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Characterization of subsets of the CD16-positive monocytes: impact of granulomatous inflammation and M-CSF-receptor mutation

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Running Title: slan as marker for monocyte subsets

Abbreviations: DC: dendritic cell; HDLS: hereditary diffuse leukoencephalopathy with axonal spheroids; MACE: massive analysis of cDNA ends; MACS: magnetic cell sorting; slan: 6-sulfo LacNAc; TLR: toll-like receptor;

Key Points

1. The slan marker can be used to define non-classical and intermediate monocytes in human blood
2. slan-negative intermediate monocytes are expanded in sarcoidosis and slan-positive non-classical monocytes are absent in HDLS

Abstract

Human monocytes are subdivided into classical, intermediate and non-classical subsets but there is no unequivocal strategy to dissect the latter two cell types. We show herein that the cell surface marker 6-sulfo LacNAc (slan) can define slan-positive CD14+CD16++ non-classical monocytes and slan-negative CD14++CD16+ intermediate monocytes. Gene expression profiling confirms that slan-negative intermediate monocytes show highest expression levels of MHC class II genes, while a differential ubiquitin signature is a novel feature of the slan approach. In unsupervised hierarchical clustering the slan-positive non-classical monocytes cluster with monocytes and are clearly distinct from CD1c-positive dendritic cells. In clinical studies we show a selective increase of the slan-positive intermediate monocytes to >100 cells/µl in patients with sarcoidosis and a 5-fold depletion of the slan-positive monocytes in patients with hereditary diffuse leukodystrophy with axonal spheroids (HDLS), which is caused by macrophage colony stimulating factor (M-CSF) receptor mutations. These data demonstrate that the slan-definition of CD16-positive monocyte subsets is informative in molecular studies and in clinical settings.

Introduction

The first clear evidence for monocyte heterogeneity was obtained in man with the use of flow cytometry. These early studies described the classical monocytes and a then novel population of CD16-positive monocytes.[1] The latter cells account for about 10% of all monocytes in healthy individuals but their number can strongly increase with mobilization from the marginal pool.[2] An increase of these cells has been reported for many inflammatory conditions including sepsis [3] and anti-inflammatory therapy with glucocorticoids can deplete these cells selectively.[4, 5] Also, after stimulation with toll-like-receptor-ligands the CD16-positive monocytes were shown to be superior producers of pro-inflammatory cytokines like TNF.[6] Based on these features these cells were termed pro-inflammatory monocytes.[7]

Using CD64 as a marker, a population of CD16+CD64+ monocytes was described, which localized between the classical monocytes and the majority of the CD16-positive monocytes.[6] Clinical studies suggested that these intermediate cells can also expand in inflammatory conditions.[8, 9] Taking these findings into account, a nomenclature proposal suggested to call these cells intermediate monocytes (CD14++CD16+) as compared to the classical monocytes (CD14++ CD16-) and the non-classical monocytes (CD14+CD16++).[10] Detailed studies have shown that these cells are intermediate with respect to the expression of many molecules but that they show selectively high levels of MHC-Class II and CD74 and of molecules involved in angiogenesis.[11, 12]

Many publications have subsequently reported on an expansion of intermediate monocytes in various conditions.[13] It, however, remained difficult to clearly separate the intermediate from the non-classical monocytes since there was no selective marker to dissect these two subsets of CD16-positive monocytes. In the present report we have asked whether the slan-marker can be used for this purpose. Slan is a 6-sulfo LacNAc carbohydrate residue that is O-linked via a 6-O-sulfotransferase to the P-selectin glycoprotein ligand (PSGL-1) on the surface of blood leukocytes.[14]

Slan-positive cells have been reported to be CD14low CD16bright, CCR2-negative monocytes that show high levels of cytokine production and have superior antigen-presentation capacity, [15] features shared with the CD16-positive monocytes. We now show that intermediate monocytes are slan-negative and we demonstrate that this marker can be used to define non-classical monocytes as CD16-positive slan-positive cells and intermediate monocytes as CD16-positive slan-negative cells.

When we compared the genome-wide gene expression data of non-classical and intermediate monocytes defined either via the level of CD14 expression or based on the expression of slan, then the analysis revealed an increased MHC class II and C1Q expression for both approaches. When using the dissection of CD16-positive monocytes

based on slan then an unexpected ubiquitin signature is discovered, suggesting a biologically relevant separation of the subsets.

We have then tested the new slan-based approach to monocyte subset definition in patients with sarcoidosis, an inflammatory disease for which an expansion in CD16-positive monocytes had been noted.[16] Our data demonstrate in these patients a selective increase in the slan-negative intermediate but not the slan-positive non-classical monocytes indicating that these two subsets can be regulated independently. In addition we have studied patients with hereditary diffuse leukodystrophy with axonal spheroids (HDLS), a neurological disease associated with mutations in the M-CSFR-gene.[17] We now show that there is a pronounced depletion of the slan-positive non-classical monocytes in HDLS. These data indicate that the slan-marker can be used for an informative separation of intermediate and non-classical monocytes in man.

Material and Methods

Blood samples

EDTA-blood samples were obtained after written informed consent from healthy volunteers and patients with sarcoidosis and with HDLS. The sarcoidosis patients were studied before diagnostic bronchoscopy and before any treatment with glucocorticoids. The HDLS cases 693, 745 and 766 have been described in Schuberth et al.[18], case 667 was reported in Karle et al.[19] The study has been approved by the local Ethics Committee of the Ludwig-Maximilians-University Muenchen.

FACS analysis

100 µl of blood was admixed with antibodies (see Table S1 for the directly conjugated monoclonal antibodies) for 20 minutes on ice in the dark. Samples were lysed in Q-prep workstation (Beckman Coulter) including formaldehyde fixation (0.1%). An equal volume of counting beads (# C36950, Molecular Probes) was added. Samples were run on a FACSCalibur (Becton-Dickinson,) for 4-color-analysis. A total of 5000 classical monocytes were acquired per sample. The instrument settings are compiled in Table S2.

Gating strategy

All monocytes and the upper right third of lymphocytes were gated in the FSC/SSC scatter plot and these events were shown in a CD14/HLA-DR plot. Here the DR-positive cells were gated and shown in a CD14/CD16 dot plot. In this plot the classical, non-classical and intermediate monocytes were separated by a vertical line to the left of the classical monocytes.[20 21] Alternatively, slan expression on the DR-positive cells was displayed in an one-color-histogram. The slan-positive and slan-negative cells were then displayed in a CD14/CD16 histogram and the slan-positive CD16-positive non-classical monocytes and the slan-negative CD16-positive intermediate monocytes were determined. Absolute counts per µl of blood were then determined with reference to counting beads.

MACS isolation of monocyte subsets and dendritic cells

Monocyte subsets were purified using microbead conjugated antibodies and MACS columns based on CD14 expression and on slan expression and dendritic cells were isolated using CD1c. Details are given in the supplement.

Generation of MACE (massive analysis of cDNA ends) libraries

MACE is the latest advancement of tag-based gene expression analysis methods.[22] MACE libraries were prepared by GenXPro GmbH (Frankfurt, Germany) as detailed in the

supplement. Ten barcoded samples were sequenced simultaneously in one lane of an Illumina Hiseq2000 (Illumina, Inc., San Diego, USA) with 1 × 100 bp.

For quantification of mRNA expression, MACE reads in each monocyte subset library were PolyA-trimmed and the marked quality region was removed. Mapping of reads to the human genome (hg19) was performed with novoalign (http://novocraft.com/). Normalization and test for differential expression was performed in the statistical programming language R (www.r-project.org) with the DEseq package.[23] Expression levels are given in tags per million (tpm). GO-enrichment analysis was performed using the GO-enrichment toolkit from http://genxpro.ath.cx, which is based on the Fisher's exact test among transcripts that are differentially expressed at a p value of <0.05. For hierarchical clustering a gene set consisting of the 427 transcripts with a minimum coefficient of variation of 0.25 was selected. The clustering was performed using the "Multiple Experiment Viewer; version 4.9.0" based on the Pearson correlation and average linkage clustering.

