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Comparison and functional characterisation of peripheral blood mononuclear cells isolated from filarial lymphoedema and endemic normals of a South Indian population

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Abstract

OBJECTIVE The underlying problem in lymphatic filariasis is irreversible swelling of the limbs (lymphoedema), which is a unique feature of lymphatic insufficiency. It is still unclear whether the natural ability of lymphatics to form functional lymphatic vasculature is achieved or attenuated in the lymphoedemal pathology. Clinical studies have clearly shown that circulating lymphatic progenitors (CLPs), a subset of bone marrow-derived mononuclear cells (PBMCs), contribute to post-natal lymph vasculogenesis. CLP-based revascularisation could be a promising strategy to bypass the endothelial disruption and damage incurred by the filarial parasites. Thus our aim was to compare and characterise the functional prowess of PBMCs in physiological and lymphoedemal pathology.

METHODS PBMCs were isolated from venous blood sample from drug-naive endemic normals (EN) and drug-deprived filarial lymphoedema (FL) individuals using density gradient centrifugation. Adhesion, transwell migration and *in vitro* matrigel assays were employed to characterise the lymphvasculogenic potential of PBMCs. CLPs were phenotypically characterised using flow cytometry; expression levels of lymphatic markers and inflammatory cytokines were quantified using qRT-PCR and ELISA, respectively.

RESULTS PBMCs from FL group display poor adherence to fibronectin ($P = 0.040$), reduced migration towards SDF-1α ($P = 0.035$), impaired tubular network ($P = 0.004$) and branching point ($P = 0.048$) formation. The PBMC mRNA expression of VEGFR3 ($P = 0.039$) and podoplanin ($P = 0.050$) was elevated, whereas integrin $\alpha 9$ ($P = 0.046$) was inhibited in FL individuals; additionally, the surface expression of CD34 ($P = 0.048$) was significantly reduced in the FL group compared to the EN group.

CONCLUSION PBMCs from filarial lymphoedema show defective and dysregulated lymphvasculogenic function compared to endemic normals.

keywords peripheral blood mononuclear cells, lymphatic endothelial progenitor cells, lymphangiogenesis, secondary lymphoedema, lymphatic filariasis

Introduction

Lymphatic filariasis (LF) is a mosquito-transmitted parasitic infection affecting public health especially in the tropics [1, 2]. The eventual problem in lymphatic filarial infection is lymphoedema; irreversible swelling of the limbs. The transport capacity of lymphatic vessels is highly compromised resulting in impaired lymphatic drainage and endothelial dysfunction [3]. The adaptive ability of lymphatic vasculature to form functional lymphatic vessels is poorly understood. In LF, the adult parasites dwell in afferent lymphatics of the lower extremities causing considerable morbidity [4, 5]. Fluid homeostasis, lipid uptake and immune surveillance are the fundamental functions of lymphatic vessels for which proper lymph flow should be maintained [6, 7]. In acute filarial infection, lymphatic drainage is slightly impaired, whereas in a chronic state, it is fully and functionally impaired impeding the lymph flow [8]. It is assumed that both parasitic products and the host inflammatory responses lead to lymphatic dysfunction and lymphangiogenesis. It is further evident from the lymphoscintigraphy results that there is a dermal backflow of lymph fluid in individuals with filarial lymphoedema and no flow in the chronic state [9].

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It is well known that in pathological conditions, the functional abilities of peripheral blood mononuclear cells are altered [10–12]. PBMCs are the primary cellular pool, comprising cells responsible for immunological, inflammatory and angiogenic functions [13–15]. Additionally, circulatory lymphatic progenitors (CLPs) are the subset of PBMCs mobilised from bone marrow in to the peripheral circulation upon certain physiological or pathophysiological stimuli. However, their circulating numbers and functional abilities also need to be addressed in filarial induced lymphoedema complication. Moreover, there is a strong association between changes in PBMCs gene expression and lymphatic vasculature, and even the secretory factors of PBMCs have been shown to improve wound healing and angiogenesis.

Several researchers have focused on the role of lymphatic endothelial cells in mediating the immune and inflammatory response for progression of filariasis. In 2003, Salven and co-workers demonstrated the post-natal lymphangiogenic potential of $CD34^{+}$, $CD133^{+}$ and $VEGFR3^{+}$ cells derived from human foetal liver, cord blood and peripheral blood [16]. This phenomenon was confirmed by Maruyama 2005 [17] and Kerjaschki 2006 [18], suggesting that these cells can differentiate into mature lymphatic endothelial cells with high functional ability to form new lymphatic vessels and thereby augment lymphatic neovascularisation in adults [19–21]. Certain inflammatory cytokines are known to promote pro-angiogenic factors. Hence this study aimed to examine the functional potential of PBMCs and enumerate circulating lymphatic progenitors in response to filarial infection and severity of the disease, keeping in mind that these cells can foster the formation of new functional lymphatic vessels to bypass the endothelial disruption incurred by the filarial parasites. The outcome of this study may lay a platform for future clinical investigation by exploring the complex mechanistic insights associated with irreversible lymphoedema and CLP-based therapeutic modalities, which is needed for effective LF management along with the prescribed drug of choice.

Materials and methods

Materials

Vacutainers for blood collection – K2-EDTA (Cat: 367863), Vacutainers for serum separation (Cat: 367812), fibronectin (Cat: 356008) and matrigel (Cat: 356231) were purchased from Becton Dickinson Biosciences. EGM-2 Bullet kits (Cat: CC-4176) were purchased from Lonza. Fluorescent antibodies, anti-human CD34-FITC (Cat: 343603) and anti-human VEGFR3-PE (Cat: 356203), and isotype control antibodies, mouse IgG2a-FITC (Cat: 400207) and mouse IgG1k-PE (Cat: 400113), were purchased from BioLegend. Transwell thin inserts (Cat: 3422) with 8.0 μm pore size were from Costar Corning. Multi-Analyte ELISA Array Kit was from Qiagen (Cat: MEH-004A). Trizol from Invitrogen (Cat: 15596-018), human SDF-1α (Cat: S190), gelatin (Cat: G1393), collagen (Cat: C0543), histopaque (Cat: 10771) and other dry chemicals were from Sigma-Aldrich. All qRT-PCR primers were purchased from Integrated DNA Technologies (IDT).

