Induction of T Regulatory Subsets from Naïve CD4+ T Cells after Exposure to Breast Cancer Adipose Derived Stem Cells

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ABSTRACT

Background: Adipose derived stem cells (ASCs) provoke the accumulation and expansion of regulatory T cells, leading to the modulation of immune responses in tumor microenvironment. **Objective:** To assess the effect of tumoral ASCs on the trend of regulatory T cells differentiation. **Methods:** Peripheral blood naïve CD4+ T cells were co-cultured with ASCs derived from breast cancer or normal breast tissues. In separate cultures peripheral blood naïve CD4+ T cells were exposed to the culture supernatants of ASCs. **Results:** Generation of CD4+CD25+Foxp3+ and CD4+CD25-Foxp3+ Treg subsets was observed after coculture of naïve CD4+ T cell with either ASCs or the related supernatant. The percentage of CD4+CD25+Foxp3+ cells increased after exposing naïve CD4+ T cells to both ASCs and their supernatants while augmentation of CD4+CD25-Foxp3+ subset mostly depended on the presence of ASCs. Similarly, upregulation of FoxP3 molecule was more significant in condition of cell to cell contact. IL-4 and IL-10 were up-regulated in the cocultured naïve CD4+ T cells after exposure to ASCs/supernatant while IFN-γ was down-regulated in the presence of ASCs. **Conclusion:** Accordingly, ASC may act as one of the major players in tumor site with immunomodulatory effects, which may mostly be carried out through direct cell-cell interaction.

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Keywords: Adipose Derived Stem Cell (ASC), Breast Cancer, Naïve CD4+ T Cell, Regulatory T Cell ,Tumor Microenvironment

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INTRODUCTION

Cancer is considered a complex disease with heterogenous multicellular interactions and a high mortality rate. The tumor microenvironment consists of various cell types, including different kinds of immune cells, pericytes, fibroblasts, mesenchymal stem cells (MSCs), endothelial cells, and carcinoma-associated fibroblasts (CAFs) (1). CAFs are generally present at the site of inflammation and have the ability to produce proteases and mediate the remodeling of the extracellular matrix through which tumor cell invasion will be launched in different types of cancers (1,2). CAFs differentiate from stromal fibroblast, bone marrow derived MSCs (3) or adipose derived stem cells (ASCs) (4). MSCs can protect breast cancer cells from host immune responses by inducing regulatory T and tolerogenic dendritic cells and decreasing the activity of cytotoxic T and natural killer (NK) cells (5-7). The immune suppressive effects of MSCs have been shown in the *in vivo* models where they prevented tumor cell rejection by the immune system, in allogenic mice mediated by regulatory T cells, and promoted tumor cell growth (8). Batten et al. proposed that MSCs can produce anti-inflammatory, but not proinflammatory, cytokines such as IL-1α, IFN-γ, and TNF-α which inhibit the proliferation and responses of primary and activated T cells (9).

MSCs have strong immunomodulatory effects on the innate and acquired immune responses through various mechanisms including direct cell to cell contact and/or the production of a number of soluble factors such as indolamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and transforming growth factor-β1 (TGF-β1) (5). MSCs also act at early stages of T cell activation by down regulating granzyme B and CD25 expression (10), or may exert modulatory functions by inducing Notch signaling in T lymphocytes (11). Some reports have also demonstrated the ability of MSCs to generate regulatory T-cells after activation of T-cells (12). On the other hand, it has been implicated that MSCs do not express MHC class II, and costimulatory molecules such as CD80, CD86, CD40, and CD40 ligand, which contributes to their immunomodulatory functions (13).

In our previous report (7), we demonstrated that distinct subtypes of Tregs are induced from peripheral blood lymphocytes in the presence of the supernatant of breast cancer adipose derived stem cells (ASCs), a mesenchymal derived stem cell. Here, we further investigate the immunosuppressive effects of human breast cancer ASCs on naïve CD4+ T cells compared to ASCs from normal subjects. As the differentiation of CD4+ T cells depends on the expression of various receptors, such as toll like receptors (TLRs), (14,15), expression of TLR4 was also assessed in naïve CD4+ T cells both before and after coculturing with ASCs. In addition, the effects of ASCs on the plasticity of naïve CD4+ T cells in a co-culture system were compared to that of ASCs supernatant. Results of this study may contribute to a better understanding of the current mechanisms of MSC-T cell cross talk in tumor microenvironment.

MATERIALS AND METHODS

Isolation and Characterization of Adipose Derived Stem Cells. The adipose tissues of fifteen breast cancer patients who had never received any therapeutic interventions, such as chemotherapy radiotherapy and surgery and whose diseases were confirmed by histological tests, were obtained by a surgeon in Shahid Faghihi Hospital, Shiraz University of Medical Sciences (Shiraz, Iran) and referred to the Stem Cell and Cancer

Biology laboratory, Shiraz Institute for Cancer Research, Shiraz-Iran. The mean and median ages of patients were 49.2 and 49 ± 13, respectively. There were 4 patients with pathological stage I, 6 with pathological stage II, and 5 with pathological stage III (Table 1). The data of breast cancer ASCs were compared to the ASCs isolated from 5 normal women, with no evidence of malignancy or autoimmune diseases, undergoing cosmetic mammoplasty surgery, from the same region. The mean and median ages of normal individuals were 33.1 and 32 ± 6, respectively. All patients and healthy individuals filled out an informed consent to take part in this study.

ASCs were extracted as previously explained (7,16-18). Briefly, fragments of adipose tissues were washed with PBS buffer, minced and digested using 0.2% collagenase type I (GIBCO, USA), and then the stromal vascular fraction (SVF) was separated using Ficoll gradient (Biosera, UK). Afterwards, the separated cells were resuspended in a DMEM medium (GIBCO, USA) containing 10% fetal bovine serum (GIBCO, USA) and penicillin/streptomycin (Biosera, UK). Adherent cells were harvested at the third passage and were subjected to immunophenotyping by flow cytometry (7,16-18). The cells were differentiated into adipocytes and used for coculturing experiments.

