Expression of VEGFA-mRNA in classical and MSX2-mRNA in non-classical monocytes in patients with spondyloarthritis is associated with peripheral arthritis.

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Research article

Abstract

Background

Spondyloarthritis (SpA) is characterized by chronic inflammation and structural damage involving spine and peripheral joints. Monocytes, as part of innate immune system, following migration into affected tissue, may play a role in the pathogenesis of SpA. Here, potential associations between osteogenesis-linked gene expression profile in particular monocyte subpopulations and clinical signs of SpA were investigated.

Methods

The 20 patients with axial and 16 with peripheral SpA were enrolled in the study. Monocyte subpopulations (classical - CD14 + + CD16-, intermediate - CD14 + + CD16 + and non-classical - CD14 + CD16++) were isolated from blood using flow cytometry and gene expression analysis was performed using real-time PCR method and TaqMan™ Array, Human Osteogenesis, Fast 96-well plates. Next, the characteristic clinical features of axial or peripheral SpA were analyzed in the context of the expression of selected genes in the three subpopulations of monocytes.

Results

We demonstrated that expression of *VEGFA* in classical and *MSX2* in non-classical monocytes were associated with the number of swollen and painful peripheral joints of SpA patients.

Conclusions

Monocytes may contribute to the development of peripheral arthritis in SpA patients. This might be possible through subpopulation specific effects, linking number of inflamed joints with expression of *VEGFA* in classical monocytes and *MSX2* in non-classical monocytes.

Background

Spondyloarthritis (SpA) represents a group of second most prevalent inflammatory rheumatic disorders (ca. 1% in Caucasians) characterized by chronic inflammation and structural damage involving axial and peripheral skeleton. Recent research in SpA has been focused on the phenotypic presentations and pathophysiology of SpA subgroups (ie. non-radiographic axial spondyloarthtitis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, arthritis in inflammatory bowel diseases and undifferentiated spondyloarthritis) exploring, whether SpA is a single disease with various clinical expression or rather a group of distinct clinical entities sharing common signs and symptoms [1].

Genetic, immunopathologic, and clinical evidence indicate that despite common downstream pathways, mediated e.g. by macrophage-derived TNFα, inflammation in SpA is driven and maintained by different cellular and molecular mediators [2, 3]. Moreover, it has been proposed that SpA is an autoinflammatory disease driven rather by innate immune cells, than a genuine autoimmune disease triggered by self-reactive T and/or B lymphocytes [4]. The phenotypic subclassification of SpA is usually based on extraarticular signs (psoriasis and inflammatory bowel disease), pathogenesis (reactive arthritis) or outcomes (ankylosing spondylitis) [1]. Nevertheless, all phenotypes share similar axial (sacroiliitis, spondylitis, back pain) or peripheral (arthritis, enthesitis, dactylitis) manifestations, and therefore SpA might be classified as one of two subforms with different pathophysiology, with predominant involvement of axial or peripheral skeleton. In other words, there is a proposal to define SpA by its pathophysiology rather than by its phenotypic presentation, since the emerging data from immunopathology studies and clinical trials suggest that axial and peripheral SpA might be driven by different mechanisms and respond differently to treatment, supporting the classification of SpA according to the presence of axial or peripheral disease [1]. As a consequence, it is interesting to explore mechanisms leading to various SpA manifestations in axial and peripheral SpA. They are likely to be most variable and at the same time most informative at an early stage of the disease, before multiple chronic inflammatory and compensatory processes occur.

In such setting, pathophysiological role of monocyte subpopulations as a source of pro- and anti-inflammatory cytokines, bone remodeling proteins and other biologically active compounds is not fully elucidated. Moreover, there is some evidence that monocytes may be the source of novel bone forming cells ("monoosteophils") [5].

The aim of our study was to link characteristic clinical features of axial or peripheral SpA with different expression of selected genes in three subpopulations of monocytes isolated from blood of SpA patients. We focused on the manifestations which are uniquely associated with axial or peripheral SpA and therefore specific only for axial or peripheral SpA. This might help to understand how monocytes and macrophages and osteoclasts possibly derived from them might drive particular pathological processes, which are then interpreted as characteristic clinical signs of axial or peripheral SpA.

