

TRANSDIFFERENTIATED MONOCYTES: A NOVEL SOURCE OF LYMPHATIC ENDOTHELIAL-LIKE CELLS

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ABSTRACT

Although monocytes have previously been demonstrated to contribute to lymphatic vessel formation in vivo, monocyte transdifferentiation into lymphatic endothelial cells and the specific conditions required remain unclear. In this study, monocyte cultures isolated from human peripheral blood were stimulated to transdifferentiate into lymphatic endothelial cells under specific in vitro induction conditions. These results demonstrate primary isolates of CD14 (+) monocytes express low levels of lymphatic endothelial cell specific markers or pan-endothelial markers under routine culture conditions. Using fibronectin (FN) coated flasks and EGM-2 supplemented culture medium, monocytes were induced to express lymphatic endothelial markers PROX-1, VEGFR-3, LYVE-1, Podoplanin, and pan-endothelial markers vWF, CD144, and VEGFR-2. Furthermore, using the FN/EGM-2/lipopolysaccharide (LPS) culture conditions, monocytes displayed dramatically increased expressions of Prox-1, VEGFR-3, Podoplanin, LYVE-1 and vWF, while the expression of CD144 and VEGFR-2 sharply decreased. In addition, VEGF-C secretion by monocytes

exposed to fibronectin coated plates with EGM-2 medium with FN/EGM-2/LPS in vitro was significantly increased over levels seen in routine culture conditions. These findings demonstrate that monocytes can be induced to undergo transdifferentiation becoming more lymphatic endothelial-like cells and increase their VEGF-C production in an FN/EGM-2/LPS environment.

Keywords: monocyte, transdifferentiation, lymphatic endothelial cell, endothelial growth factors, inflammatory factor, VEGF, in vitro

Lymphangiogenesis as a lymphatic defect has been implicated in many pathological processes including lymphatic metastasis of cancers, immune rejection, and as contributor to secondary lymphedema (1,2). Understanding the mechanisms involved in lymphangiogenesis is crucial for designing therapeutic strategies to be used in the treatment of associated lymphatic diseases.

Monocytes, an important subset of peripheral blood mononuclear cells, are known for their high plasticity and potential for transdifferentiation. Under appropriate conditions, they have been shown to

transform into macrophages and dendritic cells and to acquire the phenotypes of pluripotent stem cells such as CD34 and CD45. In addition, they have also been shown to transdifferentiate into T lymphocytes, epithelial cells, endothelial cells, nerve cells and hepatocytes (3-5). However, it remains unclear if monocytes have the ability to transdifferentiate into lymphatic endothelial cells *in vitro*.

Previous reports have explored the contribution of the monocyte-macrophage to lymphangiogenesis *in vivo*. In murine models of both tumor implantation and excisional wound healing, monocyte-macrophages were shown to directly integrate into the walls of newly formed lymphatic vessels, which expressed both lymphatic marker (LYVE-1) and macrophage markers (F4/80, CD11b and stabilin-1) (6). In mice subject to corneal transplantation, newly formed lymphatic vessels developed in the transplanted cornea and were positive for CD11b. Interestingly, new lymphatic vessels nearly disappeared when macrophages were depleted via clodronate liposomes (7,8), suggesting an organizational function derived from a monocyte-macrophage interaction. After injecting thioglycollate into the peritoneal cavities of mice, macrophages exuded from peritoneal cavities were shown to express lymphatic specific markers VEGFR-3, LYVE-1, and PROX-1, and to form tube-like structures (9). These findings taken in totality infer a monocyte to lymphatic endothelial cell transdifferentiation capability.

In this paper, we investigate the monocyte to lymphatic endothelial cell transdifferentiation and the *in vitro* induction conditions required to facilitate this process.

METHODS

Monocyte Isolation and Culture

The present study was approved by the Medical Ethics Committee of Shandong University. A written informed consent was

obtained from every donor in advance, and all experiments were carried out in accordance with relevant guidelines.

Peripheral blood mononuclear cells from healthy male volunteers were isolated by Ficoll density gradient centrifugation (Stem Cell Technologies, Vancouver, British Columbia, Canada) and washed twice with PBS. Monocytes were obtained by using attachment procedure as described previously (3,4). Briefly, the fresh mononuclear cells were suspended with DMEM (Gibco, Grand Island, New York, USA) supplemented with 10% FBS (Gibco, Grand Island, New York, USA) and then seeded at 5×10^6 per 6-ml dish and incubated for 12 h at 37°C, 5% CO₂. Floating cells were removed, dishes washed twice with DMEM, and attached cells were detached by pipetting with DMEM for plating. Cells were subsequently inoculated at 1×10^6 cells per well in 6-well plates pre-coated with 10 µg/ml fibronectin (FN) (Sigma, Santa Clara, CA, USA), and induced as follows. Control cells were cultured with DMEM supplemented with 10% Fetal Bovine Serum (FBS) in plates without FN-coating for 7 days. One group of experimental cells were cultured with endothelial growth medium-2 [(EGM-2, Lonza, Basel, Basel-Stadt, Switzerland) (EGM-2 consists of endothelial cell basal medium-2 (EBM-2) and endothelial cell growth supplement (ECGS) containing 5% FBS, VEGF, bFGF, EGF, IGF-I, apo-transferrin, hydrocortisone, retinoic acid)] in FN-coated plates for 7 days. A second group of experimental cells were cultured with EGM-2 in FN-coated plates for 6 days and then treated with 100 ng/ml Lipopolysaccharide (Endotoxin) (LPS Sigma, Santa Clara, CA, USA) for 24 h.

