

# Human Adipose-Derived Stromal/Stem Cells Induce Functional CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>-</sup> Regulatory T Cells Under Low Oxygen Culture Conditions

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Human adipose tissue stromal/stem cells (ASCs) are known to induce proliferation of resting T cells under ambient (21%) O<sub>2</sub> conditions; however, ASCs exist physiologically under lower oxygen (5% O<sub>2</sub>) conditions in adipose tissue. The effects of low oxygen levels on ASC immunomodulation of T cells are unknown. In this study, we show that ASCs stimulated proliferation of naive CD4<sup>+</sup> T cells and the percentage of CD25<sup>+</sup> T cells was significantly increased under both low and ambient O<sub>2</sub>. Forkhead box P3 (FoxP3) and transforming growth factor beta (TGF-β) mRNA expression were significantly increased when ASCs were cocultured with CD4<sup>+</sup> T cells under low compared with ambient O<sub>2</sub> conditions. The low O<sub>2</sub>-induced regulatory T cells (iT<sub>regs</sub>) exhibited functionality when added to mixed lymphocyte reactions as demonstrated by inhibition of peripheral blood mononuclear cell proliferation, and by >300-fold increase in FoxP3 mRNA, and >2-fold increase in TGF-β mRNA expression. These results demonstrate that under physiologically relevant low O<sub>2</sub> conditions, direct contact of human ASCs with naive CD4<sup>+</sup> T cells induced functional iT<sub>regs</sub>.

## Introduction

**D**UE TO THEIR INHERENT MULTIPOTENCY, relative ease of isolation from the stromal vascular fraction (SVF) of adipose tissue, and the ability to enhance vascularization, adipose tissue stromal/stem cells (ASCs) represent a favorable cell-based therapy tool for tissue engineering, regenerative medicine, and reconstructive surgery [1,2]. In a murine xenograft model using polymerized poly (ethylene) glycol-diacrylate for breast reconstruction, ASCs were reported to regenerate functional, highly vascularized adipose tissue following transplantation [3,4]. In addition, ASCs possess important immunoregulatory effects, including immunosuppression of a number of immune cells under varying conditions [5,6]. Under ambient (21%) O<sub>2</sub> conditions in vitro, ASCs inhibited proliferation of alloactivated peripheral blood mononuclear cells (PBMCs) and CD4<sup>+</sup> T cells, and stimulated proliferation of resting CD4<sup>+</sup> T cells [7,8]. T-cell proliferation continued after extended culture in the absence of ASCs [6,7,9].

Whereas they represent a small fraction of all CD4<sup>+</sup> T cells (~10%), naive T cells have the potential to differentiate into multiple functional phenotypes, including induced regulatory T cells (iT<sub>regs</sub>), T helper type I (T<sub>h1</sub>), T<sub>h2</sub>, or T<sub>h17</sub> cells [10]. Chen et al. demonstrated that naive, peripheral

CD4<sup>+</sup>CD45RA<sup>+</sup> T cells were converted into anergic/suppressor iT<sub>regs</sub>, CD25<sup>+</sup> and CD45RB<sup>-low</sup>, through costimulation of T-cell receptors (TCRs) and treatment with transforming growth factor β (TGF-β) [11]. These iT<sub>regs</sub> were not only refractory to further TCR stimulation, but they also inhibited normal T-cell proliferation in vitro when placed in mixed lymphocyte reactions (MLRs) [11].

ASC activation or suppression of one or more specific T-cell subsets may significantly affect the observed overall growth inhibition or stimulation of T cells [5–7,9,12–17]. Several studies have demonstrated that ASCs inhibit cells of both the innate and adaptive immune response through secretion of soluble factors such as prostaglandin E<sub>2</sub>, TGF-β1, hepatocyte growth factor, and production of factors that include inducible nitric oxide synthase and indoleamine 2,3-dioxygenase [7,9,12,13,20–22]. Another potential mechanism of immune interference involves ASC-mediated induction of iT<sub>regs</sub> from naive CD4<sup>+</sup> T cells by direct contact. Crop et al. demonstrated that ASCs had the potential to activate resting immune cells when directly cocultured with PBMCs [7]. The activated population was CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>-</sup>FoxP3<sup>+</sup> and possessed suppressive capacity [7]. Moreover, ASCs were demonstrated to recruit iT<sub>regs</sub> to both the lymphoid organs and adipose grafts in vivo [7,15,23]. In breast, colorectal, pancreatic, and other cancers, T<sub>reg</sub>

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activation suppresses the host immune response to tumor-specific antigens through downregulation of CD8<sup>+</sup> cytotoxic T cells [21–24]. T<sub>reg</sub> cell-mediated immune suppression is one of the more crucial tumor immune evasion mechanisms and the main obstacle to successful tumor immunotherapy [13,24–29]. Taken together, these data suggest that, when placed in direct contact with naive CD4<sup>+</sup> T cells, ASCs may induce and recruit iT<sub>regs</sub> that have the potential to suppress the cytotoxic T-cell response within adipose tissue grafts, thereby contributing to breast cancer recurrence [18,19].

Kronsteiner et al. [15] demonstrated that the stimulation method and the cellular environment may alter CD4<sup>+</sup> T-cell cytokine secretions, create an inflammatory milieu, and effectively alter ASC effects on specific cells of the immune system. Low O<sub>2</sub> content was demonstrated to significantly alter the ASC transcriptome and the secretion of pro- and anti-inflammatory cytokines that are involved in T-cell activation and suppression [8,30–33,42,45]. However, ASC immunomodulatory effects on specific subpopulations of CD4<sup>+</sup> T cells under low O<sub>2</sub> conditions are unknown.

Although most studies of ASC immune suppression have been conducted under ambient (21%) O<sub>2</sub> conditions [7,9,15,16,34–38], physiological O<sub>2</sub> levels are ~5% in intact adipose tissue and in fat grafts used for breast and other tissue reconstruction [39,40]. We hypothesized that under physiological low oxygen, ASCs would stimulate induced T<sub>regs</sub> (iT<sub>regs</sub>) from naive CD4<sup>+</sup> T cells derived from breast cancer patients. The objective of the present study was therefore to investigate the ASC immunomodulatory behavior on naive CD4<sup>+</sup> T cells under physiological conditions similar to those present in adipose tissue or in newly transplanted allogeneic fat grafts. In a newly transplanted ASC-supplemented fat graft, ASCs may contribute to the suppression of the cell-mediated immune response to tumor cells by inducing a T<sub>reg</sub> phenotype from naive CD4<sup>+</sup> T cells.

## Materials and Methods

### Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Norcross, GA) unless otherwise specified.

### Isolation, collection, and culture of human ASC

The procedures for isolation, collection, and culture of ASCs were previously described [41]. ASCs were obtained from the subcutaneous abdominal adipose tissue region of five healthy female, Caucasian patients between ages 28–61 years with a mean ± standard deviation (SD) of 42 ± 12.9 years. The patients displayed a mean body mass index (kg/m<sup>2</sup>) (±SD) of 26.4 ± 5.7. All tissues were acquired from elective procedures in local plastic surgery offices as approved by the Pennington Biomedical Research Center Institution Review Board (No. 23040), with patients' informed consent. Briefly, liposuction tissues were transported to Pennington Biomedical in a saline solution within 2 h postsurgery. The tissue was washed with phosphate-buffered saline and digested with 0.1% collagenase type I (Worthington Biochemicals, Brunswick NJ), 1% bovine serum albumin, and 2 mM CaCl<sub>2</sub> for 60 min at 37°C with intermittent shaking. The floating adipocytes were separated

from the SVF by centrifugation (300 g) for 5 min at room temperature. The supernatant containing mature adipocytes was aspirated and discarded and the remaining pellet was identified as the SVF. The SVF cells were suspended and plated immediately in T175 flasks in the stromal medium [DMEM/F-12 Ham's, 10% fetal bovine serum (FBS; Hyclone, Logan, UT, www.hyclone.com), 100 U penicillin/100 g streptomycin/0.25 g fungizone] at a density of 0.156 mL of tissue digest/cm<sup>2</sup> of surface area for expansion and culture. This initial passage of the primary cell culture was referred to as passage 0 (P0). ASCs were used between passages 2 and 3 for experiments (table in Supplementary Fig. S1a; Supplementary Data are available online at www.liebertpub.com/scd).