ASCs were stained separately with combinations of phycoerythrin (PE)-conjugated mouse anti-human CD80, CD86, CD44, CD105, CD90, CD73, CD29, and CD166 and also fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD14, CD34, and CD45 (BD Biosciences, USA). Isotype-matched irrelevant monoclonal antibodies (BD-Pharmingen, USA) were used to rule out the non specific staining of the cells. Flow cytometric analysis was performed on a FACS Calibur machine (BD Biosciences, USA) and Flow Jo software was used for the graphical presentation of the data.

Differentiation of ASCs to Adipocytes. To further characterize the isolated ASCs, cells were forced to differentiate into chondrocytes (16), osteoblasts (19), and adipocytes. For adipogenic differentiation, 1×10^5 passage 3 ASCs were cultured in 24 well -culture plates and used for differentiation when cultures were 60-80% confluent. ASCs were differentiated into adipocytes using an adipogenesis differentiation kit (STEMPRO Chondrogenesis Differentiation Kit, GIBCO, USA) and then stained with 0.2% Oil Red O (Merck, Germany) within 2-3 weeks.

Isolation of Naïve CD4+ T Cell by Magnetic Cell Sorter. Peripheral blood was obtained from a healthy donor and gently added to the same volume of Ficoll-Paque for density gradient separation of PBMCs. Then, isolated PBMCs were cultured for 2 hrs in order to exclude monocytes from the mononuclear cells. At the end of the incubation time, peripheral blood lymphocytes (PBLs) were collected and employed for isolating naïve CD4+ T cells, using a magnetic cell sorter. For magnetic bead purification, a naïve CD4+ T cell isolation kit was used according to the manufacturer's protocol (Miltenyi Biotec, Mönchengladbach, Germany). Briefly, all non-naïve CD4+ T cells were depleted over a magnetic cell separator (MACS) LD column (Miltenyi Biotech) after incubation with a cocktail of biotin conjugated mAbs against CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD36, CD34, CD56, CD123, TCR-γ/δ, HLA-DR, and CD235a. Purity of the cell subset was routinely tested using flowcytometry for the expression of CD4 and CD45RA.

Culture of Naïve CD4+ T Cells with ASCs. Naïve CD4+ T cells were cultured with ASCs from healthy individuals or patients at different stages of breast cancer at a ratio of 5 to 1, or were cultured with the culture supernatants of ASCs for 5 days. The control group consisted of T cells cultured for 5 days in RPMI culture medium (Biosera, UK) containing 10% FBS (untreated PBLs) without ASC/supernatant. Both the test and

control culture groups were supplemented with 5 ng/ml phytohemagglutinin (PHA) (GIBCO, USA).

Flowcytometric Analysis of T Cells. T cells from different experiments were washed with PBS and stained with FITC-conjugated mouse anti-human CD25, APC-conjugated mouse anti-human CD127, and Percp-conjugated mouse anti-human CD4. For intracellular staining, 400 µl of 1% cell fix was added and incubated for 5 minutes at 4°C and washed with 1 ml ice cold PBS. 500 µl of 0.2% saponin was added, incubated for 10 minutes and centrifuged. Then, cells were stained with 5 µl PE-conjugated mouse anti-human Foxp3 (BD Biosciences) antibody. After 30 minutes of incubation on ice, the cells were washed twice with PBS. Cells were also stained with isotype-matched irrelevant monoclonal antibodies (BD Pharmingen, USA) as the negative controls. Approximately 20,000 events were collected and further analyzed with the use of Flow Jo software.

Western Blot. Expression of TLR4 protein was assessed in treated and untreated T lymphocytes by western blotting. Proteins were extracted using RIPA buffer, PMSF (Fluka, USA), and protease inhibitor Coktail (Sigma, USA). The protein concentration was determined through the Bradford method, then 30 µg of protein was run on SDS-PAGE gel and blotted on PVDF membrane, which was then blocked in 5% non-fat skim milk overnight at 4°C. The blots were incubated with mouse anti-TLR4 or mouse anti-β-actin antibodies (Abcam, Cambridge, MA). Afterwards, the blots were incubated for 2 hours with horseradish peroxidase-conjugated anti-mouse secondary antibody (Abcam, Cambridge, MA). Finally, blots were washed and protein bands were observed via enzyme-linked chemiluminescence using the Super Signal West Pico chemiluminescence Kit (Pierce).

Quantitative Real-Time PCR (qRT-PCR). The abundance of IL-0, IL-4, TGF-β, IL-13, and IFN-γ gene transcripts was determined by quantitative real-time PCR (qRT-PCR), using a Bio-Rad system (Chromo 4 Real-time PCR Detector, Bio-Rad, Foster City, CA, USA) with SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). Expression of β-actin housekeeping gene was used as a reference for the level of target gene expression. Each PCR reaction was performed in a final volume of 25 µL and contained 0.5 µg of the cDNA product, 10 pmol of each primer, and 1× reaction mixture of SYBR green I. Primers were designed by the primer 3 open source software (Sourceforge, USA). Thermal cycling for all the genes was initiated with a denaturation step at 95°C for 10 min, followed by 40 cycles (denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 60°C for 60 s). The qRT-PCR amplification products were analyzed by melting curve analysis.

Statistical Analysis. The percentage of different T cell subsets and the level of IL-0, IL-4, TGF-β, IL-13, and IFN-γ gene transcripts in treated CD4+ T cells were compared to the corresponding values from the control samples and between different conditions using nonparametric Mann-Whitney U test and Kruskal-Wallis H tests, respectively, by SPSS software version 15. The relative amounts of gene transcripts were determined using the $2^{-\Delta\Delta Ct}$ formula. All graphs were plotted and evaluated by means of Prism 5 software (Inc; San Diego CA, USA, 2003). $P < 0.05$ was regarded as significant in all statistical analyses.

