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PRECLINICAL STUDY

Tumor microenvironmental plasmacytoid dendritic cells contribute to breast cancer lymph node metastasis via CXCR4/SDF-1 axis

Ramy Gadalla[1,5] · Hebatallah Hassan[1] · Sherif Abdelaziz Ibrahim[1] · Mahmoud Salah Abdullah[1] · Ahmed Gaballah[2] · Burkhard Greve[3] · Somaya El-Deeb[1] · Mohamed El-Shinawi[4] · Mona Mostafa Mohamed[1]

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Abstract

Purpose Plasmacytoid dendritic cells (PDCs) infiltration into breast cancer tissues is associated with poor prognosis. Also, CXCR4 shows compelling evidences to be exploited by cancer cells to migrate to distant sites. The present study investigated lymph node metastasis in the light of PDCs infiltration and the potential cross talk with CXCR4/SDF-1 chemokine axis.

Methods We assessed circulating PDCs proportions drained from the axillary tributaries, and the in situ expression of both CD303 and CXCR4 in breast cancer patients with positive lymph nodes (pLN) and negative lymph nodes (nLN) using immunohistochemistry and flow cytometry. We also analyzed the expression of SDF-1 in lymph nodes of pLN and nLN patients. We studied the effect of the secretome of PDCs of pLN and nLN patients on the expression of CXCR4 and activation of NF-κB in human breast cancer cell lines SKBR3 and MCF-7. TNF-α mRNA expression level in PDCs from both groups was determined by qPCR.

Results Our findings indicate increased infiltration of PDCs in breast cancer tissues of pLN patients than nLN patients, which correlates with $CXCR4^{+}$ cells percentage. Interestingly, SDF-1 is highly immunostained in lymph nodes of pLN patients compared to nLN patients. Our *in vitro* experiments demonstrate an upregulation of NF-κB expression and CXCR4 cells upon stimulation with PDCs secretome of pLN patients than those of nLN patients. Also, PDCs isolated from pLN patients exhibited a higher TNF-α mRNA expression than nLN patients. Treatment of MCF-7 cell lines with TNF-α significantly upregulates CXCR4 expression.

Conclusions Our findings suggest a potential role for microenvironmental PDCs in breast cancer lymph node metastasis via CXCR4/SDF-1 axis.

Keywords Plasmacytoid dendritic cells · CXCR4 · Breast cancer · Lymph node metastasis

Introduction

Metastasis is the major cause of breast cancer morbidity and mortality. It is a complex process in which cancer cells detach from tumor cell clusters, invade into the proximal vasculature and finally settle in a secondary organ to form a metastatic foci [1]. This occurs in response to different signals of cytokines/chemokines networks within the tumor microenvironment and the metastatic sites [2]. The regional lymph nodes are the most likely first homing site for metastasizing cancer cells followed by more distant organs [3]. The mutual interaction between breast cancer cells and infiltrating leukocytes has been proven to be a decisive factor in tumor progression and metastasis [4, 5]. This interaction can take place through cell-to-cell contact or through the secreted factors. Dendritic cells (DCs) are

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✉ Ramy Gadalla
ramy.gadalla@uhnresearch.ca

1 Department of Zoology, Faculty of Science, Cairo University, 12613 Giza, Egypt

2 Department of Clinical Oncology and Nuclear Medicine, Faculty of Medicine, Ain-Shams University, Cairo, Egypt

3 Department of Radiotherapy–Radiooncology, University Hospital Münster, Münster, Germany

4 Department of General Surgery, Faculty of Medicine, Ain Shams University, Cairo, Egypt

5 Present Address: Princess Margaret Cancer Center, University Health Network, 610 University Ave, Toronto, ON M5G 2M9, Canada

bone marrow-derived mononuclear cells that play a pivotal role in initiating adaptive immune response through antigen presentation to T-lymphocytes [6, 7]. Almost all types of carcinoma tissues, including breast cancer, are heavily infiltrated by DCs [8]. DCs comprise two distinct subsets, myeloid DCs and plasmacytoid DCs (PDCs), which share similar origin but different phenotypes and life cycles [9]. The frequency of PDCs in blood of normal human subjects represents approximately 0.2–0.5% of the total circulating leukocytes [10]. PDCs are known for its remarkable capacity of secreting type-I interferon upon stimulation, which plays an important role in mediating antiviral immunity [11]. However, infiltration of PDCs into human breast cancer tissues is strongly associated with poor prognosis and shorter overall survival [12]. Interestingly, PDCs-depleted animal models of mammary carcinoma showed a reduction in tumor growth and metastasis [13].

CXCR4/SDF-1 is a key chemokine receptor axis involved in the process of lymph node metastasis in breast cancer [14]. Naturally, CXCR4 is widely expressed on lymphocytes to mediate their migration to lymph nodes in response to its ligand SDF-1 [15]. In the tumor microenvironment, CXCR4 overexpression on cancer cells led to their attraction to the SDF-1 constitutively expressing-organs, e.g., lymph nodes, bone marrow and lungs [16]. This could explain why breast cancer metastasis incidences are usually to these specific organs [17].

Blood collected from the tumor microenvironment is well known to contain indicative cellular and soluble mediators of tumor microenvironment status [18–20] Therefore, in the present study, we utilized an innovative surgical approach [21] to collect blood sample drained from the axillary vein tributaries of breast cancer patients during modified radical mastectomy. We isolated PDCs from breast cancer patients with nLN and pLN to test their effect on lymph node metastasis. We showed that PDCs might contribute in lymph node metastasis through the upregulation of CXCR4/SDF1 axis mediated by activation of NF-κB in pLN compared with nLN breast cancer patients. Our data underscore the role PDCs play in breast cancer metastasis to lymph nodes and cancer progression.

Materials and methods

Patient's samples

For the purpose of patients' enrollment, we obtained Institutional Review Board (IRB) approval from the ethics committee of Ain-Shams University, Cairo, Egypt. Patients were enrolled from outpatient breast clinic of Ain-Shams University hospitals during the period of January 2013 till December 2017. Before participation, all patients signed a consent form including approval for publication of the study. We enrolled 35 treatment-naive women clinically diagnosed with breast cancer by physical examination, mammography, ultrasound and pathological evaluation of breast cancer biopsies. Patients with hepatitis or any other viral infection were excluded. During modified radical mastectomy, breast cancer tissues, axillary lymph nodes, and blood samples from axillary tributaries and peripheral veins were collected as previously described [21]. Lymph node biopsies were examined grossly and microscopically by a pathologist to determine patients' nodal status.