Study group, inclusion and exclusion criteria

Forty volunteers were recruited for this community-based case–control study in two groups: control group, endemic normals (endemic normals [EN], $n = 14$), and case group, filarial lymphoedema (filarial lymphoedema [FL], $n = 26$), showing irreversible lymphoedema, from an area endemic for lymphatic filariasis in Tamil Nadu, South India. The study was approved by the Institutional Ethics Committee OF Madurai Kamaraj University, as required by the Indian Council of Medical Research (ICMR). Permission from the Directorate of Public Health, Government of Tamil Nadu was also obtained. Both oral and informed written consent were obtained from all study subjects prior to the sample collection.

Ten millilitres of venous blood was collected from each study volunteer as per the ICMR guidelines in vacutainer tubes compatible for biochemical estimation, cytokine quantification, progenitor cell enumeration and functional assessments of PBMCs. Individuals with severe lymphoedema associated with active bacterial infections, cancer, acute infections or immunological disorders, known diabetes, established CAD or CVD, any form of medication, smoking and surgery (in the past 10 months) were excluded from the study.

Anthropometric measurements

Anthropometric parameters (age, height, weight, systolic and diastolic blood pressures) were recorded for all the study participants. Blood pressure was measured using mercury sphygmomanometer (Meditech India, New Delhi, India) in a sitting position for the right arm. Clinical manifestations (includes unilateral or bilateral lymphoedema and skin appearance) and clinical grades of lymphoedema (nature of swelling) were scored.

Estimation of biochemical parameters

The major biochemical parameters such as random glucose, high-sensitivity C-reactive protein (hs-CRP) and

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complete haematological profile of the study participants were measured from the collected blood sample using Automated Cell Counter and nephelometry analysis. All biochemical estimations were carried out at Lister Metropolis Laboratory as per the standard manufacturer's instructions.

Isolation of peripheral blood mononuclear cells

The PBMCs (termed as buffy coat cells) comprised of lymphocytes and monocytes were isolated from the whole blood samples using histopaque density gradient centrifugation method. The buffy coat cells were washed thrice with 1× PBS (pH 7.4) to remove platelet-rich plasma fraction. Then the cells were incubated with ACK lysing solution (ammonium chloride–potassium bicarbonate) to remove excess erythrocytes. Finally, the isolated cells were washed and resuspended with 1× PBS and checked for viability [22].

Trypan blue exclusion assay

To determine the cell viability based on the dye exclusion principle, the resuspended PBMCs from both the study groups were stained with Trypan Blue dye for 3–5 min. Stained cells were then mounted on the counting chamber and visually scored the cells, under phase contrast microscope.

Flow cytometry analysis

After confirming the viability, the freshly isolated PBMCs were used for flow cytometry analysis. Prior to staining, cells were blocked using 5% FBS and stained using fluorochrome-conjugated anti-human VEGFR3-PE and CD34-FITC antibodies. Corresponding isotype controls mouse IgG1-PE and mouse IgG2a-FITC were stained in parallel and incubated at 4 °C for 30 min in dark as per manufacturer's instructions. Cells were washed with 1× PBS and fixed using 4% paraformaldehyde. A minimum of 5×10^5 events (0.5 million) were acquired and analysed using FACS CANTO. Using the dot plot or scatter plot, gating was carried out. Upon morphological gating of the lymphomonocyte fraction based on forward and side scatter (Figure S1), $CD34^+$, $VEGFR3^+$ (single positive) and $CD34^+VEGFR3^+$ (double positive) cells were sorted following gating with appropriate fluorophore-labelled corresponding isotype control antibodies. Same quadrant settings of the isotype samples were used for test samples to obtain the positive cells. Data were analysed using FlowJo software version 7.2.5 (Tree Star Inc., USA).

Adhesion assay

The ability of a cell to adhere to another cell or to the extracellular matrix indicates its functional potential. The change in cell adhesion pattern indicates its responsiveness to its environment. In order to evaluate the adhesion ability of the isolated PBMCs, 1×10^5 cells were seeded into the wells priorly coated with extracellular matrix protein components: fibronectin (25 μg/ml), gelatin (0.2% w/v) and collagen (0.01% w/v) in EGM-2 medium without serum supplementation and allowed to adhere for 3 h. Non-adherent cells were washed with 1× PBS twice. The adhered cells were then stained using DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, 1 μg/ml) for 5 min in the dark. Images were taken using fluorescence microscope (Olympus IX51). Each sample was assessed in triplicate, and a minimum of four field views were analysed per well. Data represented as average number of cells adhered per field view.

Transwell migration assay

Migration potential of the freshly isolated PBMCs was carried out using transwell migration assay using filters of 8-micron pore size. The thin inserts of the transwell unit were coated with fibronectin (25 μg/ml) on both sides and incubated at 37 °C in 5% CO_2 for 1 h; 3×10^5 cells were seeded on the upper chamber, and recombinant human SDF-1α (100 ng/ml) was added to the endothelial basal medium (EBM2) in the lower chamber. PBMCs were incubated for 12 h at 37 °C in 5% CO_2. After incubation, the cells on the upper side of the membrane were carefully removed using a sterile cotton swab, and the migrated cells were fixed with 4% paraformaldehyde and stained with DAPI for image acquisition. The lower side of the membrane was imaged in four different field views. Data represented as the average number of cells migrated per field view [23] with and without the chemoattractant, SDF-1 α.

Matrigel tube formation assay

Growth factor-reduced, phenol red-free matrigel was coated in a 96-well plate and pre-incubated at 37 °C in 5% CO_2 for 1 h prior to cell seeding. Matrigel acts as a two-dimensional basement membrane, over which endothelial cells are able to form a tubular network. Freshly isolated PBMCs were enumerated and mixed with endothelial ECV-304 cells in an equal ratio (1:1); 2×10^4 cells were seeded on matrigel-coated wells and were incubated at 37 °C for 12 h in EBM-2 basal

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medium supplemented with VEGF (50 ng/ml). Phase contrast images were obtained for tube formation in 4× objective in four different field views of a well. The number of tubes, length of the tubes and branching points formed by cocultured ECV304 cells and PBMCs per tube were quantified using ImageJ software [24] and represented per field view.

Quantification of cytokines

Plasma levels of cytokines were quantified to determine the type of responses imparted by the filarial parasites in the host system and its influential role in lymphvasculogenic events. Circulating levels of cytokines, namely IL-1A, IL-2, IL-4 IL-6, IL-10, IL-12, TNF-α, IFN-γ and G-CSF were measured calorimetrically using the Multi-Analyte ELISArray Kit in a 96-well plate format, and the absorbance was read at 450 nm as per the manufacturer's instruction.