Immunocytochemistry/Immunofluorescence Staining

All immunocytochemical staining was performed according to the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde and treated with 3%

hydrogen peroxide for 10 min. Cells were then incubated overnight at 4°C with one of the following primary antibodies: anti-human-CD14 (1:300 dilution), anti-human-LYVE-1 (1:300 dilution), anti-human-Podoplanin (1:300 dilution), anti-human-VEGFR-3 (1:300 dilution), anti-human-VEGFR-2 (1:300 dilution) or anti-human-vWF (1:300 dilution). All primary antibodies were purchased from Abcam (Cambridge, England, UK). The diaminobenzidine (DAB) staining kit (Golden Bridge International, Los Angeles, CA, USA) was used to visualize the primary antibody using light microscopy analysis. For negative controls, the primary antibody was replaced by PBS.

Immunofluorescence staining was employed to detect the phenotype of FN/EGM-2 and/or LPS-stimulated monocytes, including the expressions of both specific lymphatic markers (VEGFR-3, LYVE-1, Podoplanin) and pan-endothelial markers (vWF, VEGFR-2). Cells were fixed with 4% paraformaldehyde and blocked with goat serum for 30 min at 37°C. Then, cells were incubated with primary antibodies (1:300 dilution) overnight at 4°C and stained with FITC or PE conjugated anti-mouse IgG (1:200 dilution) for 30 min. Nuclei were stained with DAPI. Sections were observed using a fluorescent microscope. Primary antibodies used were as described above with negative controls using only PBS.

RT-PCR

Monocyte genetic phenotyping was performed on harvested monocytes 7 days post stimulation. Total RNA was extracted using TRIzol reagent (TakaRa, Dalian, Liaoning, China) according to the manufacturer's instructions. First strand cDNAs were synthesized using prime Script RT kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. 1µL cDNAs were amplified using the following program: 5 min at 95°C for pre-denaturation, followed by 30 cycles of 30 s at

60°C, and 30 s at 72°C, with a final 5 min extension at 72°C. PCR products were visualized on a 2% agarose gel with ethidium bromide staining. The special primers were as follows: β -actin, 5'-CCA TCT ACG AGG GGT ATG CCC-3' (forward) and 5'-TCC TTA ATG TCA CGC ACG ATT TCC-3' (reverse); PROX-1, 5'-CAC CTG AGC CAC CAC CCT TG-3' (forward), 5'-GCA TTG CAC TTC CCG AAT AAG GT-3'; VEGFR-3, 5'-AAG TAC ATC AAG GCA CGC ATC-3' (forward) and 5'-GCA GTT CAG GAC CAG CTT CT-3' (reverse); LYVE-1, 5'- TTC CAT CCA GGT GTC ATG CAG-3' (forward) and 5'- AAG GGG ATG CCA CCG AGT AG-3' (reverse); Podoplanin, 5'-GCC AGC CAG AAG ATG ACA CTG-3' (forward) and 5'-GAA TGC CTG TTA CAC TGT TGA CAC-3' (reverse); CD144, 5'-GAT GCA GAG GCT CAT GAT GC-3' (forward), 5'-CTT TAC CCC AGG ATA TGG AG-3' (reverse); VEGFR-2, 5'-CCG TCA AGG GAA AGA CTA CG -3' (forward) and 5'-CTT TAC CCC AGG ATA TGG AG -3' (reverse); vWF, 5'-CAC CGT TTG CCC ACC CTT CG-3' (forward) and 5'-GCC CAC TGG GAG CCG ACA CT-3' (reverse). The experiment was repeated three times.

Flow Cytometry

The freshly isolated monocytes (control) obtained in the attachment procedure as described above and the induced monocytes (experimental) were each suspended in PBS with densities adjusted to 1×10^6 /ml. The control monocytes were marked with FITC-anti-human CD14 (Biolegend, San Diego, CA, USA) or FITC-anti-human CD14/PE-anti-human CD34 (Biolegend, San Diego, CA, USA) to determine their purity and quantify their intrinsic stem cell properties.