Isolation of PDCs and preparation of PDC-conditioned media (PDC-CM)

Blood from peripheral vein and axillary tributaries of breast cancer patients was collected as described before [21]. Blood was diluted with an equal amount of phosphate-buffered saline (PBS), pH 7.2 at room temperature. Mononuclear cells were separated by density gradient centrifugation using Ficoll-Hypaque from Lonza (MD, USA) at 1500 rpm for 30 min. We used EasySep™ Human Plasmacytoid DC Enrichment kit from STEMCELL Technologies (BC, Canada) to immunomagnetically sort out PDCs, untouched from mononuclear cells suspension, using a cocktail of magnetic-beads-coupled antibodies, according to the manufacturer's protocol. Isolated PDCs were seeded overnight in serum-free RPMI-1640 media to prepare PDC-conditioned media (PDC-CM). PDCs were then collected by centrifugation for further experiments, and CM were aspired and stored at − 80 °C for in vitro cancer cell line stimulation. Purity of isolated cells was validated by flow cytometer, and 90–95% were $CD303^+$ $CD123^+$ cells [22].

Single-cell suspension preparation

Tumor tissues were minced and digested with 5 mg/ml collagenase (SERVA, Germany) and dispase (Sigma Aldrich, MO, USA). Enzymatic dissociation was took place at 37 °C until predominantly single-cell suspension was obtained (2–4 h). Undigested cells clumps were removed by filtering through 50 µm pore size cell strainer. Single-cell suspensions were centrifuged at 800 rpm for 5 min and washed twice by PBS/1% BSA.

Cell culture and stimulation

The human MDA-MB-231, SKBR-3 and MCF-7 breast cancer cell lines (a gift from Dr. Bonnie F. Sloane, Department of Pharmacology, School of Medicine, Wayne State University, Detroit, MI, USA). MDA-MB231 and SKBR3 cells were maintained in Dulbecco's modified Eagle medium (DMEM), and MCF-7 were cultured in RPMI-1640 media.

Media were supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. For cell lines stimulation, cells were seeded at 4 × 105 density and stimulated with PDC-CM isolated from nLN and pLN breast cancer patients at concentration 1 mg/ml for 72 h. For TNF-α stimulation, a total of 300,000 MDA-MB-231 and MCF-7 cells were seeded for adhesion overnight in complete DMEM and RPMI medium, respectively. The cells were washed twice with 1X PBS and starved in 1%FBS supplemented growth media overnight. Afterwards cells were stimulated with 50 ng/ml TNFα (R&D systems) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. Finally, cells were harvested by 2 mM EDTA in PBS, washed, stained for CXCR4 expression by flow cytometry.

Flow cytometry

A volume of 100 µl of whole blood was incubated at room temperature for 30 min with a mixture of monoclonal fluorophore-conjugated antibodies; Pacific blue-lineage antibodies cocktail containing antibodies against CD3, CD14, CD16, CD19, CD20, and CD56 (Biolegend, CA, USA), phycoerythrin (PE)-conjugated anti-HLA-DR (BD Biosciences, CA, USA), Fluorescein (FITC)-conjugated anti-CD123 (Miltenyi Biotec, Bergish Gladbach, Germany). Red blood cells were lysed with Lyse/No Wash protocol using whole blood lysing buffer (LifeTechnologies, CA, USA) to minimize cell loss. Data were acquired using Attune Acoustic Focusing Cytometer (LifeTechnologies, CA, USA). PDCs were identified as lin-/lowHLA + CD123+ cells as described previously [23, 24] and counted as a percentage of total leukocytes. Similarly, 7 × 105 single-cell suspension of breast cancer tissues, SKBR3 and MCF-7 cancer cells were stained with PE-conjugated CXCR4 (eBiosciences, CA, USA) prior to acquisition using CyFlow cube 8 flow cytometer (Partec, Muenster, Germany). Gating and analysis of flow cytometry data were performed using DeNovo Software FCS Express 4 (CA, USA).

Quantitative real-time PCR

Total RNA was extracted from isolated PDCs using the GeneJET RNA purification Kit (Thermo scientific, MA, USA) and was reverse transcribed into cDNA applying the Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific, MA, USA). Quantitative PCR was performed using Step one plus real-time PCR (Applied Biosystems, CA, USA) in a 25-µl total volume containing 12.5 µl SYBR green master mix (Applied Biosystems, CA, USA), 1 µl of each upstream primer (5′-CAGCCTCTTCTCCTTCCTGA-3′) and downstream primer (5′-AGATGATCTGACTGCCTGGG-3′) of (TNF-α) gene (10 pmol/µl), 2.5 µl of cDNA and 8 µl of RNase free water. PCR thermal profile was 95 °C initial denaturation for 10 min and then followed by 40 cycle of 94 °C for 15 s and 60 °C for 1 min. Amplification specificity was verified using melting curve analysis and 2% agarose gel electrophoresis of the PCR products. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method after normalization to 18S upstream primer (5′-AACCCGTTGAACCCCATT-3′) and downstream primer (5′-CCATCCAATCGGTAGTAGCG-3′).

Immunohistochemistry

Formalin-fixed paraffin-embedded breast cancer tissues and lymph nodes were cut into 5 µm sections and mounted on positively charged slides. Tissue sections were deparaffinized and rehydrated, followed by heat-induced antigen retrieval. Endogenous peroxidase activity was blocked with Peroxide Block (BioGenex, CA, USA), while non-specific protein-binding targets were quenched with Power Block (Biogenex, CA, USA). Tissue slides were incubated overnight at 4 °C with primary antibodies CD303 and SDF-1 (R&D systems, MN, USA) at concentration 15 µg/ml and 10 µg/ml, respectively. After washing with PBS, slides were incubated with appropriate HRP-linked secondary anti-goat (KPL, MA, USA) or anti-mouse (EnVision + Dual Link System, Dako, Denmark) for 30 min in humidified chambers at room temperature as described before [25]. Negative control slides were run in parallel in which the primary antibodies were replaced with PBS. Bound antibodies were visualized by adding DAB substrate-chromogen solution (Dako, Denmark). Mayer's hematoxylin (Millipore, Germany) was used for counterstaining. Finally, slides were dehydrated through serial dilution of 70%, 95% and 100% alcohol and covered with mounting media. Signal evaluation was performed using ImageJ software as we described previously [26, 27].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting

Breast cancer cell lines stimulated by PDC-CM of nLN ($n = 18$) and pLN ($n = 17$) breast cancer patients were lysed by in radio-immunoprecipitation assay buffer (RIPA buffer) containing proteases inhibitors. Protein content was measured by Bradford assay, and 30 µg protein sample per lane was loaded onto SDS-PAGE. The samples were then electrophoresed and transferred to PVDF membrane (Millipore, Germany). The membranes were blocked for 2 h with 5% non-fat dry milk, followed by incubation with phosphor-NF-κB/p65 (ser276) antibody (cell signaling Technology, MA, USA) at 4 °C overnight. After washing, the membranes were then probed with HRP-conjugated anti-rabbit secondary antibody (1:10,000) for 1 h and the immunoreactivity were visualized using TMB substrate according to the

manufacturer's protocol (KPL, MA, USA). Density of the developed bands was quantified by ImageJ and normalized to β-actin band as we described previously [28, 29].

Statistical analysis

Statistical analysis was performed by IBM SPSS (NY, USA) version 18.0 for Windows using Student's *t* test to compare two groups, Fisher's exact test and Pearson's correlation test for correlation analysis. $P < 0.05$ was considered significant.

