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Characterization of Proangiogenic Monocytes from Blood in Patients with Chronic Ischemic Diabetic Foot Ulcers and Controls

*Ilker Uçkay^{1,4}, Beat A. Imhof², Benjamin Kressmann¹, Daniel Lew¹, Benjamin A. Lipsky¹,
Adama Sidibe^{2,3}*

¹Service of Infectious Diseases, ²Department of Pathology and Immunology, ³Department of Cell Physiology and Metabolism, Medical Faculty, University of Geneva, ⁴Balgrist University Hospital, Medical Faculty, University of Zurich, Switzerland

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Corresponding author

Ilker Uçkay, MD

Balgrist University Hospital

Forchstrasse 340, 8008 Zurich / Switzerland

Tel +41 44 386 11 11, Fax +41 44 386 37 09

E-mail: ilker.uckay@balgrist.ch

Keywords diabetic foot; ischemia; transmigration; proangiogenic monocytes; neovascularization

ABSTRACT

Many persons with diabetes mellitus have limb ischemia, which is a major clinical problem. A subset of human monocytes that expresses TIE-2, may enhance neo-vascularization. We performed 179 phlebotomies on 142 patients (or donors), including 61 patients/donors without diabetes or ischemia (controls), 39 diabetic non-ischemic patients (controls), and 42 diabetic patients with severe limb ischemia requiring amputation. We compared these groups for the presence of TIE-2-positive proangiogenic monocytes. The proportion of proangiogenic monocytes in the venous blood (on hospital admission) was significantly increased in diabetic patients without ischemia ($9.22\% \pm 1.19$); compared to controls ($6.53\% \pm 0.58$) or ischemic diabetic patients ($5.44\% \pm 0.56$) ($p < 0.05$). In this pilot evaluation, we succeeded in extracting potential proangiogenic TIE-2 monocytes from the blood of diabetic patients without ischemia, but less in patients with ischemia. The implications for therapeutic neo-angiogenesis requires further studies.

Introduction

Many persons with diabetes mellitus of long duration develop advanced peripheral arterial disease. For a substantial percent of these patients, especially those with a long-standing atherosclerotic disease or severe peripheral microangiopathy, neither vascular surgery nor angioplasty treatment is feasible. Thus, alternative strategies for improving blood supply to the lower extremities are needed. Because most previous studies of innovative methods have used angiogenic growth factors alone and reported limited efficacy [1], many research groups have turned to investigations of cell-based therapies. Results of local injections of unselected bone marrow-, or peripheral blood-, derived mononuclear cells have been encouraging but insufficiently efficacious, likely related to the limited delivery of proangiogenic cells within a complex cell population [2]. Investigations using autologous bone-marrow cell transplantation [3] have also met with limited success. Furthermore, on both methods, harvesting of angiogenic stem cells remains a cumbersome procedure. Unlike embryonic or hematopoietic stem cells, blood monocytes are abundant, readily replenished, and collected by a simple peripheral phlebotomy. A subset of monocytes endowing proangiogenic capacity is identifiable by the expression of TIE-2, a tyrosine-kinase receptor for angiopoietins that enables neo-vascularization, at least in tumors and a limb ischemia model in mice [4]. Overall, there are three major populations of circulating human monocytes: classical inflammatory ($CD14^{\text{high}}CD16^{-}$); intermediate ($CD14^{\text{high}}CD16^{+}$); and, non-classical ($CD14^{\text{low}}CD16^{+}$) [5]. Both the intermediate and non-classical monocyte types express TIE-2, and are considered as the most proangiogenic [4,6,7], even though pan-monocytes exist as a continuous spectrum rather than distinct populations; and not all $CD16^{+}$ monocytes may promote angiogenesis. Patel et al. detected increased levels of peripheral proangiogenic monocytes in three patients with critical limb ischemia [8]. However, designing therapeutic interventions requires understanding the (local) homing of these proangiogenic monocytes in diabetic patients with limb ischemia. We therefore designed a pilot study to compare the proportion of circulating proangiogenic TIE-2 monocytes in different patient populations and to characterize their recruitment capacity in presence of an inflamed vascular endothelium.

