNF family, inducer of inflammatory response          |
| MARCO       | Macrophage receptor with collagenous structure | Class A scavenger receptor, pattern recognition receptor that binds gram positive and negative bacteria |
| MICA        | MHC class I polypeptide-related sequence A | Antigen presentation, ligand for NKG2D, mediates cell lysis                     |
| MICB        | MHC class I polypeptide-related sequence B | Antigen presentation, stress induced self-antigen, ligand for NKG2D, mediates cell lysis |
| MMP1        | Matrix metalloprotease 1    | Degrade extracellular matrix, collagenase                                          |
| MMP12       | Matrix metalloprotease 12   | Degrade extracellular matrix, Elastin                                             |
| MMP2        | Matrix metalloprotease 2    | Degrade extracellular matrix, gelatinase                                           |
| MMP3        | Matrix metalloprotease 3    | Degrade extracellular matrix, stromelysin                                         |
| MMP7        | Matrix metalloprotease 7    | Degrade extracellular matrix, Matrylsin, involved in wound healing, regulates activity of defensins |
| MMP9        | Matrix metalloprotease 9    | Degrade extracellular matrix, gelatinase                                           |
| MRC1        | Mannose receptor C type 1   | Endocytosis of glycoproteins, phagocytic receptor for bacteria and fungi          |
| MSR1        | Macrophage scavenger receptor 1 | Class A scavenger receptor, endocytosis of low density lipoproteins             |
| NFKB1       | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | Pleiotropic transcription factor, essential immune regulator                     |
| PTPRC       | Protein tyrosine phosphatase receptor type C (CD45) | Signalling molecule that regulates cell growth, differentiation and mitosis, co-stimulatory molecule required for T cell activation |
| RAET1E      | Retinoic acid early transcript 1E | Ligand for NKG2D, activates NK cells                                             |
| RAET1G      | Retinoic acid early transcript 1G | Ligand for NKG2D, mediates NK cell cytotoxicity                                  |
| RAET1L      | Retinoic acid early transcript 1L | Ligand for NKG2D, antigen binding                                                |
| RARA        | Retinoic acid nuclear receptor alpha | Receptor for retinoic acid                                                        |
| RELA        | v-rel avian reticuloendotheliosis viral oncogene homolog A | Subunit of the NFκB complex, Pleiotropic transcription factor, essential immune regulator |
| S100A4      | S100 calcium binding protein A4 | Regulation of motility and tubulin polymerization                                 |
| SELL        | Selectin L (CD62L)          | Cell surface adhesion gene, C-type lectin, rolling of leukocytes on endothelial cells |
| SERPINA1    | Serpin peptidase inhibitor, Clade A | Serine protease inhibitor, primary target is elastase                            |
| SERPNB1     | Serpin peptidase inhibitor, Clade B | Inhibits urokinase-type plasminogen activator                                    |
| SIGLEC10    | Sialic acid binding Ig-like lectin 10 | Member of immunoglobulin superfamily, mediates sialic-acid dependent binding to cells, inhibitory receptor in the immune response |
| SIRPA       | Signal regulatory protein alpha | Immunoglobulin-like cell surface receptor for CD47, Mediates negative regulation of phagocytosis, prevents maturation of immature DC |
| TIMP1       | Tissue inhibitor of metalloproteinases 1 | Complexes with metalloproteinases and irreversibly inactivates them              |
| TLR2        | Toll-like receptor 2        | Mediates immune responses to bacterial lipopolysaccharides and other cell wall components |
| TLR3        | Toll-like receptor 3        | Mediates immune responses to virus by sensing double stranded RNA                |
| TLR4        | Toll-like receptor 4        | Mediates immune responses to bacterial lipopolysaccharide                        |
| TLR7        | Toll-like receptor 7        | Mediates immune responses to virus by sensing single stranded RNA                |
| TLR8        | Toll-like receptor 8        | Mediates immune responses to virus by sensing double-stranded RNA                |
| TLR9        | Toll-like receptor 9        | Mediates immune responses to bacteria by sensing unmethylated CpG dinucleotides  |
| TNF         | Tumor necrosis factor alpha | Proinflammatory cytokine that stimulates cell proliferation, differentiation, apoptosis, cytokine production and causes fever |
| TNFSF15     | Tumor necrosis factor superfamily member 15 | Ligand for decoy receptor (DR3) and DR6, activates NFκB and MAPK, promotes activation of caspases and apoptosis |
to have the highest expression of CD93, CD209, CLEC4E, which are genes involved in pathogen recognition and phagocytosis. Also genes encoding proteins involved in migration and adhesion such as the chemokine receptors, CCR1, CCR2, CCR9, CX3CR1, ITGAM and SELL were most highly expressed by the classical monocytes. The expression of genes encoding scavenger receptors was mostly expressed by the classical and intermediate monocytes. As shown earlier, the intermediate cells had the highest expression of genes involved in co-stimulation and antigen presentation, but also genes involved in NK cell and CD8 T cell activation, such as MICB, RAET1E, RAET1G, RAET1L and ULBP3 were enriched on intermediate monocytes. Furthermore, we also found that the intermediate monocytes had the highest expression of genes involved in cell differentiation and cell function, e.g. the genes encoding the transcription factors IRF5 and IRF8, the NFκB1 and RELA genes involved in heterodimer formation of the central immunologic regulatory transcription factor NFκB, and CSF1R where the encoded protein controls cell differentiation. Also, genes encoding the cytokines IL1β, IL12A, IL18 and IL23A, proteases, such as cathepsins and MMPs, and protease inhibitors, CST3, TIMP1 and SERPINA1 were highly expressed by the intermediate monocytes. In contrast, the non-classical monocytes showed the highest expression of TNF and the metalloprotease ADAM17 gene, which are involved in the processing of TNF from the cell surface. Low expression of genes involved in bacterial phagocytosis were found in the non-classical monocyte subsets, thus they had the highest expression of C1QA, a complement component, and the FcγR3A involved in antibody-mediated phagocytosis. Genes encoding the TLR8 and 9 proteins, which are classical, innate pattern recognition receptors (PRR) were also highly expressed by the non-classical monocytes.