Statistical analysis

Case control comparisons were done using Mann-Whitney U-test. Correlations were tested with Spearman's rank correlation analysis. Values of $p<0.05$ were considered significant.

Results

Definition of intermediate monocytes

For the analysis of monocytes in human blood we have used multicolour flow cytometry with antibodies against HLA-DR, CD14 and CD16. As shown in Figure 1 (upper panel) monocytes can be subdivided into classical, intermediate and non-classical cells.

Here the intermediate cells are defined within the CD14 versus CD16 dot plot in that they are separated from the non-classical monocytes using a vertical line and from the classical monocytes using a horizontal line (rectangular gating strategy).[21] These intermediate monocytes account for 16.8 cells/µl in this example while the non-classical monocytes account for 39.3 cells/µl. We then added the anti-slan antibody to the staining panel and show that this reagent can clearly separate slan-positive and slan-negative monocytes (Figure 1, middle panel). When the slan-positive monocytes are shown in a CD14 versus CD16 dot plot, then these cells are found in the area of the non-classical monocytes (Figure 1, right hand bottom panel). When the slan-negative CD16-positive monocytes are shown in a CD14 versus CD16 plot then they clearly cover the area of the intermediate monocytes as defined in the upper panel but the population also extends into the area previously thought to contain only non-classical monocytes. The slan-positive CD16-positive monocytes in this example account for 26.3 cells/µl, while the slan-negative CD16-positive monocytes amount to 31.2 cells/µl.

In average of 10 healthy male donors, the slan-positive monocytes were 36.8 ± 23.0 cells/µl and the slan-negative CD16-positive intermediate cells were 41.7 ± 24.1 cells/µl. When non-classical and intermediate monocytes were defined via the density of CD14 then the respective numbers were 48.1 ± 27.5 cells/µl and 24.0 ± 11.2 cells/µl, respectively (see Table 1).This demonstrates that the definition of intermediate monocytes as slan-negative CD16-positive monocytes results in a much larger population compared to the definition based on the CD14 expression level.

We then asked whether the definition via slan may provide an informative dissection of the CD16-positive monocytes into intermediate and non-classical cells. For this, we studied the entire transcriptome of monocyte subsets isolated by the two approaches using preparations from 5 different donors. In line with the staining pattern in Figure 1, the slan-based intermediate monocytes and non-classical monocytes extended well beyond the vertical CD14 cut-off line used for the CD14 based definition (Figure S1).

Gene expression analysis in subsets of CD16-positive monocytes

We then performed MACE of the monocyte subsets and analyzed differentially expressed genes after filtering as given under Material and Methods. When focusing on transcripts with an at least 1.2-fold difference (Table S3), then we found 676 differently expressed genes (DEG) between intermediate and non-classical monocytes for the CD14-based separation and 385 DEGs for the slan-based definition.

Among these there were 362 DEGs found only with the CD14 based separation and 71 DEGs found only with the slan-based definition of non-classical and intermediate monocytes. In addition, 314 DEGs were found in common between the two approaches.

The top genes among these common genes were (>8-fold higher in intermediate monocytes based on CD14 comparison): *STAB1, FPR3, PLA2G7, RNASE6, CLEC10A, CLEC4E, LGALS2, CD1C, MS4A6A* and (>4-fold in non-classical monocytes based on CD14 comparison) *LYPD2, VMO1* and *SCART1* (see Table S3A) and the dominant gene ontology (GO) term was “immune system process”.

The interaction analysis for these common genes revealed a strong cluster containing MHC class II genes, including *CD74, CIITA* and *CD1D*, all of which are related to antigen presentation. In addition there was a closely associated cluster containing genes *C1QA, B* and *C* and all of these class II and complement genes show a higher expression in intermediate monocytes (Figure 2A).

DEGs unique for the CD14-based separation of CD16 monocytes included (>8-fold higher in intermediate monocytes) *CATSPER1, TDRD6, ASGR2, SCD, CYP2S1, SIGLEC1, CDC42EP1* and (>4-fold higher in non-classical monocytes) *CARD11, FCRL1, C12orf7*5 and *ETS1* (see Table S3B) and the dominant GO term was “regulation of response to stimulus”. An interaction analysis revealed one cluster containing many molecules involved in signal transduction (*VAV1, FYN, SOCS3, PTK2B* and *PLCG2*) and in addition there was a ribosomal protein cluster of genes (Figure 2B).
DEGs unique to the slan-based definition of CD16 monocyte subsets included *MMP25* (>4-fold higher in intermediate monocytes) and *P2RY10* (>4-fold higher in non-classical) (see Table S3C) and the dominant GO term was “regulation of cytokine production”.

The interaction analysis revealed a set of 50 genes, which all are connected to ubiquitin C (*UBC*), a transcript that shows a higher expression level in slan-positive non-classical monocytes. The interacting genes include signaling molecules *IRAK3* and *MAP3K1* (higher in non-classical) and genes higher in slan-defined intermediate monocytes including *NFKB2,*

IRF8 and *MAP4K1* (Figure 2C). In addition, there is a cluster of three mitochondrial genes which show higher expression in non-classical monocytes.

When analyzing the genes higher in slan-defined non-classical monocytes separately then the dominant GO term was “MyD88-dependent toll-like receptor signaling pathway” and this included the genes *IRAK3, PELI1, UBC* and *MAP3K1*.

Validation of these DEGs was done by RT-PCR on altogether eight top differential genes (Table S3A and C) using the same samples that had been employed for MACE. Here the genes *STAB1, FPR3, RNASE6* and *CLEC10A* gave significant differences for both the CD14-based and the slan-based non-classical and intermediate monocytes, with a somewhat stronger effect seen with the CD14-based approach for these genes. On the other hand the genes *MMP25, CRISPLD2, DUSP2* and *NPM3* showed no difference in RT-PCR for the CD14-defined non-classical and intermediate monocytes, while the slan-based definition revealed clear and significant differences for these genes (see supplement, Figure S2). These patterns obtained in RT-PCR match the patterns seen with MACE (see Table S3).

Taken together, these data show a clearly differential gene expression pattern between non-classical and intermediate monocytes for both the CD14-based and the slan-based definition and this included genes involved in immune processes and in antigen presentation. For the CD14 based definition more signaling molecules were detected. For the slan-based definition of intermediate and non-classical monocytes unique DEGs included an ubiquitin-signature as well as genes involved in TLR signaling.

Unsupervised hierarchical clustering analysis supports the separation of non-classical and intermediate monocytes for both approaches (Figure 3). Also, all monocyte subsets including the slan-positive non-classical monocytes are clearly separated from the CD1c+ dendritic cells (DCs). This establishes that the slan-positive non-classical monocytes are distinct from dendritic cells.

Increase of slan-negative CD16-positive intermediate monocytes in sarcoidosis

We then analysed monocyte subsets based on slan and CD16 expression in sarcoidosis, a systemic granulomatous, inflammatory disease, for which an increase in CD16-positive monocytes had been shown.[8, 16] We studied our patients before diagnostic bronchoscopy and before any immunosuppressive therapy with glucocorticoids. Only cases confirmed to

have sarcoidosis based on histology showing non-caseating granulomas were included in the present analysis (Table 1).
As demonstrated in Figure 4, such patients can show a strong increase in CD16-positive monocytes. In this example the CD16-positive monocytes are increased to 224.8 cells/µl. When subdivided based on the CD14 expression level then non-classical monocytes (upper panel) account for 123.2 cells/µl and intermediate monocytes for 101.6 cells/µl. However, this subdivision appears rather arbitrary since the vertical line cuts through a homogenous population of events as is evident in Figure 4, upper panel. When subdividing the cells based on slan expression then the slan-negative CD16-positive intermediate monocytes account for 164.7 cells/µl and the slan-positive CD16-positive non-classical monocytes for only 61.0 cells/µl in this example. Hence, with the new definition the increase in CD16 monocytes in sarcoidosis is mainly due to the intermediate monocytes, while the non-classical monocytes show only a moderate rise in number in this example.

