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A defect of CD16-positive monocytes can occur without disease

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ABSTRACT

The CD16-positive monocytes have been first described in 1988 but to date no selective defect in the number of these cells in blood has been reported. We now describe a family in which three of four siblings lack both CD16-positive monocyte subsets, i.e. the nonclassical and the intermediate monocytes. All three had CD16-positive monocytes of 2 cells/μl or less as compared to 52 ± 18 cells/μl in healthy controls.

The index case was affected by recurrent pleural effusion and infections and had evidence of an auto-inflammatory condition but no mutation of any of the relevant candidate genes. The other two siblings without CD16-positive monocytes were apparently healthy. There was no defect in serum M-CSF levels and no mutation in the M-CSF and M-CSFR genes.

The data indicate that the absence of CD16-positive monocytes in blood does not lead to disease.

Introduction

Subsets of human blood monocytes have first been described in the late 1980s based on multicolor flow cytometry using antibodies against CD14 and CD16 (Ziegler-Heitbrock et al. 1988; Passlick et al. 1989). Today we define three different monocyte subsets, i.e. the classical $CD14^{++}CD16^{-}$ monocytes, the intermediate $CD14^{++}CD16^{+}$ monocytes and the nonclassical $CD14^{+}CD16^{++}$ monocytes (Ziegler-Heitbrock et al. 2010). The classical monocytes in man account for about 85% of all monocytes, the nonclassical monocytes for about 10%, and there is a low and variable number of intermediate monocytes. A detailed analysis of the intermediate monocytes has revealed that these cells represent a continuous transition from the classical to the nonclassical monocytes (Wong et al. 2011). The nonclassical and the intermediate monocytes are not always defined separately and can be addressed together as CD16-positive monocytes. These cells were shown to have a higher expression of HLA-class II molecules and a higher antigen-presenting capacity (Passlick et al. 1989; Grage-Griebenow et al. 2001) and to express higher levels of the pro-inflammatory cytokine TNF (Frankenberger et al. 1996; Belge et al. 2002; Szaflarska et al. 2004; Serbina et al. 2009; Cros et al. 2010). On the other hand, the CD16-positive cells produce less IL-10 (Frankenberger et al. 1996; Szaflarska et al. 2004) and this pattern of high pro- and low anti-inflammatory cytokines has led to these cells being called pro-inflammatory monocytes. The CD16-positive monocytes also have a unique migration response to chemokines, which is largely explained by the absence of the chemokine receptor CCR2 from these cells and by the higher level of CX3CR1 (Weber et al. 2000; Ancuta et al. 2003). Finally, these cells have been shown to express higher levels of pro-apoptotic and lower levels of anti-apoptotic molecules such that they will have a preference to enter apoptosis (Zhao et al. 2010).

Abbreviations: GPI, glycosylphosphatidylinositol; M-CSF, macrophage colony stimulating factor; M-CSF-R, macrophage colony stimulating factor receptor; SAA, serum amyloid-A; TNF, tumor necrosis factor.

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Studies into cancer, infection and inflammation have revealed that the number of these cells increases in many clinical conditions (Ziegler-Heitbrock 2007). Also, it was shown that the CD16-positive monocyte subset can decrease with immuno-suppressive therapy (Fingerle-Rowson et al. 1998; Dayyani et al. 2003; Hanai et al. 2008; Takeda et al. 2010; Siedlar et al. 2011). However, with more than two decades of clinical research into monocyte subsets, a defect in the number of these cells has never been reported.

We describe herein a defect of the CD16-positive monocytes in a patient, who presented with an auto-inflammatory disease. Since two siblings, who also lacked this monocyte subset in peripheral blood, were apparently healthy, this indicates that the absence of CD16-positive monocytes on its own does not lead to immune disease.