Methods

Patients

Thirty-six patients with SpA (20 axial and 16 peripheral SpA) according to the Assessment of SpondyloArthritis International Society classification criteria [6, 7] were enrolled in the study. Patients were under 45 years, naive to synthetic, synthetic-targeted or biologic Disease Modifying Anti-Rheumatic Drugs (DMARDs) and without administration of systemic glucocorticosteroids. Patients provided a signed informed consent and the study protocol was approved by the local bioethics committee.

Isolation of monocytes and their subsets

Monocyte subpopulations were isolated from peripheral blood mononuclear cells (PBMC) obtained from SpA patients. PBMC were isolated from EDTA-treated whole peripheral blood by the standard Pancoll human (Panbiotech, Aidenbach, Germany) density gradient centrifugation. PBMC were washed in PBS (Sigma-Aldrich, Saint Louis, USA) and then monocyte subsets (classical - $CD14^{++}CD16^{-}$, intermediate - $CD14^{++}CD16^{+}$ and non-classical - $CD14^{+}CD16^{++}$) were isolated using flow cytometry cell sorting. The following monoclonal antibodies (mAbs) were used to stain monocytes: anti-CD14-FITC (clone MφP9, BD Bioscience), anti-CD16-PE (clone 3G8, BD Bioscience) and anti −HLA -DR -PerCP (clone L243, BD Buoscience), in 1:25 dilution v/v stained and gated as previously described by us and others [8, 9]. The stained monocytes were then incubated for 30 min at 4°C after which they were sorted using the FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA). Sorter was equipped with 488 nm laser for excitation of FITC, PE and PerCP. The following band-pass filters were used for the measurement of fluorescence: 530/30 for FITC, 582/42 for PE and 695/40 for PerCP. After isolation, the cells were washed in PBS, centrifuged for 10 min at 350 x g and kept frozen at -80°C until RNA isolation.

Gene expression analysis using real-time PCR

Gene expression analysis was performed using real-time PCR method and TaqMan™ Array, Human Osteogenesis, Fast 96-well plates, # 4418741 (coverage of 92 osteogenesis associated genes and 4 endogenous control genes) (Thermo Fisher Scientific, Waltham, MA, USA). RNAs were isolated using miRVana microRNA isolation kit (Thermo Fisher Scientific); isolated RNA were transcribed into cDNA using Superscript IV VILO mastermix (Thermo Fisher Scientific). Next, cDNA was used for the assessment of gene expression profile using the TaqMan Array Human Osteogenesis plate and QuantStudioTM3 Real-Time PCR instrument (Thermo Fisher Scientific) according to manufacturer's protocol. The results were analyzed with appropriate software.

Statistical methodology

All statistical analyses, as well as data pre-processing and normalization was done in R (version 3.5.2). The expression was calculated as the Ct values and filtered according to manufacturer's instructions (only values with high confidence scores were used for further analyses). Subsequently, data was divided into three panels − each corresponding to a different subpopulation of monocytes: (1) classical, (2) intermediate, and (3) non-classical. The expression values were further normalized using the quantile normalization method as implemented in the package 'preprocessCore' (version 1.44.0). For the target statistical analysis, only genes with at least 5 observations in a given panel were used. In return, 88 genes were selected for further analysis in 37 samples of non-classical monocytes, and 38 samples of classical as well as intermediate monocytes. The association between axial or peripheral signs and gene expression in subpopulations of monocytes was tested via a simple linear model (with intercept and one predictor only) with empirical Bayes correction as implemented in the package 'limma' (version 3.38.3).

Results

Demographic, clinical and laboratory data

Table 1 presents characteristics of patients. Briefly, median age (years, IQR) of axSpA patients was 33.5 (29.7–39.7) and perSpA patients was 35.5 (31-38.5). Median disease duration (years, IQR) was 7 (5-10.7) for axSpA and 3 (2-9.5) for perSpA patients. 95% of axSpA and 38% of perSpA patients were HLA-B27 positive. Fifteen axSpA patients fulfilled mNY criteria for ankylosing spondylitis.