METHODS

Sampling and study participants

We enrolled: healthy donors (or patients with various orthopedic infections but no evidence of limb ischemia); diabetic patients without clinical evidence of limb ischemia; and, diabetic patients with critical limb ischemia requiring amputation. We defined ischemia based on clinical signs and symptoms (e.g., claudication, diminished or absent pedal pulses, delayed capillary refill time, low ankle-brachial index) present on limb examination. Included patients had diabetes mellitus, were not being treated with colony-growth factors. Patients with an infection could be enrolled if they were not bacteremic. Infected patients received antibiotic therapy according to international guidelines [1] and we did not change any of the other medications of any participants. We obtained venous blood samples on all enrolled patients from the arm; for the diabetic subjects these were collected immediately before any orthopedic surgery procedure they were scheduled to undergo. We planned that in a subset of up to 20% of the diabetic patients we would obtain blood samples multiple times, either during another hospitalization or the recovery period, to see changes over time. Our Ethical Committee (Regional Ethics Committee; République et canton de Genève, Geneva, Switzerland) approved the study (NAC 14-183). The work was performed in accordance with the Declaration of Helsinki and the guidelines for Good Clinical Practice.

Reagents and antibodies

We used Lympholyte-H (Cedarlane Laboratories) or Ficoll-Paque Plus (GE Healthcare) gradients to isolate human peripheral blood mononuclear cells (PBMC) and the Pan-Monocyte isolation kit (Miltenyi Biotec) for monocytes' isolation. We applied various commercial antibodies for flow cytometry, the live-imaging of cells, or the immune-fluorescence examinations (Table 1). We did however not specifically stain the TIE-2 expression, because they are already included in the (CD14^{high}CD16⁺) and (CD14^{low}CD16⁺) assessments, as previously described by our and other groups [4].

Analysis of the proportions of monocyte subpopulations

For targeting monocyte subpopulations, we slightly modified our previously published methods [4]. Briefly, we stored phlebotomy blood samples in refrigerators and processed them within 24 hours. The red blood cells of the venous blood samples were first lysed by

red blood cell lysis buffer (8.3g NH₄Cl, 1g NaHCO₃, 1 ml EDTA (100 mM)). Afterwards, we analyzed the peripheral blood mononuclear cells (PBMC) with a BD Accuri C6 cytometer (BD Biosciences). We incubated the various antibody combinations with FcR blocking reagents in staining buffer, which contained 0.5% bovine serum albumin (BSA) and 2 mM EDTA in phosphate-buffered saline (PBS). After staining, we washed the PBMC in a7-AAD buffer to exclude non-viable or dead cells. We identified proangiogenic monocytes by flow cytometry. Cells expressing the lineage markers CD15, CD2, CD3, CD56, NKp46, CD1c, and CD141 removed the granulocytes, lymphocytes and immature dendritic cells. We identified all the monocytes collected in the HLA-DR and CD300e-positive windows. All monocytes were negative for granulocyte, lymphocyte, natural killer (NK) and immature dendritic cell markers (CD15⁻, CD2⁻, CD3⁻, CD56⁻, NKp46⁻, CD19⁻, CD1c⁻ and CD141⁻) and positive for the monocyte markers (HLA-DR⁺ and CD300e⁺). Within the pan-monocyte population we distinguished three subpopulations according to their expression of CD14 and CD16 using the Galios cytometer (Beckman Coulter). We have previously shown that both CD14^{high}CD16⁺ and CD14^{low}CD16⁺ monocytes express TIE-2 at different levels but present similar proangiogenic activity [4]. Both markers are similar in the identification of TIE-2 blood monocytes [10,11]. However, CD16⁺ monocytes contain the primary population of TIE-2 monocytes, which are capable of promoting vascularization through the secretion of various factors [10-12]. Other groups have also reported that CD16⁺ monocytes are TIE-2-positive proangiogenic monocytes in human [6, 9-12]. Thus, in this study, we focused on CD16⁺ monocytes.

Monocyte recruitment under flow

We assessed the recruitment potential of proangiogenic monocytes according to previous reports [4,13-16]. As proangiogenic monocytes must find their way to inflamed tissues for wound healing, we tested the recruitment capacities of these proangiogenic monocytes by measuring their adhesion to experimental vascular endothelium (primary human umbilical vein endothelial cells [HUVEC]). Briefly, we coated six chambers of μ -Slide VI 0.1 ibi Treat (IBIDI) with 0.2% gelatin (Sigma-Aldrich), 1 mg/ml Collagen G (Biochrom AG) in PBS, then cultured HUVEC (3×10^4 cells/chamber) in M199 medium (20% Fetal Calf Serum, 1% Endothelial Cell Growth Supplement (Thermo-Fisher Scientific), 1% penicillin, 1% streptomycin, 0.1 mg/ml heparin sodium, 0.1 μ M hydrocortisone, 10 μ g/ml L-ascorbic acid)