To quantify the level of lymphatic marker expressed on the experimental monocytes induced under various experimental conditions, cells were incubated respectively with fluorescence labeled antibodies: FITC-anti-human CD14/PE-anti-human, VEGFR-3

(Biolegend, San Diego, CA, USA), FITC-anti-human CD14/Alexa Fluor 647®-anti-human Podoplanin (Biolegend, San Diego, CA, USA) and PE-anti-human CD14/FITC-anti-human LYVE-1 (Abcam, Cambridge, England, UK) and compared to the control antibodies (FITC Mouse IgG1, PE Mouse IgG1, Alexa Fluor® 647 rat IgG2a). Additionally, a blank standard control group was similarly incubated without any antibodies to act as autofluorescence control. Cells were incubated in the dark for 30 min at 4° C, then washed with PBS twice and suspended with 500µl PBS. Total fluorescence was quantified using a flow cytometer. The data (in triplicate) were analyzed using Cell Quest software.

ELISA

To detect the level of VEGF-C secreted by stimulated monocytes, supernatant was collected after inducing monocytes for 7 days in either DMEM with 10% FBS or EGM-2 for 7 days, or in EGM-2 for 6 days and with added LPS at 100 ng/ml for 24 h. To avoid the interference of supplements in culture medium, control groups were grouped into a blank group without samples and a DMEM group supplemented with 10% FBS; EGM-2 medium. In order to eliminate the influence of the number of cells on the final concentration, cell density was adjusted to 10⁶/ml prior to seeding. The level of VEGF-C was tested using a VEGF-C ELISA kit (eBioscience, San Diego, CA, USA). The final results were calculated by subtracting the OD value of both the blank group and the different medium control groups. Again all assays were performed in triplicate.

Statistics

Statistical analyses were performed using Student's t test by SPSS 17.0 software (SPSS Inc.). P<0.05 was considered statistically significant.

RESULTS

Purity of Monocytes

Monocytes isolated from peripheral blood using Ficoll density gradient centrifugation showed positivity for CD14 (*Fig. 1A*), a monocyte specific marker, at 96.6% ± 0.7% (*Fig. 1B*) as determined by flow cytometry (FCM).

Phenotype of Freshly Isolated Monocytes

To test the hypothesis that freshly isolated CD14+ monocytes have stem cell properties, CD34 (a stem cell marker) expression was analyzed using a double staining flow cytometric assay. CD14+/CD34+ monocytes accounted for only 0.9 % ± 0.2% (*Fig. 1C*), suggesting that monocytes rarely had stem cell properties. The freshly isolated monocytes were CD14+ (a specific monocyte marker) by immunocytochemistry (*Fig. 1A*), and were negative for lymphatic-specific VEGFR-3, LYVE-1, Podoplanin, VEGFR-2 and pan-endothelial markers vWF, VEGFR-2.

The double staining flow cytometric assay showed that CD14+/VEGFR-3+ monocytes (*Fig. 1D*) accounted for 0.1% ± 0.1% of all fresh isolated monocytes, CD14+/Podoplanin+ monocytes (*Fig. 1E*) accounted for 0.2% ± 0.1% and CD14+/LYVE-1+ monocytes (*Fig. 1F*) accounted for 4.08% ± 0.3%.

The Morphological and Phenotype Change of Induced Monocytes

Monocytes cultured on uncoated plates in DMEM were uniformly small and round under microscopic evaluation (*Fig. 2A*). After seeding on FN-coated plates and culture in EGM-2 for 7 days, monocytes became increasingly elongated and spindly or polygonal appearing consistent with endothelial cells in vitro (*Fig. 2B*).