Quantitative real-time PCR

qRT-PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems) using SYBR Green probe (Roche, Cat: 04913850001) to quantify the real-time expression of lymphangiogenic markers. Total RNA was isolated from PBMCs using Trizol method. Reverse transcription was performed with 1 μg DNAse treated RNA for 3 h. The major lymphatic endothelial markers such as VEGFR3, podoplanin and Prox-1 were quantified against *β*-actin mRNA as internal control. Relative transcript levels were determined according to the manufacturer's protocol.

Statistical analysis

All clinical and experimental data are expressed as mean ± SEM. Normality of data and their distribution were determined using Kolmogorov–Smirnov's (KS) test among the study groups. Comparisons between two groups were performed by the unpaired nonparametric Mann–Whitney U test or nonparametric anova (Kruskal–Wallis test) and followed by Dunn's multiple comparisons post test. To identify a possible link between the progenitor's roles with clinical parameters in filarial lymphoedema individuals, we carried out Spearman's rank correlation analysis between clinical variables with circulating progenitor cells. Statistical significance was accepted if the null hypothesis could be rejected at $P \leq 0.05$. Data analyses were performed using Windows-based GraphPad PRISM (GraphPad Software, Inc., San Diego, CA, USA).

Results

Selection of study location

Based on prior epidemiological survey reports, we identified and selected three villages which are endemic for lymphatic filariasis: Kanchipadi, Thomur and Velamagandigai in Thiruvallur District (formerly Chingleput), South India [25, 26] for sample collection. At the time of sample collection, Mass Drug Administration (MDA) programmes were not in implementation. Drugs were distributed until 2014, and follow-up programmes were carried out in these villages.

Characteristics of study population

Both male and female volunteers in the age between 31 and 70 years were recruited in the study. Individuals from the same ethnic origin, exposed to the same parasites and vectors with no evidence of filarial infection, were identified as endemic normals. Filarial lymphoedema group comprised of Grade II and Grade III were identified based on the medical history for evident lymphatic filariasis infection and its clinical manifestations. They included irreversible unilateral and bilateral swelling of the lower limbs. Grade II individuals exhibiting pitting oedema with no changes in skin folding and Grade III individuals exhibiting non-pitting oedema with clear thickening of the skin [27] were recruited. Grade I individuals were difficult to identify, as their lymphoedema was reversible, and sometimes, they exhibited no evident symptoms of LF infection. Grade IV individuals had a chronic infection, and some had secondary bacterial infection. Thus, we excluded both Grade I and Grade IV from this study. Trained Staff Nurses and Laboratory technicians under the supervision of Health Inspector were involved in the sample collection procedure at the Government Public Health Centres (PHCs). Staging of these patients was authenticated and well characterised by the experienced Medical Officers and Entomologist at the District Public Health Laboratory, Thiruvallur.

Clinical characteristics of study subjects are listed in Table 1. To determine whether filarial infection affects on glucose levels, we measured random plasma glucose. Circulating high-sensitivity CRP levels in the FL group was considerably lower than in EN group, suggesting that filarial infection may be in latency at this lymphoedemal stage. No significant differences in the haematological measurements were observed between the two groups (Table 2); however, monocyte counts were double those in the FL group, which may be one of the driving factors diminishing PBMCs functionality [28].

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Table 1 Clinical characteristics of study subjects

Parameters	Endemic normal (EN) (n = 14)	Filarial lymphoedema (FL) (n = 26)
Anthropometric measurements		
Median age (range)	48 (36–59)	60 (31–70)
Sex (M/F)	7/7	11/15
Height (cm)	170 ± 1.8	156 ± 2.5
Weight (kg)	72 ± 2.2	55 ± 2.3
Body mass index (kg/m^2)	25.1 ± 0.61	23.1 ± 0.99
Systolic blood pressure (mm/Hg)	124 ± 2.9	136 ± 7.0
Diastolic blood pressure (mm/Hg)	77 ± 2.0	87 ± 3.3
Lymphoedema type	None	Unilateral & bilateral
Grade II (n)	None	19
Grade III (n)	None	7
Other markers		
Plasma glucose random (mM)	6.71 ± 0.83	7.07 ± 0.61
hs-CRP (mg/l)	6.31 ± 1.37	4.59 ± 0.56

Values are expressed as mean ± SEM. hs-CRP indicates high-sensitivity C-reactive protein.

Table 2 Haematological measurements of the study subjects

Parameters	Endemic normal (EN) (n = 14)	Filarial lymphoedema (FL) (n = 26)
Total WBC count (10^9 l)	7.63 ± 0.4	8.77 ± 0.3
Lymphocytes (%)	34.21 ± 1.4	31.92 ± 1.6
Monocytes (%)	4.85 ± 0.63	5.88 ± 0.33
Eosinophils (%)	6.35 ± 1.3	4.65 ± 0.5
RBC count (10^{12} l)	4.68 ± 0.11	4.27 ± 0.08
Haemoglobin (%)	13.41 ± 0.51	12.60 ± 0.42
Platelet count (10^9 l)	0.246 ± 0.01	0.250 ± 0.01

Values are expressed as mean ± SEM. Haemoglobin and Differential cell count values are expressed in %.

Adhesion and Integrin expression of PBMCs

Circulating angiogenic cells should have to adhere to fibronectin, a component of extracellular matrix, and follow their migratory path through the ECM proteins, which was the prime event in the process of lymphatic homing and neovascularisation. Endothelial differentiation of mononuclear cells in the endothelial growth medium was regulated by integrin-mediated adhesion over fibronectin [29, 30]. To determine the adhesion potential of the isolated PBMCs, we cultured these cells over various extracellular matrices such as fibronectin, collagen and gelatin for 3 h (Figure 1a). We arrive at this time point based on the literature reports as well as our previous observations, which yield maximum cell adhesion [12, 31, 32]. Adhesion to fibronectin was significantly impaired in the FL group (P = 0.040); however, adhesion to collagen was significantly increased (P = 0.047) (Figure 1b), suggesting that the predisposed collagen in filarial lymphoedema individuals attracts these cells which can interfere with the lymphangiogenic machinery. No much changed in cell adhesion over gelatin surface. As we observed an impaired adhesion of PBMCs towards fibronectin matrix in FL group, we were interested in evaluating the expression levels of integrin, especially integrin α_9, because of its major and direct interplay in lymphatic capillary formation. Surprisingly, the expression of integrin α_9 was consistently and significantly reduced in the FL group (P = 0.046) (Figure 1c), which corroborates the finding that PBMCs exhibit poor adherence to fibronectin. Prior to adhesion, viability of the fresh PBMCs immediately after the isolation from both the study groups was assessed using trypan blue assay. It exceeded 95% for the total number of cells counted in each group.