for seven days; with medium change every other day. We then stimulated the resulting monolayers with 1 $\mu\text{g/ml}$ VEGF (Peprotech) and 1000 U/ml $\text{TNF}\alpha$ (Peprotech) for 6 hours to induce an angiogenic inflammation. For this, we generated flow over the HUVEC monolayer by perfusing washing buffer (containing 0.2% BSA in M199 pre-warmed at 37°C) over the HUVEC monolayers using a calibrated pump (74,900; Cole Parmer). The flow rate was representative of shear rates in post-capillary venules (0.05 Pa). An image of a fixed field was generated every 15 seconds during 25 minutes (Axiovert 200 microscope; Carl Zeiss). After 1 minute of recording with Openlab software, we used a switch system to deliver pre-stained pan-monocytes (3×10^6 cells/ml) under continuous uninterrupted flow for five minutes.

Transendothelial locomotion of proangiogenic blood monocytes

We furthermore evaluated the transendothelial locomotion of the blood proangiogenic monocytes. First, we isolated the CD16⁺ monocytes by using anti-CD16 (clone: 3G8) coupled with anti-mouse IgG Microbeads (Miltenyi Biotec). Proangiogenic monocytes were retained within a LS column (Miltenyi Biotec) on the magnet (Miltenyi Biotec). We then used the isolated cells in the transmigration assay by fixing them with 4% paraformaldehyde for 10 minutes and permeabilized them with 0.2% Triton-X-100 for another 10 minutes. The Slan⁺ cells were stained with anti-Slan-FITC for 30 minutes. Finally, we determined the proportions of adherent and transmigrated Slan⁺ monocytes for all three groups of patients.

Statistical analyses

Our statistical analyses were basic. Data were presented as mean $\pm$ standard deviation or mean $\pm$ standard error of mean; or dot-plot with median values. The analyses were performed using either one-way ANOVA with Holm-Sidak adjustment (Graphpad).

RESULTS

Patients

From March 2015 to December 2017, we collected blood (6 ml) from the arm veins of 142 different persons. As planned, we sampled blood from 29 participants multiple times to determine the levels of proangiogenic monocytes over time; thus, we performed a total of 179 phlebotomies. Among the enrolled patients, 61 were healthy donors (or non-ischemic patients with various orthopedic infections); 39 were diabetic patients without clinical

evidence of limb ischemia; and, 42 were diabetic patients with critical limb ischemia requiring amputation. The corresponding numbers of phlebotomies were 80, 42 and 57, respectively. Overall, 113 patients were sampled once, 22 were sampled twice, 6 three times, and 1 four times (Table 2). The patients' median age was 64 years (range, 26-84 years) and 53 were female.

Quantification of proangiogenic TIE-2 monocytes

Non-classical proangiogenic, CD16+ monocytes were distinguished from the CD16 negative/CD14 positive non-angiogenic, inflammatory monocytes by flow cytometry (Figure 1A). We renounced on supplementary staining methods for identification or quantification for practical reasons, because we judged the flow cytometry method as sufficient for our study goals. The median blood monocyte quantification showed that blood from non-ischemic diabetic patients contained 80% or more proangiogenic monocytes compared to both other study groups (Figure 1B). The proportion of proangiogenic monocytes was significantly increased in diabetic patients without ischemia ($9.22\% \pm 1.19$) compared to controls ($6.53\% \pm 0.58$) or ischemic diabetic patients ($5.44\% \pm 0.56$) (one-way ANOVA-test, $p < 0.05$). In cases of multiple sampling, this quantification of samples from the same patient did not significantly differ over time in the samples.

Recruitment capacity of TIE-2 monocytes and their transendothelial migration

For the recruitment of proangiogenic monocytes, we induced a proangiogenic inflammation to HUVEC cells by stimulating them with the combination of vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF)- α . Between the three study groups, there was no difference regarding the adhesion potential (Figure 2A). However, the number of adherent cells in diabetic patients with ischemia was twice as high as those in both of the other comparator groups. In testing the transmigration of adherent monocytes, we found that their numbers in ischemic diabetic patients were again higher compared to those in the other two groups (Figures 2B and 2C). To further explore this difference, we assessed the expression of molecules involved in the adhesion among the study groups, as described below.

