

## Hyaluronan-Binding T Regulatory Cells in Peripheral Blood of Breast Cancer Patients

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### Abstract

Regulatory T cells ( $T_{reg}$ ), both natural and induced, play an important role in maintaining immune homeostasis. Alterations in the number and functions of  $T_{regs}$  are involved in tumor growth. One of the possible regulatory mechanisms of  $T_{reg}$  functional activity involves interaction with major component of extracellular matrix hyaluronan. It has been demonstrated that high molecular weight hyaluronan promotes  $T_{reg}$  function via increased expression of FoxP3 and production of IL-10. Moreover, previous research has shown highly enhanced suppressor function of hyaluronan-binding  $CD4^+CD25^+$   $T_{regs}$  in mice. Breast cancer is characterized by upregulated production of tumor-associated hyaluronan, therefore we investigated hyaluronan-binding subset of  $T_{regs}$  obtained from peripheral blood of breast cancer patients. As a result, we showed that the majority of peripheral blood  $T_{regs}$  were able to adhere to immobilized hyaluronan, and these cells exerted superior suppressor activity, suggesting a key role in regulatory functions of these cells. The percentage of  $CD4^+FoxP3^+$   $T_{reg}$  cells binding hyaluronan, as well as  $CD39^+$  hyaluronan-binding  $T_{regs}$  were significantly increased in breast cancer patients compared to healthy donors. Enhanced number of the activated  $T_{reg}$  cells might play an important role in the suppression of antitumor immunity.

**Keywords:** Regulatory T cells; Hyaluronan; Breast cancer; CD39; CTLA-4

### Introduction

Regulatory T cells can be described as a T cell population that functionally suppresses immune response by influencing the activity of a range of effector cells, and thereby contributes to the maintenance of immune homeostasis.  $CD4^+$   $T_{regs}$  consist of two types, "natural"  $T_{regs}$  ( $nT_{regs}$ ) that constitutively express CD25 and FoxP3, and so-called adaptive or "inducible"  $T_{regs}$  ( $iT_{regs}$ ).  $iT_{reg}$  cells can be induced in the periphery from a  $CD4^+FoxP3^-$  T cell population following T cell receptor (TCR) stimulation in the presence of immunoregulatory cytokines such as TGF- $\beta$ , IL-10, and IL-4.  $nT_{regs}$  and  $iT_{regs}$  suppress immune responses through various cytokines and contact-dependent mechanisms [1,2].

One of the central mechanisms that mediate  $T_{reg}$  recruitment from the blood to sites of inflammation or tumor growth is mediated through interaction between the activated form of CD44 on peripheral  $T_{regs}$  and its ligand hyaluronan (HA) on microvascular endothelium [3]. CD44, a type I transmembrane glycoprotein, is widely expressed on T lymphocytes but requires activation for binding HA. Transition from low- to high-affinity binding state can be activated in T cells by several stimuli, such as HA-binding itself, TCR engagement, and responses to cytokines/chemokines, and one mechanism involves the enzymatic removal of terminal sialic acid from two N-linked glycans in the HA-binding domain [4-7]. Therefore, the ability of  $T_{regs}$  to interact with HA is intrinsically related to their activation state.

Previous studies raised the possibility that CD44 interactions with HA may be integrally related to  $T_{reg}$  functions. In mice HA-binding  $CD4^+CD25^+$   $T_{reg}$  cells showed highly enhanced suppressor activity *in vitro* [8].  $T_{regs}$  from CD44-deficient mice have an impaired capacity to inhibit T cell responses. *In vitro* ligation of CD44 on activated  $T_{regs}$  promotes persistent expression of FoxP3, increased production of IL-10 and expression of membrane TGF- $\beta$ , which are necessary for immunoregulatory activity. These effects on  $T_{regs}$  are shown to depend upon interaction with a high molecular weight form of HA [9].