RESULTS

ASCs Showed Stem Cell Characteristics Phenotypically and Morphologically. Adipose derived stem cells were observed with spindle-shaped appearances in the culture. Flowcytometry analysis revealed that the cultured ASCs were approximately 98% positive for the expression of several proposed stem cell markers, such as CD44, CD105, CD90, CD73, and CD166, while they showed no significant expressions of hematopoetic specific markers such as CD14, CD34, and CD45. 30% of ASCs were positive for the expression of CD29, while they were negative for the expression of HLA-DR and costimulatory molecules such as CD80 and CD86. Figure 1 demonstrates the typical staining profile of the cells.

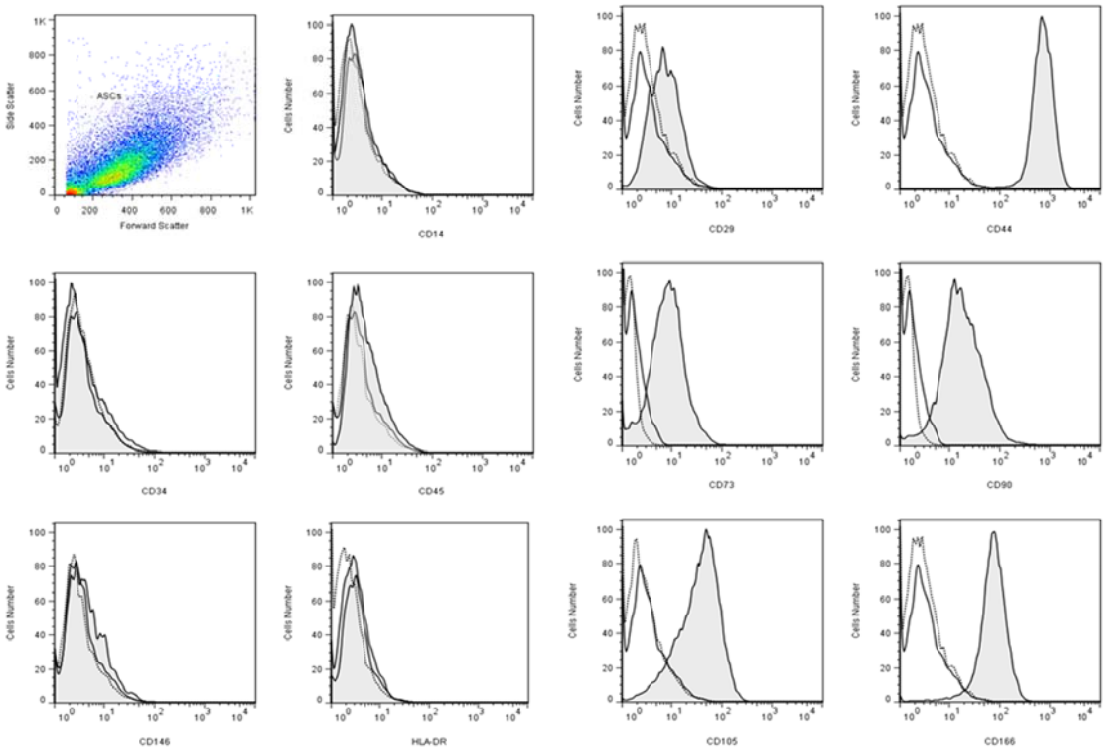

Figure 1. The schematic representation of flowcytometric analysis for the expressions of CD29, CD44, CD73, CD90, CD105, and CD166 on ASCs. ASCs were extracted from the adipose tissue of breast cancer patients and normal subjects and phenotypically assessed for the expression of stem cell specific markers. Filled histograms represent the tests (labeled ASCs), unfilled ones with a solid line are the unlabeled ASCs, and histograms with dashed lines are the isotype control.

ASCs were then further characterized by forced differentiation into adipocytes, chondrocytes, and osteoblasts. Data of differentiation into chondrocytes and osteoblasts has been published before (16,19). The adipogenic differentiation of the growing cells resulted in the formation of lipid vacuoles in ASCs (Figure 2).

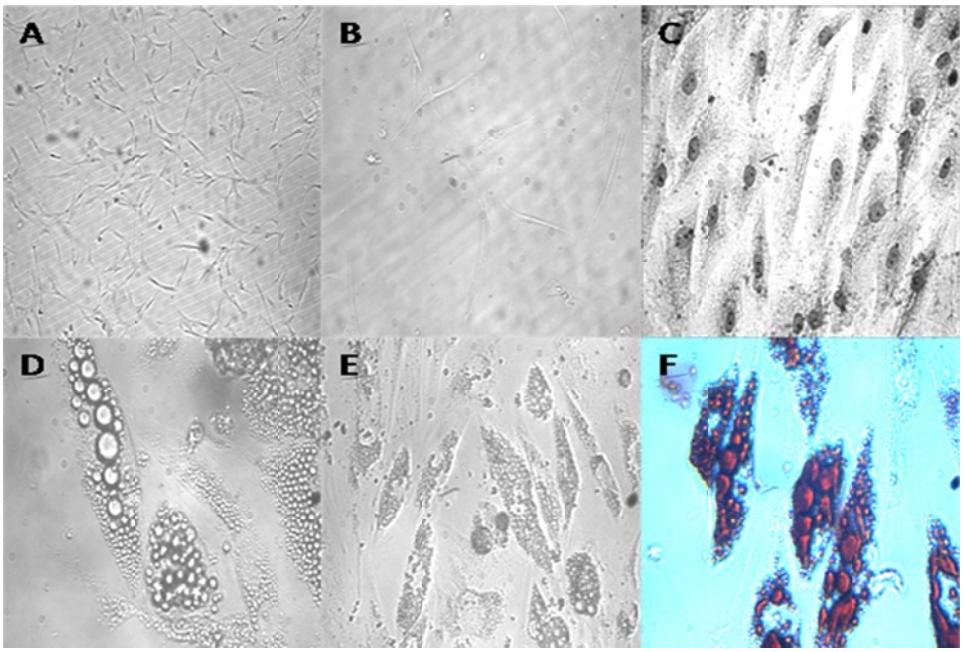

Figure 2. Differentiation of ASCs to adipocytes. A and B, ASCs before differentiation. C. control/untreated ASCs. D-F. Differentiated cells with lipid vacuoles.