Migration of PBMCs in response to SDF-1α

Stromal-derived growth factor (SDF-1α) is a strong agonist in triggering the mobilisation of progenitors or haematopoietic stem cells (HSCs) from bone marrow into peripheral circulation [33]. Generally, SDF-1α binds to its receptor CXCR4, which is expressed on mononuclear cells to assist blood angiogenesis as well as lymphangiogenesis. Hence, we next sought to determine the migratory potential of isolated PBMCs towards SDF-1α in the study groups. Migration assay was performed by placing 1×10^5 viable cells equally in both groups in the upper chamber and SDF-1α in the lower chamber in the transwell filter assembly. After overnight incubation, we observed a significant increase in the migration of PBMCs from the EN group (P = 0.028) across the fibronectin-coated membrane in response to human SDF-1α (100 ng/ml) (Figure 2a,b), whereas migration was blunted for PBMCs in FL group (P = 0.035).

Tube-forming ability of PBMCs

Further to confirm the tube-forming ability of PBMCs obtained from the study volunteers, we cocultured the freshly isolated PBMCs with endothelial cells (ECV304) over 2D matrigel matrix in the presence of VEGF (50 ng/ml). Formation of tubular network was achieved in EN group (Figure 2c), whereas cells from FL group show attenuated tubule formation, suggesting that filarial load, disease severity and PBMCs-derived secretory cytokines may inhibit the capillary tubule formation in FL group. The number of tubes (P = 0.004) and branching points

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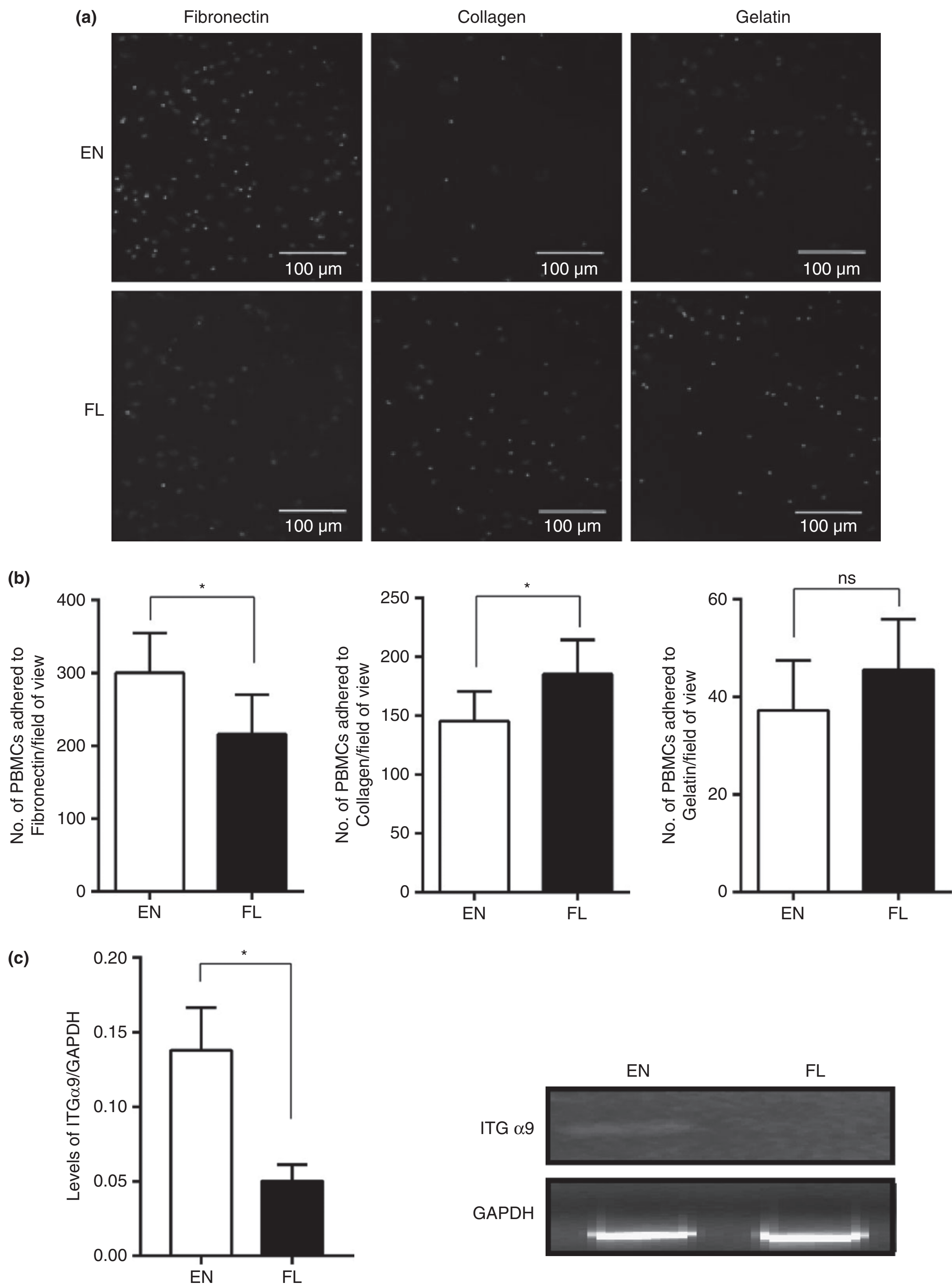
(a)
Fibronectin
Collagen
Gelatin
EN
FL
100 µm
(b)
No. of PBMCs adhered to Fibronectin/field of view
No. of PBMCs adhered to Collagen/field of view
No. of PBMCs adhered to Gelatin/field of view
ns
EN
FL
(c)
Levels of ITGα9/GAPDH
ITG α9
GAPDH

Figure 1 Assessment of adhesion and integrin expression of PBMCs. (a) Representative Image of cells adhered to the extracellular matrices; fibronectin (25 μg/ml), gelatin (0.2% w/v) and collagen (0.01% w/v) at 3 h between the study groups endemic normals (EN) and filarial lymphoedema (FL). Magnification: 10× and Scale: 100 μm. (b) Bar graph summarising total number of PBMCs adhered to the different extracellular matrices in each study group (10 individuals in each group). (c) Semi-quantitative expression analysis integrin α9 in PBMCs from EN (n = 5) and FL (n = 5) groups. Data represented as mean ± SEM. Inset is representative gel image showing the expression of integrin α9 and GAPDH ran in a 2% agarose gel. *P < 0.05 *vs.* EN.