Results

Patients' clinicopathological characteristics

Clinical and pathological characteristics of nLN and pLN patients are described in Table 1.

Carcinoma tissues of pLN patients are characterized by a higher infiltration of PDCs than that of nLN patients

We evaluated the level of PDCs infiltration in paraffin-embedded breast cancer tissues of nLN and pLN patients using immunostaining of the specific marker CD303 for PDCs. IHC results showed a statistically significant increase of the infiltrated PDCs in carcinoma tissues of pLN patients compared to that of nLN patients ($P = 0.0001$) (Fig. 1).

Higher PDCs count with more HLA-DR expression in blood isolated from axillary tributaries of pLN patients compared to those of nLN patients

Next, we evaluated the percentage of circulating PDCs in peripheral and axillary tributaries of both nLN and pLN breast cancer patients using flow cytometry. Results of flow cytometric analysis revealed a significant increase ($P = 0.034$) in percentage of circulating PDCs in axillary blood of pLN patients compared to nLN patients, whereas no significant difference in circulating PDCs of peripheral blood between the two groups was observed (Fig. 2a, b). The maturation of circulating PDCs is estimated by HLA-DR expression level [30]. The mean fluorescence intensity (MFI) of HLA-DR on PDCs gate represents HLA-DR expression level (Fig. 2c). Although flow cytometric results revealed a higher HLA-DR expression by 1.8-folds in PDCs isolated from axillary tributaries of pLN patients than those of nLN patients, the difference is not statistically significant.

Table 1 Clinical and pathological characteristics of nLN patients and pLN patients

Characteristics	nLN ($n = 18$)	pLN ($n = 17$)	*P* value
Age			
Range	27–78	34–65	
Mean ± SEM	53.11 ± 3.2	51.94 ± 2.2	0.771[a]
Tumor size (cm)			
≤4	10 (58.8%)	5 (31.3%)	
>4	7 (41.2%)	11 (68.8%)	0.107[b]
NA	1	1	
ER			
Negative	8 (47.1%)	5 (33.3%)	0.335[b]
Positive	9 (52.9%)	10 (66.7%)	
NA	1	2	
PR			
Negative	9 (52.9%)	7 (46.7%)	0.500[b]
Positive	8 (47.1%)	8 (53.3%)	
NA	1	2	
Her2			
Negative	12 (70.6%)	9 (60.0%)	0.398[b]
Positive	5 (29.4%)	6 (40%)	
NA	1	2	
Tumor grade			
G1	2 (11.8%)	0 (0.00%)	
G2	15 (88.2%)	13 (86.7%)	0.133[b]
G3	0 (0.00%)	2 (13.3%)	
NA	1	2	
Lymphovascular invasion			
Absent	16 (94.1%)	11 (78.6%)	
Present	1 (5.9%)	3 (21.4%)	0.228[b]
NA	1	3	

P value calculated by [a]Student's *t* test or [b]Fisher's exact test

A higher immunostaining of CXCR4 in carcinoma tissue and its ligand SDF-1 in lymph node of pLN than that of nLN breast cancer patients

Since the chemokine receptor CXCR4 has a well-established role in breast cancer progression and metastasis [31], we evaluated positive staining of CXCR4 cells in breast cancer tissues of nLN and pLN patients. A single-cell suspension of breast cancer tissues of both groups were stained with anti-CXCR4 antibody. Flow cytometric analysis revealed a significantly higher percentage ($P = 0.016$) of $CXCR4^+$ cells in breast cancer tissue of pLN patient by 2.1-folds than that in nLN patient (Fig. 3a). Interestingly, we detected a significantly positive correlation ($r = 0.543$, $P = 0.01$) between PDCs infiltration density and positive staining of CXCR4 in breast cancer tissue using Pearson's correlation test (Fig. 3b). The stromal cell-derived factor (SDF-1) is a major chemokine involved in hematopoietic cells trafficking

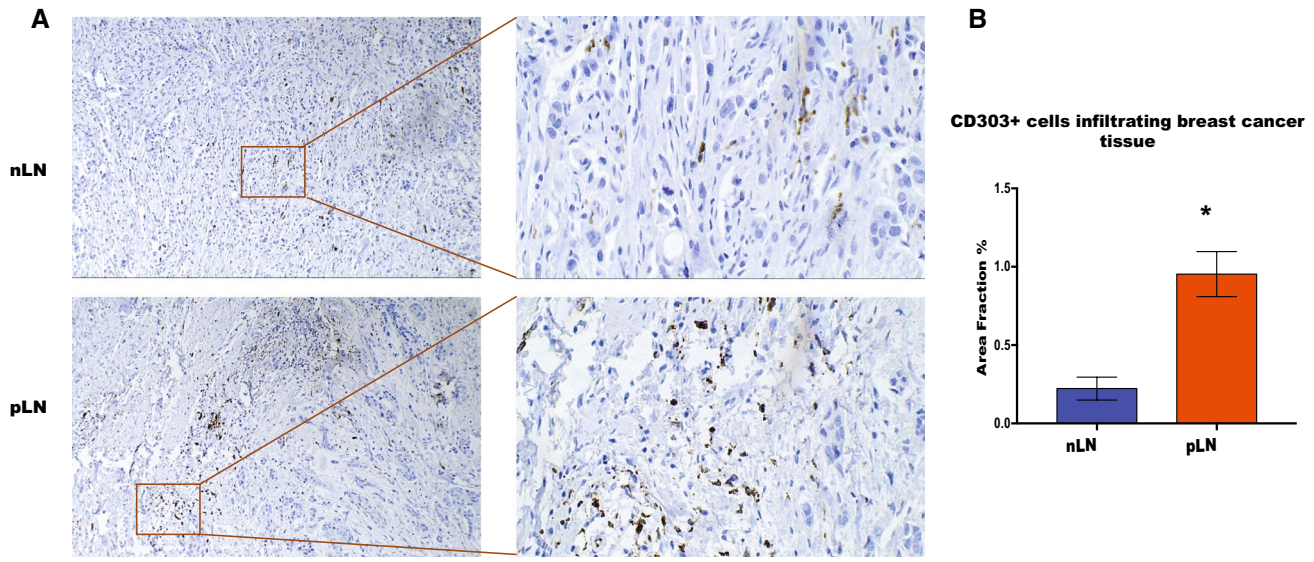


Fig. 1 An increased infiltration of PDCs in breast tumor tissues of pLN patients versus nLN patients. Paraffin-embedded tissue of nLN and pLN patients were stained immunohistochemically with antibody against. CD303 positive PDCs with brown stain appear dispersed among tumor cells. **a** Sections of cancer tissue of nLN patients (upper panel) and pLN patients (lower panel) with ×10 and ×40 magnification power. **b** Bars represent area fraction of CD303 immunostaining intensity as quantified by ImageJ software. Data represent mean ± SEM. *Significant *P* value ($P < 0.01$), as determined by Student's *t* test

through CXCR4-dependent mechanism. SDF-1 was shown to be an important factor in tumor metastasis as it exerts a chemoattractant effect on tumor cell expressing CXCR4 [30]. Therefore, we evaluated SDF-1 expression in lymph nodes to assess the chemoattraction homing toward lymph nodes. Our IHC results showed a massive positive staining of SDF-1 on the metastatic tumor cell aggregation in positive lymph node, whereas it is weakly stained in metastasis-free lymph nodes (Fig. 3b).