**Detection of inter-cellular variation**

In contrast to previous gene expression profiling studies [9,11,15], the aim of the current study was to investigate the biological genetic variability within the monocyte subsets by using the Fluidigm single-cell gene expression tool. The probes used for the PCR amplification have been tested with DNA, and were able to amplify and detect transcripts. This technique is therefore able to assess selected genes within the individual cells, and investigate possible multimodal gene expression within cell populations. Using a Hartigans dip test, we identified genes within the three subsets that had multimodal expression (Table 4, and Fig 2). Within the classical, intermediate and non-classical subsets, 37, 39 and 36 genes, respectively showed multimodal expression (Table 4). Some of the genes showed a general multimodal expression in all three subsets such as the co-stimulatory molecule CD40, the scavenger receptor MARCO, the adhesion molecules CD33, CD93 and CX3CR1, the apoptosis genes BAX and BCL2, the genes encoding the cytokines IL12A, IL15 and IL23A, TLR8 and TLR9 and the proteases, CTSK,
| Gene symbol | Gene expression values in monocyte subsets | T-test values |
|-------------|------------------------------------------|--------------|
|             | Classical | Intermediate | Non-classical | C vs I | C vs NC | I vs NC |
| ACTB        | 16.3      | 16.9         | 16.6         | 1.40E-09 | 2.40E-03 | 4.20E-03 |
| ADAM17      | 5.9       | 5.8          | 6.6          | 9.50E-01 | 3.00E-01 | 2.60E-01 |
| BAX         | 5.2       | 7.4          | 6.4          | 9.90E-04 | 8.90E-02 | 1.20E-01 |
| BCL2        | 2.6       | 3            | 2.5          | 4.80E-01 | 8.30E-01 | 3.60E-01 |
| BCL2L1      | 0.8       | 2.4          | 2.1          | 3.30E-03 | 1.80E-02 | 5.90E-01 |
| C1QA        | 0.1       | 2.7          | 3.1          | 3.80E-07 | 5.20E-09 | 5.90E-01 |
| CCR1        | 3.6       | 3.1          | 1.2          | 3.60E-01 | 1.70E-05 | 4.70E-04 |
| CCR2        | 1.4       | 0.4          | 0            | 9.50E-03 | 9.40E-05 | 4.60E-02 |
| CCR5        | 0         | 1            | 0            | 1.80E-03 | 2.30E-03 |            |
| CCR7        | 0         | 0            | 0            |            |            |            |
| CCR9        | 0.4       | 0.1          | 0.1          | 1.70E-03 | 1.70E-02 | 3.90E-01 |
| CD14        | 11.7      | 7.3          | 4.6          | 8.80E-17 | 3.40E-32 | 7.70E-05 |
| CD163       | 5.4       | 1.3          | 0            | 1.30E-09 | 8.30E-19 | 2.40E-04 |
| CD209       | 1.1       | 0.7          | 0.4          | 1.20E-01 | 3.80E-03 | 2.10E-01 |
| CD33        | 9.3       | 8            | 5.5          | 1.70E-02 | 1.50E-09 | 1.20E-04 |
| CD36        | 10.3      | 7.1          | 1            | 1.60E-06 | 1.90E-45 | 1.00E-18 |
| CD40        | 6         | 7.4          | 5            | 4.00E-02 | 1.50E-01 | 5.30E-04 |
| CD68        | 14.2      | 14.9         | 13.8         | 4.90E-10 | 2.40E-01 | 1.70E-03 |
| CD74        | 14.9      | 16.2         | 14.6         | 1.30E-16 | 2.80E-02 | 2.80E-23 |
| CD93        | 6.8       | 5            | 2.3          | 5.60E-03 | 8.10E-12 | 3.70E-05 |
| CLEC4E      | 2.2       | 0.3          | 0            | 5.40E-05 | 1.30E-06 | 9.70E-02 |
| CSF1R       | 5.4       | 10.2         | 8.9          | 2.80E-13 | 3.70E-07 | 9.70E-03 |
| CST3        | 13.4      | 14.3         | 12.7         | 3.60E-17 | 1.60E-02 | 1.60E-08 |
| CTSB        | 11.5      | 12.4         | 11.1         | 4.00E-02 | 5.00E-01 | 1.80E-03 |
| CTSK        | 2.5       | 2.4          | 1.7          | 9.60E-01 | 1.60E-01 | 1.70E-01 |
| CTSL        | 0.3       | 5.2          | 3.7          | 1.20E-13 | 4.80E-09 | 6.30E-02 |
| CTSS        | 14.7      | 14.3         | 13.8         | 2.60E-05 | 1.50E-08 | 5.00E-04 |
| CX3CR1      | 5.7       | 5.1          | 5            | 1.90E-01 | 1.20E-01 | 7.80E-01 |
| FCGR3A      | 8.1       | 11.9         | 12.3         | 1.00E-13 | 3.60E-20 | 1.50E-01 |
| HLA-DRA     | 13.2      | 14.7         | 12.4         | 4.00E-15 | 2.00E-02 | 1.70E-11 |
| HLA-DRB1    | 5.4       | 0.9          | 2            | 1.00E-09 | 2.00E-05 | 5.30E-02 |
| HMOX1       | 8.9       | 12.2         | 12           | 3.60E-10 | 5.00E-08 | 4.60E-01 |
| IL10        | 2.4       | 1.2          | 0.1          | 2.30E-02 | 1.50E-06 | 1.40E-03 |
| IL12A       | 5.8       | 7.6          | 7            | 1.60E-03 | 3.80E-02 | 3.40E-01 |
| IL15        | 2.2       | 2.5          | 2.9          | 6.60E-01 | 2.70E-01 | 5.00E-01 |
| IL18        | 2.7       | 2.9          | 0.9          | 7.50E-01 | 1.80E-03 | 4.40E-04 |
| IL1B        | 1.7       | 3.3          | 1.1          | 3.40E-02 | 2.60E-01 | 1.80E-03 |
| IL23A       | 3.3       | 3.9          | 3.9          | 2.20E-01 | 1.60E-01 | 1.00E+00 |
| IL6         | 0         | 0.1          | 0.3          | 3.10E-01 | 7.50E-02 | 3.90E-01 |
| IRF4        | 0.1       | 0.6          | 0.6          | 7.40E-02 | 1.30E-01 | 9.10E-01 |
| IRF5        | 7.1       | 9.9          | 8.6          | 4.20E-07 | 1.10E-02 | 6.90E-03 |
| IRF8        | 6.8       | 8.1          | 2.9          | 6.50E-02 | 5.20E-08 | 8.30E-15 |
| ITGAE       | 0.1       | 0.3          | 0.1          | 4.10E-01 | 6.20E-01 | 1.90E-01 |
| ITGAL       | 7.6       | 11.4         | 12           | 1.70E-10 | 3.40E-15 | 2.50E-02 |
| ITGAM       | 9.8       | 8.5          | 2.6          | 5.20E-02 | 1.70E-23 | 1.60E-15 |
| ITGAX       | 10.5      | 12.2         | 11.6         | 4.90E-09 | 1.30E-03 | 6.40E-03 |
MMP1 and MMP3. Apart from the genes with multimodal expression in all three subsets, the classical subset showed multimodal expression of the phagocytosis-associated gene CLEC4E, the scavenger receptor gene CD163 and the FcγR3A gene, adhesion and migration genes.