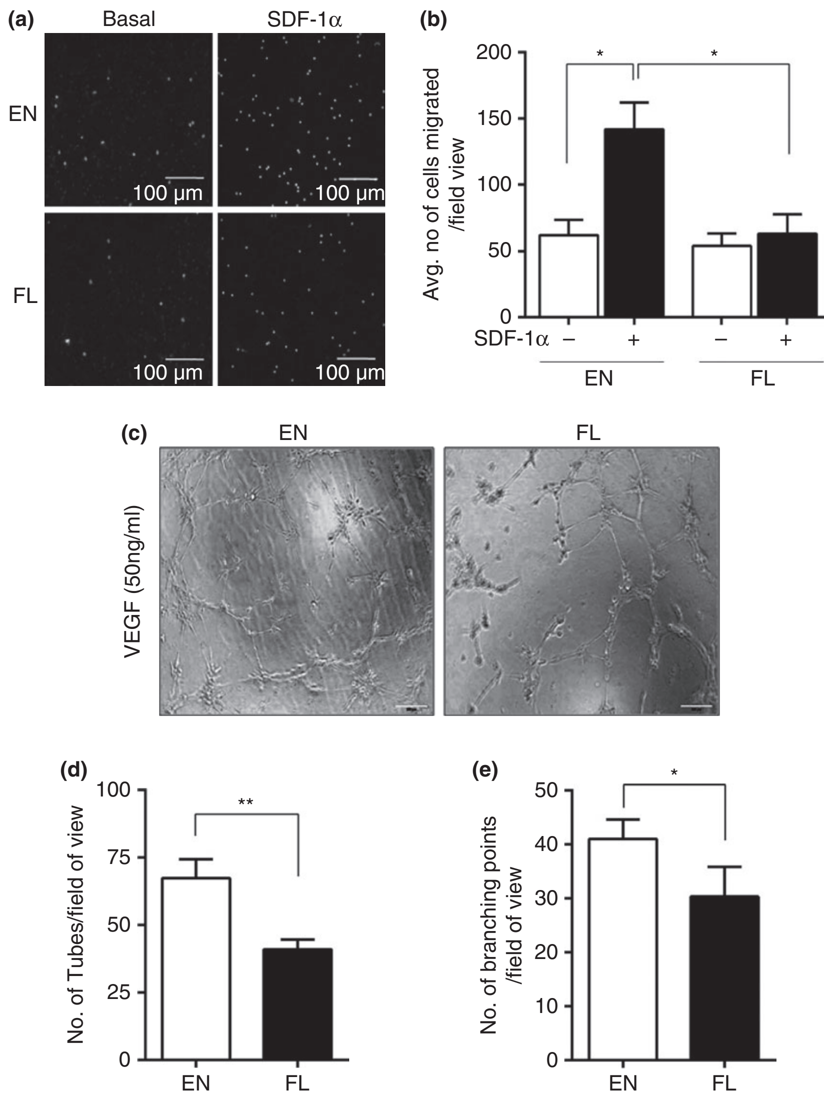

Figure 2 Assessment of migration and tube-forming potential of PBMCs. (a) Representative image of cells migrated across the fibronectin-coated membrane in a transwell filter assembly (8-micron pore size) in response to SDF-1α (100 ng/ml) in the two study groups endemic normals (EN) and filarial lymphoedema (FL). Migrated cells were stained with DAPI (1 μg/ml), and images were acquired. Magnification: 10× and Scale bar corresponding to 100 μm. (b) Bar graph summarising data for five independent experiments in the two study groups EN and FL (n = 5 individuals in each group). Data represented as mean ± SEM. (c) Representative image depicting tube formation by freshly isolated PBMCs cocultured with ECV304 cells in 1:1 ratio in response to VEGF (50 ng/ml). Images were acquired and quantified as field view. Magnification: 4× and Scale bar corresponding to 200 μm. (d) Bar graph summarising no. of tubes formed and (e) no. of branching points formed over the 2D matrigel in the study group EN and FL (n = 5 individuals in each group). Data represented as mean ± SEM, *P < 0.05 and **P < 0.01 *vs.* EN.

(P = 0.048) was significantly smaller in the FL group (Figure 2d,e).

Expression of lymphatic markers in PBMCs was altered in filarial lymphoedema

The freshly isolated PBMCs and its cellular component in the angiogenic process was determined. In this experiment we evaluated the expression of major lymphatic specific markers by sorting out from the study groups. We quantified the expression of VEGFR3, podoplanin and Prox-1 using quantitative RT-PCR. All primers used for this analysis are given in the Table S1. We observed a significant increase in the expression of VEGFR3 (P = 0.039) and podoplanin (P = 0.050), although the expression of transcription factor Prox-1

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in PBMCs showed a marginal increase in FL group *vs.* EN group (Figure 3a). Expression data revealed that PBMCs have multidifferentiation potential, based on the endogenous cue and condition *in vivo*. Also PBMCs possess markers responsible for lymphatic vasculature. Diminished type 3 VEGF receptor and podoplanin expression further leads to disruption in normal lymphatic vasculature formation and eventually causes lymphoedema.