Expression of adhesion molecules and chemokine receptors by proangiogenic monocytes

As we wished to determine the expression of adhesion molecules on the protein level, we performed flowcytometry using the windows highlighted in Figures 1A and 3A. Among possible relevant molecules, we analyzed L-selectin, the integrins LFA-1 and Mac-1, the fractalkine receptor CX3CR1 (a key chemokine receptor on angiogenic monocytes) and the selectin receptor PSGL-1. None of these showed significant differences among the different study populations. We then searched for the expression of the PSGL-1 protein that could be post-translationally modified by 6-Sulfo LacNAc glycosylation (Slan). This modification inactivates PSGL-1 as a ligand for vascular P- and E-selectin [17,18]. We found that the Slan-PSGL-1 expressions were drastically reduced in diabetic patients with ischemia compared to both of the other groups (Figures 3B and 3C). The Slan-PSGL-1 cells did not transmigrate and were encountered in the blood only, but did not adhere to endothelium. Finally, we quantified the proangiogenic monocytes in the blood, their adhesion to inflamed HUVEC, and their transendothelial migration (Figures 4A, 4B, and 4C). Figure 5 illustrates the impact of PSGL-1 and its Slan-modified version on monocytes capture on vascular endothelial cells.

DISCUSSION

In this pilot evaluation, we found that diabetic patients with severe lower limb ischemia had quantitative and functional changes of circulating blood proangiogenic monocytes that affect their local recruitment and their local proangiogenic role. Overall, we successfully derived TIE-2 monocytes from the blood of healthy donors and non-ischemic diabetic patients and expanded them *in vitro* in animal-free real-life conditions. However, the levels of these monocytes in blood were reduced in the most affected group, i.e. diabetic patients with limb ischemia, even when their individual transmigration was higher than for the two comparator groups. Previous *in vitro* or *in vivo* studies suggested a role of proangiogenic (TIE-2) monocytes in wound healing [8]. We found that in diabetic patients without ischemia, the level of circulating proangiogenic monocytes was increased, suggesting that ischemia affects the proangiogenic monocytes more than the diabetes itself [19]. While the increase of circulating proangiogenic monocytes in patients with diabetes without ischemia may be a systemic response in favor of the repair of dysfunctional vessels, this assumed advantage may be lost during ischemia.

We have previously shown that both CD14^{high}CD16⁺ and CD14^{low}CD16⁺ monocytes express TIE-2 at different levels but present similar proangiogenic activity [4]. In this study, we focused on CD16 expression to identify proangiogenic monocytes. Although TIE-2 expression is very relevant, in humans the CD14^{high}CD16⁺ and CD14^{low}CD16⁺ monocytes are similarly proangiogenic despite different levels of TIE-2 expression [4]. This suggested that in human CD16 expression is more relevant in blood circulating monocytes than TIE-2 staining. Accordingly, we quantified CD16⁺ monocytes in the present study rather than TIE-2.

Our study also provided new insights on the adhesion of proangiogenic monocytes to the inflamed endothelium and the transendothelial migration in diabetic persons. The reduction of the blood level of TIE-2 monocytes paralleled a two-fold adhesion to experimental inflamed human endothelium. The transmigration of proangiogenic monocytes requires angiogenic inflammation by the simultaneous actions of angiogenic factors and inflammatory cytokines, such as TNF- α [4,20]. Additionally, the adhesion molecule PSGL-1, critical during the capture process of monocytes, can be subject to the 6-sulfo-N-acetyl-lactosamine glycosylation in proangiogenic monocytes [17,-21, 22]. This modification usually alters the adhesion capacity of proangiogenic monocytes to vascular endothelium. In our study, we were surprised to find that both the frequency of Slan⁺ monocytes and the glycosylation level of PSGL-1 strongly decreased in diabetic patients with critical ischemia. Possibly the monocytes of ischemic diabetes patients are more adhesive to the vascular endothelium and that is the reason we see less of them circulating. In diabetic rats, the injection of autologous monocytes, together with TNF- α and/or interferon-gamma, significantly improved wound healing through angiogenesis [23]. Based on currently available scientific knowledge and in contrast to studies in rodents, future therapies in humans based on the use of autologous, proangiogenic monocytes is a complex field. We need further understanding of the proangiogenic monocyte differentiation from progenitor cells in bone marrow, their expansion *in vitro* and the prevention of PSGL-1 Slan-glycosylation in order to improve their capacity to selectively migrate to ischemic regions. Alternatively, proangiogenic monocytes may be isolated from immune compatible healthy donors and be used for therapy. For example, monocytes have been derived from human embryonic stem cells and shown to increase