Materials and methods

Clinical course of index case

There were 4 siblings in this family, three of which were healthy, while one presented with severe disease. The latter was a male patient, born in 1986 as a child of white Caucasian non-consanguineous parents, presented in 2004 on two occasions with spontaneous left pneumothorax, requiring resection of bullae and pleurodesis. In 2005 massive right-sided pleural exsudate developed (ratio of pleural fluid to blood 0.9 for protein and 1.7 for LDH). Repeated thoracocentesis could reduce but never permanently remove the pleura fluid.

During thoracoscopy white spots 2 mm in diameter were found on the pleural surface and histology revealed lympho-follicular hyperplasia; there was no evidence of malignancy. Mycobacterial infection was excluded by culture, PCR and absence of a response in the Quantiferon TB Gold® assay. In 2007 a permanent catheter was implanted to allow for continuous drainage of the pleural exsudate at home with volumes of up to 500 ml/day. Because of recurrent diarrhea without an identifiable microbe parenteral nutrition became necessary and a port system was implanted. Wound infection with *Candida albicans* was followed by nosocomial pneumonia. This required invasive ventilation and was treated successfully with a combination regimen of antibiotics and antimycotics. Polyuric kidney failure manifested with excretion of volumes of up to 14 l/day. Protein loss via kidney and gut led to albumin levels of less than 1 g/l and this was treated with albumin infusion.

Kidney and colon biopsy revealed amyloidosis type AA. Kongo-red staining of pleural tissue from 2007 was found positive, while retrospective analysis of material from 2004 was negative. Serum amyloid A (SAA) was 496 mg/l on 15.11.2007 (normal <5 mg/l). Assuming an auto-inflammatory disease the patient was treated with an IL-1 receptor antagonist (100 mg per day), beginning at 20.11.2007 until 15.02.2008 the day of death. The treatment led to a reduction of SAA (35 mg/l) four weeks after start of the treatment with a flare end of December to early January. The level again decreased to 43 mg/l at the end of January 2008.

The case was further complicated by *Clostridium difficile* colitis and *C. albicans* esophagitis. Thrombosis of the V. cava superior was associated with anti-phospolipid antibodies. The patient then developed renal failure and toxic mega-colon and died of multi-organ failure in early 2008.

Clinical laboratory

When the patient first presented at the Clinical Cooperation Group in January 2007 leukocytes and granulocytes were in the normal range, as were electrolytes, liver and kidney parameters as well as alpha 1 anti-trypsin (202 mg/dL, normal >90 mg/dL). Platelets were 685,000/μl. Throughout the course of the disease CRP (normal <3 mg/l) was elevated, peaking at 274.5 mg/l in 2007 while at the same time procalcitonin levels were negative.

ANA screenings with direct fluorescence tests on HeLa cells failed to demonstrate auto-antibodies and ELISA analyses of antibodies against MPO, PR3, mitochondrial antigens, SMA and collagen type IV were negative as was the rheumatoid factor.

Microbiological analysis found no evidence for infection with HIV-1, HIV-2, HAV, HBV, HCV, VZV, HSV and CMV or for reactivation of EBV. As mentioned above there was no evidence for a mycobacterial infection.

Since the clinical course and the persistently high SAA levels suggested an auto-inflammatory disease we sequenced the genes, known to cause such disorders. To exclude familial Mediterranean fever (FMF), cryopyrin-associated periodic syndromes (CAPS), and tumor necrosis factor receptor 1-associated periodic syndrome (TRAPS), the ten exons of the *MEFV* gene and the nine exons of the *NLRP3* gene as well as exons 2, 3, 4 and 6 of the *TNFRSF1A* gene were analyzed and found to be normal. A hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) was ruled out based on normal IgD levels (<140 mg/l).

In addition, the 3′-end of exon 5 of the *FGA* gene, all four exons of the *TTR* gene, the protein-coding exons 3 and 4 of the *APOA1* gene, exon 4 of the *APOA2* gene, and exon 2 of the *LYZ* gene showed a normal sequence thereby excluding a hereditary amyloidosis caused by aberrant proteins derived from these genes.