The rates of HA synthesis and degradation are much higher in cancer than in healthy tissues [10,11]. It has been demonstrated that in advanced cancer, aberrant synthesis and degradation of HA by transformed cells result in the formation of an extremely unusual microenvironment characterized by the accumulation of high molecular weight HA, which may facilitate the malignant transformation and survival of tumor cells, and affect functions of immune cells [12]. Several lines of evidence indicate that malignant breast tissue contains more HA than normal breast tissue or benign lesions [13-18].

These studies with other indirect evidences lead us to formulate the hypothesis that in cancer,  $T_{regs}$  bind with HA whose gradient exists in peripheral blood, and migrate to the tumor, where interaction of CD44 with high molecular weight HA increases the suppressive potential of  $T_{regs}$  and results in the suppression of antitumor immunity. To test the hypothesis, HA-binding  $T_{reg}$  cells from the peripheral blood of breast cancer patients were purified, and their phenotype related to specific suppressor activity was assessed.

## Materials and Methods

### Study Subjects

Peripheral blood samples were obtained from healthy volunteers and patients with breast cancer. Only patients without prior chemotherapy or other treatments and surgical removal of tumor were included in the study. Both the volunteers and the patients provided their informed consent for participation in the investigation. Of 16 patients, 14 carried stage II or higher disease (Table 1).

	Study subjects	
	Normal	Breast cancer patients
Donors	20	16
Average age	38.2 ± 11.7	52.8 ± 6.6
Stage I	-	2
Stage II	-	11
Stage III	-	3
Stage IV	-	0

Table 1: Patient characteristics.

### Isolation of CD4<sup>+</sup> and T<sub>reg</sub> cells from peripheral blood

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). The CD4<sup>+</sup> cell rich fraction was isolated by negative selection on a Miltenyi Biotec Vario Max separator using the CD4<sup>+</sup> T Cell Isolation Kit, human (Miltenyi Biotec AG, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the resultant CD4<sup>+</sup> cell fraction was evaluated on a BD FACS Calibur (BD Biosciences, San Jose, CA) using monoclonal antibody (mAb) anti-CD4-PerCP (Miltenyi Biotec AG, Bergisch Gladbach, Germany) and resulted in 95%. For some experiments, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells and CD4<sup>+</sup>CD25<sup>-</sup> conventional T cells were sorted on a BD FACSAria II from freshly obtained PBMC using anti-CD4-APC-Cy7 (BD Biosciences, San Jose, CA) and anti-CD25-PerCP-Cy5,5 (BD Biosciences, San Jose, CA). The purity of sorted cells was >98%.

### Separation of hyaluronan-binding CD4<sup>+</sup> cells

For 10<sup>7</sup> cells, 15µg of biotinylated hyaluronan (Sigma-Aldrich, Co., St. Louis, MO) and 50µl of anti-biotin MACSi Bead Particles (Miltenyi Biotec AG, Bergisch Gladbach, Germany) were mixed in a 50µl solution containing phosphate buffer saline (PBS) supplemented with 0.5% Bovine Serum Albumin (BSA) and 2mM EDTA, and incubated for 2 h at 4-8°C under gentle rotation. After washing by centrifugation at 300g for 15 min at 7°C the conjugate was resuspended in 100µl of the same buffer solution. Cell suspension was mixed with conjugate at a 1:1 cell-to-bead ratio and incubated for 20 min at 4-8°C. Then the cells were magnetically separated into HA-binding (HA<sup>+</sup>) and HA-nonbinding (HA<sup>-</sup>) fractions using a BD IMag Separator (BD Biosciences, San Jose, CA).