![Image](image_url)

Table 2. (Continued)

| Gene symbol | Gene expression values in monocyte subsets | T-test values |
|-------------|------------------------------------------|--------------|
|             | Classical | Intermediate | Non-classical | C vs I | C vs NC | I vs NC |
| LTB         | 0.5       | 2.8          | 3.4          | 9.80E-07 | 7.60E-09 | 3.50E-01 |
| MARCO       | 4.3       | 6.7          | 2.2          | 1.30E-03 | 2.30E-03 | 2.40E-10 |
| MICA        | 0.8       | 0.5          | 0.8          | 5.70E-01 | 9.60E-01 | 5.20E-01 |
| MICB        | 3.1       | 4.7          | 3.7          | 1.80E-02 | 3.40E-01 | 1.70E-01 |
| MMP1        | 7.3       | 7.3          | 7.5          | 9.90E-01 | 7.60E-01 | 7.80E-01 |
| MMP12       | 0         | 0            | 0            | 9.20E-01 | 7.40E-01 | 8.10E-01 |
| MMP2        | 0.3       | 0.7          | 0.5          | 8.90E-02 | 3.20E-01 | 4.50E-01 |
| MMP3        | 3.8       | 6.1          | 5.7          | 7.30E-05 | 7.50E-04 | 6.10E-01 |
| MMP7        | 2.5       | 3.4          | 2.5          | 1.40E-02 | 8.70E-01 | 9.20E-03 |
| MMP9        | 0.1       | 0            | 0.1          | 3.20E-01 | 9.50E-01 | 3.10E-01 |
| MRC1        | 0         | 0            | 0            |          |          |          |
| MSR1        | 0.8       | 3.7          | 4.4          | 2.90E-06 | 1.00E-08 | 3.60E-01 |
| NFkB1       | 4.2       | 5.8          | 4.1          | 4.10E-02 | 9.10E-01 | 2.90E-02 |
| PTPRC       | 8.8       | 11           | 10           | 5.40E-34 | 2.10E-15 | 1.10E-10 |
| RAET1E      | 0.1       | 0.4          | 0.4          | 5.20E-05 | 4.90E-04 | 4.00E-01 |
| RAET1G      | 0         | 0.1          | 0            | 1.60E-01 | 1.60E-01 |          |
| RAET1L      | 1.1       | 2.1          | 1.5          | 9.50E-03 | 3.20E-01 | 1.40E-01 |
| RARA        | 11.8      | 11.8         | 11.6         | 8.50E-01 | 6.20E-01 | 5.70E-01 |
| RELA        | 6.4       | 8.2          | 5.2          | 1.70E-02 | 1.10E-01 | 4.50E-05 |
| S100A4      | 13.1      | 13.8         | 13.6         | 1.00E-08 | 2.70E-01 | 2.50E-02 |
| SELL        | 11.9      | 7.3          | 2.4          | 7.90E-15 | 7.60E-43 | 4.30E-12 |
| SERPINA1    | 13.2      | 13.9         | 13.1         | 4.90E-09 | 7.50E-01 | 1.40E-02 |
| SERPINB2    | 0.9       | 0.1          | 0            | 5.00E-03 | 1.40E-03 | 3.20E-01 |
| SIGLEC10    | 4.6       | 10           | 10.1         | 1.50E-15 | 1.00E-06 | 9.50E-01 |
| SIRPA       | 9.4       | 7.4          | 4.1          | 3.20E-04 | 5.50E-19 | 1.10E-07 |
| TIMP1       | 11.7      | 13           | 12.2         | 2.70E-10 | 1.50E-01 | 1.20E-02 |
| TLR2        | 10        | 9            | 8.8          | 1.90E-02 | 1.40E-02 | 7.70E-01 |
| TLR3        | 0         | 0            | 0.1          | 3.10E-01 | 3.10E-01 |          |
| TLR4        | 8.4       | 8            | 7.6          | 4.20E-01 | 1.50E-01 | 5.00E-01 |
| TLR7        | 0.2       | 1.5          | 0.5          | 1.70E-03 | 3.20E-01 | 3.10E-02 |
| TLR8        | 4.7       | 5.3          | 5            | 4.40E-01 | 6.90E-01 | 7.10E-01 |
| TLR9        | 3.8       | 4.2          | 4.6          | 4.30E-01 | 2.00E-01 | 6.00E-01 |
| TNF         | 5.8       | 8.4          | 9.8          | 6.30E-04 | 8.00E-10 | 2.90E-02 |
| TNFSF15     | 5.8       | 7            | 6.4          | 2.40E-05 | 1.90E-02 | 4.50E-03 |
| TREM1       | 11.2      | 7.8          | 4.4          | 1.60E-08 | 2.40E-18 | 9.50E-05 |
| TREM2       | 0.1       | 0            | 0            | 3.20E-01 | 3.30E-01 |          |
| ULBP1       | 0         | 0            | 0            |          |          |          |
| ULBP2       | 0         | 0            | 0            | 3.10E-01 | 3.10E-01 |          |
| ULBP3       | 3.8       | 5.2          | 5            | 3.00E-02 | 8.20E-02 | 7.00E-01 |

C = Classical, I = Intermediate, NC = Non-Classical

doi:10.1371/journal.pone.0144351.t002
Table 3. Functional categorization. The data indicate relative Log2 transformed gene expression levels.