Immunophenotype

Analysis of immunoglobulins revealed increased levels for IgG 3070 mg/dl (normal 700–1600 mg/dl), which was polyclonal and showed an increase of all subclasses, IgA was 1579 mg/dl (70–400 mg/dl) and IgM was 98 mg/dl (40–230 mg/dl); complement components were in the normal range: C3 148.4 mg/dl (90–180 mg/dl) and C4 18.2 mg/dl (10–40 mg/dl).

Immunophenotyping of blood cells showed normal numbers for CD3 1150/μl, CD4 640/μl, CD8 530/μl, CD19 90/μl (kappa 65%, lambda 35%) and CD56/CD16 100/μl. There was no clonal excess for immunoglobulin light chains on the B-cells. Also mitogen responses and reactive oxygen production by granulocytes were normal.

Flow cytometry for monocyte subsets

Flow cytometry analysis for monocyte subsets was done by multicolor analysis with the following directly conjugated monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD14 clone My4 (# 6603511, Beckman Coulter, Krefeld, Germany), phycoerythrin (PE)-conjugated anti-CD16 clone Leu11c (# 347617, Becton Dickinson, Heidelberg, Germany) or clone 3G8 (# A07766, Beckman Coulter, Krefeld, Germany) and PC5-conjugated anti-HLA-DR (# PM 2659U, Beckman Coulter, Krefeld, Germany). In brief, 100 μl of the whole blood was added to a 5 ml polystyrene round-bottom tube (# 302054, BD Biosciences, Heidelberg, Germany) and 5 μl of each antigen-specific fluorochrome-labelled antibody (dilution 1:20) was added. The sample was then incubated for 20 min at 4 °C in the dark. Lysis of erythrocytes was performed using a Coulter Q-Prep® lysis instrument. For sample dilution, 800 μl aqua dest and 1600 μl of PBS/2% FCS was added and the sample was supplemented with 100 μl of Flow-Count Fluorospheres (# 7547053, Beckman Coulter, Karlsruhe, Germany) for determination of absolute numbers of cells. To ensure maximum viability, stained cells were analyzed promptly and at least 5000 monocytes were acquired per sample on an EPICS XL (Beckman Coulter, Krefeld, Germany).

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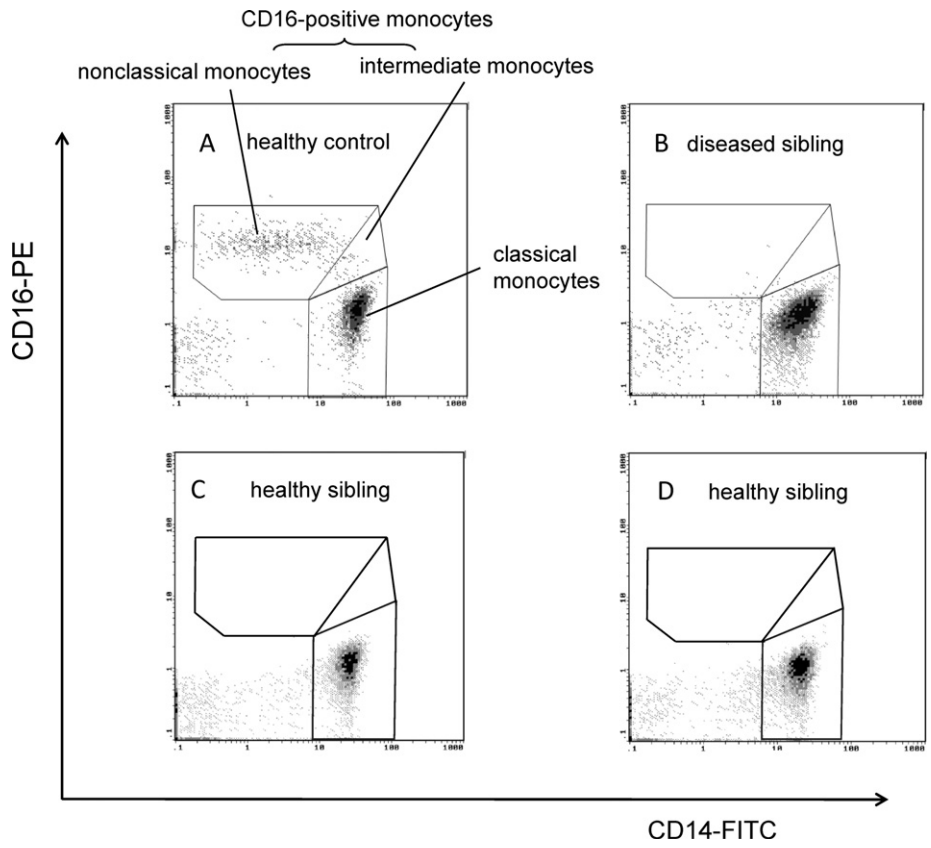