Table 1
Demographic and clinical characteristics of patient groups.

	axial SpA (n = 20)	peripheral SpA (n = 16)	p-value
Age (years), median (IQR)	33.5 (29.7−39.7)	35.5 (31−38.5)	NS
Males, n (%)	14 (70)	8 (50)	< 0.001
HLA B27 positive n (%)	18 (95%)	5 (38%)	0.002
Disease duration (years), median (IQR)	7 (5-10.7)	3 (2-9.5)	NS
ESR mm/h, median (IQR)	24 (11−33)	25 (17−37)	NS
CRP mg/l, median (IQR)	7.02 (1.4-11.98)	8.02 (3.98−13.7)	NS
BASDAI median (IQR)	2.5 (1.1−4.6)	4.9 (2.9−6.4)	NS
ASDAS (CRP) median (IQR)	2 (1.5-3.0)	3.1 (2.1−3.5)	NS
IBP (total back pain), n (%)	16 (80)	6 (40)	0.03
Number of swollen joints (out of 66), median (IQR)	0	2 (1−4)	< 0.001
Number of painful joints (out of 68), median (IQR)	0	2 (0−4)	< 0.001
DAS28 (ESR) median (IQR)	NA	4.0 (3.0-4.4)	NA
Enthesitis n (%)	7 (35)	10 (71)	NS
Dactylitis n (%)	2 (10)	14 (93)	< 0.001
Diagnosis, n (%)	5 (25)	0	
nr axSpA	15 (75)	0	
AS	0	8 (50)	
PsA	0	8 (50)	
per SpA			

BASDAI − Bath Ankylosing Spondylitis Disease Activity Index; ASDAS − Ankylosing Spondylitis Disease Activity Score; DAS28 − Disease Activity Score (ESR) 28; IBP − inflammatory back pain; CRP − C-reactive protein; ESR − erythrocyte sedimentation rate; ax − axial, nr − non radiographic; per − peripheral, SpA - spondyloarthritis; AS − ankylosing spondylitis; PsA − psoriatic arthritis.

Selected gene and probe panels are differently expressed among 3 monocyte subpopulations

To explore the expression of mRNAs tested in our panel across monocyte subpopulations in SpA we utilized microarray expression data generated in a previous study by Metcalf et al [10] (18 individuals, 3 subsets of monocytes per sample). We acknowledged 94 genes (154 probes) which constituted our mRNA SpA panel and then using a Principal Component Analysis we identified 3 clusters of samples – each corresponding to different subpopulation of monocytes as shown in Fig. 1.

Associations between mRNAs and clinical signs

We analyzed whether mRNAs identified in different monocyte subpopulations were correlated with clinical signs of axial and peripheral SpA. The following were selected: (1) inflammatory back pain (total back pain, BASDAI question 2, range 1–10) for axial, and (2) number of swollen joints (out of 66 total joint count and out of 28 joint count from DAS28 score), (3) number of painful joints (out of 68 total joint count and out of 28 joint count from DAS28 score), (4) presence of enthesitis and (5) presence of dactylitis for peripheral signs.

Expression of VEGFA in classical monocytes is associated with the number of swollen and painful joints.

We found *VEGFA* mRNA in classical monocytes to be positively associated with measurement of joint involvement, i.e. number of swollen joints from total joint count and number of swollen and painful joints from DAS28 score, with FDR < 0.05 for each feature. The most robust association, with FDR < 0.001 was observed for the number of swollen joints from DAS28 score (Table 2).

Table 2
Results of the association analysis between clinical signs (swollen/painful) and gene expression in subsets of monocytes.

gene	monocyte subset	number of joints	FDR	logFC
VEGFA	classical	swollen (total)	0.043	-0.448
VEGFA	classical	painful (total)	0.191	-0.412
VEGFA	classical	swollen (DAS28)	< 0.001	-0.674
VEGFA	classical	painful (DAS28)	0.008	-0.704
MSX2	non-classical	swollen (total)	0.002	-0.956
MSX2	non-classical	painful (total)	0.083	-0.906
MSX2	non-classical	swollen (DAS28)	< 0.001	-1.072
MSX2	non-classical	painful (DAS28)	0.008	-1.131

The false discovery rate (FDR) was estimated for each clinical variable separately due to small sample size. FDR - estimated false discovery rate based on all tests performed, logFC - logarithm of the fold change of the expression levels between experimental conditions.

Expression of MSX2 in non-classical monocytes is also associated with peripheral arthritis.