| Gene symbol | Gene expression in monocyte subsets |
|-------------|------------------------------------|
|             | Classical  | Intermediate | Non-classical |
| **Antigen presentation** | | | |
| CD40        | 6.0        | 7.4          | 5.0           |
| CD74        | 14.9       | 16.2         | 14.6          |
| HLA-DRA     | 13.2       | 14.7         | 12.4          |
| HLA-DRB1    | 5.4        | 0.9          | 2.0           |
| **Adhesion/migration** | | | |
| CD33        | 9.3        | 8.0          | 5.5           |
| CD93        | 6.8        | 5.0          | 2.3           |
| CCR1        | 3.6        | 3.1          | 1.2           |
| CCR2        | 1.4        | 0.4          | 0.0           |
| CCR5        | 0.0        | 1.0          | 0.0           |
| CCR9        | 0.4        | 0.1          | 0.1           |
| CX3CR1      | 5.7        | 5.1          | 5.0           |
| ITGA6       | 0.1        | 0.3          | 0.1           |
| ITGAL       | 7.6        | 11.4         | **12.0**      |
| ITGAM       | **9.8**    | 8.5          | 2.6           |
| ITGAX       | 10.5       | 12.2         | 11.6          |
| SEL2        | **11.9**   | 7.3          | 2.4           |
| SIGLEC10    | 4.6        | 10.0         | **10.1**      |
| **Differentiation and function** | | | |
| BAX         | 5.2        | 7.4          | 6.4           |
| BCL2        | 2.6        | 3.0          | 2.5           |
| BCL2L1      | 0.8        | 2.4          | 2.1           |
| CSF1R       | **5.4**    | **10.2**     | 8.9           |
| IRF4        | 0.1        | 0.6          | 0.6           |
| IRF5        | 7.1        | 9.9          | 8.6           |
| IRF8        | 6.8        | 8.1          | 2.9           |
| NFKB1       | 4.2        | 5.8          | 4.1           |
| PTPRC       | 8.8        | 11.0         | 10.0          |
| RARA        | 11.8       | 11.8         | 11.6          |
| RELA        | 6.4        | 8.2          | 5.2           |
| S100A4      | 13.1       | 13.8         | 13.3          |
| **Scavenger receptors** | | | |
| CD36        | **10.3**   | 7.1          | 1.0           |
| CD68        | 14.2       | **14.9**     | 13.8          |
| CD163       | **5.4**    | 1.3          | 0.0           |
| MARCO       | 4.3        | **6.7**      | 2.2           |
| MSR1        | 0.8        | 3.7          | **4.4**       |
| **Phagocytosis/bacterial clearance** | | | |
| CD93 phagocytosis | **6.8**   | 5.0          | 2.3           |
| CD209       | **1.1**    | 0.7          | 0.4           |
| CLEC4E      | **2.2**    | 0.3          | 0.0           |
| **Innate immune responses** | | | |
| ADAM17      | 5.9        | 5.8          | **6.6**       |
| C1QA        | 0.1        | 2.7          | **3.1**       |
| CD14        | **11.7**   | 7.3          | 4.6           |

(Continued)
Table 3. (Continued)

| Gene symbol | Gene expression in monocyte subsets | Classical | Intermediate | Non-classical |
|-------------|-------------------------------------|-----------|--------------|---------------|
| FCGR3A      |                                     | 8.1       | 11.9         | 12.3          |
| HMOX1       |                                     | 8.9       | 12.2         | 12.0          |
| LTB         |                                     | 0.5       | 2.8          | 3.4           |
| MICA        |                                     | 0.8       | 0.5          | 0.8           |
| MICB        |                                     | 3.1       | 4.7          | 3.7           |
| RAET1E      |                                     | 0.1       | 0.4          | 0.4           |
| RAET1G      |                                     | 0.0       | 0.1          | 0.0           |
| RAET1L      |                                     | 1.1       | 2.1          | 1.5           |
| SERPINB2    |                                     | 0.9       | 0.1          | 0.0           |
| SIRPA       |                                     | 9.4       | 7.4          | 4.1           |
| TLR2        |                                     | 10.0      | 9.0          | 8.8           |
| TLR3        |                                     | 0.0       | 0.0          | 0.1           |
| TLR4        |                                     | 8.4       | 8.0          | 7.6           |
| TLR7        |                                     | 0.2       | 1.5          | 0.5           |
| TLR8        |                                     | 4.7       | 5.3          | 5.0           |
| TLR9        |                                     | 3.8       | 4.2          | 4.6           |
| TNFSF15     |                                     | 5.8       | 7.0          | 6.4           |
| TREM1       |                                     | 11.2      | 7.8          | 4.4           |
| TREM2       |                                     | 0.1       | 0.0          | 0.0           |
| ULBP3       |                                     | 3.8       | 5.2          | 5.0           |
| **Cytokines** |                                  |           |              |               |
| IL10        |                                     | 2.4       | 1.2          | 0.1           |
| IL12A       |                                     | 5.8       | 7.6          | 7.0           |
| IL15        |                                     | 2.2       | 2.5          | 2.9           |
| IL18        |                                     | 2.7       | 2.9          | 0.9           |
| IL1B        |                                     | 1.7       | 3.3          | 1.1           |
| IL23A       |                                     | 3.3       | 3.9          | 3.9           |
| IL6         |                                     | 0.0       | 0.1          | 0.3           |
| TNF         |                                     | 5.8       | 8.4          | 9.8           |
| **Proteases and protease inhibitors** |                              |           |              |               |
| CST3        |                                     | 13.4      | 14.3         | 12.7          |
| CTSB        |                                     | 11.5      | 12.4         | 11.1          |
| CTSK        |                                     | 2.5       | 2.4          | 1.7           |
| CTL         |                                     | 0.3       | 5.2          | 3.7           |
| CTSS        |                                     | 14.7      | 14.3         | 13.8          |
| MMP1        |                                     | 7.3       | 7.3          | 7.5           |
| MMP2        |                                     | 0.3       | 0.7          | 0.5           |
| MMP3        |                                     | 3.8       | 6.1          | 5.7           |
| MMP7        |                                     | 2.5       | 3.4          | 2.5           |
| MMP9        |                                     | 0.1       | 0.0          | 0.1           |
| TIMP1       |                                     | 11.7      | 13.0         | 12.2          |
| SERPIN1     |                                     | 13.2      | 13.9         | 13.1          |