PDC-CM of pLN enhance expression of CXCR4 on breast cancer cell lines possibly via activation of NF-κB

To investigate whether secretome of PDCs had an influence on the expression of CXCR4, SKBR3 and MCF-7 breast cancer cell lines were stimulated with PDC-CM of both nLN and pLN patients for 72 h and CXCR4 expression were assessed by flow cytometry. PDC-CM isolated from pLN patients resulted in a statistically significant ($P = 0.001$) upregulation of CXCR4 expression on SKBR3 cells line compared with that isolated from nLN. However, MCF-7 stimulated with PDC-CM isolated from pLN patients showed borderline a significant upregulation of CXCR4 ($P = 0.06$) as compared to those stimulated with PDC-CM isolated from nLN patients (Fig. 4a).

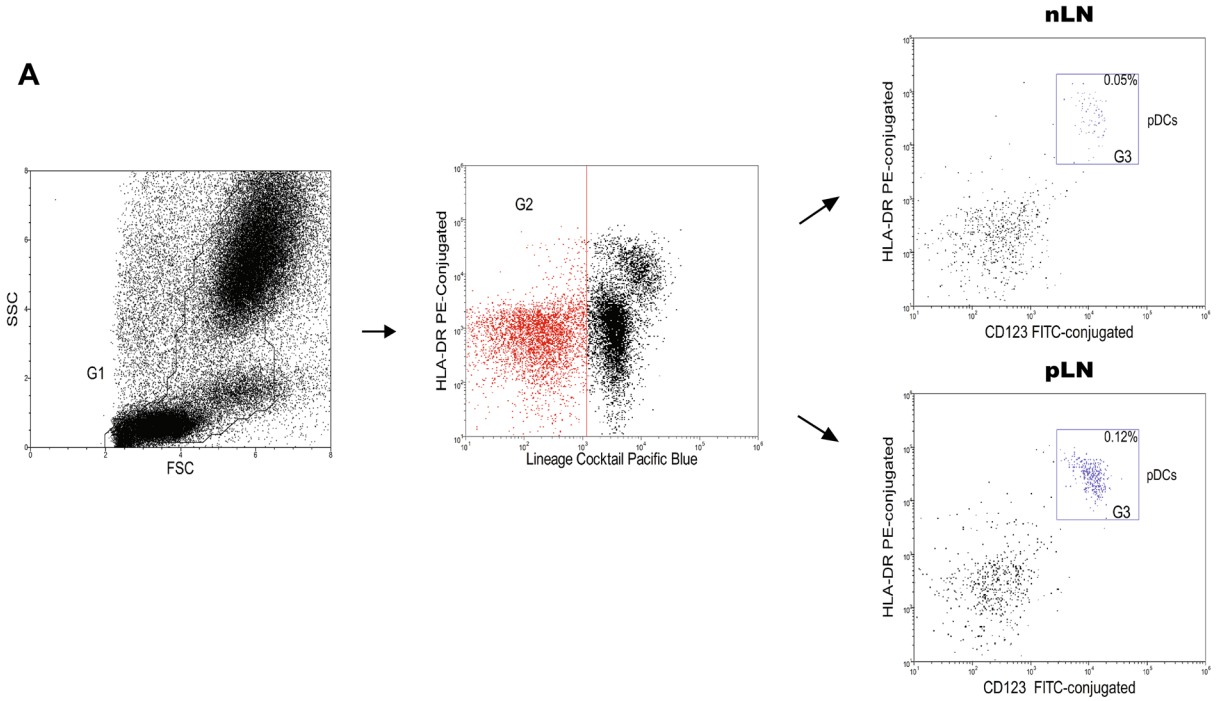
The transcription factor NF-κB plays an essential role in regulation and expression of inflammatory cytokines in the tumor microenvironment [32]. In order to address whether the secretions of PDCs contribute to the activation of NF-κB, the breast cancer cell lines SKBR3 and MCF-7 were stimulated with PDC-CM of nLN and pLN patients. Immunoblot analysis revealed a statistically significant ($P < 0.001$) higher activation of NF-κB/p65 in both cell lines stimulated with PDC-CM of pLN patients by 2.3-folds (in SKBR3) and 2.0-folds (in MCF-7) as compared to those stimulated with PDC-CM of nLN patients (Fig. 4b).

Upregulation of TNF-α mRNA expression in PDCs isolated from axillary blood of pLN compared to nLN breast cancer patients

One of the candidate inflammatory cytokines that mediate activation of NF-κB is TNF-α [33]. Thus, we assessed expression of TNF-α by qPCR in PDCs isolated from pLN and nLN patients, our qPCR data uncovered a significant upregulation of TNF-α mRNA level by 4.5-folds ($P = 0.001$) in PDCs isolated from pLN patients as compared to those of nLN patients (Fig. 5a).