### Low O<sub>2</sub> cell culture

In vitro low O<sub>2</sub> experiments were performed with a Single Set point Oxygen & CO<sub>2</sub> controller inserted in a standard incubator (Biospherix C-Chamber Hypoxia Chamber; Biospherix, Lacona, NY, www.biospherix.com) that continuously infused a calibrated gas mixture (95% N<sub>2</sub>, 5% CO<sub>2</sub>). Experiments were performed at oxygen concentrations of 21% (ambient O<sub>2</sub>) or 5% (low O<sub>2</sub>).

### ASC exposure to inflammatory conditions under low O<sub>2</sub>

To observe ASC behavior under the influence of an inflammatory milieu, cells were kept in the stromal medium supplemented with 400 U/mL recombinant human interferon-gamma (IFN-γ) (R&D Systems, Minneapolis, MN) under low O<sub>2</sub> (5% O<sub>2</sub>) or ambient O<sub>2</sub> (21% O<sub>2</sub>) culture conditions using a Single Set point O<sub>2</sub> and CO<sub>2</sub> controller as described under the Low O<sub>2</sub> Cell Culture section. After 5 days of culture, untreated or IFN-γ-treated cells were subjected to flow cytometry analysis, used for direct or indirect coculture experiments, or the cytokine secretion profile was determined by applying conditioned supernatants onto the Human Proteome Profiler Array Kit, panel A (R&D Systems).

### Western immunoblot

ASCs were cultured 3 days under 5% O<sub>2</sub> or 21% O<sub>2</sub>. The total protein was quantified using a Biorad protein assay (Life Science Research, Hercules, CA). Fifty micrograms of protein was loaded onto a 15% denaturing acrylamide gel, separated by electrophoresis, transferred to nitrocellulose membrane, and probed sequentially with primary murine monoclonal antibodies corresponding to amino acids 432–528 of human hypoxia-inducible factor-1 alpha (HIF-1α; Abcam HIF1A67) and human GAPDH (ab8245). Images were scanned using a LI-COR Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

### Flow cytometry for ASC expression of immune-stimulatory markers on ASCs

ASCs were treated with 400 U/mL IFN-γ under low O<sub>2</sub> or ambient O<sub>2</sub> culture, as described under the ASC Exposure to Inflammatory Conditions Under Low O<sub>2</sub> section. Adherent cells were removed from culture flasks by incubation with

0.25% trypsin-EDTA at 37°C for 5 min, washed two times with the stromal medium, and resuspended in a cell staining buffer (Biolegend, San Diego, CA) at a final concentration of  $1 \times 10^6$  cells/mL. Cell suspensions were incubated with antibodies against CD86-phycoerythrin (PE), CD80-fluorescein isothiocyanate (FITC), CD40-PE, human leukocyte antigen A,B,C-related (HLA-A,B,C)-allophycocyanin (APC), or human leukocyte antigen D-related (HLA-DR)-FITC (BD Biosciences, San Jose, CA) at room temperature for 30 min, protected from light. After two washes with a cell staining buffer (Biolegend), flow cytometric analysis was performed using a BD Biosciences fluorescence-activated cell sorter (FACS) Beckman-Coulter Galios flow cytometer with two lasers and eight detectors running the Galios acquisition software (BD Biosciences).

### Isolation of PBMCs

PBMCs ( $n=60$ ) were obtained through the Louisiana Cancer Research Consortium (LCRC) Biospecimen Core. Peripheral blood samples were collected from patients with breast cancer (Supplementary Fig. S2). PBMCs were isolated from the collected heparinized peripheral blood by density gradient centrifugation using Ficoll Isopaque ( $d=1.077$ ; Amersham Biosciences; Upsala, Sweden) and frozen at  $-150^\circ\text{C}$  until use. The demographic characteristics of the patients are also presented in Supplementary Table S1.

### Isolation of naive $\text{CD4}^+$ T cells from PBMCs by QuadroMACs

Naive  $\text{CD4}^+$  T cells were isolated from PBMCs (Miltenyi Biotech, Auburn, CA) by negative selection using QuadroMACs (Miltenyi Biotech). PBMCs were thawed and plated in a complete medium [RPMI 1640, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% (v/v) nonessential amino acids (all media supplements obtained from Gibco, Gaithersburg, MD)]. To check for purity, both fractions (negatively selected naive  $\text{CD4}^+$  T cells and the flow-through fraction) were stained with CD4-PE and CD45RA-FITC (Biolegend). Flow cytometric analysis was performed on a FACS Beckman-Coulter Galios flow cytometer.

### Direct coculture of ASCs with naive $\text{CD4}^+$ T cells under low $\text{O}_2$

For direct coculture, ASCs were seeded in the stromal medium in 6-well plates at concentrations ranging from 1,000 cells/well to 100,000 cells/well. Following 24 h of attachment, ASCs were treated with 400 U/mL human recombinant IFN- $\gamma$  (Sigma-Aldrich) for 5 days, as described above under the ASC Exposure to Inflammatory Conditions Under Low  $\text{O}_2$  section. ASCs were treated with 50  $\mu\text{g}/\text{mL}$  mitomycin c (Sigma-Aldrich) for 1 h to inhibit proliferation at mitosis. Isolated naive  $\text{CD4}^+$  T cells were then plated at 100,000 cells per well with the ASC monolayers, yielding ratios of 1:100 (1,000 ASC:100,000 T cells), 1:10 (10,000 ASC:100,000 T cells), and 1:1 (100,000 ASC:100,000 T cells). ASC-naive T-cell cocultures were cultured under low  $\text{O}_2$  and ambient  $\text{O}_2$  culture conditions

for 3 days. Naive T-cell proliferation was assessed using the bromodeoxyuridine (BrdU) assay.

### Flow cytometry for regulatory T-cell immunophenotype

Naive  $\text{CD4}^+$  T cells were cocultured with IFN- $\gamma$ -treated ASCs under low  $\text{O}_2$  or ambient  $\text{O}_2$  culture as described under the Direct Coculture of ASCs with naive  $\text{CD4}^+$  T Cells Under Low  $\text{O}_2$  section. T cells were then collected and cell suspensions were incubated with antibodies against CD4-FITC, CD127-PE, and CD25-PE for 30 min. Cells were fixed and permeabilized using the cell fixation/permeabilization buffer (Biolegend) and resuspended with antibodies against Forkhead box P3 (FoxP3)-Alexa Fluor 647 at 4°C. Flow cytometric analysis was performed using a Beckman-Coulter Galios flow cytometer (BD Biosciences).

### Functional analysis of T cells cultured previously with ASCs

Naive T cells were cocultured with IFN- $\gamma$ -treated ASCs for 3 days under low  $\text{O}_2$  or ambient  $\text{O}_2$  culture, as described under the Direct Coculture of ASCs with naive  $\text{CD4}^+$  T Cells Under Low  $\text{O}_2$  section. T cells were collected and added into mixed lymphocyte reactions (MLRs) at a ratio of 1:10 with 2  $\mu\text{M}$  PKH-26-labeled PBMCs (Sigma-Aldrich) for 7 days. PBMC proliferation was assessed by the BrdU assay. PBMC suspensions from functional assays were incubated with antibodies against CD4-FITC, CD127-PE, and CD25-PE for 30 min, and CXCR3-APC-cyanin 7 (Cy7) for 30 min. Flow cytometric analysis was performed using a BD FACSARIA.