### Surface and Intracellular Staining

The following mouse mAbs were used: anti-CD4-PerCP, anti-CD25-PE, anti-FoxP3-PE, anti-FoxP3-APC, anti-CD39-PE, anti-CD39-FITC, anti-CTLA4-APC, anti-IL-17-FITC, anti-LAP-APC, anti-GITR-APC (Miltenyi Biotec AG, Bergisch Gladbach, Germany), anti-CD25-PerCP-Cy5.5, anti-CD25-FITC (BD Biosciences, San Jose, CA), anti-TGF-β-FITC, anti-IL-10-PE, anti-IL-10-FITC, anti-IL-35-APC (R&D Systems, Inc., Minneapolis, MN) and their relevant control isotypes. Cells were incubated with mAbs specific for surface markers for 20 min at 4-8°C in the dark and then fixed in Cytofix buffer (BD Biosciences, San Jose, CA). For intracellular staining, after surface labeling cells were permeabilized with paraformaldehyde/saponin solution (Cytofix & Cytoperm kit, BD Bioscience, San Jose, CA). Permeabilized cells were stained with mAbs specific for intracellular markers for 30 min at 4°C in the dark. FoxP3 Staining Buffer Set (Miltenyi Biotec AG, Bergisch Gladbach, Germany) was used for intracellular labeling of FoxP3 according to the manufacturer's instruction. Afterward cells were analyzed by BD FACSCalibur with CellQuest Pro software (BD Bioscience, San Jose, CA).

For detection of HA-binding T<sub>reg</sub> cells, freshly purified PBMC were stained with biotinylated hyaluronan (15µg for 10<sup>7</sup> cells), mixed well and incubated for 20 min at 4-8°C. Then cells were washed, labeled with streptavidin-PE (R&D Systems, Inc., Minneapolis, MN) and T<sub>reg</sub>-associated markers and analyzed by flow cytometry.

### In vitro cell culture

All the cultures were maintained in RPMI-1640 (Sigma Chem. Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS), penicillin (100U/ml), streptomycin (100mg/ml) and L-glutamine (2mM) at 37°C, 5% CO<sub>2</sub> and 95% humidity. Freshly isolated HA<sup>+</sup> and HA<sup>-</sup> fractions of CD4<sup>+</sup> cells were activated with anti-CD3/anti-CD28/anti-CD2 bound to beads (T<sub>reg</sub> Suppression Inspector assay, TSI, Miltenyi Biotec AG, Bergisch Gladbach, Germany) at a cell-to-bead ratio of 2:1 for 18 h. After washing cells were stained with fluorochrome-labeled mAb and analyzed.

### Suppression of CD4<sup>+</sup>CD25<sup>-</sup> conventional T lymphocytes

The suppressor effect of HA<sup>+</sup> and HA<sup>-</sup> T<sub>regs</sub> was determined by the ability to inhibit proliferation of autologous CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> conventional T lymphocytes. In brief, sorted conventional T cells were resuspended at 2-3×10<sup>6</sup>/ml in PBS with 0.1% BSA and kept on ice. CFSE solution (5µM) was added at volume equivalent to cell suspension and incubated at room temperature without agitation for 10 min. The reaction was quenched as quickly as possible with ice-cold fetal bovine serum (FBS). Subsequently, cells were immediately put on ice for 2 min, washed twice and resuspended at 5×10<sup>5</sup>/ml. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were fractionated into HA<sup>+</sup> and HA<sup>-</sup> using biotinylated HA conjugated with anti-biotin MACSi Bead Particles. The obtained fractions were cultured in 96-well plates with CFSE-labeled conventional T cells at different effector-to-target ratios (1:1, 3:1, 5:1) in the presence of 10µg/ml PHA (Sigma-Aldrich, St. Louis, MO) for 72 h at 37°C.