Purity of Isolated Naïve CD4+ T Cells. The purity of naïve CD4+ T cells was checked based on the expression of CD4 and CD45RA after isolating them by magnetic cell sorter using flowcytometry. Results showed a purity of 93 ± 3.1 % for CD4+CD45RA+ cells.

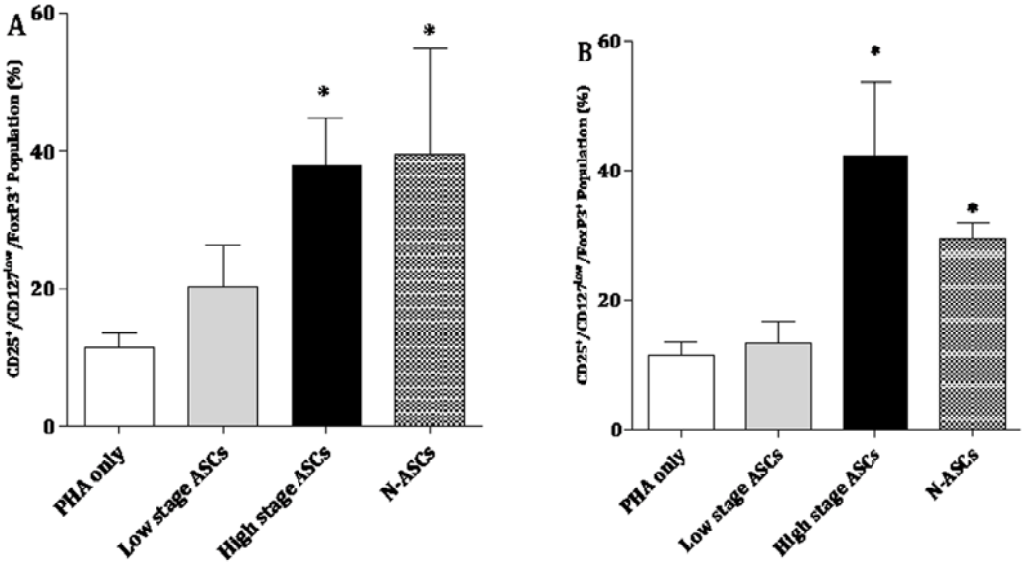

Figure 3. Expansion of $CD4^{+}CD25^{+}CD127^{Low}FoxP3^{+}$phenotype in naïve CD4+ T cell population after exposure to A. ASCs and B. ASCs cultured supernatants. Data is shown as mean ± SEM of cell percentages. *shows p< 0.05 compared to T cells which were cultured without ASC/supernatant but with PHA.

Induction of T Regulatory Cells from Naïve CD4+ T Cells Treated with Breast Cancer or Normal ASCs or the Culture Supernatants of these Cells. Naïve CD4+ T cells were separately co-cultured with different stages of breast cancer and normal ASCs and their supernatants in order to determine if there is a difference between the effects of normal and the different stages of cancer ASCs. Then, the expression of CD4, CD25, FoxP3, and CD127 were assessed 5 days post culture. Results are presented based on the different subsets of T regulatory cells including $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ and $CD4^{+}CD25^{-}CD127^{low}Foxp3^{+}$. Expression of FoxP3 in the presence and absence of ASCs was also compared.

$CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ Lymphocytes: The percentage of $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ as a subset of T regulatory cells was assessed in different conditions. All data was presented as mean ± SEM. Based on flowcytometry analyses, 11.5 ± 2.2% of naïve T lymphocytes showed this phenotype in the absence of ASCs, while 20.2 ± 6.1% and 37.9 ± 7% of cells co-cultured with low pathological stages (stages I and II) and also with the high stage (stage III) of breast cancer ASCs showed this phenotype. Co-culturing with normal ASCs produced a shift of 39.6 ± 15.3 %of naïve CD4+ T cells into $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ cells (Figure 3A). These differences were statistically significant for stage III and normal ASCs (P= 0.003 and p=0.04, respectively). The expansion of $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ cells was also observed when the supernatants of ASCs were used. The supernatant of the low and high stages of cancer ASCs caused up regulations of $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ phenotype up to 13.4 ± 3.3% and 42.3 ± 11.3%, respectively (p=0.022 for stage III ASCs). The percentage of these cells reached a value of 29.4 ± 2.4% in the presence of normal ASCs culture supernatant (p=0.009) (Figure 3B).