### Quantitative real time polymerase chain reaction (qRT-PCR) for TGF- $\beta$ and FoxP3 gene expression

Total RNA was purified from human naive  $\text{CD4}^+$  T cells that were cocultured with ASCs under low  $\text{O}_2$  and ambient  $\text{O}_2$  conditions for 3 days using the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Inc., Valencia, CA; www.qiagen.com) according to the manufacturer's specifications. One-step reverse transcriptase-polymerase chain reactions were performed with  $\sim 500$  ng of total RNA using the iScript One-Step RT-PCR Kit for probes (Bio-Rad Laboratories, Hercules, CA). The complete reaction mix was incubated with the oligonucleotide primer and probe sets specific for human TGF- $\beta$  (forward: 5'-TCGCCTACCAG CTCATGCATAACA-3'; reverse: 5'-TGAAGCTCTCCA GGTGTCAACGA-3'; probe: 5'-/56-FAM/TCAGGGACATT GACGTGATCATGGG/3BHQ\_1/3') and FoxP3 (forward: 5'-GGT TCA CAC GCA TGT TTG CCT TCT-3'; reverse: 5'-GCA CAA AGC ACT TGT GCA GAC TCA-3'; probe: 5'-/56-FAM/AGA AAC CAT/ZEN/CCT GCC ACC TGG AAG AA/3BHQ\_1/3') in a real-time thermal detection system (Bio-Rad Laboratories). All results were normalized relative to GAPDH expression control.

### Statistical analyses

Statistical analysis of data was performed using Graphpad Prism v5.0 software; the level of  $P$  value was set at 0.05. The statistical analyses performed were as follows: two-way analysis of variances (two-way ANOVAs), followed by



Bonferroni's post-tests were used for CyQUANT and BrdU proliferation experiments, differentiation, and array experiments. Linear regression analyses were performed on T-cell proliferation following direct coculture, and the determination coefficients ( $R^2$  values) were given to evaluate the relationship between the ASC:naive T-cell coculture ratio and T-cell proliferation. The coefficient of determination was such that  $0 \leq R^2 \leq 1$ , and denoted the strength of the association between the ASC:naive T-cell coculture ratio and proliferation, where 1 represents the strongest correlation. Results were reported as a positive or negative correlation. A positive correlation reflected an increase in growth as the number of ASCs plated increased. A negative correlation reflected a decrease in growth as the number of ASCs plated increased.

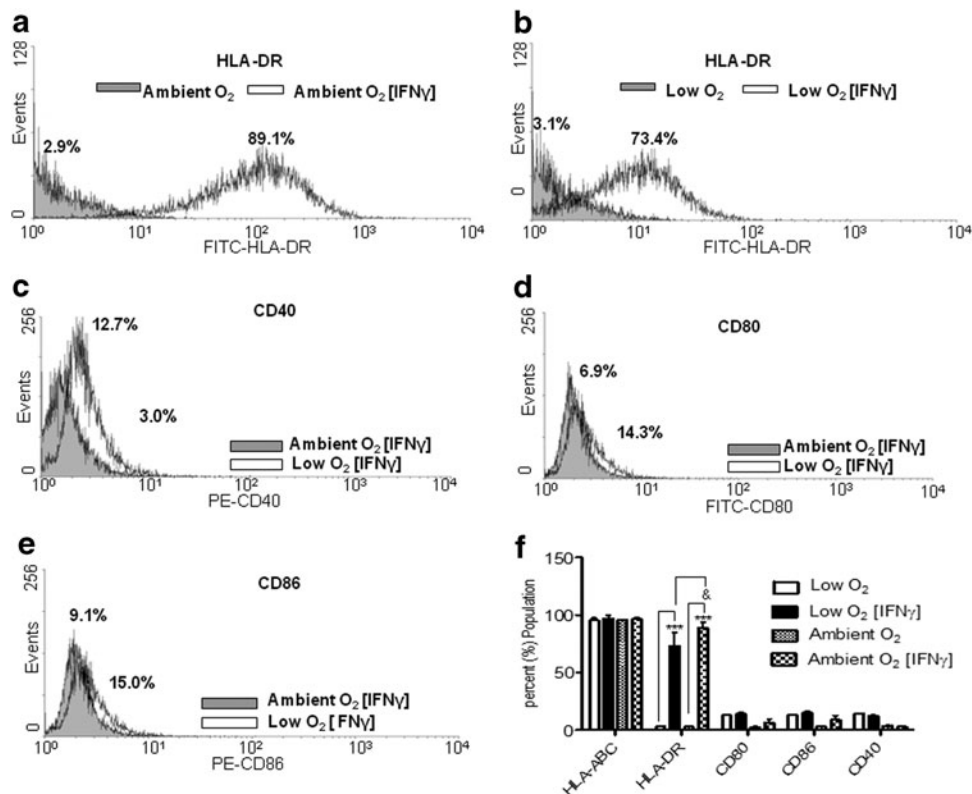
## Results

### Effect of low O<sub>2</sub> on ASC and naive CD4<sup>+</sup> T-cell secretomes

ASCs isolated from five healthy female, Caucasian donors (Supplementary Fig. S1a) were cultured under ambient O<sub>2</sub> (21%) or low O<sub>2</sub> (5%) conditions. Naive CD4<sup>+</sup> T cells were isolated from the peripheral blood of five donors who

were diagnosed with breast cancer (Supplementary Table S1) and cultured 1, 3, and 5 days in low O<sub>2</sub> or ambient O<sub>2</sub> culture. Low O<sub>2</sub> culture did not affect viability or proliferation of ASCs or naive T cells (data not shown, Supplementary Fig. S1b, c). The percent purity for naive T-cell isolation using the MACs separation procedure averaged 92.5% (Supplementary Fig. S1d, e). To demonstrate that low O<sub>2</sub> exposure stimulates hypoxia-induced pathways in ASCs, ASCs from five different donors were exposed to low O<sub>2</sub> for 3 days. The protein was extracted and probed for HIF-1 $\alpha$  by western immunoblot (Supplementary Fig. S1f).

We previously demonstrated that exposure to low physiological O<sub>2</sub> levels for 3 days altered ASC secretion of extracellular matrix remodeling proteins and type I cytokines that are involved in tissue fibrosis [41]. To determine the effects of low O<sub>2</sub> on the secretome of naive CD4<sup>+</sup> T cells alone, the conditioned medium from naive CD4<sup>+</sup> T cells cultured in ambient O<sub>2</sub> or low O<sub>2</sub> was used to probe a human Proteome Profiler array that contained 36 different cytokines, chemokines, and acute-phase proteins important in T-cell growth, differentiation, gene expression, migration, immunity, and inflammation. All 36 proteins present on the array were detected in the secretomes of naive CD4<sup>+</sup> T cells cultured in both ambient O<sub>2</sub> and low O<sub>2</sub> (Supplementary Figs. S2



**FIG. 1.** Expression of mixed lymphocyte reaction class I/II and costimulatory molecules on adipose tissue stromal/stem cells (ASCs) following IFN- $\gamma$  treatment. Human leukocyte antigen D-related (HLA-DR) expression on the surface of ASCs following interferon-gamma (IFN- $\gamma$ ) treatment under (a) ambient (21% O<sub>2</sub>) conditions and (b) low (5% O<sub>2</sub>) culture conditions for 5 days. (c, d, and e) Expression of costimulatory molecules CD40, CD80, and CD86, respectively, on ASCs under ambient and low O<sub>2</sub> culture conditions. (f) Quantification of flow analyses following ASC culture under low or ambient O<sub>2</sub> culture conditions. Three independent sets of experiments were performed. Data are reported as mean ( $\mu$ )  $\pm$  standard error (SE). \*\*\* $P < 0.0001$  comparing HLA-DR expression in an unstimulated ASC to IFN- $\gamma$ -stimulated ASC, &  $P < 0.05$  comparing HLA-DR expression in ASCs under low O<sub>2</sub> to ambient O<sub>2</sub>.

and S3). Thirteen proteins were not significantly altered when comparing culture in ambient  $O_2$  to culture in low  $O_2$ , including interleukin (IL)-4 and IL-5, which are involved in  $T_{H2}$  differentiation, and IL-12p70, which is involved in  $T_{H1}$  differentiation [24]. Seven proteins were significantly decreased under low  $O_2$  culture, including the cytokines IL-6 ( $P < 0.05$ ) and IL-23 ( $P < 0.001$ ) that stimulate  $T_{H17}$  differentiation, while inhibiting  $T_{reg}$  cell differentiation/expansion [24,43]. Other protein secretions that were significantly decreased under low  $O_2$  culture included soluble intercellular adhesion molecule-1 ( $P < 0.001$ ), IL-8 ( $P < 0.001$ ), monocyte chemoattractant protein-1 (MCP-1,  $P < 0.001$ ), and macrophage inhibitory protein-1 $\alpha$  and  $\beta$  (MIP-1- $\alpha$ ,  $P < 0.01$ ; MIP-1 $\beta$ ,  $P < 0.01$ ). Sixteen proteins were significantly increased under low  $O_2$  culture, including IL-10 ( $P < 0.001$ ) that is involved in  $T_{reg}$  differentiation [44] (Supplementary Figs. S2 and S3). These data suggested that culture of naive T cells in low  $O_2$  may favor a  $T_{reg}$  secretory profile.