Immunofluorescence

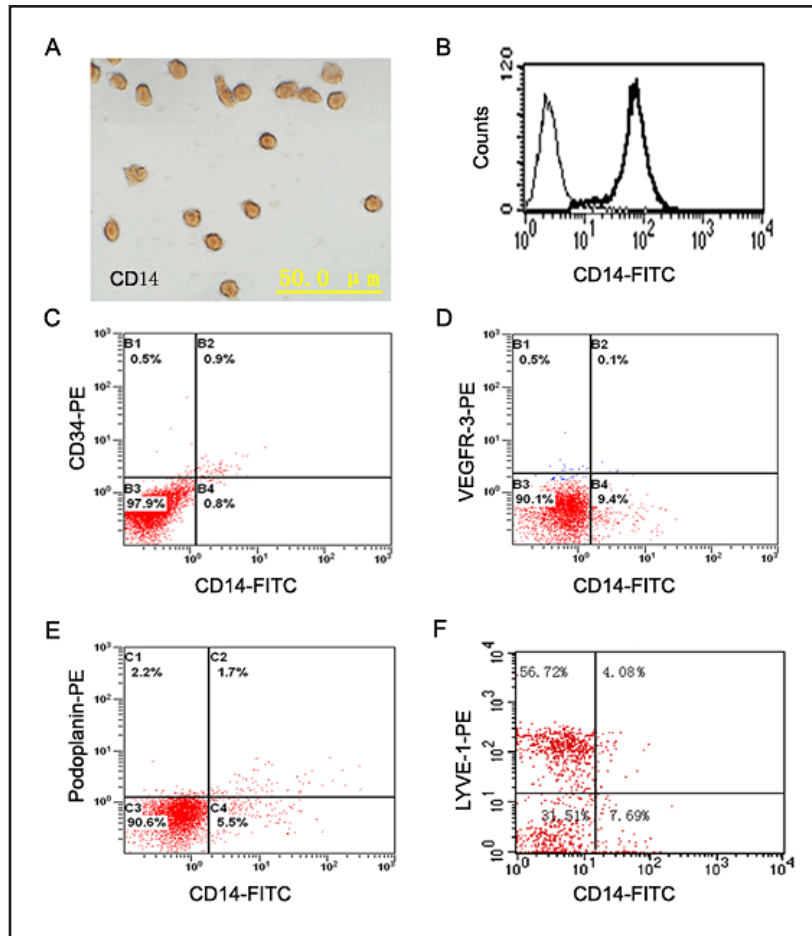


Fig. 1. Phenotype of freshly isolated monocytes. CD14, a specific monocyte marker, was expressed in monocytes by immunocytochemistry (A) and 96.6% were positive (right peak) by flow cytometry (B). The co-expression of CD14 with CD34 (C), VEGFR-3 (D), Podoplanin (E), and LYVE-1 (F) was determined by double staining flow cytometric assay.

Specific lymphatic markers VEGFR-3, LYVE-1, Podoplanin, and pan-endothelial markers vWF, and VEGFR-2 were all positive in FN/EGM-2-induced cells (*Fig. 2C*).

RT-PCR

The mRNAs of markers were at the lower threshold of detection in monocytes cultured in DMEM with 10% FBS for 7 days. When monocytes were cultured in FN-coated plates with EGM-2, mRNAs of PROX-1, VEGFR-3, Podoplanin, LYVE-1, vWF,

CD144, and VEGFR-2 were expressed. When monocytes were cultured in EGM-2 for 6 days and then treated with LPS for the last 24 h, mRNA of VEGFR-3, LYVE-1, Podoplanin, and vWF was significantly increased, while mRNA of CD144 and VEGFR2 sharply decreased or even disappeared (*Fig. 3*).

Flow Cytometry

Positive marker expression for VEGFR-3, LYVE-1, and Podoplanin was $0.40 \pm 0.2\%$,

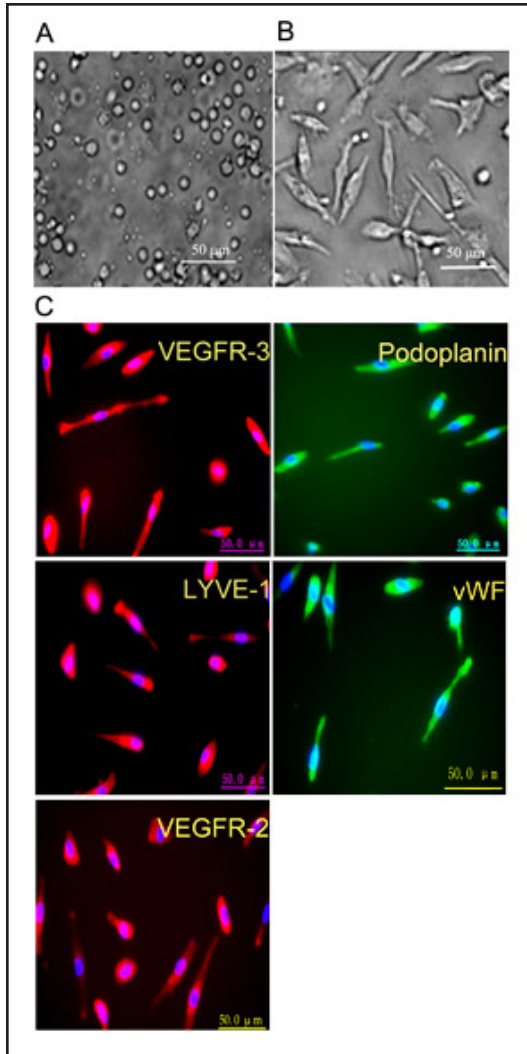


Fig. 2. Morphological evaluation using light microscopy of monocytes cultured in DMEM for 7 days exhibit a normal monocyte morphology (A) while those cultured in FN/EGM-2 exhibit an elongated morphology (B). FN/EGM-2 treatment induced monocytes to express VEGFR-3, LYVE-1, Podoplanin, vWF, and VEGFR-2 by immunofluorescence (C).