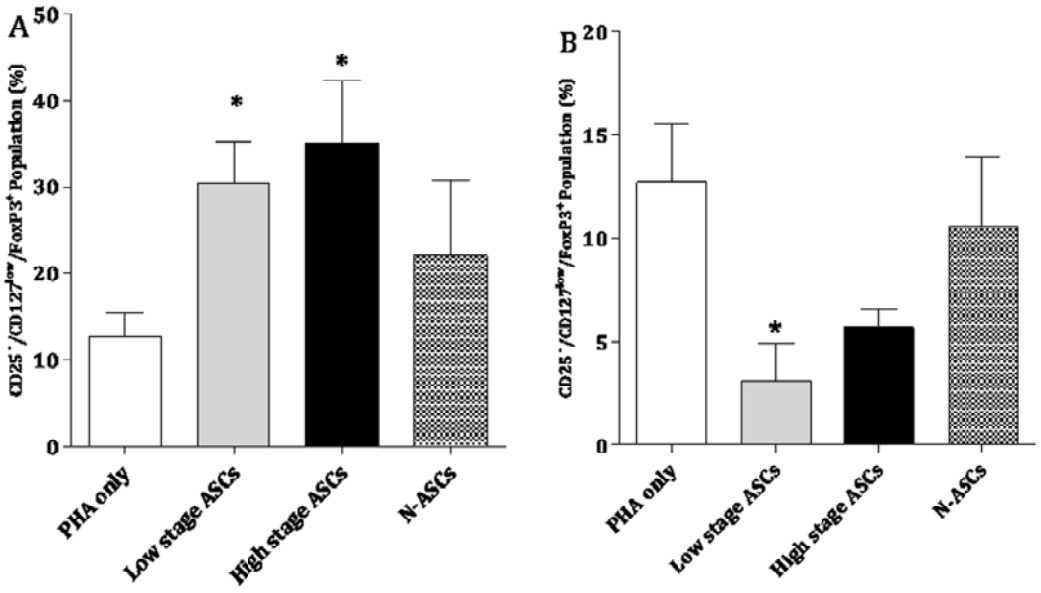

Figure 4. Flowcytometric analysis of CD4+ T cell population. The percentage of $CD25^{-}$ $CD127^{Low}FoxP3^{+}$ phenotype after culturing of naïve CD4+ T cell population with A. breast cancer and normal ASCs and B. ASCs culture supernatants. Data is shown as mean ± SEM of cell percentages. *represents p< 0.05 compared to T cells which were cultured without ASC/supernatant but with PHA.

CD4+CD25-CD127LowFoxP3+ Phenotype: Co-culture of naïve CD4+ T cells with breast cancer ASCs resulted in a significant increase in the percentage of $CD25^{-}D127^{Low}FoxP3^{+}$ subset. As shown in Figure 4, presence of low and high stage cancer ASCs induced this subset to reach values of 30.5% ± 4.8 and 35.1% ± 7.3 (p= 0.006 and p=0.044), respectively, while the percentage of this subset in the absence of ASCs was only 13% ± 2.8. $CD25^{-}CD127^{Low}FoxP3^{+}$ subset reached a level of 22.1% ± 8.7, which is in the numerical vicinity of normal ASCs (p> 0.05) (Figure 4A).

There was no significant change when the cells were cultured with the supernatant of either high stage breast cancer or normal ASCs (p> 0.05, Figure 4B). However, a significant decrease was observed in the percentage of this subset when the culture supernatant of low stage ASCs was used (p< 0.05, Figure 4B).

Induction of FoxP3 Expressing CD4+ **T Cells in the Presence of ASCs.** FoxP3 expressing T cells were assessed in different groups, in the presence of ASCs and their culture supernatants, and when the CD4+ cells were cultured with PHA. Presence of all types of ASCs, low (50.7 ± 7%), high (73 ± 13.1%), and normal (76.5 ± 11%), and the supernatants of the high (48 ± 11.2%) and normal (40 ± 5.5%) ASCs, resulted in an upregulation in the frequency of FoxP3 expressing CD4+ T cells, compared with PHA treated ones (24.2 ± 4.6).

These differences were statistically significant for various ASCs but not for their culture supernatants (p<0.01). Comparison of ASCs with their corresponding supernatants showed that existence of ASCs had a more apparent effect on increasing the frequency of FoxP3 expressing CD4+ T cells compared to its culture supernatants. This upregulation was statistically significant for the low stage ASCs and their supernatant (50.7 ± 7 vs. 16.5 ± 4.3%) (p=0.004, Figure 5).

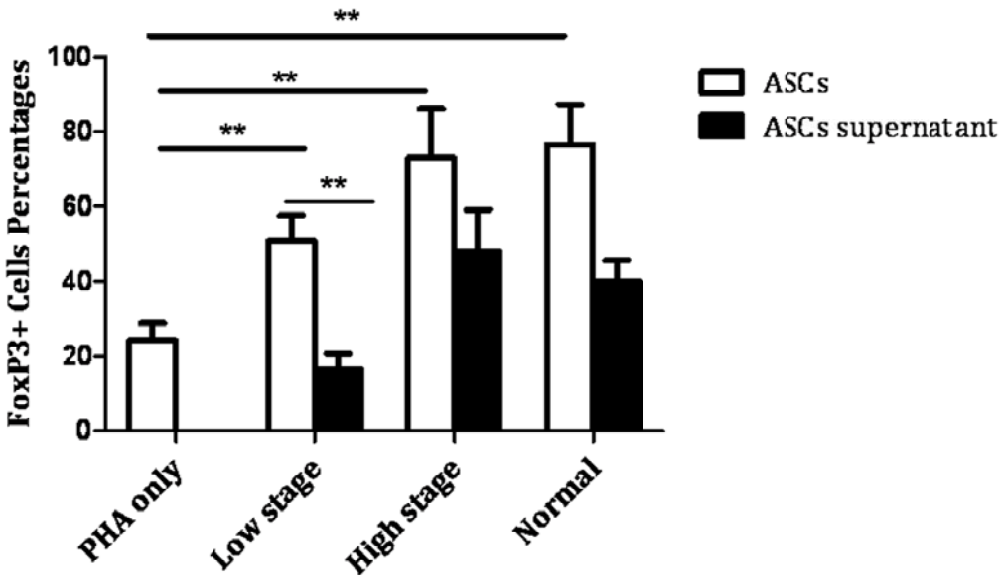

Figure 5. Flowcytometric analysis of CD4+Foxp3+ T cell population after culturing of naïve CD4+ T cell population with either breast cancer and normal ASCs or ASCs culture supernatant. Data is shown as mean ± SEM of cell percentages. **shows p<0.01.

Expression of TLR4 Protein in Naïve T Lymphocytes. Expression of TLR4 was assessed in naïve CD4+ T cells before and after the co-culture with breast cancer and normal ASCs by the western blotting method. As depicted in Figure 6, a 1.18 and 1.32-fold higher expressions of TLR4 were observed in T cells co-cultured, respectively, with either normal or stage II ASCs compared with the PHA treated naïve T cells. However, a 50% decrease in the TLR4 expression was observed when T cells were co-cultured with stage III ASCs (Figure 6).