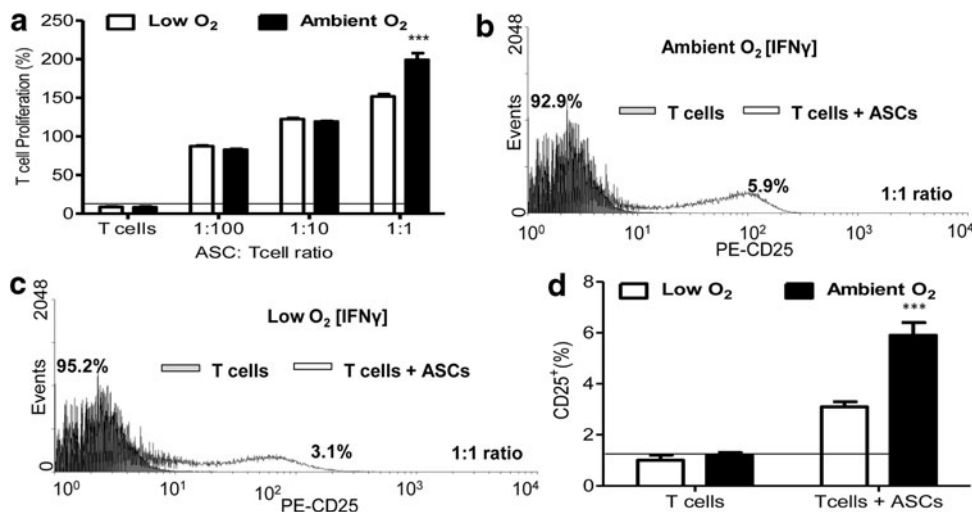
#### Low $O_2$ treatment reduced expression of HLA-DR on IFN- $\gamma$ -stimulated ASCs

Following culture of ASCs in ambient or low  $O_2$  for 5 days concurrent with IFN- $\gamma$  exposure, flow cytometry was performed to measure surface expression of mixed lymphocyte reaction class II receptor HLA-DR, the MHC class I receptor HLA-A,B,C, and costimulatory molecules CD40, CD80, and CD86. As expected, significant HLA-A,B,C surface expression was detected on ASCs cultured under both ambient  $O_2$  (untreated:  $96.1\% \pm 0.1\%$ ; treated:  $96.8 \pm 3.0$ ; Fig. 1f) and low  $O_2$  (untreated:  $95.5\% \pm 4.1\%$ ; treated:  $97.1\% \pm 5.6\%$ ; Fig. 1f).

Two-way ANOVAs reflected no significant difference in HLA-A,B,C expression on IFN- $\gamma$ -treated ASCs under ambient  $O_2$  or low  $O_2$ . HLA-DR expression was significantly increased under both ambient  $O_2$  (untreated:  $3.1\% \pm 1.6\%$ ; treated:  $89.1\% \pm 10.1\%$ ;  $P < 0.001$ ; Fig. 1a) and low  $O_2$  (untreated:  $3.1\% \pm 0.8\%$ ; treated:  $73.4\% \pm 25\%$ ;  $P < 0.001$ ; Fig. 1b) following IFN- $\gamma$  treatment. Analyses of mean fluorescent intensities of ambient  $O_2$  and low  $O_2$  demonstrated that a higher percentage of IFN- $\gamma$ -stimulated ASCs expressed HLA-DR under ambient  $O_2$  culture (Fig. 1f). IFN- $\gamma$  treatment did not significantly alter surface expression of the costimulatory molecules CD40, CD80, and CD86 on ASCs cultured in ambient  $O_2$  or low  $O_2$  (Fig. 1c–f). These data indicated that low  $O_2$  culture did not significantly affect IFN- $\gamma$ -induced expression of costimulatory molecules CD40, CD80, and CD86 on ASCs; however, low  $O_2$  culture did reduce IFN- $\gamma$ -induced expression of HLA-DR.

#### ASC stimulated naive $CD4^+$ T-cell proliferation

Increasing numbers of IFN- $\gamma$ -stimulated ASCs were cocultured with naive  $CD4^+$  T cells (Fig. 2a, ASCs: T cells; 1:100, 1:10, and 1:1) for 3 days under ambient  $O_2$  or low  $O_2$ . ASCs significantly induced T-cell proliferation at all ratios (Fig. 2a). This stimulation of proliferation was cell number dependent, as T-cell proliferation increased as the ASC number increased (Fig. 2a). The highest T-cell proliferation was observed following a 1:1 ratio of coculture, and a higher percentage of T cells proliferated following coculture with ASCs under ambient  $O_2$  at this ratio [mean ( $\mu$ )  $\pm$  SD: ambient  $O_2$ ,  $199.1\% \pm 8.7\%$ ; low  $O_2$ ,  $151.7\% \pm 2.9\%$ ;



**FIG. 2.** Naive T-cell proliferation and activation following direct coculture with ASCs under low  $O_2$  and ambient  $O_2$  culture conditions. Naive  $CD4^+$  T cells were isolated from peripheral blood of breast cancer patients. ASCs were treated with IFN- $\gamma$  for 5 days, and then treated with  $50 \mu\text{g/mL}$  mitomycin c to inhibit growth. Naive  $CD4^+$  T cells were cocultured with IFN- $\gamma$ -treated ASCs at cell concentrations of 1:100 (1,000 ASCs:100,000 T cells), 1:10 (10,000 ASCs:100,000 T cells), and 1:1 (100,000 ASCs:100,000 T cells) for 3 days. T-cell proliferation was quantified through bromodeoxyuridine (BrdU) incorporation.  $CD4^+$  T-cell proliferation (%) =  $(E_{T_{cell}+ASC}/E_{T_{cell}}) \times 100$ , where  $E_{T_{cell}+ASC}$  = proliferation absorbance measurement for naive T-cell + ASC group, and  $E_{T_{cell}}$  = proliferation absorbance measurement for T-cell group. (a) Percent (%) T-cell proliferation following coculture at varying ratios. Percent (%)  $CD25^+$  T cells were evaluated following 1:1 coculture with ASCs by flow cytometry under (b) ambient and (c) low  $O_2$  conditions. (d) Quantification of flow cytometric analyses in panels b and c for  $CD25^+$  expression of T cells following coculture of ASCs with naive T cells. Three independent sets of experiments were performed for each treatment. Data are reported as mean ( $\mu$ )  $\pm$  SE. Significance: \*\*\* $P < 0.001$ .