### Statistical analysis

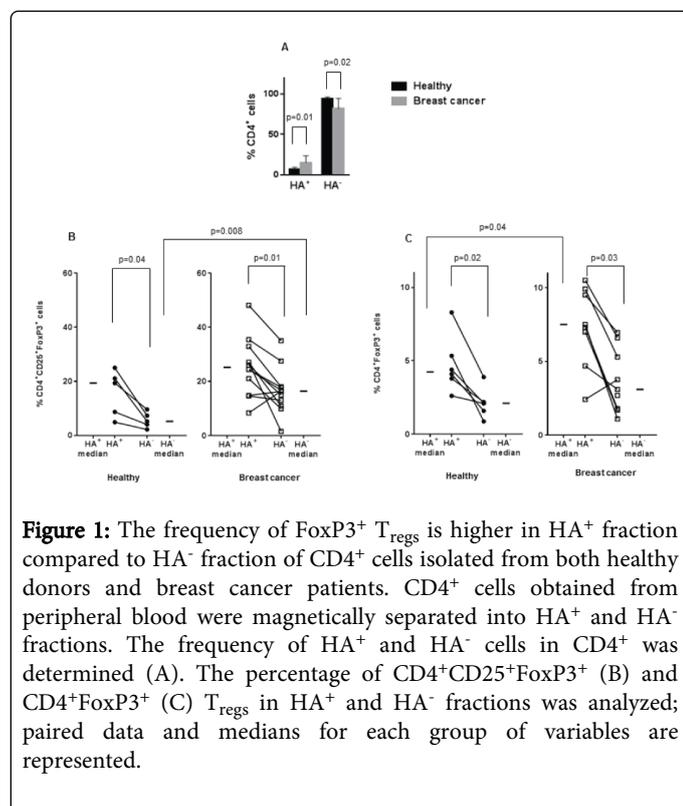
Data are expressed as mean ± SD or median and inter quartile range, p25-p75. The Wilcoxon signed-rank test was used to determine pairwise differences. The Mann-Whitney U test and Student t-test were used to determine differences between groups. A probability

value of equal to or less than 0.05 ( $p \leq 0.05$ ) was considered statistically significant.

## Results

### Increased number of FoxP3<sup>+</sup> cells in hyaluronan-binding CD4<sup>+</sup> T cell population in breast cancer patients

Although soluble HA failed to detect HA<sup>+</sup> T<sub>regs</sub> in freshly obtained PBMC, HA<sup>+</sup> and HA<sup>-</sup> fractions of CD4<sup>+</sup> cells were separated using immobilized high molecular weight HA. In healthy individuals, the fraction of CD4<sup>+</sup> cells able to bind HA was small ( $6.5 \pm 2.6\%$ ), but it was significantly increased in breast cancer patients ( $14.8 \pm 8.3\%$ ,  $p=0.01$ ) (Figure 1A). In healthy donors, the prevalence of CD4<sup>+</sup>CD25<sup>+</sup> cells was significantly higher in HA<sup>+</sup> fraction compared to HA<sup>-</sup> fraction (median 9.9%, IQR 6.6-12.6 and median 4.6%, IQR 3.3-8.7, correspondingly,  $p=0.03$ ). There was no significant difference in the percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells between HA<sup>+</sup> and HA<sup>-</sup> fractions in breast cancer patients (data not shown).



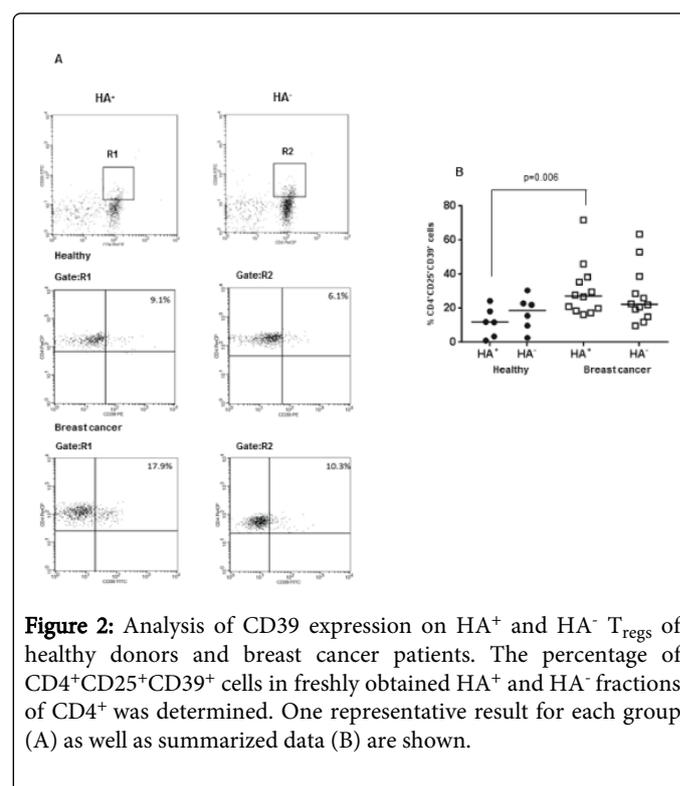
While CD25 can be expressed on activated conventional T cells "contaminating" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell subpopulation, transcription factor FoxP3 has a central role in T<sub>reg</sub> identification. As a highly characterized marker of T<sub>regs</sub>, FoxP3 has been shown to be essential for their suppressive activity [19,20]. With gating on CD4<sup>+</sup>CD25<sup>+</sup>, the frequency of FoxP3<sup>+</sup> cells was higher in HA<sup>+</sup> compared to HA<sup>-</sup> both in healthy donors and breast cancer patients (Figure 1B). The difference in the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> between HA<sup>+</sup> and HA<sup>-</sup> fractions of breast cancer patients was not associated with stage of the disease. The percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in HA<sup>-</sup> fraction was significantly increased in the breast cancer group compared to the healthy donor group (Figure 1B).