Fig. 1. Flow cytometry for monocyte subsets. Monocyte subsets were determined by flow cytometry of whole blood stained with CD14 (My4) conjugated to fluoresceine-isothiocyanate (FITC) or with CD16 (Leu11c) conjugated to phycoerythrin (PE). Absolute numbers were determined with respect to absolute counting beads. (A) Healthy control, (B) diseased sibling (case), (C) healthy sibling, and (D) healthy sibling.

DNA sequencing of candidate genes

PCR amplification products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced with the ABI PRISM Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Genotyping

Genotyping was done using the high resolution Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, California) and the Affymetrix Genotyping Console Software (Version 3.0.2). The familial relationship was analyzed with the software packages GRR (Abecasis et al. 2001) and Pedcheck (O'Connell and Weeks 1998). For linkage analysis we used Allegro (Gudbjartsson et al. 2000) and EasyLinkagePlus (Hoffmann and Lindner 2005).

Results

A male patient, aged 19, in 2004 presented with spontaneous left pneumothorax, followed by massive right-sided pleural exsudate. Amyloidosis type AA was diagnosed and the patient eventually died of multi-organ failure in 2008. Details of the clinical course are given in the "Materials and methods" section.

Laboratory findings

There was no evidence of auto-immune disease, of any persistent viral or mycobacterial infection and DNA sequencing revealed no mutation in genes invoked in auto-inflammatory disease (for details see "Materials and methods" section). Immunophenotyping showed a normal lymphocyte phenotype and function and no defect in immunoglobulins and complement.

Analysis of blood monocyte subpopulations

Analysis of blood monocytes by flow cytometry revealed a deficiency in the number of CD16-positive monocytes. As seen in Fig. 1A in a normal donor there is a major population of cells with high levels of CD14 and with no CD16. These $CD14^{++}CD16^{-}$ classical monocytes accounted for 326 cells/μl in this example. In addition there is a minor population of cells with low level expression of CD14 and high levels of CD16. The number of these $CD14^{+}CD16^{++}$ nonclassical monocytes was 82 cells/μl for the control donor, i.e. they accounted for 20.5% of all monocytes. Then there is a population of $CD14^{++}CD16^{+}$ intermediate monocytes with 5 cells/μl. The two subsets together form of the CD16-positive monocytes and these are 87 cells/μl in this example. In average of 40 healthy controls the CD16-positive monocytes account for 52 ± 18 cells/μl.