We identified the *MSX2* mRNA expression in non-classical monocytes as positively associated with measurements of peripheral arthritis, i.e. number of swollen joints from total joint count and number of swollen and painful joints from DAS28 score, under the FDR < 0.08 for each feature (Table 2).

There were no significant associations between the expression of selected genes in the intermediate subset of monocytes with the number of swollen and painful joints. There were either no associations between the expression of *VEGFA* or *MSX2* mRNAs in subsequent monocytes subpopulations with other SpA clinical features, e.g. inflammatory back pain, enthesitis or dactylitis.

Discussion

Here, we provide evidence that monocytes, cells of innate immune system, may contribute to the development of arthritis in peripheral SpA and therefore might be at play when considering chronic inflammation, one of the most significant features of this disease.

There is a substantial evidence concerning the role of the innate immune system in the pathogenesis of a variety of SpA features, including chronic inflammation, repair and new bone formation [11, 12]. It seems

that peripheral blood monocytes in SpA are functionally primed and/or functionally reprogrammed by not yet well identified factor(s) and exhibit molecular or cellular features characteristic for SpA [13–15]. Monocytes are classified into three subpopulations – classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and nonclassical (CD14$^+$CD16^{++}); the latter two subpopulations being referred to as "proinflammatory" [16]. The "proinflammatory" subsets form app. 5–15% of circulating monocytes, express and secrete upon stimulation by various factors large amounts of pro-inflammatory cytokines, e.g. TNFα, interleukins (IL) – IL-12 and IL-1, but insignificant amount of anti-inflammatory IL-10. Contrariwise, classical monocytes produce relatively small amounts of TNFα, but they are a robust source of IL-10 [17]. Under physiological conditions most of classical monocytes (ca. 80–90%) leave the bloodstream after a circulating lifespan of 1 day, whereas remaining fraction further mature into intermediate cells and finally convert (in app. 12 days) to nonclassical monocytes before leaving the circulation [18]. Then extravasated monocytes supplement the population of resting tissue macrophages, but they may differ functionally in respect to their stage of maturation, pre-priming, and the type of target tissue mileu they migrate to. Therefore, these cells may influence inflammatory processes by factors released inside blood vessels or locally, upon their migration.

Currently, there are only a few studies (based on the analysis of single blood samples) that investigated differences in transcriptome/proteome profile of monocyte subpopulations isolated from healthy individuals [e.g. 19–26]. These studies indicate, on molecular basis, the significant differences in genetic profiles between these three monocyte subpopulations, confirming and extending most of the former phenotypic and functional observations. However, there is no data so far comparing gene expression in monocyte subsets in SpA patients exhibiting axial or peripheral signs.

Vascular endothelial growth factor-A (VEGFA) is one of the most important growth factors engaged in vascular development and angiogenesis. Since bone is a highly vascularized organ and angiogenesis plays an important role in osteogenesis, it has been established that VEGFA also influences skeletal development and postnatal bone repair [27]. Process of bone remodeling is based on a balance between bone formation and resorption [28]. Disturbance of this balance may strongly depend on both osteoclastic and osteoblastic activity. Liu et al [29] suggested, that VEGFA and TNFα might directly participate in the differentiation of fibroblasts into osteoblasts, and anti-VEGFA has been suggested as a possible new therapy preventing osteogenesis in SpA patients [28].

We have shown that *VEGFA*-mRNA expression in classical monocytes positively correlated with the number of swollen and painful joints. Our results also confirmed higher VEGFA level in SpA patients' sera when compared to healthy blood donors (data not shown) supporting the previous findings by Lin et al [30]. This may identify the classical monocytes subpopulation as the main source of VEGFA, which, upon migration to the synovium of peripheral joints, may promote local inflammation in SpA patients. It was already shown that VEGFA serum and synovial fluid levels are elevated in patients with ankylosing spondylitis expressing features of peripheral arthritis [31, 32]. Moreover, VEGFA may be secreted by various cell types, including macrophages, which are present in the synovial membrane and entheses in

patients with SpA, but no association with particular monocyte subpopulation (as a source of particular tissue macrophages) has been specified [32].