TNF-α treatment upregulates surface expression of CXCR4 protein on MCF-7 cells

TNF-α in vitro stimulation of MCF-7 cells resulted in a statistically significant upregulation of CXCR4 ($P = 0.005$), whereas no significant effect was observed in MDA-MB-231 cells (Fig. 5b).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G1
HLA-DR PE-Conjugated
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G2
nLN
0.05%
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G3
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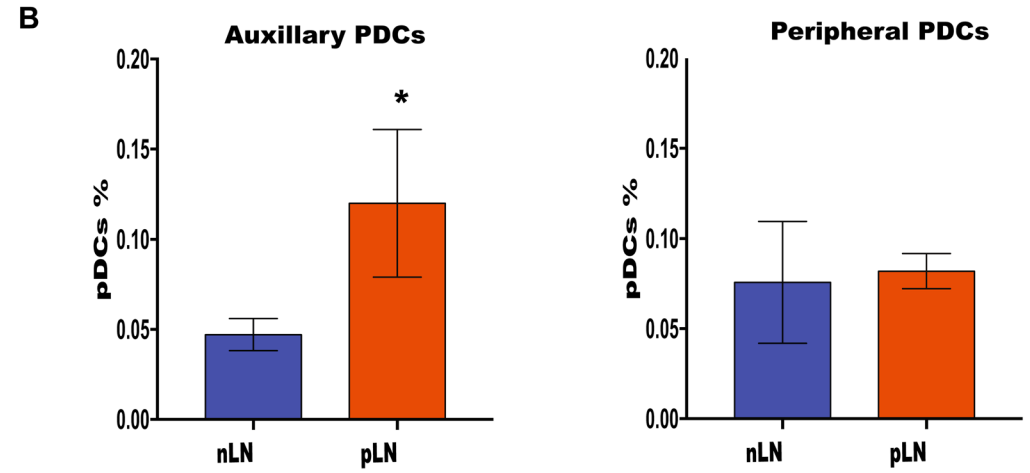
CD123 FITC-conjugated
pLN
0.12%
pDCs
G3
HLA-DR PE-conjugated
CD123 FITC-conjugated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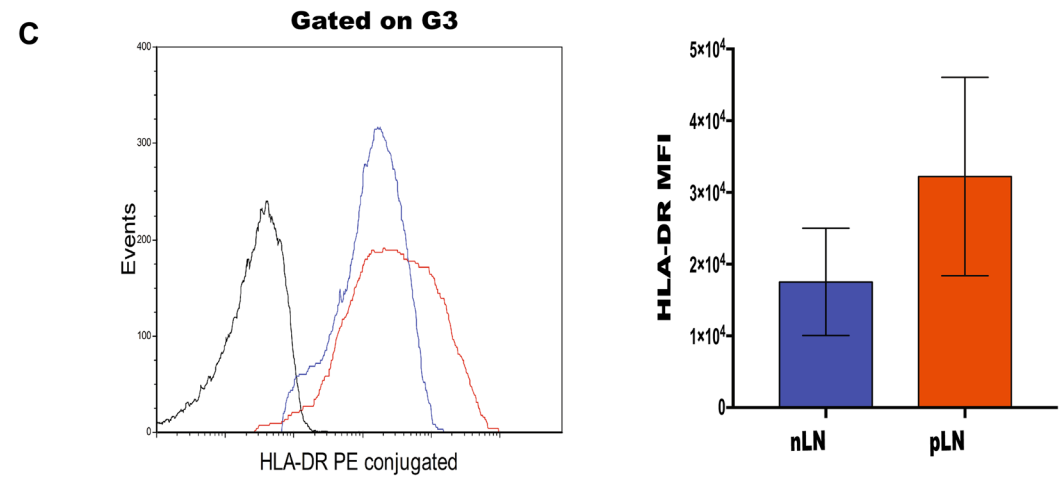
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◂**Fig. 2** Flow cytometric analysis showing a higher percentage of tumor microenvironmental PDCs of breast tumor in pLN patients. **a** Demonstration of the gating strategy. Gate G1 was determined on the light scatter plot to include live cells and to exclude debris and outlier cells. A daughter plot gated on G1 shows HLA-DR versus lineage markers axes. Gate G2 was determined to include $HLA^+Lin^{-/low}$ cells. Another daughter plot gated on G2 shows HLA-DR versus CD123 axes. Gate G3 was drawn to define PDCs as HLA^+CD123^+ cells. **b** Bars represent circulating PDCs drained from axillary and peripheral blood of nLN and pLN patients. **c** Overlay histogram showing the mean fluorescence of HLA-DR in PDCs (G3) of nLN (blue) and pLN patients (red), unstained cell was used as negative control (black). Bars represent the mean fluorescence intensity of HLA-DR in nLN and pLN patients. Data represent mean ± SEM. *Significant *P* value ($P < 0.01$), as determined by Student's *t* test

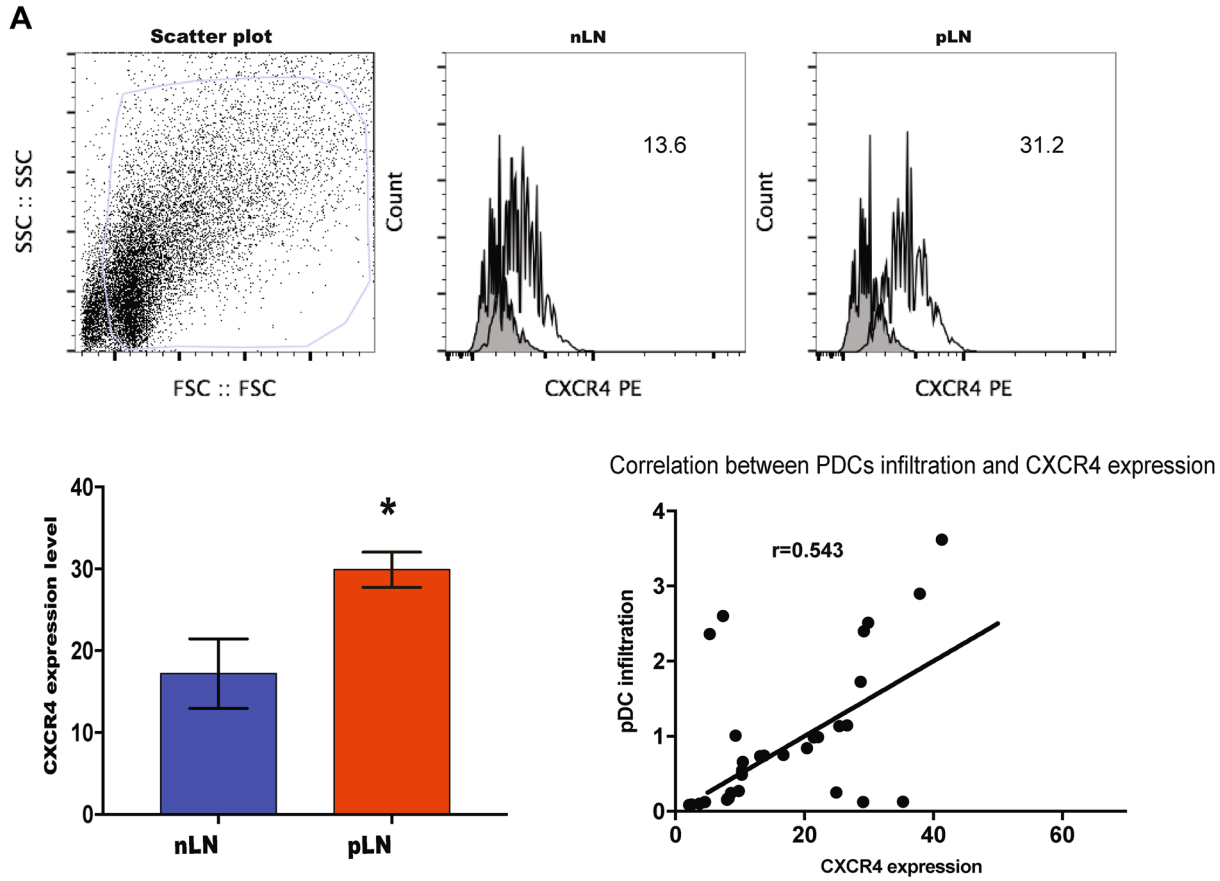
Discussion

The cross talk between DCs and other tumor microenvironment components including tumor cells is a significant determinant of the efficacy of the subsequent immune response against tumor cells [34]. DCs are known to be highly plastic immune cells that are able to change its phenotype and functional status as a result of interaction with other stromal cells and in response to microenvironment stimuli [35]. The plasticity characteristics of DCs are well exploited by tumors to repress DC effectiveness in elimination of cancer cells. For example, a study on humanized mice grafted with the human breast cancer cell line demonstrated that DCs recruited CD4+T cells to the graft and instigated it to secrete IL-13 which facilitate tumor development through activating STAT6 signaling, which are known to be implicated in metastasis process [36, 37]. Another study showed that the primary suppressive CD8+ regulatory T cells were induced by tumor-associated PDCs in ovarian cancers [38]. Moreover, it has been shown that sentinel lymph nodes with metastasis have higher arrest of maturation of dendritic cells compared to sentinel lymph nodes without metastasis in breast cancer patients [39].