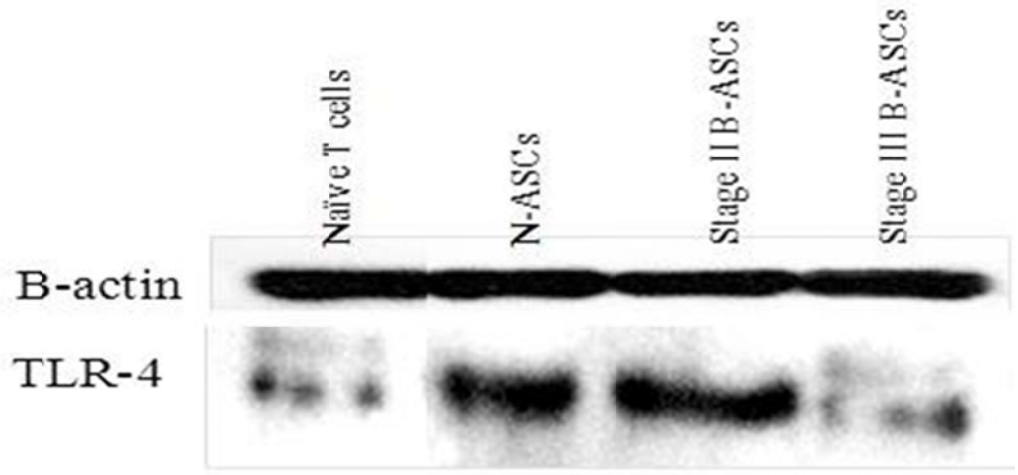

Figure 6. Expression of β-actin and TLR4 in CD4+ T cells in the presence or absence of breast cancer and normal ASCs.

MRNA Expressions of IL-10, IL-4, IL-13, TGF-β1 and IFN-γ in Naïve CD4+ T Cells before and after Co-Culturing with ASCs. To show the immunomodulatory effects of ASCs on T cells, Expression of anti-inflammatory cytokines such as IL-10 ، IL-4 ،IL-13, and TGF-β1, and inflammatory cytokine IFN-γ were assessed in both treated and untreated T cells and the results are shown in Figures 7 and 8. Although the differences in the expression of mentioned cytokines were not statistically significant, we observed an upregulation in anti-inflammatory cytokines after exposing naïve CD4+ T cells to either ASCs or their culture supernatants.

Compared to PHA treated T cells, expression of IL-10 transcript in T cells increased after exposure to either stage II ASCs (5.2-fold) or its culture supernatant (5.7-fold). In T cells exposed to stage III ASCs or its supernatant, expression of IL-10 transcript was either 60- or 380- fold higher than PHA treated T cells, respectively. Normal ASCs and their culture supernatants led to a 126- or a 9- fold increase, respectively, in the expression of IL-10 in T cells compared to the PHA treated ones (Figure 7A).

Expression of IL-4 transcript in T cells increased after culturing with either ASCs or its supernatants. As depicted in Figure 7B, in T cells cultured with stage II ASCs or its culture supernatant, the level of IL-4 transcripts increased 1.4- and 13.6-fold, respectively, compared to PHA treated T cells. Also, IL-4 transcript had a 5.3- or a 22.4-fold, respectively, higher expression in T cells exposed to either stage III ASCs or their culture supernatant compared to PHA treated T cells. Normal ASCs and their

supernatant showed respective values of 9.3- and 14.8-fold higher expression of IL-4 in naïve T cells compared to PHA treated ones.
Expression of IL-13 transcript in T cells showed 30% decrease after exposing the T cells to both stages II and III ASCs. However, an 18.7-fold increase in the IL-13 transcript expression was observed after exposure to normal ASCs. In contrast, the supernatant of both stages II and III caused 29.4- and 2.6-fold higher expression of IL-13 mRNA, respectively (Figure 7C).