Analysis of a total of 14 patients demonstrates increases of the slan-negative intermediate monocytes to above 100 cells/ µl in five patients (see Figure 5).
Of these five cases four were male and one was female. When looking at the entire group of male sarcoidosis patients (Table 1) then the intermediate monocytes defined via slan-negativity gave clearly higher average values at 95.7 ± 55 cells/µl when compared to the intermediate monocytes defined based on CD14 expression level (56.0 ± 32.2 cells/µl). The male cases with high values of slan-negative intermediate monocytes (>100 cells/µl) all had increased levels of the soluble IL2R (> 710 U/mL) but at only n=7 the correlation was not significant (p= 0.071, Spearman linear correlation test).
When looking at non-classical monocytes then in male sarcoidosis patients there was a trend towards an increase of these cells when defined via CD14 density (mean 87.3 cells/µl versus 48.1 cells/µl in controls) while with definition via slan-expression the non-classical monocytes did not show any rise (mean 36.8 cells/µl versus 36.8 cells/µl in controls) (Table 1).
These data show that the use of slan for subset definition in sarcoidosis reveals a selective expansion of intermediate monocytes while non-classical monocytes remain unchanged.

Impact of M-CSF-R mutation on monocyte subsets

M-CSF is crucial to development of monocytes and macrophages as has been demonstrated in mutant mouse models that show reduced numbers and function of these cells.[24] Mutations of the human M-CSF-receptor gene have only recently been reported in man in patients with a late onset neurodegenerative disease termed hereditary diffuse leukodystrophy with axonal spheroids.[17] We have asked whether blood monocyte subsets might be altered in these patients. As shown in Figure 6 in case 667 with the c.2342C>T, p.A781V *M-CSF-R* there is a

strong reduction of both non-classical slan-positive monocytes and intermediate slan-negative monocytes (compare the case in B to control donor in A).

The other carriers of M-CSF mutations also showed a strong reduction of non-classical monocytes but the intermediate monocytes appeared unaffected (see case 745 in Figure 6 C and Table 1). As shown in this table, the depletion of non-classical monocytes was less apparent when these cells were defined via CD14 rather than the differential slan expression. The absolute numbers for the slan+ non-classical monocyte compared to healthy controls are summarized in Figure 7, which illustrates the strong depletion of these cells in HDLS.

All four cases had different mutations in the cytoplasmic part of the M-CSF-receptor and this may affect the clinical presentation and the degree of monocyte subset depletion. However, the slan-positive non-classical monocytes are most sensitive and are decreased by all of the four mutations studied herein.

Discussion

The nomenclature proposal for monocyte subsets, published in 2010 [10], has enabled a clearer communication in the field and the definition of the intermediate monocytes in that paper was followed by a host of publications on the prevalence of intermediate monocytes in various disease states (see Wong et al.[13] for review). However, the dissection of intermediate monocytes from the non-classical monocytes is still a matter of debate since setting a cut-off in the CD14 CD16 dot plot at a selected CD14 level often appears arbitrary and can vary between studies. To resolve this it has been suggested to use an additional marker that would reliably separate the intermediate monocytes from the non-classical monocytes. It had been shown earlier that the slan marker is expressed on CD16 monocytes with very low CD14 [25, 26] but this molecule has not been tested for its suitability as a marker for definition of non-classical monocytes. We show herein that slan can be used for this purpose since it can clearly distinguish slan-positive and slan-negative subsets of the CD16-positive monocytes (Figure 1). Using the slan approach instead of the CD14 approach many more cells are assigned to the intermediate monocytes rather than the non-classical monocytes such that the absolute number of slan-negative intermediate monocytes is higher compared to the non-classical (see Table 1). Hence, with the slan approach a monocyte subpopulation previously thought to be a minor population becomes a sizable subset.

The slan-based definition in common with the CD14-based definition has identified differentially expressed genes related to antigen presentation and this is in line with earlier reports that have shown a higher expression of these genes in intermediate monocytes.[11, 12] In addition the slan-based definition of non-classical and intermediate monocytes has uncovered an unique UBC-signature (Figure 2C). Ubiquitination has various roles within the cell and it impacts on protein degradation, intracellular trafficking and signal transduction.[27] For the slan-defined non-classical and intermediate monocytes there were many differentially expressed genes interacting with ubiquitin C that are, in fact, involved in signal transduction. This indicates that the slan-marker can define subsets of CD16-positive monocytes with a distinct biological repertoire.

Analysis of monocyte subset gene expression has been done in the past using micro-array and SuperSAGE technologies.[**11**, **12**, **28**, **29**] For the present study we have used the highly sensitive MACE approach for determination of the transcriptome.[22] When comparing the present results to the previous SuperSAGE approach [11] we found a similar gene expression pattern for monocyte subsets. However, due to the higher sequencing depth in the present study, we detected additional DEGs and this included the mitochondrial genes *MT-ND1* and *MT-ND5* (Table S4). As expected we also found expression of the CD16a gene in the intermediate and non-classical monocytes at a similar level for these two subsets (data not

shown). For CX3CR1, however, slan-positive non-classical monocytes had a significantly higher expression level (717 tpm) than the slan-negative intermediate monocytes (560 tpm, Table S3A), while classical monocytes show a low level expression of this gene (255 tpm).

Slan was originally described as a marker of dendritic cells although the slan-positive cells were shown to have monocyte morphology.[30] While some studies maintain that the slan-positive cells are dendritic cells, it was shown early on that slan-positive cells overlap with the CD16-positive monocytes. [15,25] The assignment of the slan-positive cells to monocytes rather than DC is also underlined by the expression of this marker on typical macrophages, i.e. on alveolar macrophages harvested from the lung.[25]

Also, slan-positive cells were shown to have properties similar to the CD16-positive monocytes, i.e. they show high levels of TNF production [31] and are CCR2-negative.[15]

It has been clearly shown earlier that the CD16-positive monocytes co-cluster with monocytes and not with DCs.[29, 32, 33] Still one could hypothesize that the slan-positive subset of the CD16-positive monocytes might have a gene expression pattern matching that of DCs. Evidence supporting the concept that slan-positive cells and DC are clearly distinct have been published earlier in that TLR3, 4 and 10 transcripts did show a reciprocal expression pattern in slan-positive cells and dendritic cells.[34] In the comprehensive approach taken herein we have compared the transcriptome of monocyte subsets and CD1c+ blood DCs in unsupervised hierarchical clustering. This demonstrated that the slan-positive CD16-positive cells co-clustered with monocytes and not with CD1c DCs (Figure 3). Hence, the slan-positive non-classical cells appear to represent bona fide monocytes and not dendritic cells. Consistent with this conclusion is the expression of the M-CSF-R in these cells in that classical monocytes had a low level of CD115 transcripts (459 tpm), intermediate monocytes had higher levels (1349 tpm) and slan-positive non-classical monocytes were highest (1513 tpm) (FDR < 10E20 for all comparisons). Analysis of cell surface expression of the CD115 M-CSF-R demonstrated a mean level of specific fluorescence intensity of 706.2 +/- 422.4 channels (mean +/-SD) for classical monocytes, a 2.5-fold higher level on intermediate monocytes (1754.8+/- 848.7 channels, $p>0.05$ compared to classical monocytes) and a 3-fold higher expression on non-classical monocytes (2106.2 +/- 624.4 channels, $p<0.05$ compared to classical monocytes, $p>0.05$ compared to intermediate monocytes). This suggests that the CD16-positive monocyte subset may be more responsive to the effects of M-CSF.