Circulating levels of CD34⁺ and VEGFR3⁺

Circulating CD34⁺ and VEGFR3⁺ cell counts was enumerated from the PBMCs fraction based on the surface

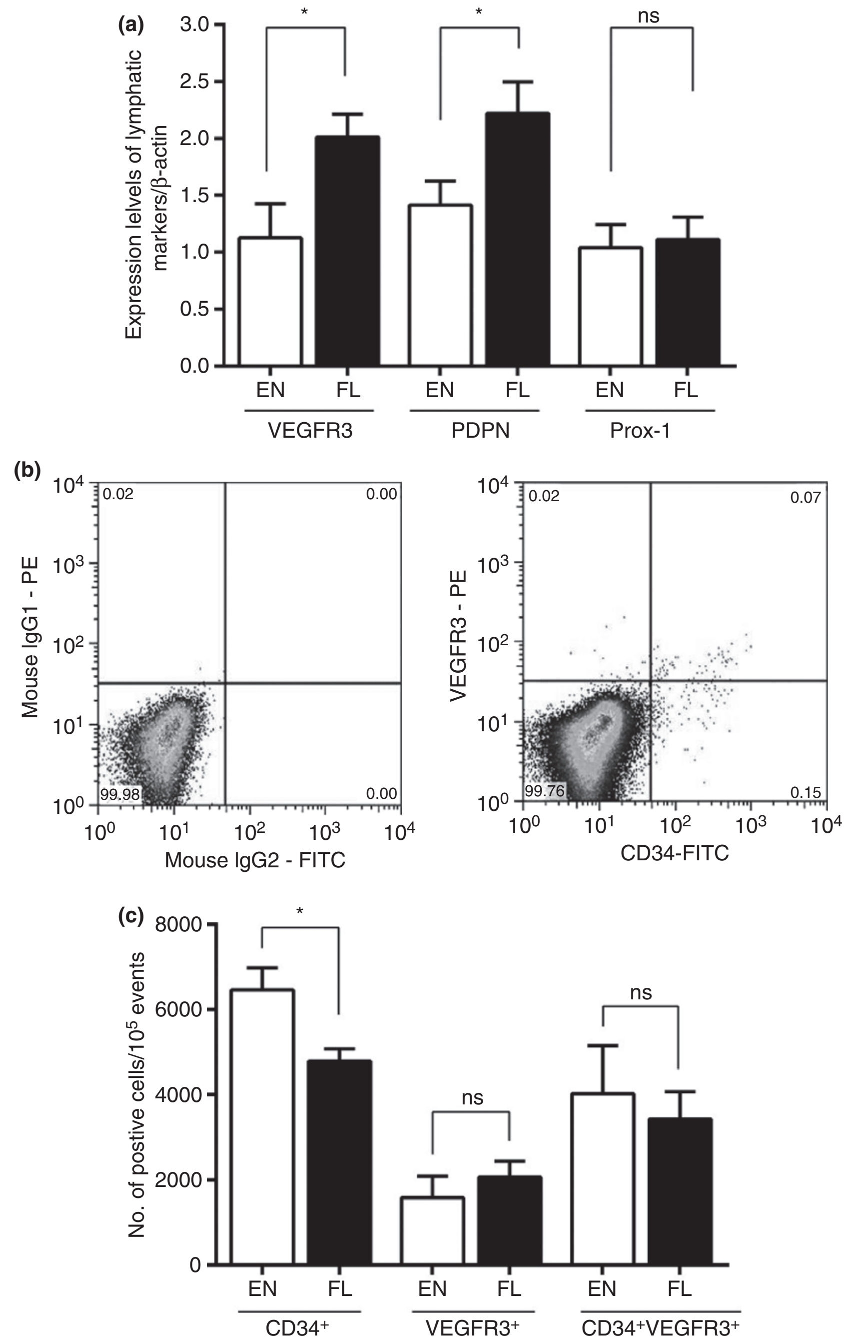

Figure 3 Expression analysis of lymphatic markers and circulating progenitor counts in PBMCs. (a) Bar graph showing the levels of expression of lymphatic-specific markers; VEGFR3, PDPN and Prox-1 between the study subjects endemic normals (EN) (n = 5) and filarial lymphoedema (FL) (n = 5) using quantitative RT-PCR. β-Actin was used as an internal control to normalise the levels of expression. (b) Representative image showing the quadrant settings of isotype control and test sample stained with their respective antibodies for flow cytometry enumeration of CD34⁺ and VEFGR3⁺ cell counts. (c) Bar graph showing the circulating CD34⁺, VEFGR3⁺ (single positive) and CD34⁺VEFGR3⁺ (double positive) cell counts in the study group, EN and FL (n = 10 individuals in each group). *P < 0.05 *vs.* EN.

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expression using flow cytometry analysis (Figure 3b). These circulating angiogenic cells have the potential to differentiate into mature lymphatic endothelial cells with high functional ability to form new lymphatic capillaries in adults. We observed a phenomenal reduction in the circulating CD34$^+$ counts (P = 0.048), and at the same time, VEGFR3$^+$ counts do not change significantly (P = 0.484) (Figure 3c), suggesting that PBMCs display inherent angiogenic ability; however, the CD34$^+$ cell counts in the circulation and its contribution in lymphatic vessel growth may be inadequate, and stimuli for its mobilisation from bone marrow may be poor in this chronic pathological setting.

Th2 cytokines

The influential role played by pro- and anti-inflammatory cytokines in the activation of mononuclear cells in the pathological state was studied using plasma samples collected from the study individuals by ELISA method. The circulating levels of various cytokines were measured calorimetrically using plate reader. The circulating levels of cytokines, namely IL-1A (P = 0.257), IL-2 (P = 0.455) and IL-12 (P = 0.401) show a marginal increase in FL individuals (Figure 4a), suggesting the pro-inflammatory response imparted by Th1 cytokines may be suppressed. Simultaneously, we observed a significant increase in the circulating levels of IL-4 (P = 0.035), IL-6 (P = 0.041) and IL-10 (approaching significance, P = 0.061), indicating the Th2 response was predominant in the filarial lymphoedema (Figure 4b) *vs.* the EN group. The levels of IFN-γ (P = 0.120) and TNF-α (P = 0.720) were not significantly changed in FL group; however, the circulating G-CSF levels (P = 0.055) was significantly decreased in FL group compared to EN group (Figure 4c), indicating that the stimuli for triggering the mobilisation of lymphatic endothelial progenitor cells into circulation may be weak, and concurrently, the elevated Th2 cytokines confer humoral immune responses by inhibiting inflammation which may in turn affect lymphangiogenic factors in the FL individuals.

Correlation analysis

We employed Spearman's rank (ρ) correlation analysis to identify the possible correlates between the circulating progenitor cells (CD34$^+$, VEGFR3$^+$ and CD34$^+$VEGFR3$^+$) and clinical variables. A strong and significant inverse correlation was observed for CD34$^+$VEGFR3$^+$ (double positive) cells and high-sensitivity C-reactive protein (hs-CRP) levels (ρ = −0.714, P = 0.020) and total platelet counts (ρ = −0.587, P = 0.049). Also these double-positive cells were positively correlated with CD34$^+$ cells significantly (ρ = 0.829, P = 0.058). However, no significant correlation was obtained for both single-positive cells with other clinical variables.