Previous studies have demonstrated that FoxP3 does not always correlate with CD25 expression [21,22]. We also observed that not all FoxP3<sup>+</sup> cells expressed CD25. FACS analysis showed that the majority of CD4<sup>+</sup>FoxP3<sup>+</sup> cells were able to bind HA in both groups. The percentage of HA<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells was significantly elevated in breast cancer patients compared to healthy subjects (Figure 1C).

### Higher number of CD39<sup>+</sup> cells in circulating hyaluronan-binding CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> subpopulation

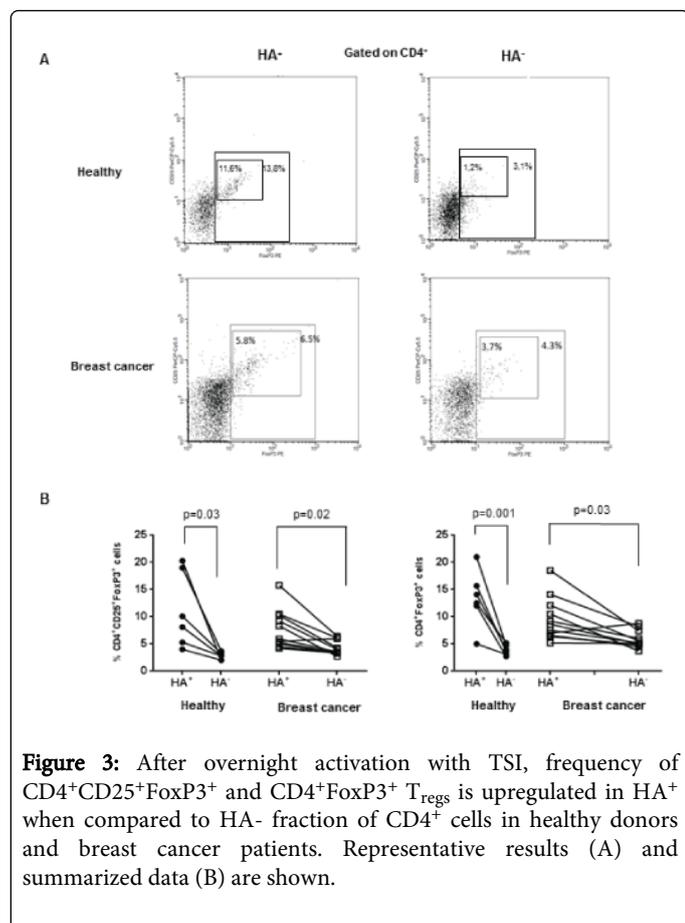
Recent findings reveal an important role of CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> T<sub>regs</sub> in cancer pathogenesis [23-26]. Considering the importance of this cell population, frequency of CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> T<sub>regs</sub> in HA<sup>+</sup> and HA<sup>-</sup> fractions of CD4<sup>+</sup> was evaluated. No difference in CD39 expression between HA<sup>+</sup> and HA<sup>-</sup> fractions either in healthy donors and breast cancer patients was obtained (Figure 2).