Among the DEGs for non-classical versus intermediate monocytes we found several signaling molecules that act downstream of the M-CSF-R. [35] These include the *lyn* and *dusp5* genes, which show higher expression in non-classical monocytes and thus may contribute to a more efficient M-CSF signal transduction.

The slan cell surface marker can be induced by M-CSF plus GM-CSF in-vitro.[36] The question is whether these cytokines initiate a differentiation program that includes up-regulation of slan or whether this is a direct effect of the cytokines on the regulation of the slan-marker. The slan residue can be added to PSGL-1 by a 6-O-sulfotransferase, specifically by carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 (CHST2).[37] Of note, the CHST2 transcripts are differentially increased in the non-classical monocytes as compared to the intermediate monocytes (see Table S3,A: common slan CD14). Whether the induction of the slan-marker by M-CSF plus GM-CSF is via induction of this enzyme is currently unknown.

An increase in CD16 monocytes in sarcoidosis patients had been noted earlier.[8, 16] With the recent further subdivision of the CD16-positive monocytes into a non-classical and an intermediate monocyte population, we now asked which of these subsets is responsible for the expansion in sarcoidosis. Here we have shown that only the slan-negative intermediate monocytes and not slan-positive non-classical monocytes are expanded in these patients. In our study we noted 4 out of 7 sarcoidosis cases with increased slan-negative intermediate monocytes among males compared to 1 out of 7 among females. While this might suggest a gender effect, additional studies with higher numbers of cases are required in order to resolve the question whether females show less of an expansion of slan-negative intermediate monocytes in sarcoidosis.

Sarcoidosis is a systemic inflammatory disease affecting various tissues in the body most frequently with manifestations in the lung.[38] Depending on the degree of inflammation serum parameters like CRP and sIL2R are increased in these patients [39, 40, 41, 42, 43] and this was also observed in some but not all of the patients herein (see Table 1).

While in the lungs of sarcoidosis patients activated macrophages have been described [39,44, 45], the role of monocytes especially of the expanded intermediate blood monocytes remains unclear.

Based on intra-vital microscopy in the mouse model non-classical monocytes have been described to migrate into tissue rapidly in response to inflammatory stimuli.[46] Also, recent studies in a murine model of arthritis have shown that Ly6C^{low} monocytes can migrate into joint tissue and drive inflammation.[47] Similar studies on migration into tissue for intermediate monocytes are lacking, but we speculate that in sarcoidosis these expanded, slan-negative intermediate monocytes with their pro-inflammatory repertoire will migrate into the various tissues, develop into macrophages and contribute to the granulomatous inflammation.

The data in the M-CSF-R mutant HDLS patients show that both subsets of CD16-positive monocytes are under control of the M-CSF signalling pathway. Here the slan-positive non-classical monocyte subset is most sensitive to a disruption of this pathway, since these cells were reduced in every patient, while the intermediate monocytes were clearly reduced in only

one case. Of note, while slan-positive non-classical monocytes were reduced in HDLS, the numbers for CD1c+, CD141+ and CD303+ dendritic cells were similar to controls (data not shown). This further supports the contention that slan-positive non-classical monocytes are independent of the dendritic cell lineage.

All of the four HDLS patients had a different single nucleotide mutation in the cytoplasmic part of the M-CSF-R gene (see Table 1). The case with the strongest decrease of CD16-positive monocytes had a mutation within the tyrosine kinase domain of the receptor, but patient 4, with a decrease of the non-classical monocytes only, had a mutation within the same domain. In any event it appears that different mutations can lead to different levels of depletion of non-classical slan-positive monocytes and we suggest that the flow cytometric determination of this monocyte subset can be used as an in-vivo measure of the degree of impairment of receptor function in-vivo. Also, it remains to be determined whether the depletion of non-classical blood monocytes, which can be seen early in life in the absence of neurological symptoms (see Table 1, case 1), plays a role in the pathophysiology and in the progression of disease manifestation in the brain.

Taken together we show herein that the dissection of the CD16-positive monocytes into a slan-negative intermediate monocyte subset and a slan-positive non-classical monocyte subset reveals a differential gene expression pattern with an ubiquitin signal transduction signature. Using the slan approach we show that slan-negative intermediate monocytes are increased in sarcoidosis and slan-positive non-classical monocytes are depleted in HDLS. It remains to be shown whether the slan-approach can define distinct roles of the two CD16-positive monocyte subsets in other diseases.

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Contributions: Thomas P. Hofer performed research and analyzed data and wrote the paper, Adam M. Zawada performed research and analyzed data and wrote the paper, Marion Frankenberger performed research and analyzed data, Kerstin Skokann performed research, Anna A. Satzl, Wolfgang Gesierich, Madeleine Schuberth, Johannes Levin and Adrian Danek identified appropriate patients and contributed vital research samples , Björn Rotter performed experiments and analyzed data, Gunnar H. Heine designed the study and analyzed data, Loems Ziegler-Heitbrock designed the study, performed experiments, analyzed data and wrote the paper.

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Table 1 Demographic, clinical and laboratory data