Discussion

Different disease pathologies alter the inherent functional abilities of peripheral blood mononuclear cells [10–12]. Lymphocytes and monocytes are the major cellular components of PBMCs, which play central roles in angiogenesis/lymphangiogenesis, immunity, wound healing, cellular repair and regeneration. We report the attenuated and dysregulated vasculogenic functions of PBMCs obtained from filarial-induced lymphoedema individuals. The lymphomononuclear cells exhibit poor tubular network formation; their adhesion to fibronectin and migration towards stromal-derived factor (SDF-1α) is also significantly attenuated. Concomitantly decreased expression of integrin α9 and reduced circulating levels of granulocyte colony-stimulating factor (G-CSF) were evident in this pathological condition. This case–control study addresses and identifies these impairments in PBMCs from a filarial endemic South Indian population. It was carried out without any exogenous induction of PBMCs (upon culture), to capture and better understand the original physiological and pathophysiological events that exist in the study groups. Thus freshly isolated lymphomononuclear cell fraction was used for the functional, phenotypical and molecular characterisation with regard to vasculogenic processes and gene expression. Moreover, PBMCs form the major surrogate cellular pool for bone marrow-derived circulating lymphatic progenitors (CLPs). Salven and co-workers reported that lymphatic endothelial progenitor cells (LEPCs) promote post-natal lymphangiogenesis in haematopoietic organ [16]. These cells are characterised by the surface expression of CD34, CD133 and VEGFR3 markers [34]. Jiang *et al.* studied the potential contribution of haematopoietic stem cells in lymphatic endothelial cell repair [35]. These cells are also capable of differentiating into mature lymphatic endothelial cells when there is a cue.

Mobilisation, adhesion, migration, differentiation and tube formation are the major physiological events involved in lymph vasculogenesis. SDF-1α and Integrin contribute to stimulating the mobilisation of lymphatic progenitors from bone marrow into circulation [36, 37] and subsequent adhesion over the extracellular matrix proteins. SDF-1α-induced mobilisation of CD34$^+$ cells depends upon integrin-mediated signalling [38, 39]. Similarly Integrin α9 does contribute to the functional lymphatic vessel formation. Significant reduction in the expression of integrin α9, reduced circulating G-CSF

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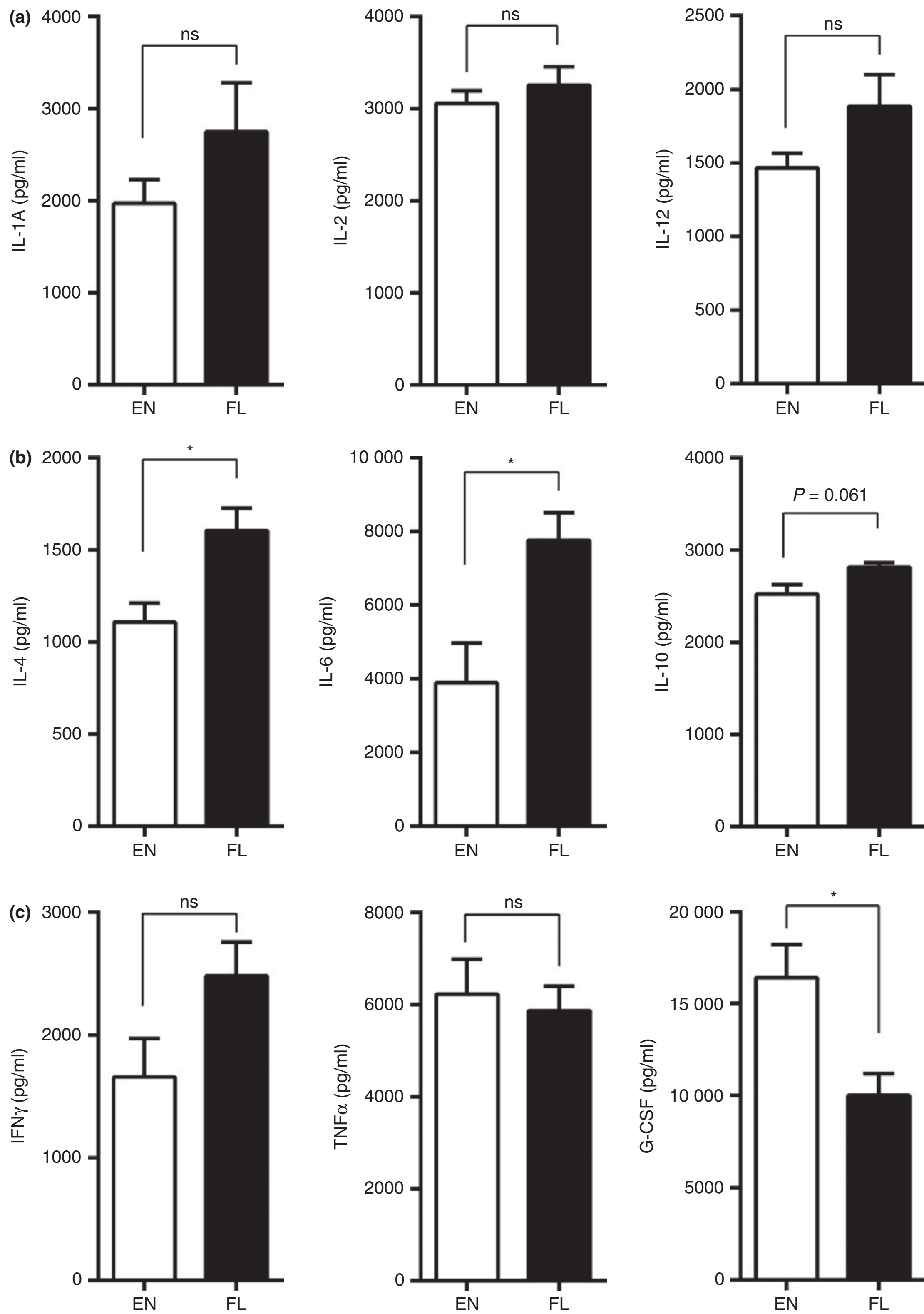