**Bold** = highest gene expression, **Standard** = medium gene expression, **Italic** = lowest gene expression among the three monocyte subsets

doi:10.1371/journal.pone.0144351.t003
Table 4. Multimodal expression in the three monocyte subsets.

| Gene symbol | Gene expression modality | Gene symbol | Gene expression modality |
|-------------|--------------------------|-------------|--------------------------|
|             | Classical | Intermediate | Non-Classical | Classical | Intermediate | Non-Classical |
| ACTB        | 0.86      | 0.68         | 0.97         | IRF8      | 0.00        | 0.00         | 0.00         |
| ADAM17      | 0.00      | 0.00         | 0.00         | ITGA1E    | 1.00        | 1.00         | 1.00         |
| BAX         | 0.00      | 0.00         | 0.00         | ITGAL     | 0.00        | 0.83         | 0.95         |
| BCL2        | 0.00      | 0.00         | 0.00         | ITGAM     | 0.00        | 0.00         | 0.00         |
| BCL2L1      | 0.94      | 0.00         | 0.00         | ITGAX     | 0.99        | 0.86         | 0.95         |
| C1QA        | 1.00      | 0.00         | 0.00         | LTB       | 0.99        | 0.00         | 0.00         |
| CCR1        | 0.00      | 0.00         | 0.31         | MARCO     | 0.00        | 0.00         | 0.00         |
| CCR2        | 0.03      | 1.00         | 1.00         | MICRA     | 0.98        | 1.00         | 0.99         |
| CCR5        | 1.00      | 0.99         | 1.00         | MICB      | 0.00        | 0.00         | 0.00         |
| CCR9        | 1.00      | 1.00         | 1.00         | MMP1      | 0.00        | 0.00         | 0.00         |
| CD14        | 0.86      | 0.00         | 0.00         | MMP2      | 1.00        | 1.00         | 0.99         |
| CD163       | 0.00      | 0.15         | 1.00         | MMP3      | 0.00        | 0.00         | 0.00         |
| CD209       | 0.94      | 0.99         | 1.00         | MMP7      | 0.34        | 0.03         | 0.22         |
| CD33        | 0.02      | 0.00         | 0.00         | MMP9      | 1.00        | 1.00         | 1.00         |
| CD36        | 0.11      | 0.00         | 0.49         | MSR1      | 0.92        | 0.00         | 0.00         |
| CD40        | 0.00      | 0.00         | 0.00         | NFKB1     | 0.00        | 0.00         | 0.00         |
| CD68        | 0.70      | 0.99         | 0.99         | PTPRC     | 0.96        | 0.72         | 0.95         |
| CD74        | 0.76      | 0.95         | 0.98         | RAET1E    | 1.00        | 0.97         | 0.99         |
| CD93        | 0.00      | 0.00         | 0.00         | RAET1G    | 1.00        | 1.00         | 1.00         |
| CLEC4E      | 0.00      | 1.00         | 1.00         | RAET1L    | 0.86        | 0.12         | 0.67         |
| CSF1R       | 0.00      | 0.94         | 0.00         | RARA      | 0.98        | 1.00         | 0.83         |
| CST3        | 0.52      | 0.62         | 0.88         | RELA      | 0.00        | 0.00         | 0.00         |
| CTSB        | 0.25      | 0.75         | 0.70         | S100A4    | 0.82        | 0.95         | 0.91         |
| CTSK        | 0.00      | 0.00         | 0.00         | SELL      | 0.97        | 0.00         | 0.00         |
| CTSL        | 1.00      | 0.00         | 0.00         | SERPINA1  | 0.99        | 0.96         | 0.99         |
| CTSS        | 0.85      | 0.99         | 0.99         | SERPINB2  | 0.34        | 1.00         | 1.00         |
| CX3CR1      | 0.00      | 0.00         | 0.00         | SIGLEC10  | 0.00        | 0.10         | 0.70         |
| FCGR3A      | 0.05      | 0.96         | 0.95         | SIRPA     | 0.25        | 0.00         | 0.00         |
| HLADRA      | 0.90      | 0.94         | 0.61         | TIMP1     | 0.96        | 0.93         | 0.90         |
| HLADRB1     | 0.00      | 0.92         | 0.02         | TLR2      | 0.85        | 0.72         | 0.04         |
| HMOX1       | 0.00      | 0.84         | 0.98         | TLR3      | 1.00        | 1.00         | 1.00         |
| IL10        | 0.00      | 0.49         | 1.00         | TLR4      | 0.00        | 0.00         | 0.00         |
| IL12A       | 0.00      | 0.00         | 0.00         | TLR7      | 1.00        | 0.02         | 1.00         |
| IL15        | 0.00      | 0.00         | 0.00         | TLR8      | 0.00        | 0.00         | 0.00         |
| IL18        | 0.00      | 0.00         | 0.24         | TLR9      | 0.00        | 0.00         | 0.00         |
| IL1B        | 0.07      | 0.00         | 0.67         | TNF       | 0.00        | 0.00         | 0.70         |
| IL23A       | 0.00      | 0.00         | 0.00         | TNFSF15   | 0.83        | 0.80         | 0.75         |
| IL6         | 1.00      | 1.00         | 1.00         | TREM1     | 0.66        | 0.00         | 0.00         |
| IRF4        | 1.00      | 0.98         | 0.99         | TREM2     | 1.00        | 1.00         | 1.00         |
| IRF5        | 0.00      | 0.72         | 0.00         | ULBP3     | 0.00        | 0.00         | 0.00         |

**Bold** = Multimodal expression $P < 0.05$, **Italic** = Unimodal expression

doi:10.1371/journal.pone.0144351.004

CCR1, CCR2, ITGAL, and SICLEG10, the cytokines IL10, IL18 and TNF, and genes involved in differentiation such as the transcription factor IRF5. The intermediate monocytes showed generally multimodal expression of the genes involved in apoptosis and adhesion such as...
Furthermore, it was in particular genes involved in regulating immune responses such as C1QA, CD14, IL1β, IL18, TNF, TLR7, TREM1, and SIRPα, and genes encoding the proteases, CTSL and MMP7 that showed multimodal expression. The non-classical monocytes exhibited, in addition to the genes which also showed multimodal expression by the classical and intermediate monocytes, multimodal expression of genes involved in apoptosis, differentiation and activation such as BCL2L1, CSF1R, and IRF5. Similar to the intermediate subset, the non-classical monocytes also showed multimodal expression in a range of immune responsive genes such as ADAM17, C1QA, CD14, LTB, TREM1 and SIRPα as well as the genes encoding the proteases, CTSL, CTSL, MMP1 and MMP3.