However, CD39 expression was significantly increased on HA<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> in breast cancer patients when compared to healthy donors. Notably, there was no difference in the number of CD39<sup>+</sup> T<sub>regs</sub> in HA<sup>-</sup> fractions between the healthy and breast cancer groups (Figure 2).

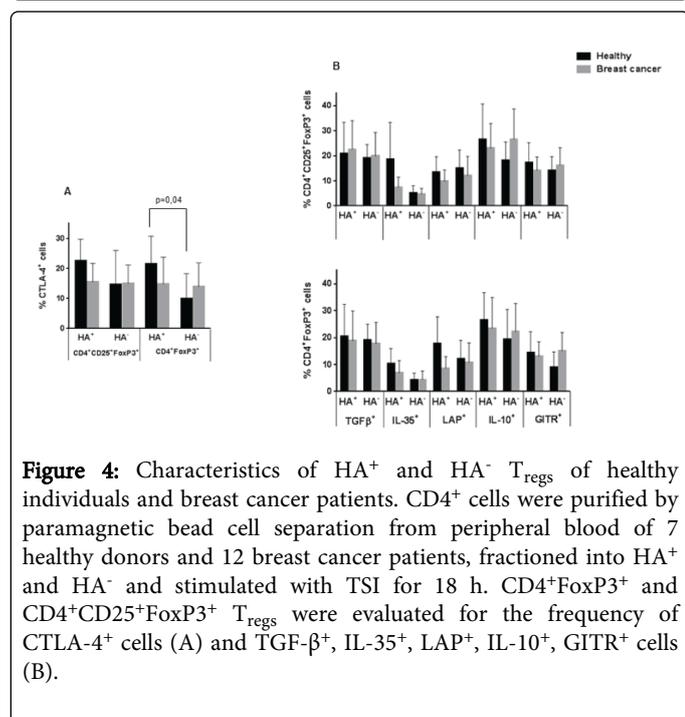


### Increased level of CTLA-4 in hyaluronan-binding CD4<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub>

It is well known that the T<sub>reg</sub> cell population bears elevated expression of suppression markers. Therefore, we investigated whether the expression of such markers is associated with the ability to bind HA. HA<sup>+</sup> and HA<sup>-</sup> fractions of CD4<sup>+</sup> T cells were activated with TSI and analyzed for expression of suppression markers. After overnight activation, higher prevalence of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> in HA<sup>+</sup> fraction compared to HA<sup>-</sup> fraction was observed both in healthy individuals and breast cancer patients (Figure 3).



**Figure 3:** After overnight activation with TSI, frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> is upregulated in HA<sup>+</sup> when compared to HA<sup>-</sup> fraction of CD4<sup>+</sup> cells in healthy donors and breast cancer patients. Representative results (A) and summarized data (B) are shown.