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								CD16-positive monocytes								clinical laboratory				
disease/ control	Proband ID	gender (m/f)	age (y)	radiological stage	classical monocytes (cells/µl)	classical monocytes (%)	total classical intermediate (CD14+ non-classical (CD14-)	CD14++ intermediate monocytes (cells/µl)	CD14++ intermediate monocytes (%)	CD14+ non-classical monocytes (cells/µl)	CD14+ non-classical monocytes (%)	slan- intermediate monocytes (cells/µl)	slan- intermediate monocytes (%)	slan+ non-classical monocytes (cells/µl)	slan+ non-classical monocytes (%)		ACE (U/L)	sIL2RCD25 (U/mL)	CRP (mg/L)	BAL CD4/CD8 (ratio)	BAL lymphocytes (%)
sarcoidosis	2062	m	66	III	494.2	75.3	655.9	64.0	9.8	97.7	14.9	140.4	21.4	45.6	7.0		69.3	787	3.7	7.8	21
sarcoidosis	2061	m	63	II	1220.0	84.9	1437.6	46.9	3.3	170.8	11.9	164.3	11.4	63.8	4.4		14.4	770	7.5	5.6	
sarcoidosis	2065	m	53	I	338.7	87.5	387.0	23.2	6.0	25.2	6.5	36.6	9.4	24.4	6.3		48.5	603	3.8	3.5	27
sarcoidosis	2073	m	45	I	388.2	84.6	458.7	33.6	7.3	36.9	8.0	46.2	10.1	25.8	5.6		32	577	48	4.6	14
sarcoidosis	2074	m	28	I	427.6	65.6	652.3	101.6	15.6	123.2	18.9	164.7	25.3	61.0	9.4		<6.0	2109	2.3	12.6	59
sarcoidosis	2078	m	35	II	374.7	83.9	446.4	27.1	6.1	44.6	10.0	58.5	13.1	13.1	2.9		<6.0	462	2.3	1.7	25
sarcoidosis	2131	m	53	II	351.6	62.8	560.1	96.0	17.1	112.5	20.1	128.5	22.9	48.2	8.6		78,4	1373	4.8	3.2	24
mean			49		513.6	77.8	656.9	56,0	9.3	87.3	12.9	95,7	16.2	36.8	6.3		41.1	954.4	10.3	5.6	28.3
SD			13.96		315.9	10.1	359.5	32.2	5.2	53.6	5.3	55.0	6.7	18.8	2.2		23.4	588.6	16.7	3.7	15.7
								p=0.032	p=0.040			p=0.015	p=0.025							
sarcoidosis	2079	f	31	II	328.5	82.4	398.9	31.8	8.0	38.5	9.7	60.9	15.3	16.7	4.2		71	1786	1.5	6.5	50
sarcoidosis	2080	f	36	0 §	349.0	80.7	432.4	35.4	8.2	48.0	11.1	65.4	15.1	17.9	4.1		<6.0	562	1.1	2.4	10
sarcoidosis	2088	f	62	I	351.2	85.9	408.9	19.6	4.8	38.1	9.3	51.0	12.5	6.5	1.6		34.5	898	4.1	4.3	37
sarcoidosis	2094	f	50	II	283.7	79.1	358.6	23.3	6.5	51.6	14.4	44.4	12.4	28.6	8.0		<82	499	2.8	4.6	19
sarcoidosis	2102	f	71	II	628.7	95.9	655.7	16.8	2.6	10.2	1.6	24.9	3.8	2.6	0.4		80	1341	1.6	11.9	20
sarcoidosis	2112	f	34	II	222.8	89.2	249.7	11.0	4.4	15.9	6.4	15.0	6.0	11.8	4.7		66	676	5.5	23.3	59
sarcoidosis	2146	f	58	I	248.6	67.8	366.4	42.0	11.5	75.8	20.7	100.6	27.5	23.5	6.4		<6.0	2660	16.4	5.9	23
mean			48.9		344.7	83.0	410.1	25.7	6.6	39.7	10.4	51.7	13.2	15.4	4.2		62.9	1203.1	4.7	8.4	31.1
SD			15.56		134.7	8.8	123.3	11.1	3.0	22.2	6.0	28.2	7.7	9.2	2.6		19.8	791.5	5.4	7.2	18.0
																normal range	12,4-81,6	223-710	up to 3	0,7-3,5	< 15
								CD16-positive monocytes								neurological impairment and imaging""				
disease	Proband ID	gender (m/f)	age (y)	mutation	classical monocytes (cells/µl)	classical monocytes (%)	total classical intermediate (CD14+ non-classical (CD14-)	CD14++ intermediate monocytes (cells/µl)	CD14++ intermediate monocytes (%)	CD14+ non-classical monocytes (cells/µl)	CD14+ non-classical monocytes (%)	slan- intermediate monocytes (cells/µl)	slan- intermediate monocytes (%)	slan+ non-classical monocytes (cells/µl)	slan+ non-classical monocytes (%)		motor impairment	cognitive and memory impairment	histology	MRI	PET
HDLS	667	f	22	2342C > T, A781V	433.0	98.4	439.8	3.0	0.7	3.7	0.8	4.2	1.0	2.6	0.6		none	none	none	hyperintensities	not done
HDLS	692	m	47	1745T>C, L582P	295.0	90.6	325.5	14.0	4.3	18.0	5.5	23.0	7.1	7.5	2.3		wheel-chair	severe	demyelination	hyperintensities	not done
HDLS	745	f	47	1897G>A, E633K	625.0	91.6	682.3	39.0	5.7	24.0	3.5	50.0	7.3	7.3	1.1		wheel-chair	severe	demyelination	hyperintensities	hypometabolism
HDLS	766	m	58	2512G>C, V838L	284.0	91.3	309.4	13.0	4.2	14.0	4.5	21.0	6.8	4.4	1.4		wheel-chair	severe	demyelination	hyperintensities	hypometabolism
mean			43.5		409.3	93.0	439.3	17.3	3.7	14.9	3.6	24.6	5.6	5.5	1.4						
SD			15.2		159.0	3.6	172.1	15.3	2.1	8.5	2.0	18.9	3.0	2.4	0.7						
						p=0.011				p=0.005	p=0.005			p=0.002	p=0.002						
								CD16-positive monocytes													
control	Proband ID	gender (m/f)	age (y)		classical monocytes (cells/µl)	classical monocytes (%)	total classical intermediate (CD14+ non-classical (CD14-)	CD14++ intermediate monocytes (cells/µl)	CD14++ intermediate monocytes (%)	CD14+ non-classical monocytes (cells/µl)	CD14+ non-classical monocytes (%)	slan- intermediate monocytes (cells/µl)	slan- intermediate monocytes (%)	slan+ non-classical monocytes (cells/µl)	slan+ non-classical monocytes (%)						
controls	n=10	m																			
mean			38.80		392.8	85.2	465.0	24.0	5.0	48.1	9.8	41.7	8.5	36.8	7.5						
SD			12.81		84.5	4.8	116.1	11.2	1.6	27.5	3.7	24.1	3.2	23.0	3.4						
controls	n=10	f																			
mean			48.1		433.6	85.5	504.7	21.8	4.5	49.3	10.0	46.2	9.6	26.6	5.2						
SD			11.1		93.9	5.9	91.8	7.4	1.8	19.9	4.4	18.2	4.7	16.3	3.0						
controls																					
mean	n=20	m+f	43.5		413.2	85.3	484.8	22.9	4.7	48.7	9.9	44.0	9.1	31.7	6.4						
SD			12.6		89.4	5.3	103.9	9.3	1.7	23.4	4.0	20.9	4.0	20.1	3.3						
SD																					

*p<0.05 compared to gender matched controls (Mann-Whitney U-Test)

histology in all sarcoidosis patients showed non-caseating epitheloid granulomas

§ granulomas in bronchial wall

"" no sensory impairment was seen in any of the HDLS patients

Legends to Figures

Figure 1

Use of slan for dissection of CD16-positive monocyte subsets

Whole blood from a healthy control (#2059) was stained with monoclonal antibodies to CD14, CD16, HLA-DR and slan and analyzed in four color flow cytometry. Monocyte subsets defined in the CD14 CD16 dot plot (top) are classical 417.6/µl; intermediate monocytes 16.8/µl and non-classical 39.3/µl. Separation based on slan (bottom) gives slan-positive non-classical monocytes 26.3/µl and slan-negative intermediate monocytes 31.2 /µl.

Figure 2

Interaction analysis of the differential genes between non-classical and intermediate monocytes. Shown are interactions at a confidence score of 0.5. A) DEGs found for both the slan-based and the CD14-based definition of subsets. B) DEGs found for the CD14-based definition of subsets. C) DEGs found for the slan-based definition of subsets.

Figure 3

Unsupervised hierarchical clustering of monocyte subsets and CD1c+ DCs

The analysis is based on 427 transcripts with a minimum coefficient of variation of 0.25.

Figure 4

Slan-based CD16-positive monocyte subsets in sarcoidosis

Whole blood from a sarcoidosis patient (#2074) was stained with monoclonal antibodies to CD14, CD16, HLA-DR and slan and analyzed in four color flow cytometry. Monocyte subsets defined in the CD14 CD16 dot plot (top) are classical 427.6/µl; intermediate monocytes 101.6/µl; non-classical 123.2/µl. Separation based on slan (bottom) gives slan- positive non-classical monocytes at 61.0/µl and slan-negative intermediate monocytes at 164.7/µl.

Figure 5

Absolute number of slan-based intermediate monocytes in sarcoidosis

Given are the respective values for male and female controls and sarcoidosis patients as determined in whole blood by flow cytometry using absolute counting beads. * $p<0.05$

Figure 6

CD16 monocyte subsets in hereditary diffuse leukodystrophy with axonal spheroids (HDLS). A) control donor 665; B) patient 667 C) patient 745. The slan-positive non-classical monocytes are depleted in every HDLS patient.