In the present study, we demonstrated higher infiltration of PDCs ($CD303^+$ cells) in breast cancer tissues of pLN patients compared to nLN patients, with a positive correlation to CXCR4+ cells within breast cancer tissues. This is consistent with the observation that PDCs-infiltrating human primary breast tumors is an independent prognostic factor and is associated with poor outcome [40]. When we collected blood directly from the tumor microenvironment using surgical approach described above, we found a higher PDCs proportion in blood drained from tumor microenvironment of pLN patients in comparison with nLN patients. On the other hand, no statistical significant difference was observed in PDCs count collected from peripheral blood of both pLN and nLN patients. This could be attributed to the fact that PDCs population is known to use hematogenous route to home from tissues to lymph nodes, unlike most of leukocytes using lymphatics system [41]. This could render axillary blood PDCs count significantly higher and a more reflective parameter to the tumor microenvironment than those circulating peripheral blood. Additionally, we found that circulating PDCs of pLN patients exhibits more mature phenotype than those of nLN patients, as evidenced by a higher HLA-DR surface expression, which could be indicative of the higher inflammatory/stimulatory status in pLN patients' tumor microenvironment [42]. This suggests that breast tumor exerts a local effect on PDCs proportion.

Tumor cell migration and metastasis share many similarities with leukocytes trafficking, which is governed mainly by chemokines and cytokines network [43]. This is even more evident when the target site of metastasis is the lymph node. Normally, CXCR4/SDF-1 axis is used by hematopoietic cells to home to lymphoid organs [44], whereas in colorectal and gastric cancer CXCR4/SDF-1 axis has been shown to be involved in lymph node metastasis and progression through the promotion of epithelial-mesenchymal transition viaWnt/β-catenin signaling pathway [45, 46]. Indeed, *in vitro* experiments and animal models studies demonstrated that silencing of CXCR4 with interfering RNAs impaired invasion of breast cancer cells MDA-MB-231 in Matrigel invasion assay and inhibited the lung metastasis of MDA-MB-231 cells in an animal model [47]. We suggest a similar mechanism may exist in the patients' cohort of the present study. Flow cytometric analysis revealed a higher percentage of $CXCR4^+$ cells in tumor tissues of pLN patients than that of nLN patients. In order to examine the whole CXCR4/SDF-1 chemoattraction axis, the expression of SDF-1 was assessed with immunohistochemical staining in lymph nodes. Results showed that metastatic deposits of breast cancer cells in lymph nodes intensively express SDF-1, which triggers more $CXCR4^+$ cells to migrate, i.e., positive feedback of metastatic cascade. Metastasis-free lymph nodes showed low expression of SDF-1 expression. Kang et al. has shown higher expression of SDF-1 in breast cancer tissue of node-positive lymph nodes than node-negative patients [48]. Altogether, this suggests that carcinoma cells of pLN patients have a higher tendency to home to organs expressing SDF-1 including lymph nodes. Due to the internalization and the ligand-dependent degradation of CXCR4 upon binding to SDF-1 as reported in previous studies [49, 50], assessment of CXCR4 expression in the lymph nodes cannot be quantified reliably.

In a previous study, we demonstrated the effect of secretions of tumor-associated leukocytes on promoting lymph node metastasis [51]. Most studies investigate PDCs in the context of T-cells priming, and not much attention has been given to the potential modulatory effect of PDCs on tumor microenvironment through their secretions, although it is characterized by a massive secretory capacity that is equivalent to lymphocytes capacity [52]. Herein, we demonstrated


A
Scatter plot
SSC :: SSC
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Correlation between PDCs infiltration and CXCR4 expression
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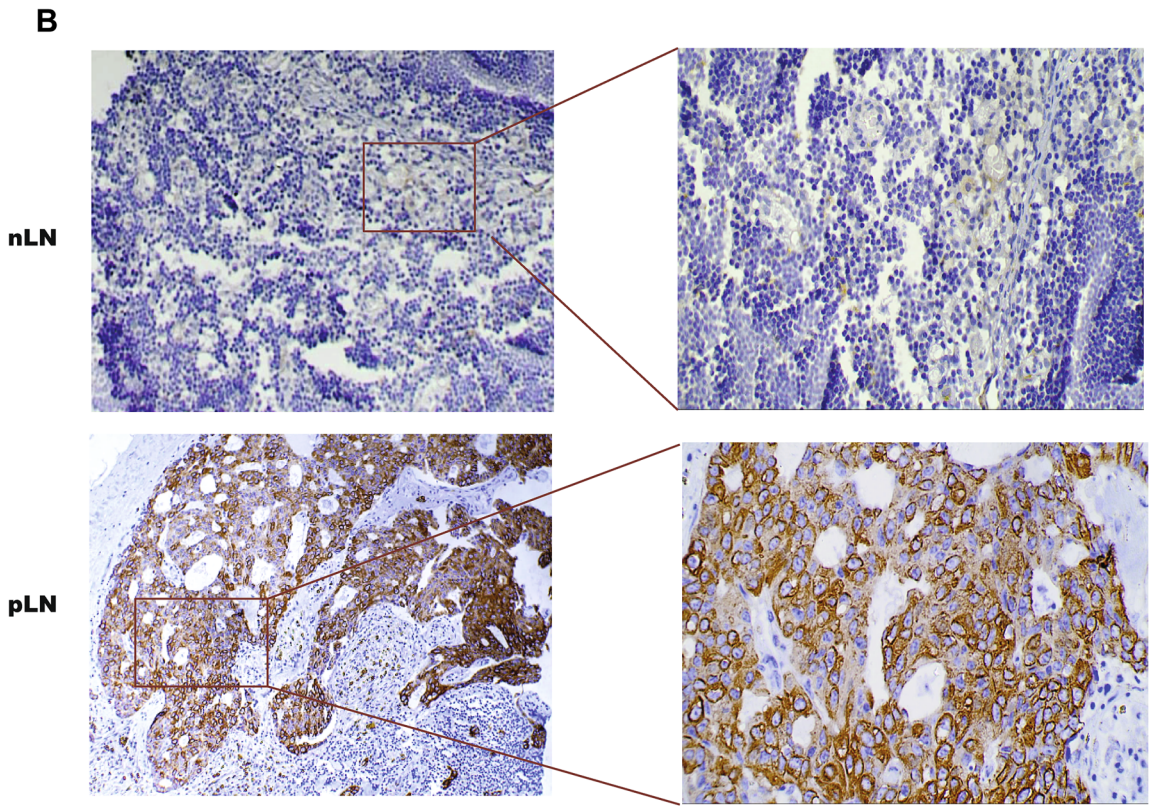
B
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pLN