When looking at the patient, the $CD14^{+}CD16^{++}$ nonclassical monocytes were <1% of all monocytes at 1 event/μl and the $CD14^{++}CD16^{+}$ intermediate monocytes also were <1% (1 event/μl), while the number of classical monocytes was high at 580 cells/μl (Fig. 1B). Hence both CD16-positive subpopulations were essentially absent in this case. This depletion of CD16-positive monocytes to <1% of all CD14-positive monocytes was seen on 4 independent occasions. In one sample these cells increased to 2% of all monocytes during an inflammatory period with leukocytosis and a CRP of 205 mg/l (normal range 0–3 mg/l). Glucocorticoids have been shown to deplete the $CD14^{+}CD16^{++}$ monocytes (Fingerle-Rowson

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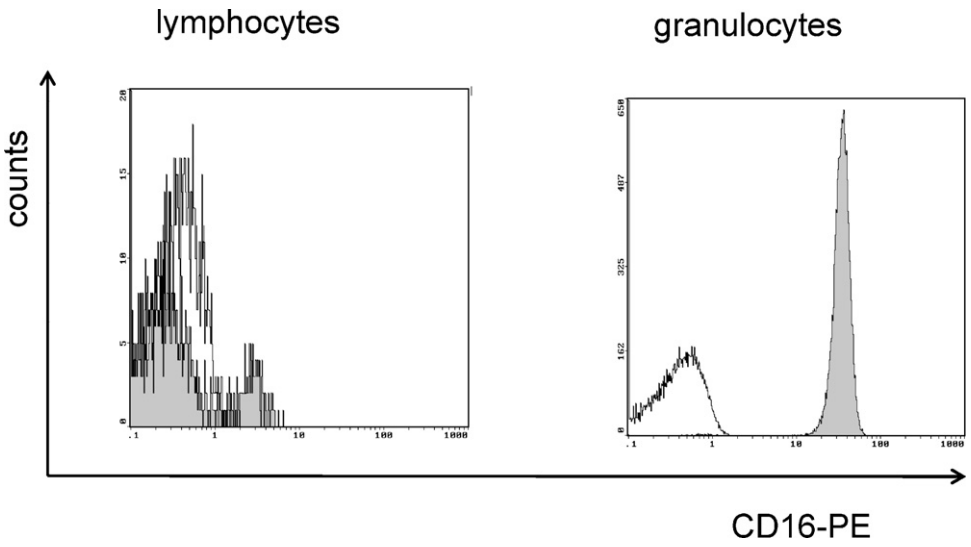

Fig. 2. CD16 expression on lymphocytes and granulocytes. Whole blood from the index case was stained as in Fig. 1 and then analyzed for CD16 expression on lymphocytes and granulocytes. 11.6% of the lymphocytes and all of the granulocytes are CD16 positive.

et al. 1998; Dayyani et al. 2003) but there was no such therapy in this patient.

Lack of CD16-positive monocytes was seen both with the CD16 antibodies Leu11c (Fig. 1B) and 3G8 (not shown). In order to exclude that the nonclassical monocytes were present but evade detection because of lack of CD16, we identified these monocytes via staining for CD14 and HLA-DR. These studies demonstrated the absence also of CD14 low DR high nonclassical monocytes in the patient (data not shown).

Also, CD16 expression was normal for NK cells and for granulocytes (Fig. 2).

The Pedigree

The index case had 3 siblings and we had access to the parents and three of the grand parents. Two of the three siblings had a lack of CD16-positive monocytes similar to the index case (see Fig. 1C and D). For all other family members analysis of monocyte subsets revealed numbers in the normal range or above (see Fig. 3). The two siblings lacking CD16-positive monocytes were clinically well, had no clinical or laboratory evidence of immunodeficiency and had normal values for CRP and SAA. This indicates that the absence of the nonclassical and intermediate monocytes on its own will not lead to disease. It is, however, possible that this defect leads to disease when coinciding with additional genetic or environmental factors.

Furthermore, levels of M-CSF were tested in serum. Here we found increased levels for the index case (1270 pg/ml) while the two affected healthy siblings showed levels of 256 and 315 pg/ml, which is clearly within the normal range (289 ± 77 pg/ml).