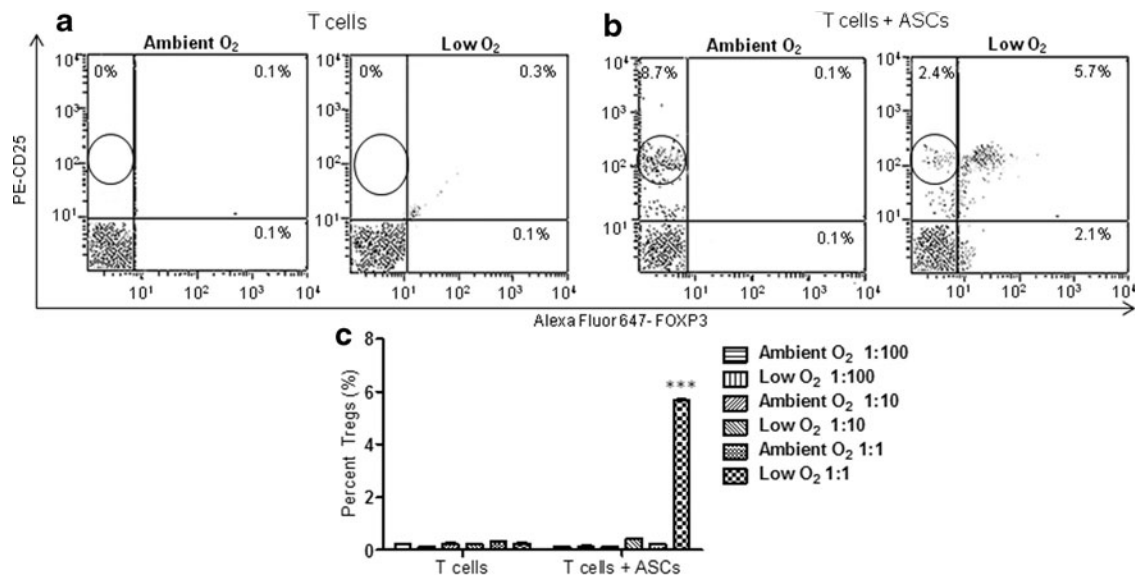
$P < 0.0001$ , Fig. 2a]. T cells were collected and flow cytometry was performed for expression of T-cell early activation marker CD25. The percentage of CD25<sup>-</sup> T cells was designated in the gray peaks and labeled. The percentage of CD25<sup>+</sup> T cells was designated in the clear peaks (Fig. 2) and labeled accordingly. Flow cytometric analyses revealed that the percentage of CD25<sup>+</sup> T cells was significantly increased ( $P < 0.001$ ) when naive CD4<sup>+</sup> T cells were cocultured with ASCs at a 1:1 ratio under both low O<sub>2</sub> and ambient O<sub>2</sub>, and CD25 expression was higher in cells under ambient O<sub>2</sub> conditions (Fig. 2b–d). In contrast to direct coculture, indirect coculture of ASCs with naive CD4<sup>+</sup> T cells in a Boyden chamber showed no significant difference in T-cell proliferation under low O<sub>2</sub> or ambient O<sub>2</sub> culture (data not shown), demonstrating that ASCs stimulated proliferation and activation of naive T cells under both ambient O<sub>2</sub> and low O<sub>2</sub>, and that these effects were cell number and cell contact dependent.

#### ASCs induced CD4<sup>+</sup> CD25<sup>high</sup> FoxP3<sup>+</sup> iT<sub>reg</sub> phenotype from naive CD4<sup>+</sup> T cells

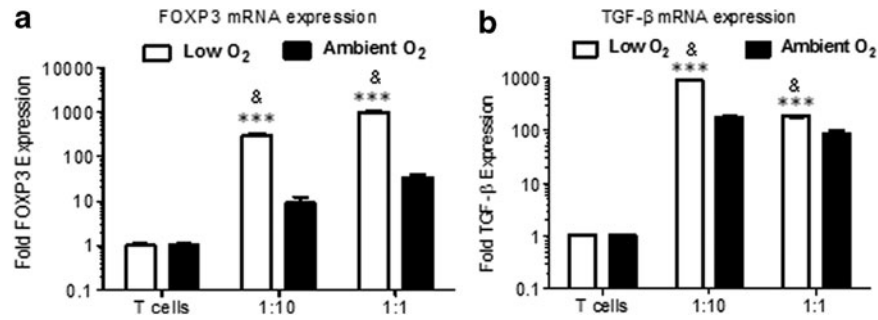
Naive CD4<sup>+</sup> T cells were cocultured with IFN- $\gamma$ -stimulated ASCs at ratios of 1:100, 1:10, and 1:1, under ambient O<sub>2</sub> and low O<sub>2</sub> culture. Flow cytometric analyses were performed for detection of CD4, CD25, and FoxP3, markers of iT<sub>regs</sub>. Consistent with the CD25 results in Fig. 2, the surface expression of CD25<sup>high</sup> on T cells was significantly increased when naive T cells were directly cocultured with ASCs at a 1:1 ratio under both ambient O<sub>2</sub> (8.7%  $\pm$  0.03%) and low O<sub>2</sub> (2.4%  $\pm$  0.07%) conditions (Fig. 3a, b). Remarkably, the percentage of cells

coexpressing CD25 and FoxP3 significantly increased following coculture with ASCs at a 1:1 ratio under low O<sub>2</sub>, but not under ambient O<sub>2</sub> (ambient O<sub>2</sub>: T alone, 0.3%  $\pm$  0.03%; T+ASC, 0.21%  $\pm$  0.03%; low O<sub>2</sub>: T alone, 0.3%  $\pm$  0.03%; T+ASC, 5.7%  $\pm$  0.07%;  $P < 0.0001$ , Fig. 3a–c).

Coexpression of TGF- $\beta$  and FoxP3 has been associated with the T<sub>reg</sub> phenotype [46]. To confirm that the proliferating population induced by ASCs was iT<sub>regs</sub>, mRNA expression of FoxP3 and TGF- $\beta$  in naive T cells was measured by qRT-PCR following coculture at ASC/T-cell ratios of 1:1 and 1:10. FoxP3 mRNA in T cells was 200-fold higher in cocultures under low O<sub>2</sub> compared with ambient O<sub>2</sub> at the 1:10 cell ratio ( $2^{\Delta\Delta Ct}$ : ambient O<sub>2</sub>, 9.0  $\pm$  3.2; low O<sub>2</sub>, 302.3  $\pm$  32.4;  $P < 0.001$ ), and 300-fold higher at the 1:1 cell ratio ( $2^{\Delta\Delta Ct}$ : ambient O<sub>2</sub>, 33.6  $\pm$  5.1; low O<sub>2</sub>, 982.3  $\pm$  99;  $P < 0.001$ ; Fig. 4a). Similarly, TGF- $\beta$  mRNA expression was more than sevenfold higher in cocultures under low O<sub>2</sub> compared with ambient O<sub>2</sub> in the 1:10 cell ratio ( $2^{\Delta\Delta Ct}$ : ambient O<sub>2</sub>, 177.3  $\pm$  7.5; low O<sub>2</sub>, 883.2  $\pm$  9.8;  $P < 0.001$ ), and twofold higher in the 1:1 cell ratio ( $2^{\Delta\Delta Ct}$ : ambient O<sub>2</sub>, 85.5  $\pm$  13.1; low O<sub>2</sub>, 186.5  $\pm$  7.5;  $P < 0.001$ ; Fig. 4b). Taken together, these data demonstrated that following direct contact for a brief period of time, ASCs induced a T<sub>reg</sub> phenotype from naive CD4<sup>+</sup> T cells. Taken together, these data demonstrated that following direct contact for a brief period of time, ASCs induced a T<sub>reg</sub> phenotype from naive CD4<sup>+</sup> T cells. These data also suggested that extended culture may have induced higher detectable percentages of iT<sub>regs</sub> by flow cytometric analyses. In addition, extended culture may have induced higher detectable percentages of iT<sub>regs</sub> by flow cytometric analyses.



**FIG. 3.** Expression of CD25 and Forkhead box P3 (FoxP3) from naive CD4<sup>+</sup> T cells following coculture with ASCs under ambient and low O<sub>2</sub> culture conditions. IFN- $\gamma$ -treated ASCs were cocultured with naive CD4<sup>+</sup> T cells for 3 days under low or ambient O<sub>2</sub> culture conditions at ratios of 1:100 (1,000 ASCs:100,000 T cells), 1:10 (10,000 ASCs:100,000 T cells), and 1:1 (100,000 ASCs:100,000 T cells). T cells were collected and stained for fluorescein isothiocyanate (FITC)-CD4, phycoerythrin (PE)-CD25, and Alexa Fluor 647-FoxP3 (AF-FoxP3) and flow cytometry was used to calculate percent T<sub>reg</sub> cells present in the gated CD4<sup>+</sup> population. Representative flow cytometric analyses of CD25- and FoxP3-positive (a) T cells alone, and (b) T cells following coculture with ASCs at a 1:1 ratio under low and ambient O<sub>2</sub> culture conditions. (c) Representative quantification of percentage of T<sub>regs</sub> following coculture of ASCs with T cells. Three independent sets of experiments were performed for each treatment. Data are reported as mean ( $\mu$ )  $\pm$  SE. \*\*\* $P < 0.001$ .