◂**Fig. 3** Higher expression of CXCR4 cells in breast cancer tissue of pLN patients compared to nLN patients. Flow cytometric quantitation analysis of $CXCR4^+$ cells in single-cell suspension of breast cancer tissue of nLN and pLN patients. **a** Upper panel shows overlaid histograms of single-cell suspension of breast cancer of nLN and pLN patients. The numerical values inside each plot denote the mean fluorescence intensity, which was calculated by subtracting mean of the unstained cell peak (tinted) from the stained peak (white). Left lower panel showing bars chart representing CXCR4 expression in both groups. Data represented as mean ± SEM. Right lower panel shows a scatter chart with a trend line shows the positive correlation relationship between PDCs infiltration density into breast cancer tissue and CXCR4 expression ($P < 0.05$, Pearson's correlation coefficient = 0.546). **b** Immunohistochemical staining of SDF-1 in lymph node of breast cancer patients showing weak expression of SDF-1 in nLN patients (upper panel) in comparison with the massive expression of SDF-1 on metastatic tumor deposits in pLN patient (lower panel) (left ×10, right ×40 magnification). *Significant *P* value ($P < 0.01$), as determined by Student's *t* test

that secretions of PDCs isolated directly from tumor-microenvironment of breast cancer patients might contribute to lymph node metastasis via stimulation of CXCR4 expression by carcinoma cells. This was proven by stimulating SKBR3 and MCF-7 breast cancer cell lines with media conditioned by PDCs-derived factors, which resulted in an increase of the percentage of $CXCR4^+$ cells upon stimulation with PDC-CM of pLN patients versus that of nLN patients. This result is in line with our previous observation where we demonstrated the effect of secretions of tumor-associated leukocytes on promoting lymph node metastasis [51]. To prove the principle in vivo, interestingly we found that PDCs infiltration density was positively correlated with percentage of cells expressing CXCR4 in breast carcinoma tissues. HER2-overexpressing SKBR3 cell line exhibited a higher tendency to upregulate CXCR4 than MCF-7 cells. This may be ascribed to the observation that HER2 expression enhances the expression and function of CXCR4 [53].

One of the mechanistic clues of CXCR4 expression is the transcription factor NF-κB. The promoter of CXCR4 contains binding sites for NF-κB, and it has been proven at the transcriptional level that CXCR4 expression in breast cancer cell line MDA-MB-231 is directly mediated by NF-κB [54]. Also, butein, a tetrahydroxychalcone compound—known to interfere of NF-κB pathway—was reported to downregulate CXCR4 expression in breast cancer cell lines [55, 56]. Indeed, we observed a higher phospho-NF-κB expression in both cell lines stimulated with PDC-CM of pLN than that of nLN patients. TNF-α is known to directly activate NF-κB pathways through the stimulation of TNF receptors [57]. Therefore, we verified expression of TNF-α mRNA level as a possible PDCs-derived factor that contributes to CXCR4 and NF-κB upregulation. Our qPCR results indicated a higher mRNA expression of TNF-α in PDCs isolated from axillary blood of pLN patients in comparison with nLN patients. To further support our hypothesis, we showed that direct TNF-α stimulation upregulates CXCR4 expression in breast cancer cell line MCF-7 but not in the invasive MDA-MB-231 cells. A high expression of TNFRII in breast cancer is associated with advanced clinical stage and poor clinical outcomes [58]. We hypothesize that the PDCs-derived TNF-α, contributed in part along with the other inflammatory mediators in the tumor microenvironment to activate the NF-κB pathway, which in turn induced upregulation of CXCR4 and to promote breast cancer progression [54].

In conclusion, our findings demonstrate that the higher infiltration of PDCs contributes to upregulation of CXCR4 expression in breast cancer tissues possibly through TNF-α-mediated NF-κB activation, which ultimately led to cell migration toward lymph node via CXCR4/SDF-1 chemoattractive axis. Demonstrating the pro-tumor effect of PDCs, this study suggests a further complete investigation for the factors that promote PDCs recruitment and accumulation within breast cancer tissue is needed in order to formulate therapeutic modality in an attempt to inhibit breast cancer progression and lymph node metastasis.