Figure 7

Absolute number of slan-positive non-classical monocytes in HDLS

Given are the absolute numbers for all patients with hereditary diffuse leukodystrophy with axonal spheroids (HDLS) as determined in whole blood by flow cytometry using absolute counting beads. * $p<0.05$

Figure 1

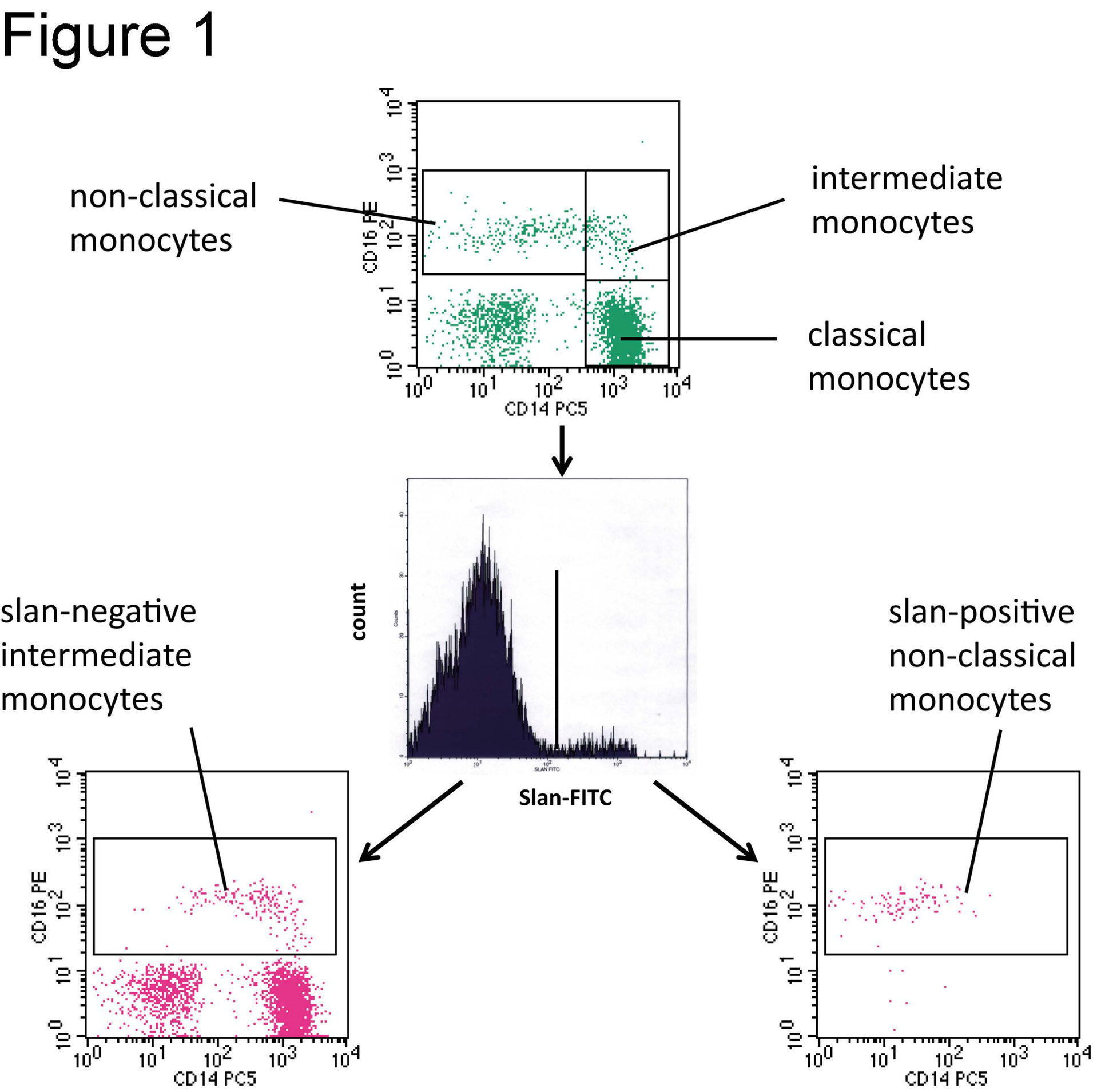

non-classical monocytes
intermediate monocytes
classical monocytes
CD16 PE
CD14 PC5
count
Slan-FITC
slan-negative intermediate monocytes
slan-positive non-classical monocytes
CD16 PE
CD14 PC5
CD16 PE
CD14 PC5

Figure 2

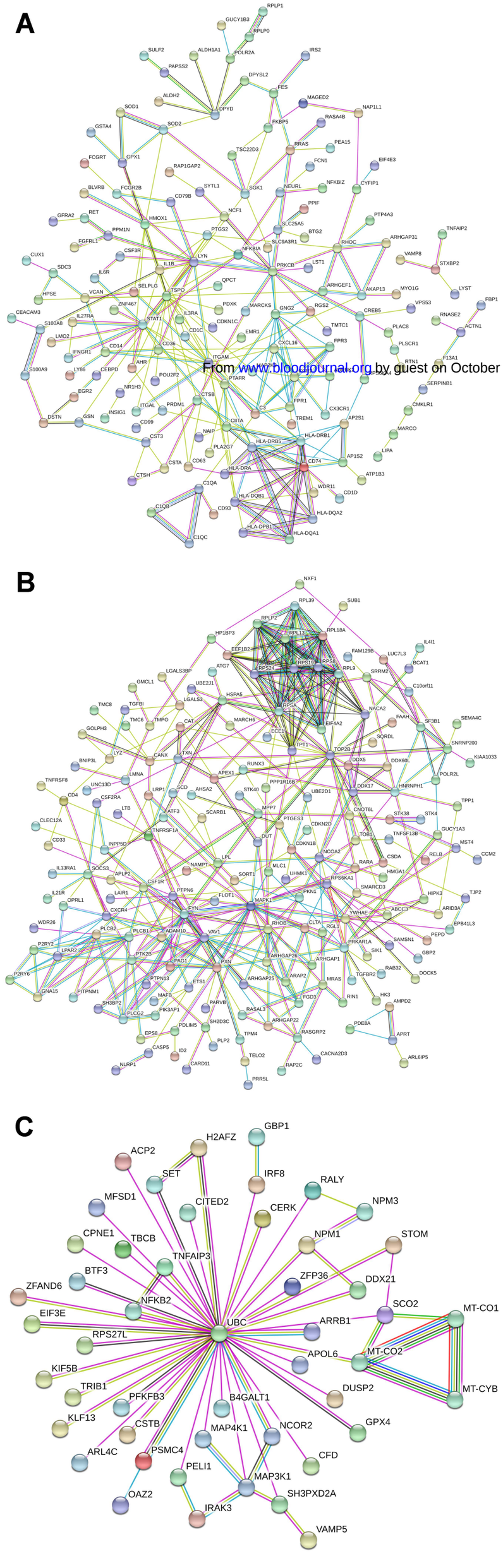


Figure 3

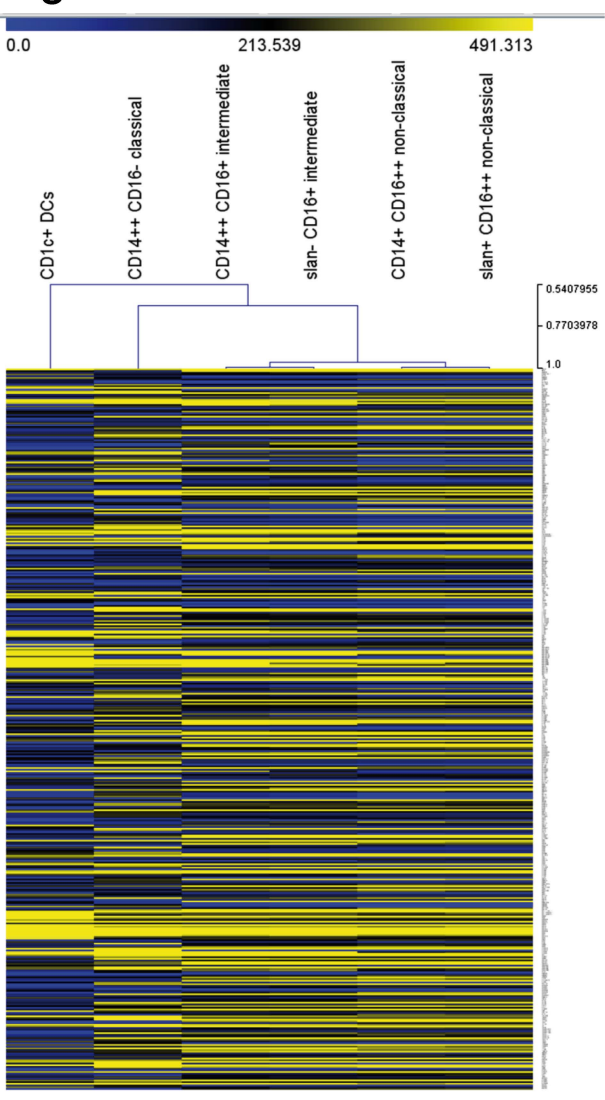

0.0
213.539
491.313
CD1c+ DCs
CD14++ CD16- classical
CD14++ CD16+ intermediate
slan- CD16+ intermediate
CD14+ CD16++ non-classical
slan+ CD16++ non-classical
0.5407955
0.7703978
1.0