$1.99 \pm 1.0\%$, and $0.7 \pm 0.3\%$ respectively when monocytes were cultured in DMEM with 10% FBS for 7 days. When monocytes were cultured in FN/EGM-2 for 7 days, positivity increased to $3.50 \pm 1.0\%$, $14.00 \pm 1.9\%$ and $2.53 \pm 0.4\%$ respectively. Monocytes

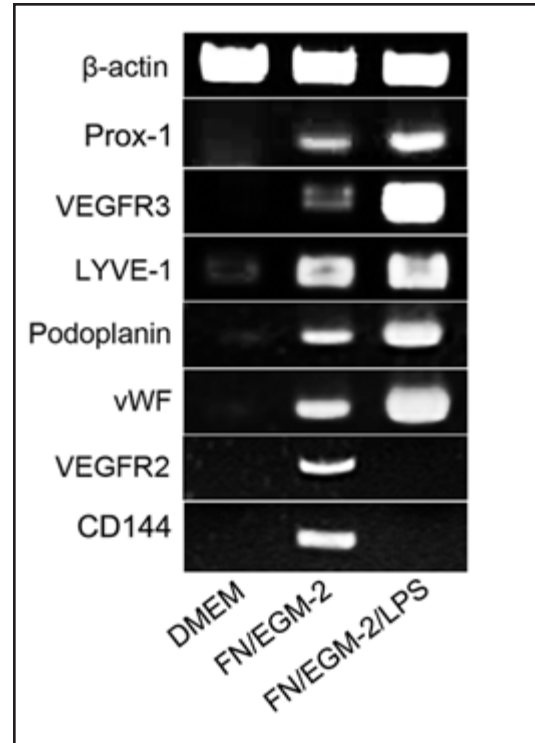


Fig. 3. Phenotype changes in induced monocytes analyzed by RT-PCR. mRNA expression of Prox-1, VEGFR-3, LYVE-1, Podoplanin, vWF, CD144 and VEGFR-2 were barely detected in monocytes cultured in DMEM. VEGFR-3, Podoplanin, LYVE-1, vWF, and VEGFR-2 levels were expressed in monocytes cultured in FN/EGM-2 while Prox-1, VEGFR-3, LYVE-1, Podoplanin, and vWF were dramatically expressed and both CD144 and VEGFR-2 disappeared in monocytes cultured in FN/EGM-2/LPS.

cultured in EGM-2 for 6 days and then with LPS for 24 h demonstrated $31.07 \pm 2.1\%$, $70.6 \pm 2.3\%$, and $51.03 \pm 1.6\%$ positivity, respectively. Comparing two different treatment groups, there were significant differences in positivity for VEGFR-3, LYVE-1, and Podoplanin (Fig. 4, $p < 0.05$).

ELISA

ELISA assay results revealed the level of VEGF-C secreted by monocytes was 6.3 ± 11.1 pg/ml in the DMEM group,