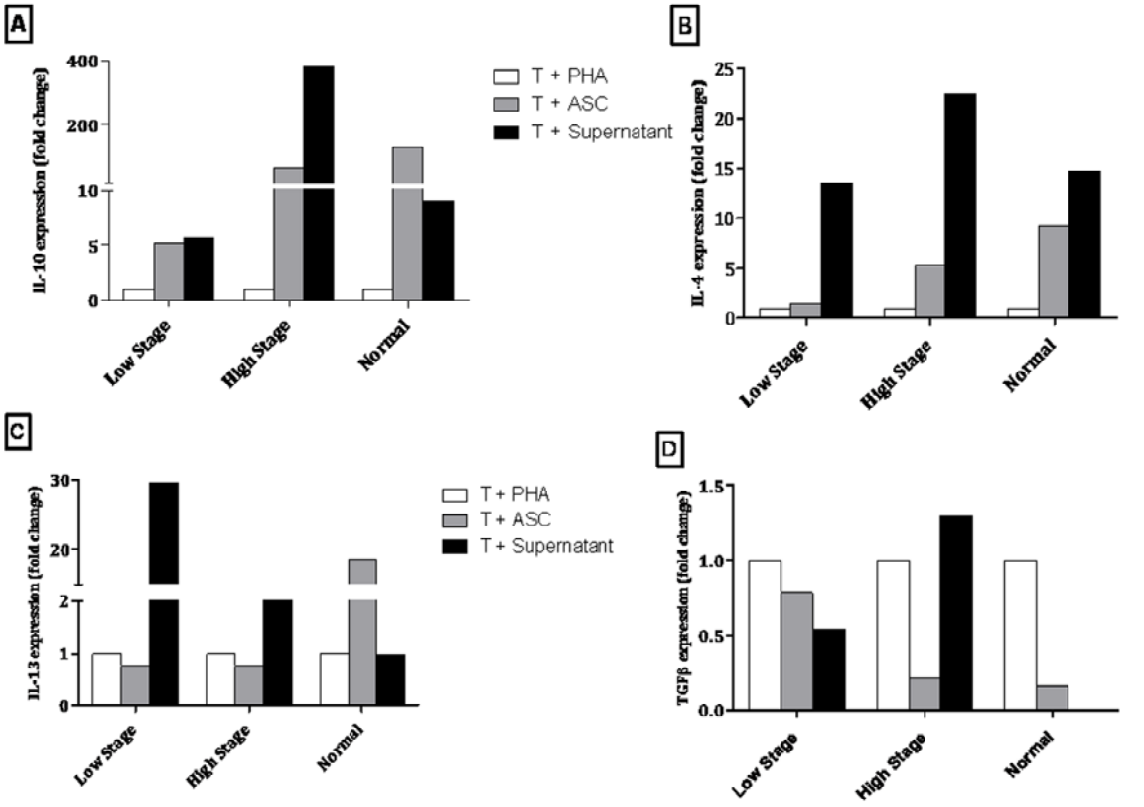

Figure 7. Results of qRT-PCR for IL-10 (A), IL-4 (B), IL-13 (C) and TGF-β1 (D) gene transcripts in naïve CD4+ T cells co-cultured with ASCs or its supernatant. The data is shown as the fold change of gene expression compared to naïve CD4+ T cells cultured with PHA but without ASCs. P>0.05 for all cytokines.

TGF-β1 mRNA expression showed 30%, 80% and 80% decrease in T cells cultured with stage II, stage III and normal ASCs, respectively. However, when T cells were cultured with the supernatant of ASCs, upregulation of TGF-β1 was only observed when stage III ASC culture supernatant was used. This upregulation was approximately 1.3-fold higher than PHA treated T cells (Figure 7D).
Data of qRT-PCR for expression of IFN-γ transcript in T cells showed 80%, 70% and 90% decrease after exposing the T cells to stage II, stage III and normal ASCs, respectively. However, when we cultured T cells with ASCs supernatant, a decrease in the expression of IFN-γ transcript was only seen with normal ASCs culture supernatant

(80% decrease), 3.8- or 99-fold, respectively, while increase in its expression was observed ($p<0.05$) when the T cells were exposed to culture supernatants of either stage II or stage III ASCs (Figure 8).

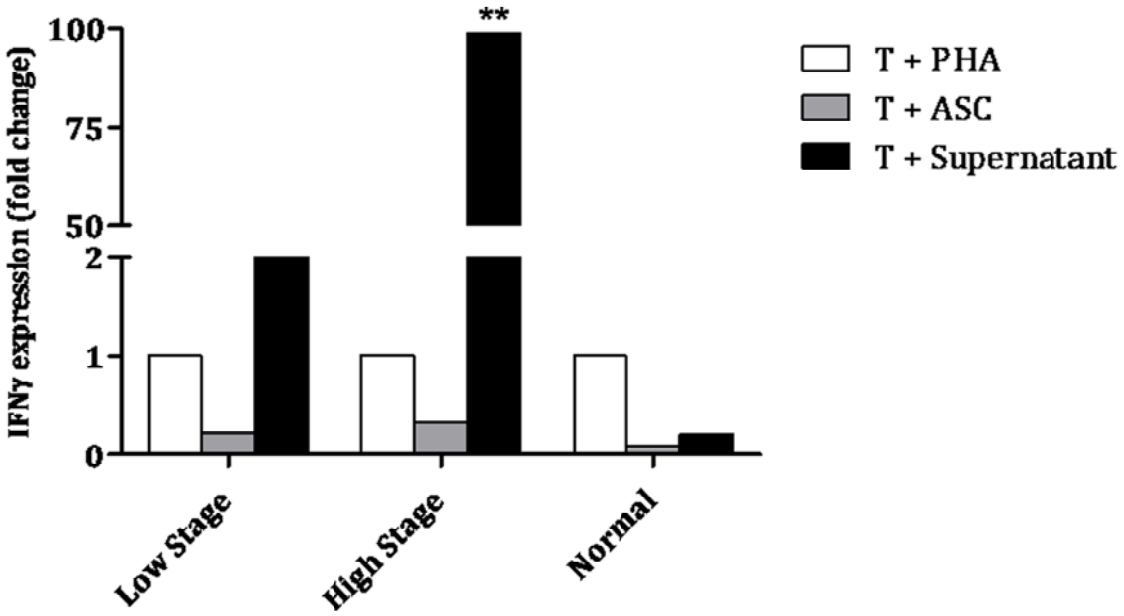

Figure 8. IFN-γ gene transcript in naïve CD4+ T cells which were co-cultured with or without ASCs and ASCs supernatant. The data are shown as the fold change of IFN-γ gene expression compared to PHA treated cells. **represents $p<0.01$.

DISCUSSION

Several evidences have supported the hypothesis that tumors evade immunological rejection by the creation of an immunosuppressive microenvironment leading to tumor cell growth and invasion (20-23).

There have been a variety of studies describing the presence of MSCs in the vicinity of tumors and their ability to promote tumor progression (4,24). Karnoub *et al*. reported the tumor promoting ability of MSCs through secreting RANTES and augmenting the metastatic potential of tumor cells (24). SDF-1, TGF-β, VEGF, IGF-1, and IL-8 which are acknowledged for their tumor promoting effects are also produced by MSCs (24,17). Moreover, MSCs are introduced as important regulators of the epithelial mesenchymal transition (25), a crucial mechanism for tumor cell metastasis.

The relationship between MSCs and immune cells is currently a controversial issue. MSCs have been shown to promote tumor progression through immune modulation. These cells inhibit the maturation of monocytes into DCs (26) and impair their antigen presenting function (27). MSCs inhibit the proliferation, IFN-γ production, and cytotoxic activity of NK cells by downregulating the expression of activating receptors (28,29). Although little is known about the effect of MSCs on B- cells, a number of studies suggest that MSCs have the ability to modulate B-cell proliferation and function

(30). In the case of T cells, shifting from Th1 to Th2 and the generation and proliferation of Tregs in the presence of MSCs has been reported (31,32). In pathologic situations such as cancer, inducible (i)Tregs are dominant subsets of Tregs, which develop in response to distinctive microenvironmental stimuli and regulate various immunological responses. Present knowledge about the origin of human iTregs and their suppressing mechanisms are limited and the extent to which the local microenvironment regulates Treg activity is of considerable prevailing interest (33). In this regard, we had previously demonstrated that the supernatant of breast cancer ASCs, a kind of MSC, has the ability to induce Treg phenotype from peripheral blood lymphocytes (7).