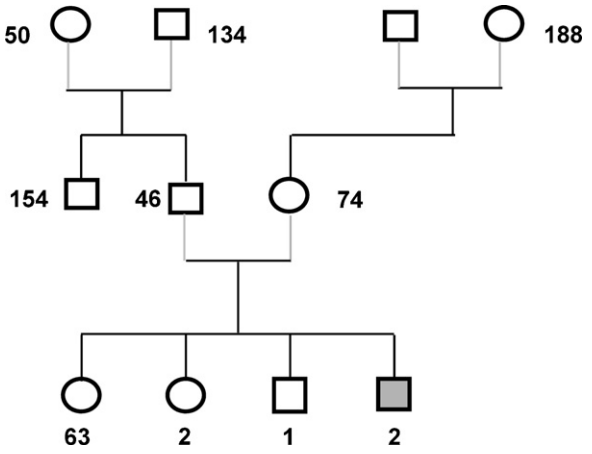

Fig. 3. Pedigree and numbers of CD16-positive monocytes. Circle: female, square: male, filled square: index case. The numbers given are the CD16-positive monocytes/μl of blood. Please note that the index case and two of his siblings had 2 or less CD16-positive events. Blood for the maternal grandfather was not available.

Genetic analysis

Since M-CSF has been shown to promote development of the $CD14^{+}CD16^{++}$ monocytes (Weiner et al. 1994; Schmid et al. 1995; Saleh et al. 1995) we considered a mutation of this candidate cytokine or the respective M-CSF-receptor. Sanger sequencing of the coding sequences amplified from cells of the index case, however, failed to demonstrate any abnormality. Exome sequencing also failed to show any mutation in the M-CSF and M-CSF receptor genes.

Using GRR and Pedcheck software packages the familial relationship as shown in Fig. 3 could be confirmed. SNP genotyping was done on DNA of all family members shown in the figure, except for the maternal grandfather. Due to the limited power of the present family we have, however, not been able to define the gene(s) responsible for the absence of the CD16-positive monocytes, as yet.

Discussion

Only recently a few reports have described patients lacking blood monocytes and in these cases both the classical and the nonclassical CD16-postive monocytes were affected. This included a syndrome with lack of circulating monocytes, NK-cells and B cells associated with infections by *Mycobacterium avium*, fungi and papilloma virus (Vinh et al. 2010; Bigley et al. 2011), which recently could be attributed to mutations of GATA2 (Hsu et al. 2011) and a defect of all circulating monocytes and dendritic cells with susceptibility to BCG disease due to IRF8 mutations (Hambleton et al. 2011). Given that the CD16-positive monocyte subset has been described more than 20 years ago it is astounding that a selective defect of these cells has not been described so far.

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We now describe three individuals with such a defect. The absence of a cell type in blood can simply be due to the absence of the cell surface marker used for identification of the cell. An example for this is the failure to detect CD4 T-helper cells with the antibody OKT4 because of the lack of the epitope recognized by the antibody (Bach et al. 1981). For the individuals described herein, this is not the case since the CD16-positive monocytes were not detected with antibodies to two different epitopes. Also, NK cells, which express the same transmembrane CD16a gene as monocytes, were readily identified with these antibodies as were granulocytes, which express the GPI-anchored CD16b gene (see Fig. 2). Furthermore, the lack of the nonclassical monocytes was apparent when these cells were identified via their low level CD14 expression and high HLA-DR.

These data indicate that the CD16-positive monocytes are really absent from blood in these individuals. There are different possibilities for such an absence and this includes (a) a reduced influx of these monocytes into blood either due to a failure of the monocytes to mature or to egress from bone marrow, (b) an increase in apoptosis and (c) an increased efflux of these monocytes from blood into tissue. The CD16-positive monocytes have been described to preferentially reside in the marginal pool (Steppich et al. 2000) and preferential location to this compartment could explain the lack of the cells in blood. A preliminary attempt to mobilize these cells by excessive exercise in one of the affected siblings, however, failed to increase the number of the CD16-positive monocytes (data not shown).