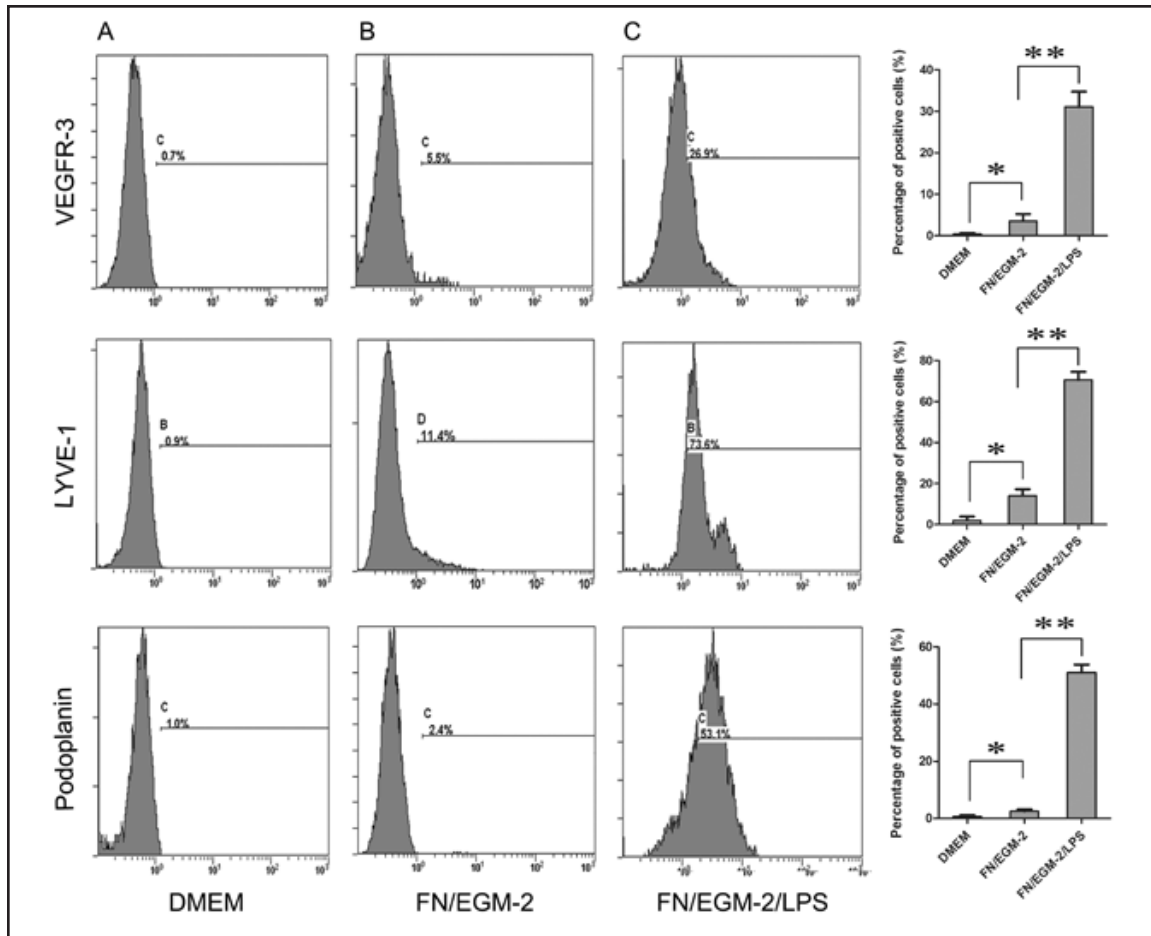


Fig. 4. Phenotype changes in induced monocytes measured by flow cytometry. Lymphatic markers, VEGFR-3, LYVE-1, and Podoplanin expression from monocytes cultured in DMEM were at the lower level of detection (A). Monocytes cultured in FN/EGM-2 showed modest expression (B, * $p < 0.05$). Monocytes cultured in FN/EGM-2/LPS showed significant expression when compared to controls (C, ** $p < 0.01$).

98.2 ± 11.9 pg/ml in the FN/EGM-2 group, and 211.4 ± 10.5 pg/ml in the FN/EGM-2/LPS group. These results were statistically significant for both treatment groups compared to control cells and demonstrate effective induction and up-regulation of VEGF-C production in response to FN/EGM-2 and LPS exposure *in vitro* (Fig. 5).

DISCUSSION

The present study was designed to evaluate the potential for monocyte trans-

differentiation into lymphatic endothelial cells. *In vivo* lymphatic endothelial cells express pan-endothelial biomarkers VEGFR-2, CD144, and vWF and lymphatic specific markers Prox-1, Podoplanin, and LYVE-1 (10,11).

We have previously demonstrated that monocytes stimulated by FN, VEGF-C, and Lipopolysaccharide (LPS) express the specific lymphatic endothelial cell markers (LYVE-1, Podoplanin, and Prox-1) but do not express the pan-endothelial markers vWF or eNOS (12).

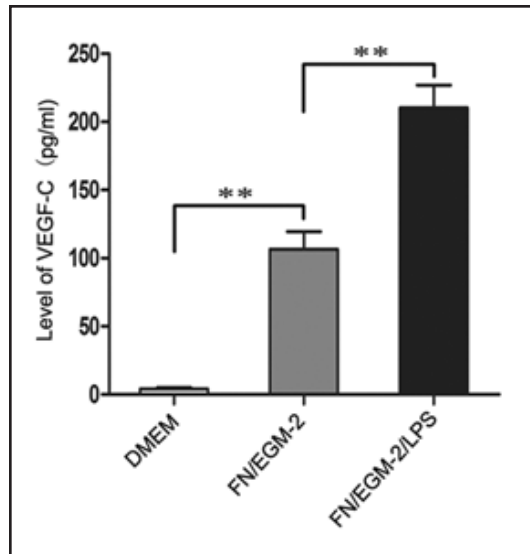


Fig. 5. ELISA evaluation of VEGF-C secretion by monocytes in culture. DMEM control cells demonstrated a VEGF-C secretion that was barely detectable. A statistically significant increase was seen in monocytes cultured in FN/EGM-2, and a dramatic increase was seen in those cultured with FN/EGM-2/LPS (** $p < 0.01$).

We initially analyzed the gross phenotypic features of freshly isolated human monocytes confirming a uniform spherical morphology for these primary isolates. FCM analysis demonstrated 97% of these primary isolates expressed CD14+. We also demonstrated that CD14+/CD34+ monocytes rarely exist in primary isolates suggesting inactivated circulating monocytes do not routinely express these pluripotent markers. In addition, our data demonstrate circulating monocytes rarely express lymphatic endothelial markers. These data establish the baseline inactivated monocyte expression values used to test the hypothesis that monocyte have the potential, under specific conditions, to transdifferentiate into lymphatic endothelium cell.

Numerous studies have established FN as a critical component in extracellular matrix and its involvement in messaging, cell adhesion, proliferation, differentiation and

tissue function (13). FN is critical in monocyte to pluripotent cell differentiation *in vitro* (3,4). EGM-2, a specialized medium supplemented containing several growth factors, including VEGF, hEGF, hFGF-B, R3-IGF-1, and FBS, etc., has been previously shown to be a beneficial medium for endothelial progenitor cell transformation to endothelial-like cells (4,14).