We demonstrate here that single-cell gene-expression analysis is a valuable tool in detecting multi-modality within cell populations. To exclude that the bi-modality we observed is not caused by a so-called “drop out effect” due to technology artefact, we calculated the relationship between log-transformed transcript expression data and Hartigan’s dip test p-value. When we include the cells with an expression we found no difference comparing uni- and bi-modal genes (p = 0.54). If genes with low expression were more prone to drop out effects we would have expected an overrepresentation of these amongst the bi-modal genes.
Fig 3. Expression level of genes deviating in identified subgroups. Subgroups of cells were identified based on the PCA of single-cell PCR gene expression analysis data. One subgroup among the classical monocytes, intermediate monocytes and non-classical monocytes, and co-expression of genes within the subgroups were assessed using a student T test ($P < 0.05$). A) Bar graph demonstrating the differentially expressed genes by the subgroup within the classical monocyte subset identified on the PCA score plot. The
Classification of subpopulations

With single-cell gene expression profile we are able to study the heterogeneity between and within cell populations. In addition to the multimodal analysis we also analysed co-expression of genes within the classical monocytes, intermediate monocytes and non-classical monocytes in order investigate potential subgroups with distinct gene expression profiles. Using the PCA of single-cell PCR gene expression analysis data it was possible to identify one subgroup of monocytes that diverged from the main populations (Fig 3 and S1 Table). Within the population of classical monocytes, we found one subgroup that showed differential expression of 22 genes (Fig 3A). Of these genes, only 2 genes showed a higher expression than the main population, namely TNFSF15 and TREM1. The remaining genes were all found to have lower expression than in the main population. Within the intermediate subset, we also identified a subgroup of cells displaying differential gene expression of eight genes (Fig 3B). Compared to the main population of intermediate monocytes, only IL10 was found to have lower expression whereas LTB, PTPRC, HMOX1, CSF1R, FCGR3A, RAETL1 and TNF were all found to be significantly more highly expressed. Likewise, within the non-classical monocytes we found a subgroup of cells with a co-expression of IRF8 and RAET1E at higher levels than the main population but showing lower gene expression of CTSS, IL123A, IRF5 and TNF (Fig 3C).

Comparing the expression of genes within the subgroup of cells identified by the PCA plot, we demonstrate that the classical, intermediate and non-classical human blood monocytes each contain a subgroup of cells characterized by distinct gene signatures. This highlights the great diversity and possible plasticity within the human monocyte sub-populations.

Discussion

Comprehensive genome-wide analyses have shown distinct heterogeneity within human monocytes [9,11,15]. Three subsets have been identified in humans, namely the classical, intermediate and non-classical monocytes. In this study we have used the single-cell PCR gene analysis technique from Fluidigm, and confirmed existing data defining three monocyte subsets and demonstrated differential gene expression among the classical, intermediate and non-classical monocytes. Moreover, the differential expression of genes encoding cell surface molecules identified, for instance, the expression of CD163 and TREM2 in classical monocytes and the expression of CCR5 and RAET1G in intermediate monocytes, could be useful when discriminating the three monocyte subsets by methods such as flow cytometry. Furthermore, we have shown inter-cellular variation of genes within each subset, which highlights the heterogeneity of monocytes as a diverse group of innate immune leukocytes containing possible further functional subclasses. Thus, by our study, performed with one donor, we here demonstrate the possibilities of subgrouping the monocytes using single-cell PCR gene expression analysis.

The single-cell gene expression analysis presented here demonstrates high phagocytic capacity (CD93, CD209, CLEC4E, and SIRPA) of the classical monocytes, which is in agreement with previous studies. Also, a high expression of a broad range of innate sensing receptor genes, pro-inflammatory genes and genes linked to innate immune responses (CD14, TLR2, TLR4 and TREM1) are observed for the classical monocytes [9,11,15]. In addition to these findings we have previously shown that the classical monocytes secrete high levels of IL-1β, IL-...
10, and TNFα, and that most IL-6 and MMP1 is produced in response to LPS and immune-complex activation by the classical subset compared to the intermediate and non-classical subset [12]. In accordance to earlier micro-array studies, we also saw the highest expression of genes involved in migration. These findings, together with our previous results showing the highest migratory capacity of the classical monocytes towards CCL2 [12] may underline the capacity of classical monocytes to support inflammation and mount an immune response towards microbial pathogens.

The intermediate monocytes had high antigen presenting potential, which has also been demonstrated by their property to induce CD4⁺ T cell proliferation [15]. Moreover, the intermediate monocytes were the only subset that expressed CCR5, a chemokine receptor responsible for recruiting dendritic cell (DC) precursors from blood to the draining lymph nodes [17]. In addition, our data demonstrate higher gene expression of several cytokines (IL1β, IL12A, IL18, and IL23A) which are important in inducing functionally distinct CD4⁺ T helper (Th) cells. IL12 plays a role in the differentiation of Th1 cells, whereas IL6 and IL23 are important in driving and sustaining the differentiation of Th17 cells [18]. Moreover, IL12, IL15, IL18 and TNFSF15 are involved in the induction of T cell receptor-independent cytokine production by CD4⁺ T cells [19–21]. The intermediate monocytes also showed a higher expression of genes linked to the activation status such as apoptosis regulation (BAX and BCL2), cell differentiation and regulation (CSF1R, IRF5, IRF8, NFκB1, RELA and PTPRC). This may suggest that these cells are more activated than the classical monocytes.