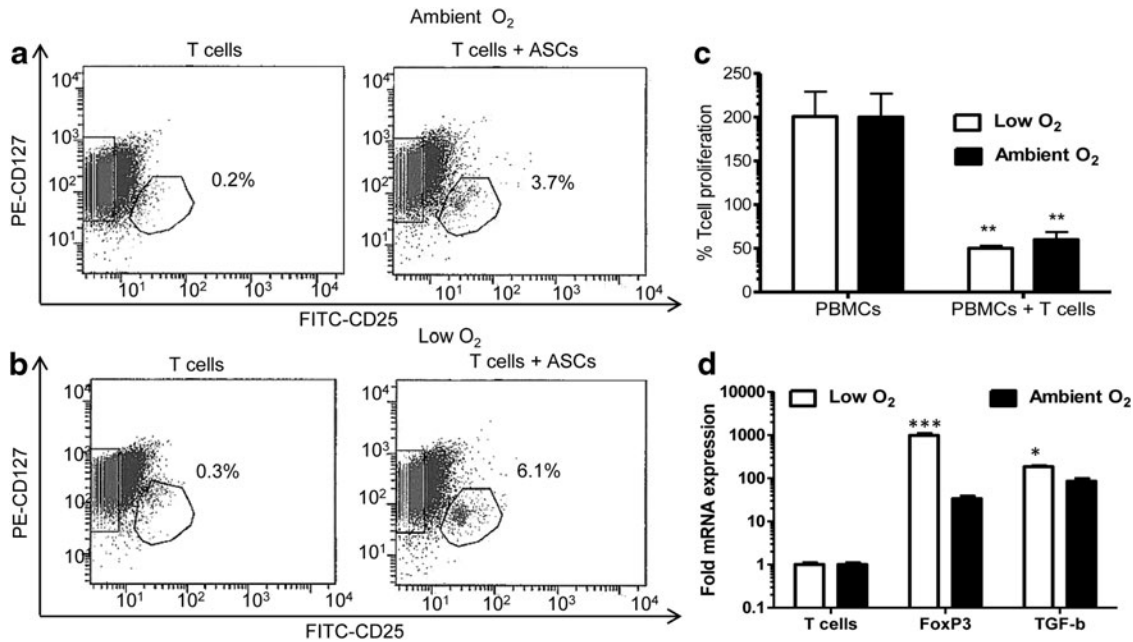


**FIG. 4.** FoxP3 and transforming growth factor beta (TGF-β) gene expression in T cells following coculture with ASCs. IFN-γ-treated ASCs were cocultured with naive CD4<sup>+</sup> T cells for 3 days under low or ambient O<sub>2</sub> culture conditions at ratios of 1:10 (10,000 ASCs:100,000 T cells) and 1:1 (100,000 ASCs:100,000 T cells). T cells were collected, RNA was isolated, and one-step quantitative real-time polymerase chain reaction (qRT-PCR) was performed following coculture of ASCs with T cells using forward and reverse primers and probes for human (a) FoxP3 and (b) TGF-β. Three independent sets of experiments were performed for each treatment. Data are reported as mean (μ) ± SE. \*\*\*P < 0.0001 comparing low O<sub>2</sub> to ambient O<sub>2</sub> culture for each group. &P < 0.001 comparing T cells alone under low O<sub>2</sub> culture to T cells + ASCs under low O<sub>2</sub> culture, and T cells alone under ambient O<sub>2</sub> culture to T cells + ASCs under ambient O<sub>2</sub> culture.

*ASCs induced CD127<sup>-/low</sup> CD4<sup>+</sup> CD25<sup>+/high</sup> FoxP3<sup>+</sup> functionally active iT<sub>regs</sub>*

To determine whether iT<sub>regs</sub> induced by ASCs were functional, and to assess the impact of low O<sub>2</sub>, naive T cells were cocultured with IFN-γ-treated ASCs under low O<sub>2</sub> or ambient O<sub>2</sub> culture at a 1:1 ratio. T cells were then collected,

sorted for the CD127<sup>-</sup> CD25<sup>+</sup> fraction (Fig. 5a, b), and added to MLRs with PBMCs at a 1:10 ratio for 7 days under low O<sub>2</sub> or ambient O<sub>2</sub> conditions. CD25<sup>+</sup> CD127<sup>-</sup> T cells that were cocultured with ASCs under low O<sub>2</sub> and ambient O<sub>2</sub> significantly inhibited PBMC proliferation by 40% and 30%, respectively (low O<sub>2</sub>: PBMCs alone, 200% ± 28.6%; PBMCs + iT<sub>regs</sub>, 50.1% ± 2.9%; ambient O<sub>2</sub>: PBMCs alone,



**FIG. 5.** Expression of human adipose tissue-specific markers on induced regulatory T cells (iT<sub>regs</sub>) following coculture with ASCs. IFN-γ-treated ASCs were cocultured with naive CD4<sup>+</sup> T cells for 3 days under low or ambient O<sub>2</sub> culture conditions at ASC:T-cell ratios of 1:10 (10,000 ASCs:100,000 T cells) and 1:1 (100,000 ASCs:100,000 T cells). T cells were collected, incubated with antibodies for FITC-CD25 and PE-CD127, and subjected to flow cytometry to sort for CD127<sup>-/low</sup> CD25<sup>+</sup> iT<sub>regs</sub> under (a) ambient and (b) low O<sub>2</sub> culture conditions. Isolated iT<sub>regs</sub> were cocultured for 7 days with allogeneic PBMCs isolated from human breast cancer patients at a 10:1 ratio (100,000 PBMCs:10,000 iT<sub>regs</sub>) under low and ambient culture conditions. (c) T-cell proliferation was quantified by BrdU analysis in parallel experiments. % T-cell proliferation = (E<sub>Tcell+ASC</sub>/E<sub>Tcell</sub>) × 100 where E<sub>Tcell+ASC</sub> = proliferation absorbance measurement for the naive T-cell + ASC group, and E<sub>Tcell</sub> = proliferation absorbance measurement for the T-cell group. (d) mRNA was isolated and one-step qRT-PCR was performed following ASC-T-cell coculture using forward and reverse primers and probes for human FoxP3 and TGF-β. Three independent sets of experiments were performed for each treatment. Data are reported as mean (μ) ± SE. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.0001.



200.1% ± 26.8%; PBMCs + T<sub>regs</sub>, 59.9% ± 8.7%;  $P < 0.01$ , Fig. 5c). FoxP3 mRNA in previously cocultured T cells was 300-fold higher following cocultures under low O<sub>2</sub> compared with ambient O<sub>2</sub> ( $2^{\Delta\Delta Ct}$ : ambient O<sub>2</sub>, 31.6 ± 3.1; low O<sub>2</sub>, 990.3 ± 98;  $P < 0.001$ ; Fig. 5d). TGF- $\beta$  mRNA expression was more than twofold higher ( $2^{\Delta\Delta Ct}$ : ambient O<sub>2</sub>, 84.5 ± 12.1; low O<sub>2</sub>, 180.5 ± 6.5;  $P < 0.05$ ; Fig. 5d).

## Discussion

In the present study, we demonstrated that ASCs stimulated proliferation of naive CD4<sup>+</sup> T cells from breast cancer patients under both low O<sub>2</sub> and ambient O<sub>2</sub>. The low O<sub>2</sub> iT<sub>regs</sub> exhibited functionality when added to MLRs as demonstrated by 40% inhibition of PBMC proliferation, and by >300-fold increase in FoxP3 mRNA and >2-fold increase in TGF- $\beta$  mRNA.