Despite these reports, the relationship between MSC and naïve CD4+ T cells is not well established. Therefore, in the current study, we examined whether the presence of ASCs from breast cancer patients and normal subjects affect naïve CD4+ T cells and if there is a difference between the impact of direct cell to cell contact and the supernatant of ASCs on naïve CD4+ T cells differentiation.

Based on the results of this study, presence of ASCs or its supernatant, from both cancerous and normal individuals, had crucial roles in changing T cell subsets and inducing distinct types of Tregs. Accordingly, we observed both $CD4^{+}CD25^{+}CD127^{Low}Foxp3^{+}$ and $CD4^{+}CD25^{-}CD127^{Low}Foxp3^{+}$ Treg subsets after coculturing of naïve CD4+ T cells with either ASCs or the culture supernatant. $CD4^{+}CD25^{+}CD127^{Low}Foxp3^{+}$ phenotype increased after exposure to both ASCs and their culture supernatant, while augmentation of CD4+CD25-Foxp3+ subset seems mostly dependant on the presence of ASCs and cell to cell contact compared to the supernatant alone. This result was also observed for the expression of FoxP3 molecule which was upregulated in the presence of both ASCs and supernatants but was more significant when naïve CD4+ T cells were co-cultured in a cell to cell contact condition. Similarly, in a study by Frazier *et al*. ASCs were demonstrated to stimulate the proliferation of naive CD4(+) T cells and to augment the percentage of CD25(+) T cells. Induction of functional iTreg with the ability of upregulating FoxP3 and TGF-β expression was observed in direct contact with ASCs but under a low O2 condition [34]. Numerous studies have shown the suppressive effect of CD4+CD25+Foxp3+ subset but the significance of CD4+CD25-Foxp3+ T cells, particularly in tumor biogenesis, is currently unclear. It was shown that $CD25^{-}$ T cells are a subset of Tregs induced by tumor in mice and are characterized with augmented expression of IL-10 and Foxp3 and suppressive functions (35). Yang *et al.* and his colleagues found that a proportion of intratumoral CD4+ T cells were CD4+CD25-Foxp3+ Treg with the ability of suppressing CD8+ T cell proliferation. They reported that the existence of this subtype depends on T cell-B cell contact and expression of CD70 molecule on lymphoma B cells was necessary for Foxp3 upregulation in this subset (36). Thus, based on these reports and the results of our study, the importance of the existence of ASCs in the tumor site and cell to cell communication would be more provoking of the upregulation FoxP3 and Treg cells induction, especially CD25- Tregs. As CD70 is also expressed by ASCs (37,38), this molecule may also play key roles in ASCs-T cell communication and induction of distinct subtypes of Tregs. However, further studies are undoubtedly needed to clarify the exact molecular mechanisms.

Development, activation, and expansion of different T cell subsets including Tregs may be associated with distinct signalling pathways such as TLRs. TLRs are known to be

important in determining the differentiation of CD4+ T cells to Th1, Th2, Th17, and Treg cells (14,15).

Herein, assessment of the expression of TLR4 in naïve CD4+ T cells showed a higher expression of this receptor after co-culturing with normal and stage II ASCs, while stage III ASCs caused a significant reduction in TLR4 expression in T cells. Reynolds *et al*. reported that the TLR4 signaling pathway increased the proliferation and survival of naïve T cells. They found that TLR4−/− CD4+ T cells had a remarkable decrease in Th1 and Th17 cytokines. Thus, based on our results, it seems that stage III ASCs reduce proliferation and survival of T cells, and consequently reduced the proinflammatory responses in tumor microenvironment.

We have demonstrated that the pattern of the cytokine profile of T cells was modified after exposing naïve CD4+ T cells to ASCs/supernatant. Among the anti-inflammatory cytokines studied, IL-4, IL-10, TGF-β and IL-13, IL-4, and IL-10 upregulated in co-cultured T cells while TGF-β upregulated only in the presence of stage III ASCs culture supernatant. Thus, it is concluded that besides IL-10 producing Tregs (inducible (i)Treg), a subset of IL-4 producing Th2 cells may also be induced in the presence of ASCs or ASCs supernatant. Whiteside *et al*. showed that the plasticity of iTregs, expanding in response to cytokines, including TGF-β or IL-10, is controlled by the tumor microenvironment. These cells are likely to be responsible for tumor escape as a result of the suppression of anti-tumor immune responses (33). As an inflammatory cytokine, we assessed IFN-γ and, as expected, downregulation of this cytokine was observed in all conditions except in the presence of cancer ASC's supernatants. Based on our results, it seems that the effect of ASCs for decreasing inflammatory cytokines mostly depends on the presence of ASCs and cell to cell contact compared to secretory factors of the culture supernatant alone. Correspondingly, it has been reported that ASCs are proficient to enhance TGF-β but reduce IFN-γ and the Th1 related transcription factor, T-bet, in T cells (39). Also, suppression in proliferation of CD4(+) and CD8(+) activated T cells, induction of T regulatory cells with CD4(+)CD25(+)CTLA-4(+) phenotype, and an increased production of IL-10, PGE2 and also IFN-γ, under the influence of bone marrow MSCs, has been previously reported (40).

From the data presented here, it can be concluded that ASCs may act as major immunomodulatory players in the tumor microenvironment and their activity largely depends on direct cell to cell contact. In this scenario, the interactions of ASCs, tumor cells and Tregs seem more significant since these players create a vicious triangle resulting in tumor cell survival and dissemination.

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