angiogenesis in xeno-transplanted human tumors [24]. The long-term risks of these proangiogenic monocyte enhancements in humans have not been determined. In the future it could be possible to use these cells to improve wound healing in diabetic patients with limb ischemia, if we can find ways of avoiding the potential long-term neoplastic effects of these cell lines. In healthy donors, we estimate infusing about 250-1000 proangiogenic monocytes/ml would be necessary for neo-angiogenesis in ischemic areas [24], but the available literature lacks benchmark numbers. Moreover, we currently ignore the minimal, or optimal, quantity for monocyte therapy in diabetic patients. We also do not know if we should inject proangiogenic monocytes alone or concomitantly with colony-stimulating factors, as many basic researchers advocate [25]. Likewise, we ignore so far how these monocytes behave inside specific patient populations such as diabetic patients with and without ischemia and controls; or if the cells remain basically the same. Our literature search did not find any particular hints that the blood monocytes would be altered *per se* in their structure, only because of the underlying chronic co-morbidity; which however, would be logical given the differences in our group comparisons. Finally, the future will show if the approach of autologous pro-angiogenic monocyte administration via the venous blood phlebotomy is a better way to go than for example the classical angiogenesis-factor harvesting from pluripotent (adipose) stem cells. This approach is another field of increasing research, more advanced than monocyte harvesting, but also much costlier and more cumbersome [26].

In conclusion, we clearly succeeded in extracting proangiogenic TIE-2 monocytes from the venous blood of diabetic patients without clinical ischemia, but were less successful with the target population of diabetic patients with critical ischemia. Possibly the monocytes of severely ischemic diabetes patients are more adhesive to the vascular endothelium and that is the reason we see less of them circulating. The implication of these findings for therapeutic neo-angiogenesis will require further studies. There is equally still room for further preliminary studies before the first therapeutic trials.

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Author Disclosure Statement

All authors declare that there is competing financial interest associated with their contribution to this manuscript.

Data availability

Anonymized data can be obtained on reasonable request upon the corresponding author.

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Table 1 - List of antibodies used in the study

<u>Names, -conjugation, clone</u>	<u>Manufacturer</u>	<u>Origin</u>	<u>Reactivity</u>
CD19 (B4)-VioBlue, (clone : LT19)	Miltenyi Biotech	Mouse	Human
CD2-VioBlue, (clone: LT2)	Miltenyi Biotech	Mouse	Human
CD3-VioBlue, (clone: BW264/56)	Miltenyi Biotech	Mouse	Human
CD15-VioBlue, (clone: VIMC6)	Miltenyi Biotech	Mouse	Human
CD56-Biotin, (clone: REA196)	Miltenyi Biotech	Human	Human
CD335 (NKp46)-Biotin, (clone: 9E2)	Miltenyi Biotech	Mouse	Human
CD141 (BDCA-3)-VioBlue, (clone: AD5-14H12)	Miltenyi Biotech	Mouse	Human
CD1c (BDCA-1)-Vioblue, (clone: AD5-8E7)	Miltenyi Biotech	Mouse	Human
CD300e (IREM-2)-PE-Vio770, (clone: UP-H2)	Miltenyi Biotech	Mouse	Human
CD300e (IREM-2)-APC-Vio770, (clone: UP-H2)	Miltenyi Biotech	Mouse	Human
HLA-DR-PerCP, (clone: AC122)	Miltenyi Biotech	Mouse	Human
HLA-DR-APC-Cy7, (clone: L243)	Biolegend	Mouse	Human,
CD16-FITC, recombinant (clone: REA423)	Miltenyi Biotech	human	Human
CD16-PE, recombinant (clone: REA423)	Miltenyi Biotech	human	Human
CD16-PE, (clone: 3G8)	BD Biosciences	Mouse	Human
CD14-APC, (clone: TÜK4)	Miltenyi Biotech	Mouse	Human
CD14-FITC, (clone: M5E2)	BD Biosciences	Mouse	Human
CX3CR1-APC, (clone : 2A9-1)	Miltenyi Biotech	Mouse	Human
CD62L (L-SELECTIN) -FITC, (clone :DREG-56)	BD Biosciences	Mouse	Human
CD11a (LFA-1)-FITC, (clone: HI111)	BD Biosciences	Mouse	Human
CD11b (MAC-1)-FITC, (clone: ICRF44)	Biolegend	Mouse	Human
CD162 (PSGL-1)-PE-Vio770, (Clone: REA319)	Miltenyi Biotech	Human	Human
Slan (M-DC8)-FITC, (clone: DD-1)	Miltenyi Biotech	Mouse	Human