MSX2 is a transcription factor with a homeobox domain, presumably involved in bone development and ectopic calcifications, although its role in these processes is still controversial. Furuichi et al. examined a total of 45 single nucleotide polymorphisms (SNPs) in 15 genes by sequential screening and reported promising evidence for the association between *MSX2* polymorphisms and SpA in Japanese population [33]. Moreover, in the basic studies involving animal models, the *MSX2* knockout mice display remarkable decrease in mineralization of the axial skeleton, reduced proliferation of osteoprogenitors defecting skull ossification and abnormal calvarial development [34], whereas transgenic mice overexpressing *MSX2* show enhanced proliferation of calvarial cells [35, 36]. A loss-of-function mutation of *MSX2* in humans, which reduces DNA binding activity, causes defect in skull ossification [37]. These observations contrast with a gain-of-function mutation of *MSX2*, which results in an autosomal dominant disorder, Boston-type craniosynostosis [38, 39]. These findings demonstrate that *MSX2* expression is critical for human skull development and suggest its positive ossific role in bone development. However, MSX2 protein suppresses the expression of bone marker genes, including RUNX2 (a master regulator of osteoblast differentiation) and osteocalcin, and negatively regulates bone development and ectopic calcification [40–42]. The roles of MSX2 may vary depending on cell type and/or cell differentiation stage.

We have demonstrated that *MSX2*-mRNA overexpression in non-classical monocytes is positively associated with the number of swollen and painful joints, and therefore question the canonical role of this protein expressed preferentially in non-classical subset of monocytes. It was shown that TNFα may induce *MSX2* expression and MSX2 mediates the inhibitory action of TNFα in osteoblast differentiation [43, 44]. It may not be excluded that MSX2 expression in non-classical monocytes is secondary to their proinflammatory functions related to TNFα auto- or paracrine action. Moreover, upon migration into soft tissue, non-classical monocytes may contribute to local inhibition of the BMP2-regulated osteoblast differentiation within inflamed peripheral joints [44] and therefore being possibly involved in new bone formation and joint remodeling. These concepts must be verified applying the appropriate mouse model (e.g. SKG mice) of SpA associated peripheral arthritis, which is currently underway.

Our study has some limitations. Obviously, the number of patients is small, but it is our belief that this pilot observation is interesting although requires further investigation. Also, this is a cross-sectional study and we do not know whether the discovered findings are durable enough to be attributed to chronic pain and synovitis characteristic for peripheral SpA. Finally, we were exploring the peripheral blood monocytes only, having no matching data considering the local environment of synovial tissue.

Conclusions

Our data suggest that monocytes may contribute to the development of arthritis in peripheral SpA patients. This might be due to the subpopulation specific effects, linking the number of swollen and painful joints with the expression of VEGFA in classical monocytes and MSX2 in non-classical

monocytes. We argue for the first time that overexpression of both proteins in classical and non-classical subsets of monocytes may be linked with the inflammatory process within joints in peripheral SpA patients.

List Of Abbreviations

axSpA   axial spondyloarthritis

BASDAI   Bath Ankylosing Spondylitis Disease Activity Index

DAS   disease activity score

DMARDs   Disease Modifying Anti-Rheumatic Drugs

IL   interleukin

mAbs   monoclonal antibodies

PBMC   peripheral blood mononuclear cells

perSpA   peripheral spondyloarthritis

SNPs   single nucleotide polymorphisms

SpA   Spondyloarthritis

TNFα   tumor necrosis factor alpha

VEGFA   vascular endothelial growth factor-A

Declarations

Ethics approval and consent to participate

The study protocol was approved by the local bioethics committee (KBET/252/B/2012, Bioethics Committee of the Jagiellonian University, Podwale 3 Str., 31-118 Krakow, Poland). All included patients gave their informed written consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest.

Authors' contributions

M. St. performed experiments, collected data and wrote the manuscript; M. Se. analyzed results, performed statistical analysis and wrote the manuscript; M. K. recruited patients, analyzed results and edited the manuscript; Z. G. recruited patients and analyzed results; R. S. collected data and edited the manuscript; K. W. performed experiments and collected data; M. R-Z. performed experiments and collected data; M. L. performed experiments and collected data; M. C. discussed and edited the manuscript; J. C. discussed and edited the manuscript; J. B. discussed and edited the manuscript; A.G. performed experiments; K. WL. performed experiments; P. W. analyzed results and edited the manuscript; M. Si. developed the scientific concept of the research, supervised the project, analysed results and edited the manuscript.