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Figure 4 Quantification of circulating pro and anti-inflammatory cytokines. A) Bar graph depicting the circulating levels of pro-inflammatory cytokines (IL-1A, IL-2 and IL-12) which imparts Th1 responses conferring cellular immunity and B) anti-inflammatory cytokines (IL-4, IL-6 and IL-10) which imparts Th2 responses conferring humoral immunity in the healthy (n = 12) and filarial lymphoedema (n = 12) individuals were quantified as per the manufacturer's instruction. C) Other inflammatory markers, namely IFN-γ, TNF-α and G-CSF were quantified for all the plasma samples from the study individuals using Multi-Analyte ELISA kit. Data represented as mean ± SEM. *P < 0.05 *vs*. endemic normals (EN).

levels and the defective fibronectin adherence of PBMCs in FL individuals clearly explain the moderate involvement of these players in lymphangiogenic process. It seems that low levels of circulating G-CSF may be one of the reasons behind poor mobilisation of these cells into circulation [40, 41]. Also, impaired adhesion is likely to play a role in the CLPs dysfunction. However, the molecular mechanism utilised by CLPs in this chronic state in regulating the expression of integrin α9 remains to be elucidated.

Immediately after adhesion, these lymphomononuclear cells have to migrate and differentiate into mature state. Migration potential was studied using transwell membrane filter set-up, where the membrane in the upper chamber was pre-coated with fibronectin and SDF-1α, and a potent chemo-attractant in the lower chamber mimics exactly how the circulating cells invade and migrate towards the site of action by SDF-1α gradient *in vivo*. Migration potential of lymphomononuclear cells in response to SDF-1α was greatly attenuated in the filarial infected group, suggesting the involvement and interplay of SDF-CXCR4 axis [42] in this chronic condition may impaired. Another hypothesis states that the influence of pro-inflammatory cytokines in these individuals also contributes to impairments of PBMCs and their progenitor subset. To confirm the lymphangiogenic gene expression in freshly isolated PBMCs, we determined the expression of Prox-1, VEGFR3 and podoplanin genes. Prospero-related homeobox domain 1 (Prox-1) is considered to be the master regulator in lymphatic development, lymphatic endothelial cell (LEC) specification and maintenance. It is a constitutive marker of LECs and expressed in tissues of both healthy individuals and lymphoedema patients. However, bone marrow-derived blood vascular endothelial cells (BECs) poorly express Prox-1 compared to markers such as CD31 and CD34 which are predominant [43]. Notably, the expression of Prox-1 confers LEC identity and fate, and at the same time, loss of Prox-1 expression leads to arrested lymphangiogenesis as shown in mice models by Wigle and Oliver [44]. Thus, Prox-1 is necessary for lymphangiogenesis and contributes to the expression of other lymphatic-specific genes, which play a role in the differentiation of lymphatic progenitor cells into functional lymphatic endothelial cells. By contrast, increased expression of Prox-1 was reported in inflammatory settings mediated by the activation of NF-$_{K}$B in lymphatic endothelium. However, the mechanisms behind the regulation of Prox-1 expression are presently unclear in normal physiological conditions. Our data explain that the activation of humoral immune responses is achieved by Th2 cytokines by inhibiting inflammation and in turn affects lymphangiogenic factors in FL individuals.

Certainly, the lymphatic endothelial makers VEGRF3 and podoplanin (PDPN) directly contribute to the formation of lymphatic vasculature [21]. Significant elevation in the expression of VEGFR3 and PDPN indicates a higher prevalence of these positive cell populations in the freshly isolated, bone marrow-derived mononuclear cells, as demonstrated by Tan and Lee, respectively [45, 46]. Although Prox-1 clearly plays central functions in lymphatics, it is most likely that the specialised fate and maintenance of lymphatic endothelial cells is a process that is ultimately governed by several interrelated transcriptional pathways and downstream target genes. Also there may be a need to differentially recruit certain cellular components during the lymphoedemal pathology. It was well known that humoral immune response against extracellular parasites, bacteria or toxins is mediated by Th2 cells. We observed an elevated Th2 response in the filarial-induced lymphoedema group, which again confers the lymphangiogenic inhibitory effects. Recent findings by Shin *et al.* [47] and Savetsky *et al.* [48] proved that Th2 cells and their cytokines regulate the lymphatic vessel function and inhibit lymphangiogenesis, respectively. Our results were consistent with these findings and also in sync with the previous findings by Clavin *et al.*, and Avraham *et al.*, demonstrating the endogenous inhibition of lymphangiogenesis by elevated TGF-β and IFN-γ expression [4, 49–51]. The attenuated lymphangiogenic ability of freshly isolated PBMCs to form a tubular network over 2D matrigel in response to VEGF clearly portrays the influence of secretory products of PBMCs and their paracrine effects [24, 52]. Also, the inhibitory factors of Th2 cells potentially inhibit tube formation *in vitro*. Yet the function of progenitor cells in varied inflammatory state and their

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ultimate contribution in lymphatic neovascularisation need to be studied in detail for therapeutic interventions.

The major limitation of our study was its small sample size. Although the region is prevalent for filariasis, ethically we were permitted only to collect samples from the individuals with evident filarial lymphoedema. In addition, most infected individuals had severe chronic infections and were taking drugs for other complications. As smoking strongly influences circulating progenitor cell counts, smokers were omitted from the study even after recruitment. Large-scale screening of individuals for filarial prevalence or for surveillance requires Government Health Authorities' approval. Recruiting enough subjects to carry out mechanistic studies was beyond the scope of this work. Furthermore, ethical constraints limit the volume of blood that can be collected for detailed studies. Certain lymphatic endothelial functional parameters were lacking in the current study, which are necessary to arrive at a mechanistic conclusion, and we did not carry out detailed surface profiling of PBMCs for expression of other lymphatic-specific markers and receptors.

Conclusion

We attempted to characterise the functional potential of PBMCs in response to filarial infection and its severity given that these cells can foster the formation of new functional lymphatic vessels to bypass the endothelial disruption and damage incurred by the filarial parasites. We observed that in chronic pathology, PBMCs exhibit attenuated vasculogenic potential with elevated levels of proinflammatory cytokines and increased expression of lymphatic markers. Further studies with suitable animal models and cell culture systems could provide effective leads and mechanistic insights.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gating strategy applied for isolated PBMCs. Morphological gating of the lympho-monocyte fraction using dot-plot based on forward and side scatter.

Table S1. List of Real Time primers used in the study.

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