The CD16⁺ monocytes have been shown to adhere to the endothelium and mediate arrest through the CX3CL1-CX1CR3 interaction [22]. Additionally, the non-classical monocytes are thought to patrol the vasculature and selectively respond to viral infected- or damaged cells. In line with previous data, in non-classical monocytes we found considerably higher expression of genes coupled to complement (C1QA) and FcR-mediated phagocytosis (FcRy3A), adhesion (ITGAL and SIGLEC10) and TLR9. However, we find that the genes encoding TLR7 and TLR8, which sense nucleic acids and viruses, are mostly expressed by the intermediate subset. Moreover, we find the highest expression of the CX3CR1 gene in the classical subset, albeit with small variation among the three subsets. This latter finding conflicts with previous data showing high cell surface expression of CX3CR1 on non-classical monocytes and their capacity to respond to damaged cells and viral infections. However, these observations in gene expression do not always correspond to the actual protein expression. Thus difficulty in functional interpretation of genes is a limitation to transcriptome analysis and may explain the discrepancies between observed protein expression and gene analysis data.

The advantage of single-cell gene expression analysis compared to micro-array is that it provides the possibility to analyse gene expression within single cells, facilitating the discovery of multimodal expression on single cells and possible new subpopulations in cell populations identified to date [23,24]. Therefore, in addition to comparing our results with previous micro-array data, we also investigated the intercellular variation among the monocyte subtypes and analysed data for co-expression of genes within each monocyte subset. Interestingly, we could demonstrate that multimodal gene expression is present in all three subsets. The transcription factors IRF8, NFκB1 and RELA are among the genes that show multimodal expression in all three subsets. Also genes involved in apoptosis regulation (BAX and BCL2) and cell adhesion (CD33, CD93, CX3CR1 and ITGAM) are expressed multimodally in all three subsets. This multimodality seen in the monocyte subsets may be a reflection of differential maturation and immune activation status. In addition, the classical monocytes also show multimodal expression of the chemokine receptor genes (CCR1 and CCR2), adhesion genes (ITGAL and ITGAM) and innate immune response genes (TLR4, TLR8-9, IL10, IL12A, IL15, IL18, and IL23A), favouring evidence for potentially immune activated cells. However, further data are
needed to establish if this is in fact the case, thus these potentially different activation states could be of importance in light of the monocytes ability to extravasate into tissues and respond to pathogenic stimulation. For example, cells expressing high levels of CCR2 together with CLEC4E, TLR4, and TNF-a might be more prone for migration and response to bacteria, in contrast to monocytes expressing low levels of CCR2. Also, cells expressing high levels of FCGR3A, CD36 and CD163 might be more prone for scavenging and phagocytosis. Several genes encoding immune responses also show multimodal expression within the intermediate subset together with genes encoding the proteases. Though our study has been carried out using monocytes from a healthy donor, the data presented here serve as a basis for discovering targets on certain subpopulations of cells that may be implicated in disease pathogenesis. In addition, single-cell PCR analyses have revealed that colon cancer tissues contain cell populations distinct from healthy colon, which were not identified by immunohistochemistry or flow cytometry [25]. Moreover we identified a subgroup of cells within the classical group of monocytes that may be less activated given lower expression of, for example genes within the NFκB complex, IRF8, and genes encoding several cytokines such as IL1b, IL12A, IL15, IL23A and TNF. In contrast, we describe here a subgroup of cells among the intermediate monocytes, showing higher gene expression of TNF and FCGR3A and lower expression of IL10, suggesting that the cells of this subgroup are more differentiated towards an immune-activated phenotype. The transcription factor IRF8 is known to be involved in the differentiation of monocytes and DCs [26–28]. The subgroup identified in the non-classical cells showed higher gene expression of IRF8 and lower gene expression of IRF5, IL23A and TNF. This could imply that this subgroup of cells is more differentiated towards dendritic cells. Of note, non-classical monocytes are likewise shown to have a higher propensity to become dendritic cells [29].

The advantage of using single-cell gene expression profiling methods compared to microarray is the ability to distinguish potential target cells or subgroups of cells implicated in disease pathogenesis. However, the limitation of the single-cell gene expression technique is its being dependent on a predesigned panel of primers compared to the broad dataset obtained using micro-array, which is not limited to investigating predesigned genes only.

**Materials and Methods**

**Monocyte Purification**

A buffy coat from a healthy donor was purchased from the Clinical Immunology Blood Bank, The State University Hospital, Copenhagen. Peripheral Blood Mononuclear Cells (PBMC) were obtained using Ficoll-paque Plus (GE-Healthcare Bio-sciences AB, Uppsala, SE) density centrifugation. Untouched monocytes were isolated from PBMC by negative selection using antibody-coated magnetic bead separation (EasySep Human Monocyte Enrichment Kit without CD16 depletion, Stemcell Technologies, Vancouver, Canada) according to the manufacturers’ instructions. The study was approved by the Regional Ethics Committee in Lund, Sweden.

**FACS**

Monocytes and LPC were subsequently washed in PBS containing 2% fetal calf serum (FCS) (all from Gibco, Paisley, UK) prior to staining for 15 minutes at 4°C in the dark. The following antibodies were used for surface staining: APC-conjugated anti-CD3 (UCHT1), Pacific blue-conjugated anti-CD14 (M5E2), PerCP-conjugated anti-CD45 (2D1), FITC-conjugated anti-CD64 (10.1), PE-conjugated anti-HLA-DR (TU36) (all from BD Pharmingen), PerCP-conjugated anti-CD16 (3G8), Live/Dead Fixable Near-IR (all from Invitrogen Molecular Probes). The three monocyte subpopulations, classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16++) were single-cell sorted according to the expression of CD14 and
CD16. Each cell population was sorted into a 96-well plate using fluorescence-activated cell sorting (FACS) on a BD FACS ARIAII.

Single-cell gene expression analysis

The monocytes were sorted into 96-well plates by FACS with the target of one cell in each well containing 5 μl RT Mix solution (mixture of VILO reaction mix, SUPERase-In and 10% NP40 according to the manufacturer’s protocol). Samples were frozen on dry ice and after thawing, synthesis of cDNA was performed with SuperScript VILO (Invitrogen). Specific targets amplification (STA) was done with a mixture of 85 PCR primers (see Table 1) using Taqman preamp master mix (Invitrogen) running 22 cycles. The probes used have all been tested with DNA and were able to amplify and detect transcripts. Residual primers were subsequently removed by treatment with Exonuclease I (New England Biolabs).