Table 2- Key demographic variables of the study population (hospitalized patients and controls)

Total phlebotomies, n = 179	Diabetes with ischemia, n = 57	Diabetes without ischemia, n = 42	Controls & non-diabetic patients, n = 80
Males: N, (%)	45 (79%)	30 (71%)	51 (64%)
Females: N, (%)	12 (21%)	12 (29%)	29 (36%)
Median age sample episodes (years)	75 (range, 56-84)	65 (range, 34-82)	39 (range, 26-83)
Total persons, n = 142	Diabetes with ischemia, n = 42	Diabetes without ischemia, n = 39	Controls & non-diabetic patients, n = 61
- Sampled twice	6	1	15
- Sampled three times	3	1	2
- Sampled four times	1	0	0

Figure legends

Figure 1 A

Figure 1 B

Figure 1. Analysis of human proangiogenic monocytes in blood of healthy donors and patients.

A. Example of analysis by flow cytometry of human CD16⁺ proangiogenic monocytes in blood. The red blood cells were lysed in whole blood by red blood cell lysis buffer (8.3 g NH₄Cl, 1 g NaHCO₃, 1 ml EDTA (100 mM)). Cells were then analyzed for their morphology (side scatter) and size (forward scatter), as well as for expression of the markers for granulocytes, lymphocytes, natural killer (NK) cells, immature dendritic cells (CD15, CD2, CD3, CD56, NKp46, CD1c and CD141), antigen presenting cells (HLA-DR) and monocytes (Mo: CD300e⁺). Human proangiogenic monocytes (HPMo) and non-angiogenic monocytes (NMo) were identified by the presence of CD16⁺ and CD16-CD14⁺, respectively. B. Quantification of the percentage of circulating proangiogenic monocytes in 63 blood samples from healthy donors, 29 samples from diabetic patients without ischemia and 43 samples from diabetic patients with critical limb ischemia. Data are presented as a dot plot of the percentage of CD16⁺ monocytes in total monocytes. Each dot represents one blood sample. The median is indicated by black line. P-value tested by Wilcoxon-rank sum-test.

Figure 2 A

Figure 2 B

Figure 2 C

Figure 2. Recruitment capacity of human proangiogenic monocytes derived from healthy donors and patients. A. Analysis of the adhesion under flow of non-angiogenic and proangiogenic monocytes to human umbilical vein endothelial cells (HUVEC). B. Analysis of the absolute number of transendothelial migrating non-angiogenic and proangiogenic monocytes under flow. C. Capacity of transmigration of non-angiogenic and proangiogenic monocytes under flow expressed as transmigration rate (percentage).

Figure 3 A **Figure 3 B** **Figure 3 C**

Figure 3. Analysis of the expression of adhesion molecules and chemokine receptor by human proangiogenic monocytes derived from healthy donors and patients. A. Examples of analysis by flow cytometry of the expression of MAC1, PSGL1 and 6-Sulfo LacNAc (Slan)-PSGL1 on proangiogenic monocytes from healthy donors and patients. B. Percentage of human proangiogenic monocytes expressing adhesion molecules (L-selectin, LFA1, MAC1, PSGL1 and Slan-PSGL1) and the chemokine receptor CX3CR1. C. Mean fluorescence intensity of used in B.

Figure 4. Effect of PSGL1 inactivation by 6-Sulfo LacNAc glycosylation of human proangiogenic monocyte on adhesion and transmigration. A. Characterization of monocytes for PSGL1 modification by 6-Sulfo LacNAc (Slan) glycosylation. B. Percentage of Slan-modified PSGL1+ cells in blood and among adherent and transmigrated human proangiogenic monocytes of healthy donors and patients. C. Analysis of the absolute number of human proangiogenic monocytes adherent to HUVEC under flow.

Figure 5. Scheme illustrating the effect of PSGL-1 and its SLaN-modified version on monocytes capture by E-Selectin on vascular endothelial cells (EC).