A low O<sub>2</sub> concentration (5%) is representative of O<sub>2</sub> levels in tissues *in vivo* during T-cell activation, clonal expansion, and differentiation [48–50]. Atkuri et al. [48] demonstrated that primary T cells cultured at ambient O<sub>2</sub> (21%) significantly altered the intracellular redox state (stimulated production of an abnormally elevated amount of oxidized intracellular glutathione), whereas T cells cultured at an O<sub>2</sub> level of 5% maintained the balance of intracellular glutathione and its oxidized form that more closely resembled the *in vivo* status. Toussaint et al. [50] demonstrated that the observed increased T-cell proliferative response in many studies following extended culture under ambient O<sub>2</sub> conditions may be a skewed T-cell response. Moreover, based on the proliferation index and T-cell yield, Duggan et al. [49] demonstrated that CD4/CD28 antibody-mediated T-cell proliferation was higher at ambient O<sub>2</sub> than at low O<sub>2</sub>. This was partly due to intracellular nitric oxide (NO) levels, which were skewed higher at ambient O<sub>2</sub> [49]. Because high levels of intracellular NO and sustained CD69 tend to downregulate T-cell responses *in vivo*, the lower proliferative T-cell responses *in vitro* under low O<sub>2</sub> may be considered as a reflection of *in vivo* regulatory mechanisms.

Crop et al. demonstrated that ASCs stimulated PBMC proliferation under ambient oxygen conditions, independent of cell contact [7]. The present study demonstrated that when placed in direct contact with naive CD4<sup>+</sup> T cells from breast cancer patients under ambient O<sub>2</sub> culture conditions, ASCs induced iT<sub>regs</sub> *in vitro*. Both soluble mediators from the ASCs, as well as direct contact with ASCs may contribute to iT<sub>reg</sub> induction. Of note, there were several differences in the experimental design in the present study compared with previous studies. The present study selected for the naive CD4<sup>+</sup> T-cell subpopulation from breast cancer patients before coculture analyses. We cannot conclude whether the gene expression changes identified would also occur in CD4<sup>+</sup> T cells from healthy donors. It is possible that naive CD4<sup>+</sup> T cells from healthy patients may not respond to ASC cytokine/chemokine/growth factor secretions in the same manner, and further investigation on T-cell activation in breast cancer is warranted. Multiple studies cocultured MSCs with PBMCs for 1 week before measuring effects on iT<sub>reg</sub> induction [7,11,12]. Because of the setup for the hypoxia c-chamber and to prevent ischemia/reperfusion variable interference, the present study measured proliferation following only 3 days of coculture. The present study

supports and further extends the observations of Kronsteiner et al. [15], who reported that ASC immunomodulatory effects on different immune cells may vary significantly depending on the culture conditions utilized [51–52].

These data are consistent with previously published reports that, under ambient O<sub>2</sub> conditions, ASCs cocultured with peripheral blood leukocytes from breast cancer patients induced expression of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T cells [38,53–55]. In addition, recent studies have reported a relationship between leukocyte infiltration, an increased ratio of iT<sub>reg</sub> to CD4<sup>+</sup> T cells in primary tumors, and tumor recurrence in breast cancer patients [38,47,56]. In a retrospective study on triple negative breast cancer (stage I to III), Kim et al. [56] found that an increased number of FoxP3<sup>+</sup> T<sub>reg</sub> cells were significantly correlated with tumors that exhibited lymph node metastases. There were also significant correlations between the increased ratio of FoxP3<sup>+</sup> T<sub>reg</sub> to CD4<sup>+</sup> T cells with lymph node metastasis and expression of the estrogen receptor (ER), p53, and Ki-67 positivity. A lower ratio of FoxP3<sup>+</sup> T<sub>reg</sub> to CD4<sup>+</sup> T cells was significantly associated with triple-negative breast cancer. Disease-free survival of analyzed patients was also significantly associated with the number of FoxP3<sup>+</sup> T<sub>regs</sub> [56].

Taken together, data from these previous studies and the present study suggest that ASC usage to supplement subcutaneous adipose tissue transplanted grafts could possibly contribute to breast cancer recurrence by inducing an iT<sub>reg</sub> cell phenotype from circulating naive CD4<sup>+</sup> T cells and subsequent downregulation of cytotoxic T-lymphocyte activity. Further studies are required to explore changes in gene expression in healthy donors compared with breast cancer patients.

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## Author Disclosure Statement

All authors declare that they have no competing interests except for J.M.G. who is the cofounder of LaCell, LLC, a company focusing on the development of ASCs for research and clinical applications.

## References

1. Cherubino M and KG Marra. (2009). Adipose-derived stem cells for soft tissue regeneration. *Regen Med* 9:109–117.
2. Gimble JM, AJ Katz and BA Bunnell. (2007). Adipose-derived stem cells for regenerative medicine. *Circ Res* 2007, 100:1249–1260.
3. Moiola EK, M Chen, R Yang, B Shah, J Wu and JJ Mao. (2010). Hybrid adipogenic implants from adipose stem cells for soft tissue reconstruction *in vivo*. *Tissue Eng Part A* 16:3299–3307.
4. Yoshimuro K. (2010). Hybrid adipogenic implants from adipose stem cells for soft tissue reconstruction *in vivo*. *Tissue Eng Part A* 16:3299–3307.



5. Abumaree M, JM Al, RA Pace and B Kalionis. (2011). Immunosuppressive properties of mesenchymal stem cells. *Stem Cell Rev* 2:375–392.
6. Singer NG and AI Caplan. (2011). Mesenchymal stem cells mechanisms of inflammation. *Annu Rev Pathol* 6:457–478.
7. Crop MJ, CC Baan, SS Korevaar, JN Ijzermans, W Weimar and MJ Hoogduijn. (2010). Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation. *Stem Cells Dev* 19:1843–1853.
8. Wang JA, A He, X Hu, Y Jiang, Y Sun, J Jiang, C Gui, Y Wang and H Chen. (2009). Anoxic preconditioning: a way to enhance the cardioprotection of mesenchymal stem cells. *Int J Cardiol* 133:410–412.
9. Delarosa O, B Sanchez-Correa, S Morgado, C Ramirez, RB Del, R Menta, E Lombardo, R Tarazona and JG Casado. (2011). Human adipose-derived stem cells impair natural killer cell function and exhibit low susceptibility to natural killer-mediated lysis. *Stem Cells Dev* 21:1333–1343.
10. Murphy KM. (2011). *Janeway's Immunobiology*, 8th edition. Garland Science, New York, NY.
11. Chen W, W Jin, N Hardegen, KJ Lei, L Li, N Marinos, G McGrady and SM Wahl. (2003). Conversion of peripheral CD4<sup>+</sup>. *J Exp Med* 198:1875–1886.
12. Ghannam S, J Pene, G Torcy-Moquet, C Jorgensen and H Yssel. (2010). Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a t regulatory cell phenotype. *Immunology* 185:302–312.
13. Hegyi B, G Kudlik, E Monostori and F Uher. (2012). Activated T-cells and pro-inflammatory cytokines differentially regulate prostaglandin E2 secretion by mesenchymal stem cells. *Biochem Biophys Res Commun* 419:215–220.
14. Hoogduijn MJ, MJ Crop, AM Peeters, GJ Van Osch, AH Balk, JN Ijzermans, W Weimar and CC Baan. (2007). Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem Cells Dev* 16:597–604.
15. Kronsteiner B, S Wolbank, A Peterbauer, C Hackl, H Redl, M van Griensven and C Gabriel. (2011). Human mesenchymal stem cells from adipose tissue and amnion influence T-cells depending on stimulation method and presence of other immune cells. *Stem Cells Dev* 20:2115–2126.
16. Marconi S, G Castiglione, E Turano, G Bissoletti, S Angiari, A Farinazzo, G Constantin, G Bedogni, A Bedogni and B Bonetti. (2012). Human adipose-derived mesenchymal stem cells systemically injected promote peripheral nerve regeneration in the mouse model of sciatic crush. *Tissue Eng Part A* 18:1264–1272.
17. Zhou Y, J Yuan, B Zhou, AJ Lee, AJ Lee, M Ghawji, Jr. and TJ Yoo. (2011). The therapeutic efficacy of human adipose tissue-derived mesenchymal stem cells on experimental autoimmune hearing loss in mice. *Immunology* 133:133–140.
18. Muehlberg FL, YH Song, A Krohn, SP Pinilla, LH Droll, X Leng, M Seidensticker, J Ricke, AM Altman, et al. (2009). Tissue-resident stem cells promote breast cancer growth and metastasis. *Carcinogenesis* 30:589–597.
19. Uccelli A, L Morretta and V Pistoia. (2008). Mesenchymal stem cells in health and disease. *Nat Rev* 9:726–736.
20. De Miguel MP, S Fuentes-Julián, A Blázquez-Martínez, CY Pascual, MA Aller, J Arias and F Arnalich-Montiel. (2012). Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Curr Mol Med* 12:574–591.
21. Singer NG and AI Caplan. (2011). Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol* 6:457–478.
22. Yanez R, A Oviedo, M Aldea, JA Bueren and ML Lamana. (2010). Prostaglandin E2 plays a key role in the immunosuppressive properties of adipose and bone marrow tissue-derived mesenchymal stromal cells. *Exp Cell Res* 316:3109–3123.
23. Kuo YR, CC Chen, S Goto, IT Lee, CW Huang, CC Tsai, CT Wang and CL Chen. (2011). Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model. *Plast Reconstr Surg* 128:661e–672e.
24. Bilate AM and JJ Lafaille. (2012). Induced CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells in immune tolerance. *Annu Rev Immunol* 30:733–758.
25. Engelhardt BG, SM Jagasia, JE Crowe, Jr., ML Griffith, BN Savani, AA Kassim, P Lu, JH Weitkamp, DJ Moore, et al. (2012). Predicting posttransplantation diabetes mellitus by regulatory T-cell phenotype: implications for metabolic intervention to modulate alloreactivity. *Blood* 119:2417–2421.
26. Ichihara F, K Kono, A Takahashi, H Kawaida, H Sagai and H Fuji. (2003). Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 9:4404–4408.
27. Lanca T and B Silva-Santos. (2012). The split nature of tumor-infiltrating leukocytes implications for cancer surveillance and immunotherapy. *Oncoimmunology* 1:717–725.
28. Xu L, W Xu, Z Wen and S Xiong. (2011). In situ prior proliferation of CD4<sup>+</sup>CCR6<sup>+</sup> regulatory T cells facilitated by TGF- $\beta$  secreting DCs is crucial for their enrichment and suppression in tumor immunity. *PLoS One* 6:e20282.
29. Zou W. (2006). Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6:295–307.
30. Henegar C, J Tordjman, V Achard, D Lacasa, I Cremer, M Guerre-Millo, C Poitou, A Basdevant, V Stich, et al. (2008). Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. *Genome Biol* 9:R14.
31. Lee EY, Y Xia, WS Kim, MH Kim, TH Kim, KJ Kim, BS Park and JH Sung. (2009). Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. *Wound Repair Regen* 17:540–547.
32. Leroux L, B Descamps, NF Tojais, B Seguy, P Oses, C Moreau, D Daret, Z Ivanovic, JM Boiron, et al. (2010). Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. *Mol Ther* 18:1545–1552.
33. Rosova I, M Dao, B Capoccia, D Link and JA Nolte. (2008). Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* 26:2173–2182.
34. Choi EW, IS Shin, SY Park, JH Park, JS Kim, EJ Yoon, SK Kang, JC Ra and SH Hong. (2012). Reversal of serologic, immunologic, and histologic dysfunction in mice with systemic lupus erythematosus by long-term serial adipose tissue-derived mesenchymal stem cell transplantation. *Arthritis Rheum* 64:243–253.
35. Koenen TB, R Stienstra, LJ van Tits, LA Joosten, JF van Velzen, A Hijmans, JA Pol, JA van der Vliet, MG Netea, et al. (2011). The inflammasome and caspase-1 activation: a new mechanism underlying increased inflammatory activity in human visceral adipose tissue. *Endocrinology* 152:3769–3778.
36. O'Rourke RW, MD Metcalf, AE White, A Madala, BR Winters, II Maizlin, BA Jobe, CT Roberts Jr., MK Slifka