Figure 4

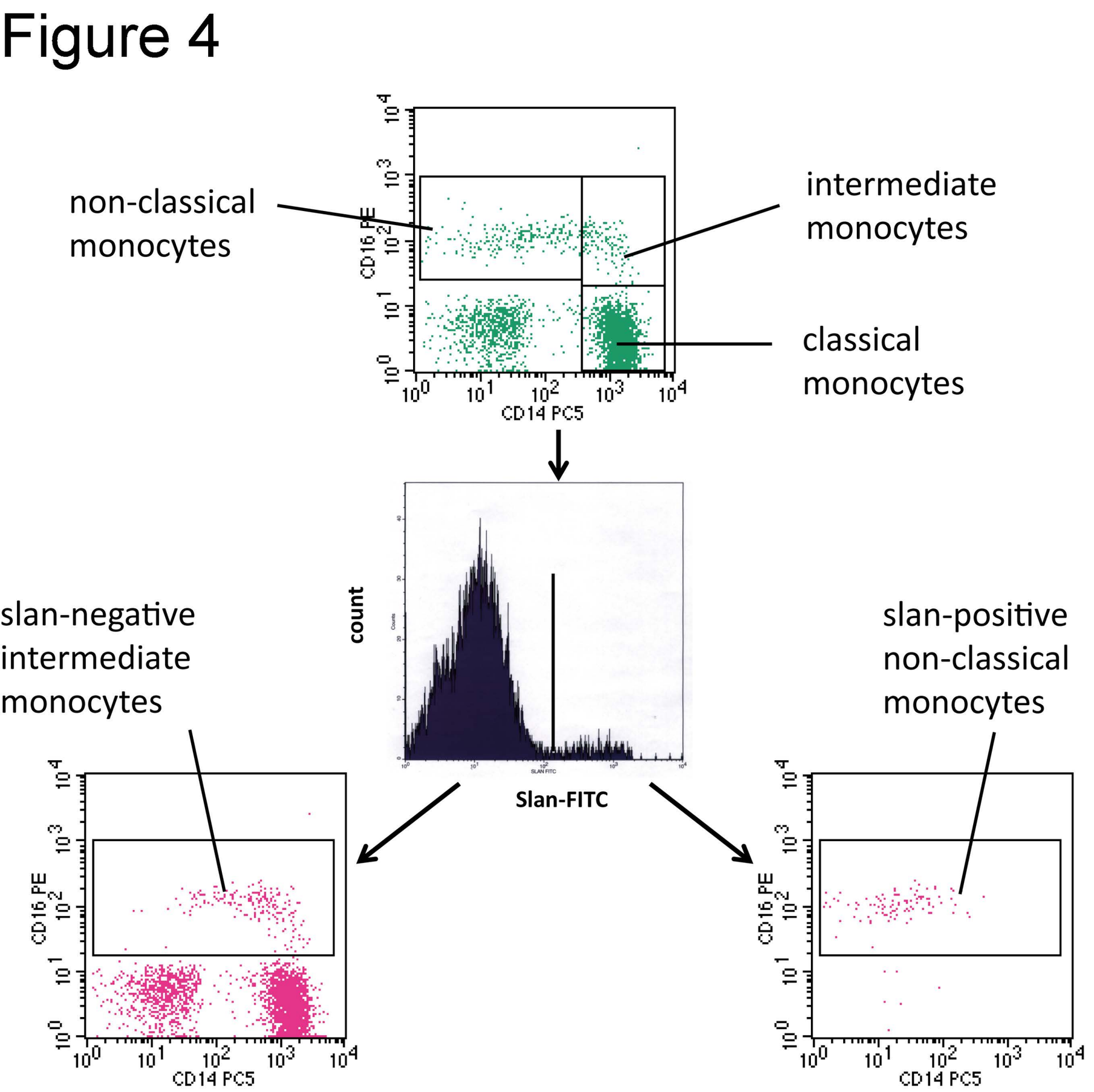

non-classical monocytes
intermediate monocytes
classical monocytes
CD16 PE
CD14 PC5
count
Slan-FITC
slan-negative intermediate monocytes
slan-positive non-classical monocytes
CD16 PE
CD14 PC5
CD16 PE
CD14 PC5

Figure 5

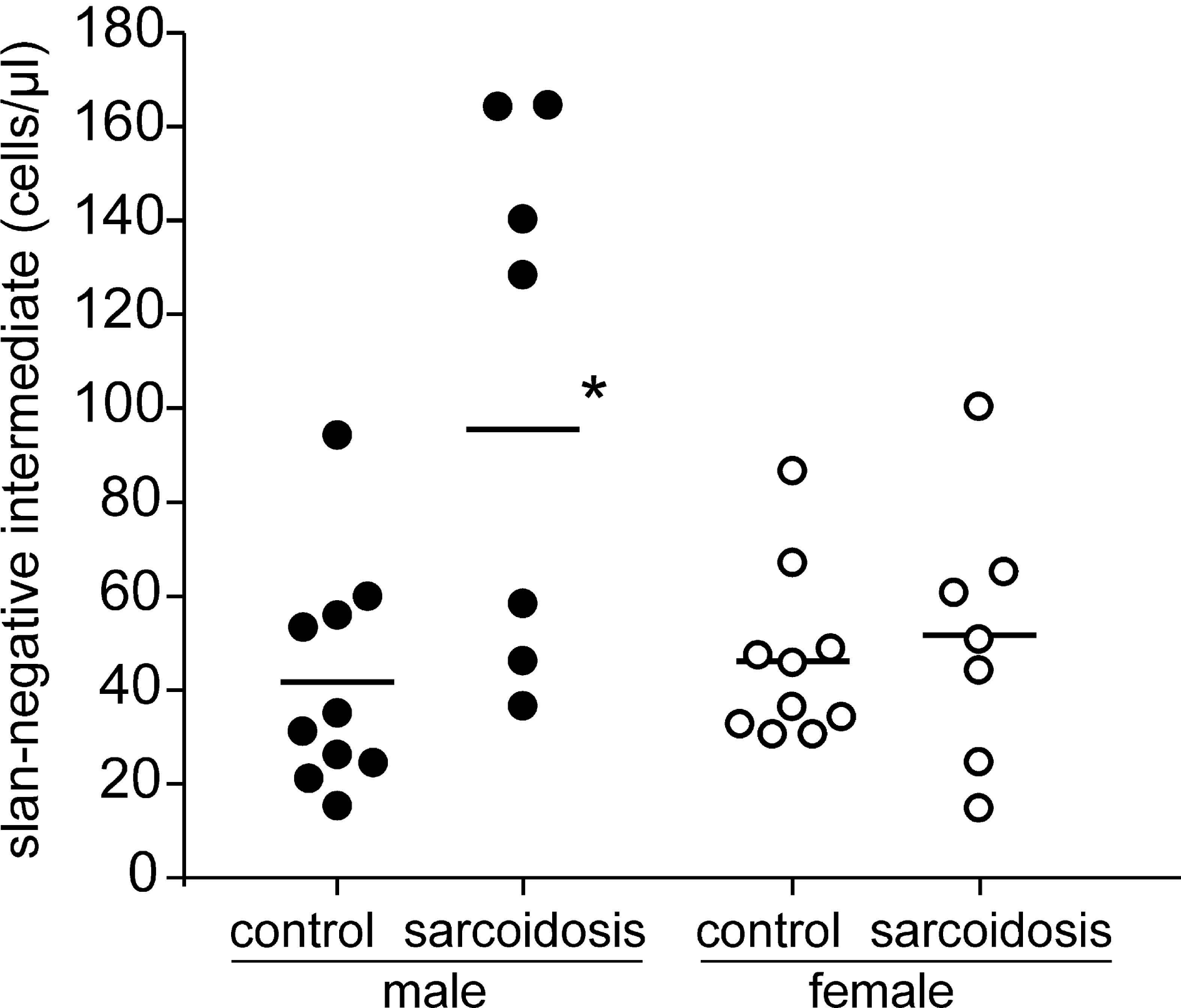

slan-negative intermediate (cells/µl)
180
160
140
120
100
80
60
40
20
0
*
control
sarcoidosis
control
sarcoidosis
male
female