Although previous publications have reported FN/EGM-2 cultured monocytes could express progenitor-cell markers and pan-endothelial makers (4,14), there is a lack of specific data on lymphatic endothelial cell marker expression in cultured monocytes. Consequently, we seeded isolated human monocytes on FN-coated 6 well culture plates and exposed them to EMG-2 *in vitro* for 7 days. Monocytes exposed to FN/EMG-2 appeared to transform their morphology becoming more endothelial cell-like with spindle form and polygonal in shape when examined by light microscopy. In contrast, control monocytes cultured in DMEM in uncoated culture dishes did not exhibit a change in morphology. A weakness of our experiments is that we did not try to transdifferentiate these cells back using DMEM medium alone to see if the changes could be reversed. Additionally, further experiments need to be carried out to test functional characteristics of the purported lymphatic endothelial cells (for example, can they form capillary-like structures on Matrigel? Do they duplicate and expand?).

In addition, RT-PCR demonstrated monocytes exposed to FN/EGM-2 in culture expressed not only the pan-endothelial markers such as VEGFR-2, CD144, and vWF but also lymphatic endothelial markers such as VEGFR-3, LYVE-1, Podoplanin, and Prox-1. These cells also secreted more VEGF-C, which is the growth factor specific for lymphatic endothelial cells. We defined these vWF+/VEGFR-3+/LYVE-1+ /Podoplanin+ positive cells as lymphatic endothelial like cells. Some investigators have reported that dendritic cells can secrete VEGF-C and

further experiments need to be carried out to ensure exclusion of dendritic cells from our analysis.

While previous data suggest FN and VEGF-C play a key role in monocyte lymphatic endothelial cell transformation, we attempt here to explore the phenomena in an inflammatory environment through the use of LPS.

The theory of inflammation-induced lymphangiogenesis is well established in the scientific literature. Inflammatory cytokines lead to enhanced lymphatic marker expressions in lymphatic endothelial cells in vitro presumably through NF- κ B as demonstrated in a lymphangiogenesis in vivo mouse peritonitis model. There is a dose-dependent increase in lymphatic vessel density and a concomitant infiltration of CD11b+ macrophages in the experimental mice (15,16). The monocyte marker CD14 has been shown to act as a receptor for lipopolysaccharides. By treating FN/EGM-2 induced monocytes for 24 h with LPS, there was an increase in the percentage of cells exhibiting a lymphatic endothelial-like phenotype and a concomitant increased expression of VEGFR-3/LYVE-1/Podoplanin Prox-1/vWF. In addition, there was an up-regulation of VEGF-C secretion in LPS exposed FN/EGM-2 stimulated cells when compared with unstimulated controls. Interestingly, the expression of VEGFR-2 and CD144 (a protein of the cadherin family that plays an important role in maintaining endothelial cell phenotypic expression by mediating intercellular adhesion and controlling the organization of the intercellular junctions) declined post LPS stimulation. Previous reports have shown VEGFR-2, a tyrosine-protein kinase and receptor for VEGF or VEGF-C, plays a key role in endothelial cell proliferation. In recent years, it has also been demonstrated that inflammatory cytokines can induce endothelial to mesenchymal phenotypic transition (17), which is characterized by the loss of certain endothelial phenotypic expression products, i.e., loss of CD144 (VE-cadherin) and

VEGFR2. In light of these data, we postulate that the reversal of the monocytic expression of endothelial-like phenotypic characteristics post LPS stimulation in our study is the result of this mesenchymal transition in direct response to LPS stimulation.

VEGF-C is widely regarded as a specific lymphatic endothelial growth factor. Bonding with its receptor VEGFR-3, VEGF-C/VEGFR-3 plays a critical role in lymphatic development in the embryonic stage (18) as well as in adult cancer-associated lymphangiogenesis (19) and in lymphatic re-anastomosis post-lymph node dissection and transplantation (20,21). Monocyte-macrophages have also been shown to be the most abundant producers of VEGF-C, VEGF-D, and VEGF-A, which can initiate lymphangiogenesis in inflamed or tumor tissue (22). In the present study, we demonstrate activated monocytic secretion of VEGF-C in both experimental arms of the study significantly increased over control. These data demonstrate monocyte-derived lymphatic endothelial-like cells can act as either a direct structural contributor to endothelialization of lymphatic vessel walls or as a potent secretor of VEGF-C aiding in lymphangiogenesis.

Our data demonstrate human peripheral blood monocytes are capable of phenotypic transdifferentiation exhibiting a lymphatic endothelial-like phenotype post FN/EGM-2 exposure suggesting endothelial cells are one point in the complex immune cascade. In addition VEGF-C secretion up regulation in a FN/EGM-2 environment supports functional as well as phenotypic transformation in monocytic activation.