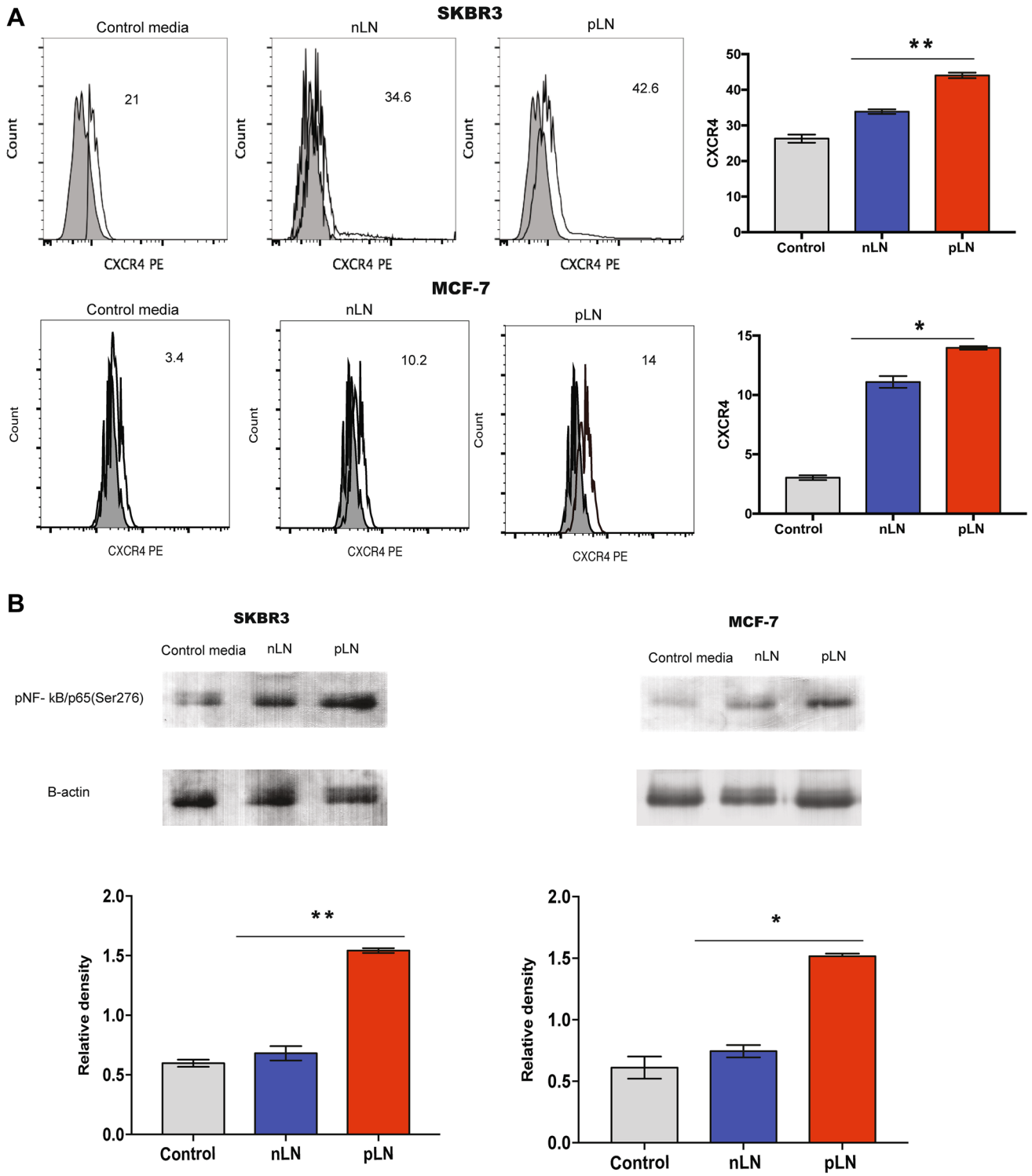

Fig. 4 Effect of PDC-CM isolated from of pLN than nLN patients on expression of CXCR4 and NF-κB /p65 activation in SKBR3 and MCF-7 cells. **a** Upregulation of CXCR4 on breast cancer cell lines upon stimulation with PDC-CM of pLN than nLN patients. CXCR4 was determined by flow cytometry on SKBR3 and MCF-7 cell lines 72 h after stimulation with PDC-CM of nLN and pLN patients. Left panel: histograms of unstimulated cells (serum-free RPMI culture media), PDC-CM of nLN and pLN patients. Right panel: bars represent differential CXCR4 upregulation upon stimulation with PDC-CM of nLN and pLN patients. $*P<0.01$, $**P<0.001$, as determined by Student's *t* test. **b** Western blot for phospho-NF-κB /p65$^{(ser276)}$ in SKBR3 and MCF-7 cells cultured with media conditioned by PDCs isolated from axillary tributaries of nLN and pLN patients. Equal protein concentration of total cell lysate was loaded, electrophoresed and immunoblotted. The membrane was probed with the phosphorylated form of NF-κB. Immunoblot band intensities were normalized to β-actin as loading control

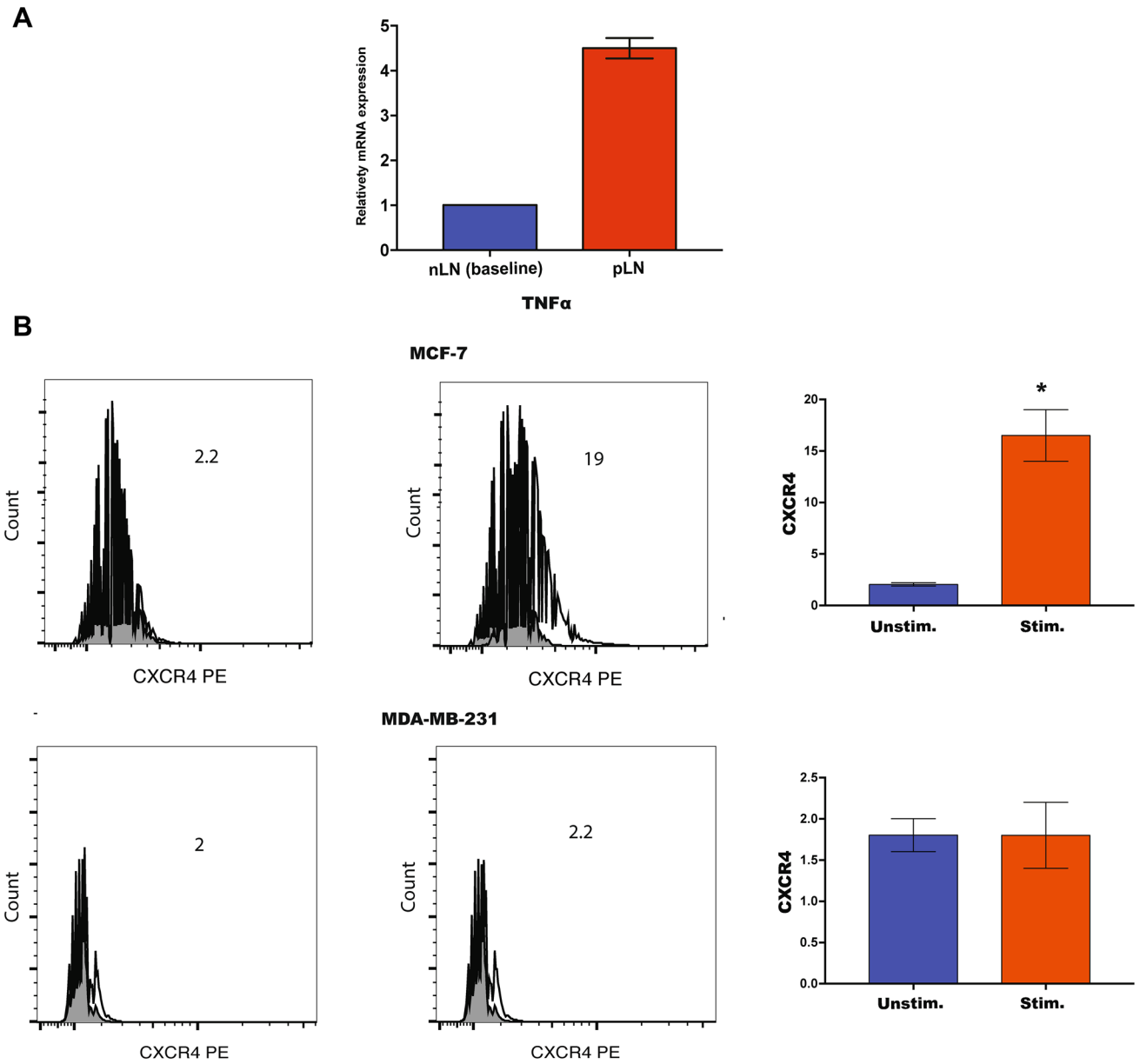

Fig. 5 **a** Higher TNFα transcript level in PDCs isolated from of axillary blood of pLN patients as compared to nLN. Relative TNFα mRNA expression was determined by qPCR. Data shown are a single experiment representative of three independent experiments. Data are expressed as mean ± SEM. *Significant *P* value ($P < 0.01$), as determined by Student's *t* test. **b** Overlaid histograms for TNFα stimulated and unstimulated MCF-7 and MDA-MB-231 cell lines. (Dashed peak represents isotype, tinted peak represents unstimulated cells, and white peak represents stimulated cells.) Data are expressed as mean ± SEM. *Significant *P* value

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Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest regarding the publication of this article.

Ethical approval All procedures performed in the study were in accordance with the ethical standards of Ain shams University research ethics committee, the Egyptian national research committee and with the 1964 Helsinki Declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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