- and DL Marks. (2009). Depot-specific differences in inflammatory mediators and a role for NK cells and IFN-gamma in inflammation in human adipose tissue. *Int J Obes (Lond)* 33:978–990.
37. O'Rourke RW, AE White, MD Metcalf, AS Olivas, P Mitra, WG Larison, EC Cheang, O Varlamov, CL Corless, CT Roberts Jr. and DL Marks. (2011). Hypoxia-induced inflammatory cytokine secretion in human adipose tissue stromovascular cells. *Diabetologia* 54:1480–1490.
  38. Razmkhah M, M Jaberipour, N Erfani, M Habibagahi, AR Talei and A Ghaderi. (2011). Adipose derived stem cells (ASCs) isolated from breast cancer tissue express IL-4, IL-10 and TGF- $\beta$ 1 and upregulate expression of regulatory molecules on T cells: do they protect breast cancer cells from the immune response? *Cell Immunol* 2:116–122.
  39. Keith B and MC Simon. (2007). Hypoxia-inducible factors, stem cells, and cancer. *Cell* 129:465–472.
  40. Tremolada C, G Palmieri and C Ricordi. (2010). Adipocyte transplantation and stem cells: plastic surgery meets regenerative medicine. *Cell Transplant* 19:1217–1223.
  41. Frazier TP, JM Gimble, I Kheterpal and BG Rowan. (2013). Impact of low oxygen on the secretome of human adipose-derived stromal/stem cell primary cultures. *Biochimie* 20:1–11.
  42. Crop MJ, CC Baan, SS Korevaar, JN Ijzermans, MSAP Pescatori, WFJ van IJcken, MH Dahlke, E Eggenhofer, W Weimar and MJ Hoogdijjn. (2010). Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. *Clin Exp Immunol* 3:474–486.
  43. Bettelli E, Y Carrier, W Gao, T Korn, TB Strom, M Oukka, HL Weiner and VK Kuchroo. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235–238.
  44. O'Garra A, PL Vieira, P Vieira and AE Goldfeld. (2004). IL-10-producing and naturally occurring CD4<sup>+</sup> Tregs: limiting collateral damage. *J Clin Invest* 114:1372–1378.
  45. Crop MJ, CC Baan, SS Korevaar, JN Ijzermans, IP Alwayn, W Weimar and MJ Hoogdijjn. (2009). Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. *Transplantation* 87:896–906.
  46. Dons EM, G Raimondi, DK Cooper and A Thomson. (2012). Induced regulatory T cells: mechanisms of conversion and suppressive potential. *Hum Immunol* 73:328–334.
  47. Cipolletta D, D Kolodin, C Benoist and D Mathis. (2011). Tissue-resident Foxp3<sup>+</sup>CD4<sup>+</sup> T cells that impacts organismal metabolism. *Semin Immunol* 23:431–437.
  48. Atkuri KR, LA Herzenberg, AK Niemi, T Cowan and LA Herzenberg. (2007). Importance of culturing primary lymphocytes at physiological oxygen levels. *Proc Natl Acad Sci U S A* 104:4547–4552.
  49. Duggan O, P Hyland, K Annett, R Freeburn, C Barnett, G Pawelec and Y Barnett. (2004). Effects of a reduced oxygen tension culture system on human T cell clones as a function of *in vitro* age. *Exp Gerontol* 39:525–530.
  50. Toussaint O, G Weemaels, F Debacq-Chainiaux, K Scharfetter-Kochanek and M Wlaschek. (2011). Artefactual effects of oxygen on cell culture models of cellular senescence and stem cell biology. *J Cell Physiol* 226:315–321.
  51. Wion D, T Christen, EL Barbier and JA Coles. (2009). PO(2) matters in stem cell culture. *Cell Stem Cell* 5:242–243.
  52. Csete M. (2005). Oxygen in the cultivation of stem cells. *Ann N Y Acad Sci* 1049:1–8.
  53. Sakaguchi S, N Sakaguchi, M Asano, M Itoh and M Toda. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor  $\alpha$ -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151–1164.
  54. Shevach EM. (2000). Regulatory T cells in autoimmunity. *Annu Rev Immunol* 18:423–449.
  55. Vieira PL, JR Christensen, S Minaee, EJ O'Neill, FJ Barrat, A Boonstra, T Barthlott, B Stockinger, DC Wraith and A O'Garra. (2004). IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *J Immunol* 172:5986–5993.
  56. Kim ST, H Jeong, OH Woo, JH Seo, A Kim, ES Lee, SW Shin, YH Kim, JS Kim and KH Park. (2012). Tumor-infiltrating lymphocytes, tumor characteristics, and recurrence in patients with early breast cancer. *Am J Clin Oncol* 36:224–231.

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