Since monocytes are quite abundant in blood (accounting for 3%-8% of all cells in peripheral blood), our data demonstrate the potential for a wide range of potential clinical applications including how monocytes may provide a novel strategy for the treatment and prevention of lymphatic associated diseases. Thus, the potential now exists for local stimulation of monocytes and by

association, macrophages facilitating trans-differentiation up-regulating VEGF-C secretion and improving tissue healing and lymphatic endothelialization. In addition, monocytes now present the potential for extracorporeal lymphatic vessel formation for transplantation without the need for anti-rejection therapy as well as the potential for a monocyte pathway in tumor vessel formation.

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CONFLICT OF INTEREST AND DISCLOSURE

All authors declare that no competing financial interests exist.

REFERENCES

- Zheng, W, A Aspelund, K Alitalo: Lymphangiogenic factors, mechanisms, and applications. *J. Clin. Invest.* 124 (2014), 878-887.
- Liersch, R, M Detmar: Lymphangiogenesis in development and disease. *Thromb. Haemost.* 98 (2007), 304-310.
- Zhao, Y, D Glesne, E Huberman: A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc. Natl. Acad. Sci. USA.* 100 (2003), 2426-2431.
- Kuwana, M, Y Okazaki, H Kodama, et al: Endothelial differentiation potential of human monocyte-derived multi-potential cells. *Stem Cells* 24 (2006), 2733-2743.
- Seta, N, Y Okazaki, H Miyazaki, et al: Platelet-derived stromal cell-derived factor-1 is required for the transformation of circulating monocytes into multipotential cells. *PLoS One* 8 (2013), e74246.
- Schledzewski, K, M Falkowski, G Moldenhauer, et al: Lymphatic endothelium-special hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumors and wound healing tissue in vivo and in bone marrow cultures in vitro: Implications for the assessment of lymphangiogenesis. *J. Pathol.* 209 (2006), 67-77.
- Maruyama, K, M Ii, C Cursiefen, et al: Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J. Clin. Invest.* 115 (2005), 2363-2372.
- Maruyama, K, T Nakazawa, C Cursiefen, et al: The maintenance of lymphatic vessels in the cornea is dependent on the presence of macrophages. *Invest. Ophthalmol. Vis. Sci.* 53 (2012), 3145-3153.
- Schoppmann, SF, P Birner, J Stöckl, et al: Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am. J. Pathol.* 161 (2002), 947-956.
- Kriehuber, E, S Breiteneder-Geleff, M Groeger, et al: Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J. Exp. Med.* 194 (2001), 797-808.
- Hou, WH, IH Liu, CC Tsai, et al: CRSBP1/LYVE-ligands disrupt lymphatic intercellular adhesion by inducing tyrosinephosphorylation and internalization of VE-cadherin. *J. Cell Sci.* 124 (2011), 1231-1244.
- Changming, W, L Xin, T Hua, et al: Monocytes can be induced to express lymphatic phenotypes. *Lymphology* 44 (2011), 48-53.
- Fan, CL, Y Li, PJ Gao, et al: Differentiation of endothelial progenitor cells from human umbilical cord blood CD 34+ cells in vitro. *Acta Pharmacol. Sin.* 24 (2003), 212-218.
- Krenning, G, PY Dankers, D Jovanovic, et al: Efficient differentiation of CD14+ monocytic cells into endothelial cells on degradable biomaterials. *Biomaterials* 28 (2007), 1470-1479.
- Flister, MJ, A Wilber, KL Hall, et al: Inflammation induces lymphangiogenesis through up-regulation of VEGFR-3 mediated by NF- κ B and Prox1. *Blood* 115 (2010), 418-429.
- Chaitanya, GV, SE Franks, W Cromer, et al: Differential cytokine responses in human and mouse lymphatic endothelial cells to cytokines in vitro. *Lymphat. Res. Biol.* 8 (2010), 155-164.
- Good, RB, AJ Gilbane, SL Trinder, et al: Endothelial to mesenchymal transition contributes to endothelial dysfunction in pulmonary artery hypertension. *Am. J. Pathol.* 185 (2015), 1850-1858.

18. Karkkainen, MJ, P Haiko, K Sainio, et al: Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* 5 (2004), 74-80.
19. Sun, L, J Duan, Y Jiang, et al: Metastasis-associated in colon cancer-1 upregulates vascular endothelial growth factor-C/D to promote lymphangiogenesis in human gastric cancer. *Cancer Lett.* 357 (2015), 242-253.
20. Tammela, T, A Saaristo, T Holopainen, et al: Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation. *Nat. Med.* 13 (2007), 1458-1466.
21. Schindewolf, L, G Breves, M Buettner, et al: VEGF-C improves regeneration and lymphatic reconnection of transplanted autologous lymph node fragments: An animal model for secondary lymphedema treatment. *Immun. Inflamm. Dis.* 2 (2014), 152-161.
22. Ji, RC: Macrophages are important mediators of either tumor- or inflammation-induced lymphangiogenesis. *Cell Mol. Life Sci.* 69 (2012), 897-914.

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