After the clean-up step, 6 μl of STA PCR product from each sample was transferred to a new microtitre plate and a standard qPCR (TaqMan 2x Universal PCR Master Mix, Applied Biosystems) with a Taqman assay (Invitrogen) directed against 18S, was performed to identify empty wells. Wells with no 18S Ct value or with an 18S Ct value above 40 were considered empty.

Single-cell qPCR was performed on a Fluidigm BiomarkHD instrument using SSO Fast EvaGreen SUpermix (Bio-Ras Laboratories) according to the manufacturer’s protocol. All primer pairs from genes listed in Table 1 were used. The data were analysed using the SINGuLAR R package (Fluidigm) and expression values were normalised to that of ACTB.

Sub-groups for each monocyte sub-type were defined from the initial principal component analysis (PCA) plot (Fig 1B). Differentially regulated genes in each sub-group were identified by comparing gene expression in the subgroup versus the rest of the cells in the sub-type using a Students t-test. Differentially regulated genes were defined as those with a p-value < 0.05 and a log2 fold change of at least 1.

Supporting Information

S1 Table. List of genes differentially expressed by the monocyte subgroups.

(DOCX)

Acknowledgments

The authors would like to thank Anette Klestrup for valuable technical assistance.

Author Contributions

Conceived and designed the experiments: STG SJ KH OG. Performed the experiments: STG TBR. Analyzed the data: STG TBR JGG. Contributed reagents/materials/analysis tools: STG TBR. Wrote the paper: STG OG JGG SJ TBR KH.

References

1. Auffray C, Sieweke MH, Geissmann F (2009) Blood monocytes: development, heterogeneity, and relationships with dendritic cells. Annu Rev Immunol 27: 669–692. doi: 10.1146/annurev.immunol.021908.132557 [pii]. PMID: 19132917

2. Ziegler-Heitbrock L (2014) Reprint of: Monocyte subsets in man and other species. Cell Immunol. S0008-8749(14)00108-7 [pii]; doi: 10.1016/j.cellimm.2014.06.008

3. Passlick B, Flieger D, Ziegler-Heitbrock HW (1989) Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood 74: 2527–2534. PMID: 2478233
4. Ancuta P, Liu KY, Misra V, Wacleche VS, Gosselin A, Zhou X, et al. (2009) Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and. BMC Genomics 10: 403. 10.1186/1471-2164-10-403 [pii]; doi:10.1186/1471-2164-10-403 PMID: 19712453

5. Castano D, Garcia LF, Rojas M (2011) Increased frequency and cell death of CD16+ monocytes with Mycobacterium tuberculosis infection. Tuberculosis (Edinb) 91: 348–360. S1472-9792(11)00065-5 [pii]; doi:10.1016/j.tube.2011.04.002

6. Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler-Heitbrock HW (1993) The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. Blood 82: 3170–3176. PMID: 7693040

7. Grip O, Bredberg A, Lindgren S, Henriksson G (2007) Increased subpopulations of CD16(+) and CD56 (+) blood monocytes in patients with active Crohn's disease. Inflamm Bowel Dis 13: 566–572. doi:10.1002/ibd.20025 PMID: 17260384

8. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, et al. (2002) CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum 46: 2578–2586. doi:10.1002/art.10545 PMID: 12384915

9. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. (2010) Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity 33: 375–386. S1074-7613(10)00317-1 [pii]; doi:10.1016/j.immuni.2010.08.012 PMID: 20832340

10. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. (2010) Nomenclature of monocytes and dendritic cells in blood. Blood 116: e74–e80. blood-2010-02-258558 [pii]; doi:10.1182/blood-2010-02-258558 PMID: 20628149

11. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U (2012) The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. Arthritis Rheum 64: 671–677. doi:10.1002/art.33418 PMID: 22006178

12. Schmidl C, Renner K, Peter K, Eder R, Lassmann T, Balwierz PJ, et al. (2014) Transcription and enhancer profiling in human monocyte subsets. Blood 123: e90–e99. blood-2013-02-484188 [pii]; doi:10.1182/blood-2013-02-484188 PMID: 24671955

13. Papadakis KA, Prehn JL, Landers C, Han Q, Luo X, Cha SC, et al. (2004) TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells. J Immunol 172: 7002–7007. PMID: 15153521

14. Sattler A, Wagner U, Rossol M, Sieper J, Wu P, Krause A, et al. (2009) Cytokine-induced human IFN-gamma-secreting effector-memory Th cells in chronic autoimmune inflammation. Blood 113: 1948–1956. blood-2008-02-139147 [pii]; doi:10.1182/blood-2008-02-139147 PMID: 19104082

15. Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Lucinskas FW, et al. (2003) Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. J Exp Med 197: 1701–1707. doi:10.1084/jem.20022156 jem.20022156 [pii]. PMID: 12810688
23. Shalek AK, Satija R, Adiconis X, Gertner RS, Gaublomme JT, Raychowdhury R, et al. (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498: 236–240. nature12172 [pii]; doi: 10.1038/nature12172 PMID: 23685454

24. Kippner LE, Kim J, Gibson G, Kemp ML (2014) Single cell transcriptional analysis reveals novel innate immune cell types. PeerJ 2: e452. doi: 10.7717/peerj.452 [pii]. PMID: 25024920

25. Dalerba P, Kalisky T, Sahoo D, Rajendran PS, Rothenberg ME, Leyrat AA, et al. (2011) Single-cell dissection of transcriptional heterogeneity in human colon tumors. Nat Biotechnol 29: 1120–1127. nbt.2038 [pii]; doi: 10.1038/nbt.2038 PMID: 22081019

26. Yamamoto M, Kato T, Hotta C, Nishiyama A, Kurotaki D, Yoshinari M, et al. (2011) Shared and distinct functions of the transcription factors IRF4 and IRF8 in myeloid cell development. PLoS One 6: e25812. doi: 10.1371/journal.pone.0025812 PONE-D-11-09702 [pii]. PMID: 22003407

27. Tamura T, Tailor P, Yamaoka K, Kong HJ, Tsujimura H, O’Shea JJ, et al. (2005) IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. J Immunol 174: 2573–2581. 174/5/2573 [pii]. PMID: 15728463

28. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. (2014) Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol 14: 571–578. nri3712 [pii]; doi: 10.1038/nri3712 PMID: 25033907

29. Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schakel K (2002) The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. J Exp Med 196: 517–527. PMID: 12186843