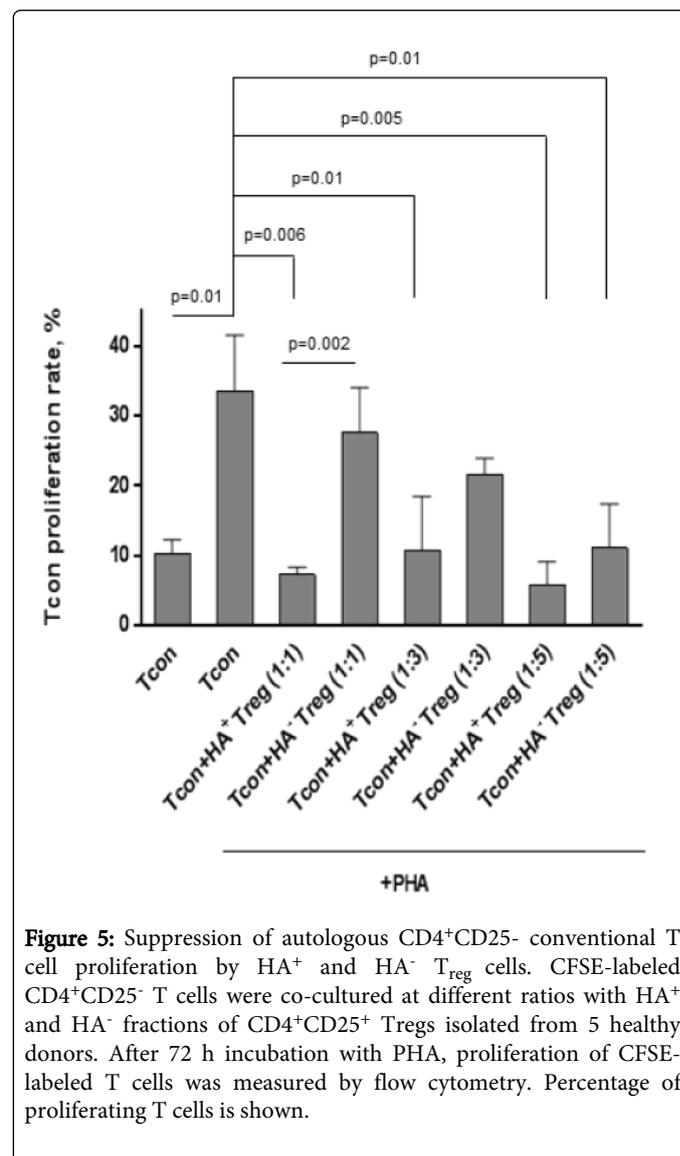
**Figure 4:** Characteristics of HA<sup>+</sup> and HA<sup>-</sup> T<sub>regs</sub> of healthy individuals and breast cancer patients. CD4<sup>+</sup> cells were purified by paramagnetic bead cell separation from peripheral blood of 7 healthy donors and 12 breast cancer patients, fractionated into HA<sup>+</sup> and HA<sup>-</sup> and stimulated with TSI for 18 h. CD4<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> were evaluated for the frequency of CTLA-4<sup>+</sup> cells (A) and TGF-β<sup>+</sup>, IL-35<sup>+</sup>, LAP<sup>+</sup>, IL-10<sup>+</sup>, GITR<sup>+</sup> cells (B).

In healthy donors, the prevalence of CTLA-4<sup>+</sup> cells was higher in HA<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> compared to HA<sup>-</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cell fraction: mean ±SD percentage 21.4 ± 6.2% and 9.9 ± 6.2% correspondingly, p=0.04.

In contrast, there was no difference in the expression of CTLA-4 on HA<sup>+</sup> and HA<sup>-</sup> fractions of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> (Figure 4A). When we analyzed the levels of TGF-β, IL-35, LAP, IL-10 and GITR, no difference was seen between HA<sup>+</sup> and HA<sup>-</sup> fractions, either in CD4<sup>+</sup>FoxP3<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> subsets in breast cancer patients and healthy donors (Figure 4B).

### Hyaluronan-binding CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> have enhanced suppressive activity

To determine whether the ability to bind HA is correlated with distinctive functional activity, freshly sorted CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> from five healthy donors were fractionated into HA<sup>+</sup> and HA<sup>-</sup> cells and co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> conventional T cells in an *in vitro* suppressor assay. HA<sup>+</sup>T<sub>regs</sub> suppressed conventional T cell proliferation even at 1:1 suppressor to target ratio. While HA<sup>-</sup> T<sub>regs</sub> were suppressive at 5:1 suppressor to target ratio, they lost the suppressive capacity as the ratio decreased (3:1 and 1:1) (Figure 4). These results suggest that two subsets of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> are functionally distinct; HA<sup>+</sup> T<sub>regs</sub> are more suppressive.