Figure 6

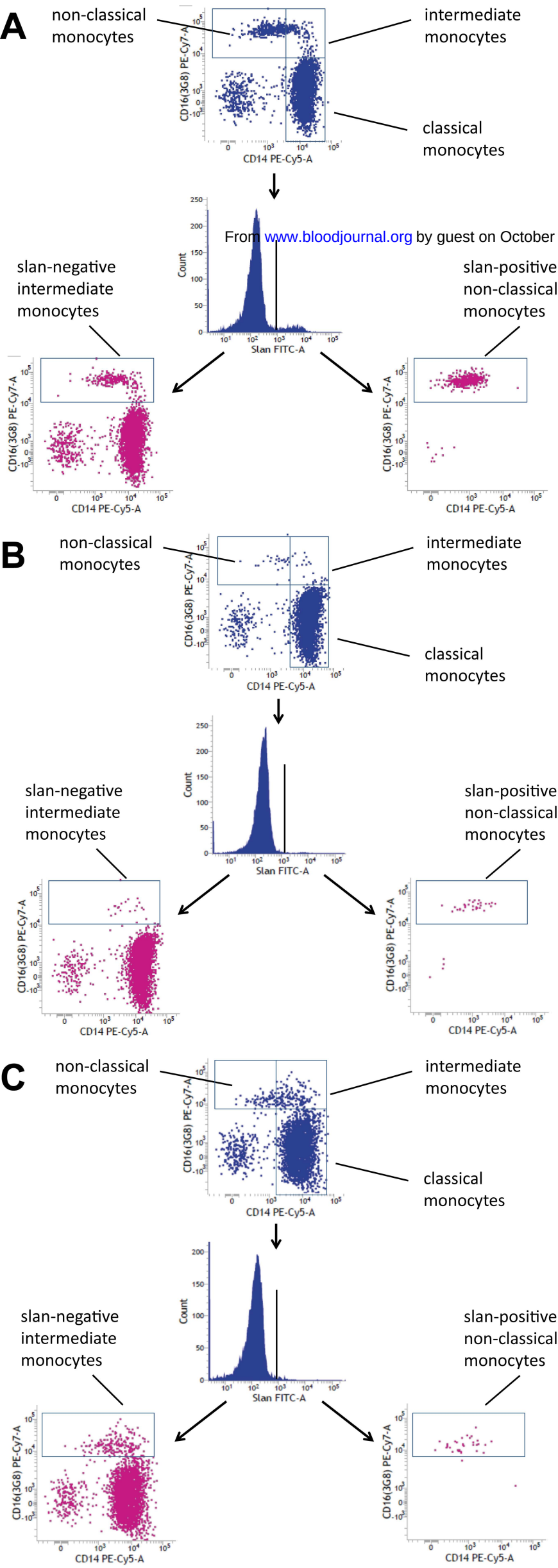
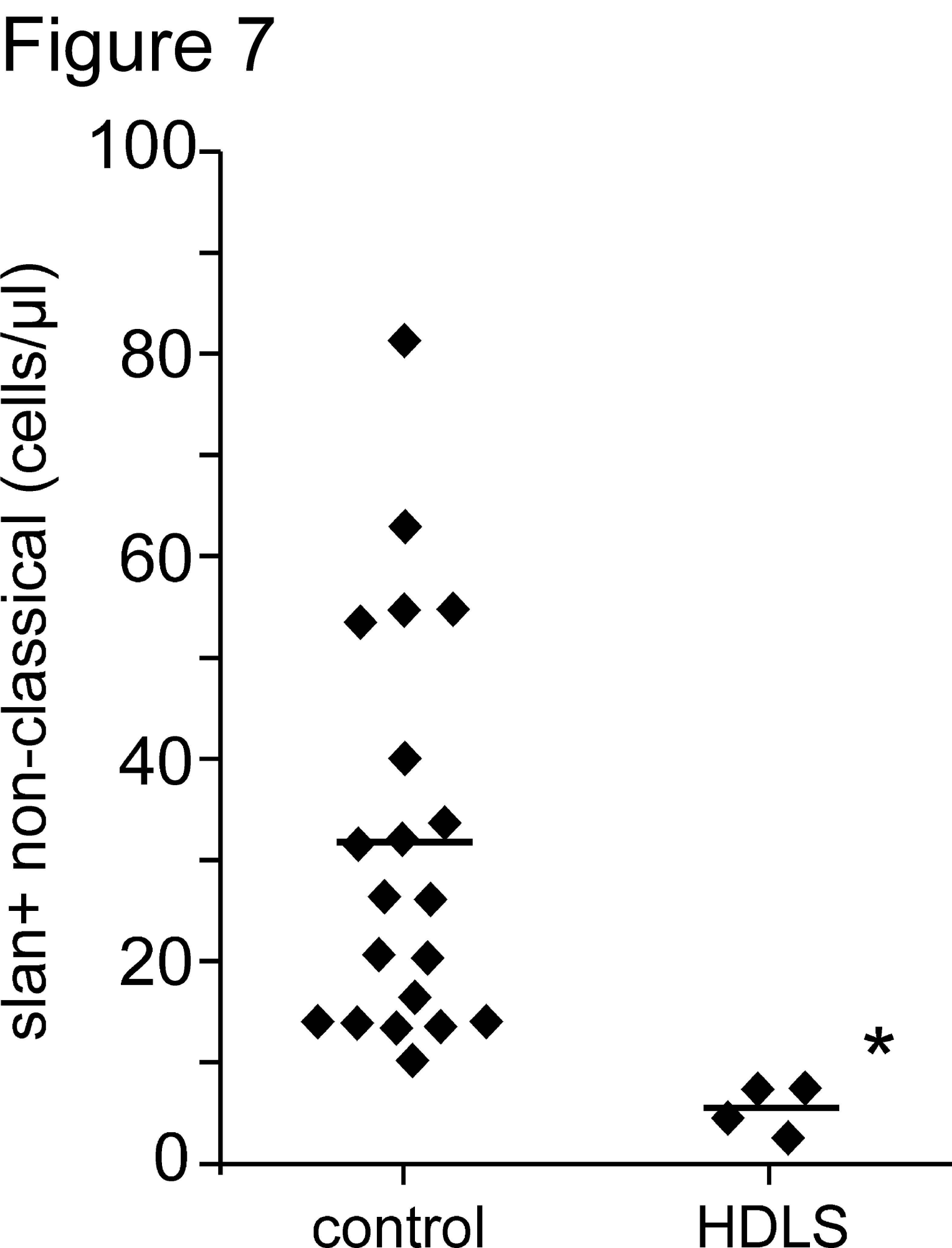


Figure 7



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Characterization of subsets of the CD16-positive monocytes: impact of granulomatous inflammation and M-CSF-receptor mutation

Thomas P. Hofer, Adam M. Zawada, Marion Frankenberger, Kerstin Skokann, Anna A. Satzl, Wolfgang Gesierich, Madeleine Schuberth, Johannes Levin, Adrian Danek, Björn Rotter, Gunnar H. Heine and Loems Ziegler-Heitbrock

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