The defects in monocytes reported thus far are going along with increased susceptibility to infection (Vinh et al. 2010; Bigley et al. 2011; Hsu et al. 2011; Hambleton et al. 2011). These defects resulted in a low number of total monocytes while in our family including the patient the absolute monocyte numbers were in the normal range. The index case in our report was suffering from various infections including nosocomial pneumonia. These infections were, however, considered secondary to the severe medical condition, which included continuous protein loss associated with pleural exsudate formation and diarrhea. The dominant pathophysiological process in the case described herein appears to be an auto-inflammatory disease, which eventually led to amyloidosis.

Surprisingly, the two siblings, who also have a defect of the CD16-positive monocytes, were of good health without any evidence of infection and inflammation and with normal blood levels for CRP and SAA. This indicates that on its own the absence of this monocyte subset from blood does not lead to disease. We assume that confounding factors are required to allow for manifestation of disease. We also have to consider the scenario that the absence of these cells from blood does not lead to any disease at all. In this scenario the lack of these cells and the novel type of auto-inflammatory disease are two totally unrelated events.

Independent of the clinical implications of the lack of CD16-positive monocytes the family we describe herein offers the possibility to define the gene(s) responsible for the lack of these monocytes. Since the development of these monocytes is governed by the M-CSF pathway (Weiner et al. 1994; Schmid et al. 1995; Saleh et al. 1995) we have analyzed cell surface expression of the receptor on classical monocytes and found expression levels similar to healthy controls (data not shown). We then sequenced the genes for M-CSF and its receptor but we found no mutation.

Genotyping has not been able to pinpoint the causative gene. Although we had access to samples from three generations of the family, this was not sufficient to identify genetic loci. Also, exome sequencing has not revealed a clear candidate as yet. A recent report on NR4A1 knock-out has shown a decrease on the percentage of nonclassical monocytes in the mouse, due to an increased apoptosis of these cells (Hanna et al. 2011), which are prone to apoptosis (Wong et al. 2011). We have looked at the human homologue of NR4A1 and found a normal sequence in our family. Also, while in the mouse a 7-fold higher expression of the gene is seen in the nonclassical monocytes, our transcriptome analysis found no differential expression for the genes of NR family in the human subsets. The gene responsible for the defect of the CD16-positive monocytes in the family described herein may well be involved in apoptosis but it may also govern processes of maturation and migration.

Blockade of the M-CSF receptor in the mouse was shown to lead to increased M-CSF levels in serum (Lenzo et al. 2011) probably because the M-CSF protein cannot be bound, internalized and degraded (Pixley and Stanley 2004). When we assume that the M-CSF receptor in the individuals without CD16-positive monocytes is unable to bind and remove M-CSF, then increased M-CSF can be expected. Only in the index case levels were increased, the likely reason being that infection and inflammation will induce M-CSF (Blumenstein et al. 1997; Shimoda et al. 1993). The two affected siblings without inflammatory disease had serum levels in the normal range, providing indirect evidence that the M-CSF receptor is intact and can bind and internalize the M-CSF protein.

Taken together we describe herein a defect of the CD16-positive nonclassical plus intermediate monocytes in three siblings from non-consanguineous parents. It is hoped that more reports on families with a lack of the CD16-positive monocytes will be published in the future such that with increased statistical power the genetic defect can be identified.

Authors' contributions

MF designed, performed experiments and analyzed data and contributed to the study design. ABE designed, performed experiments and analyzed data. MWA provided clinical samples and data. HH performed laboratory analysis and contributed to the study design. TPH performed experiments and analyzed data. IH provided clinical samples and data. PM and PL performed experiments and analyzed data. MW designed experiments and analyzed data. KH provided clinical samples and data and contributed to the study design. AR designed experiments and analyzed data. LZH conceived the project, designed and analyzed experiments and wrote the paper.

Conflicts of interest

None of the authors declares any competing financial interests or other conflicts of interest.

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