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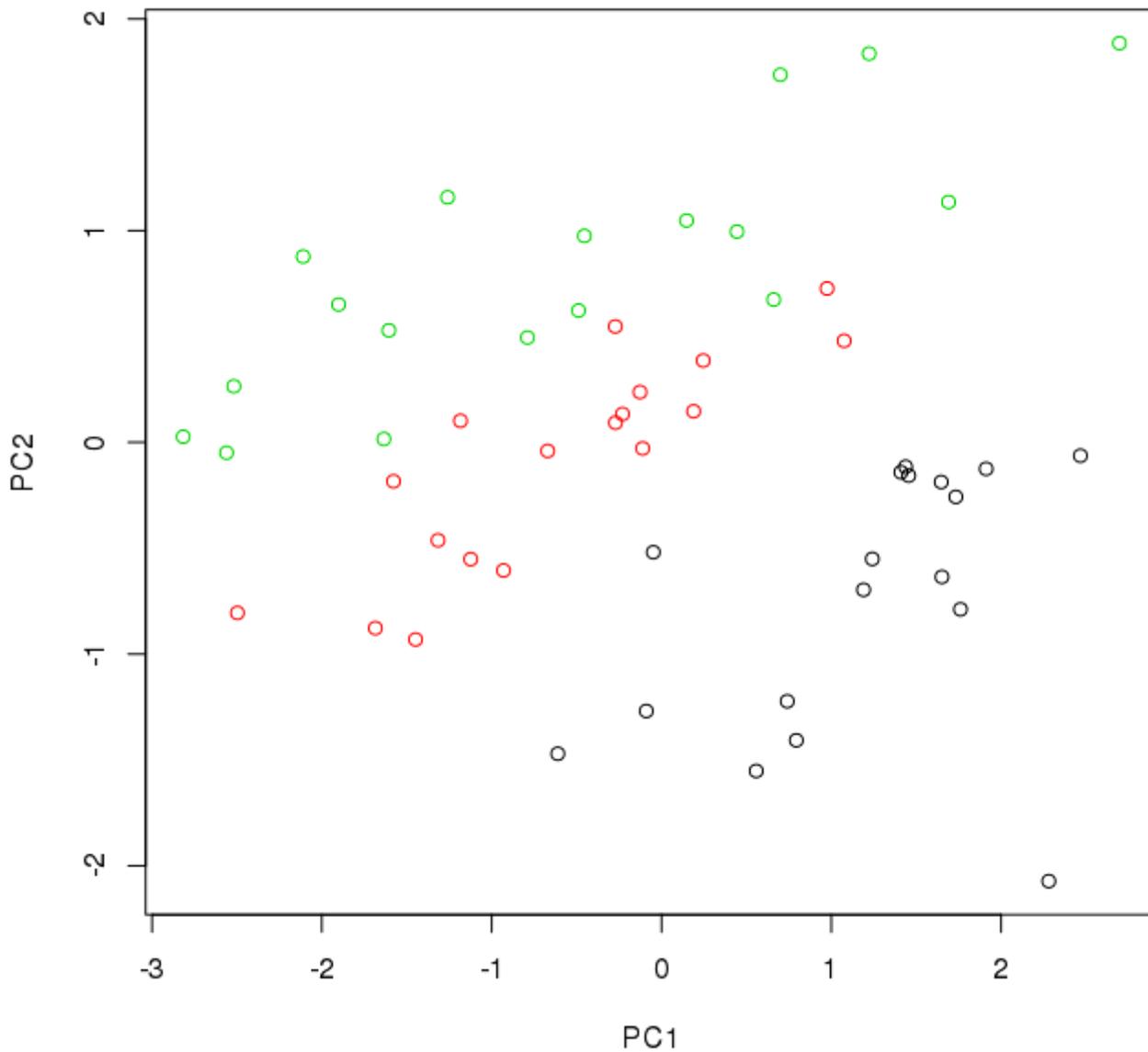
Figures

Figure 1

The results of PC analysis in microarray expression data in classical (black dots), non-classical (green dots) and intermediate (red dots) monocytes. Only probes in genes whose expression was initially measured in (and expressed well on the array) were selected for this analysis. PC1 and PC2 are the first two principal components estimated based on the expression data with the aid of the prcomp function in R