**Figure 5:** Suppression of autologous CD4<sup>+</sup>CD25<sup>-</sup> conventional T cell proliferation by HA<sup>+</sup> and HA<sup>-</sup> T<sub>reg</sub> cells. CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> T cells were co-cultured at different ratios with HA<sup>+</sup> and HA<sup>-</sup> fractions of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> isolated from 5 healthy donors. After 72 h incubation with PHA, proliferation of CFSE-labeled T cells was measured by flow cytometry. Percentage of proliferating T cells is shown.

## Discussion

Hyaluronic acid is a negatively charged glycosaminoglycan that is abundantly present on endothelial cells and in extracellular matrix [27]. Its production is increased at the tumor-stroma interface, including breast cancer [14,17]. It has been demonstrated that interaction of HA with CD44 which is widely regarded as the major receptor for HA, mediates entry of T cells including immunosuppressive  $T_{regs}$  to target sites, as well as cell motility within these tissues [4].  $T_{regs}$  are well known to play a crucial role in inhibiting anticancer defenses in the tumor microenvironment, resulting in tumor progressive growth and metastatic dissemination [28]. M. Firan et al. have shown that the ability to bind HA discriminates mouse  $T_{regs}$  with enhanced suppressive function and state of activation [8], but if these cells are present in human peripheral blood under normal physiological conditions and if this  $T_{reg}$  subset is distinct in cancer is unclear.

To investigate the ability of peripheral blood  $T_{regs}$  to bind HA we used biotinylated HA, which was subsequently identified with streptavidin-PE.  $T_{regs}$  did not stain with soluble HA. On the contrary, we were able to obtain  $T_{regs}$  that actively bound immobilized HA. For this purpose, we developed a method of cell separation using a commercially available biotin-conjugated high molecular HA in combination with paramagnetic beads coated with anti-biotin Ab, which allowed us to isolate cells that were able to adhere to HA. Apparently, the large beads with coated HA were imitating extracellular matrix; therefore, the ability of these cells to interact with immobilized HA was more pronounced when compared to soluble HA.

Earlier studies have demonstrated that around 1% of naïve peripheral lymph node T lymphocytes bind soluble HA without any stimulation [8]. Ariel et al. have shown that adhesion of freshly purified human T cells to immobilized HA was always between 5 and 15% [6]. Here we show that the HA<sup>+</sup> fraction of unstimulated CD4<sup>+</sup> T cells is enriched with naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>  $T_{regs}$ . It should be noted that evaluated characteristics did not correlate with age of the individuals involved in the study. Thus, it appears that a large proportion of naturally arising  $T_{regs}$ , key players of the immune regulation, is capable of HA-mediated rolling. The higher prevalence of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>  $T_{regs}$  in HA<sup>+</sup> fraction was also observed after overnight activation. Moreover, freshly separated HA<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>  $T_{regs}$  showed upregulated suppressor activity. The reason for persistence of HA<sup>+</sup> n $T_{regs}$  in the peripheral blood is unclear; we suppose that it is likely to be important for the maintenance of immune tolerance to self-antigens. The obtained data are consistent with the results received by Levine et al. They report that just as in the conventional CD4<sup>+</sup> T cell compartment,  $T_{regs}$  consist of CD44<sup>lo</sup>CD62L<sup>hi</sup> 'naïve-like' and CD44<sup>hi</sup>CD62L<sup>lo</sup> 'effector-like' populations [29]. It is assumed that transition to the activated state is induced in the thymus and in the periphery upon TCR activation presumably by self-peptide [30]. The question if CD44<sup>hi</sup>CD62L<sup>lo</sup>  $T_{regs}$  are self-reactive, remains to be elucidated.

We observed that FoxP3 was expressed in a significant percentage of CD4<sup>+</sup> T cells independently of CD25 expression. The importance of the CD4<sup>+</sup>FoxP3<sup>+</sup> population in cancer and its negative impact on antitumor therapy has been demonstrated [31]. Analysis showed that a major portion of circulating CD4<sup>+</sup>FoxP3<sup>+</sup> cells was able to bind HA, and their content was significantly higher in the peripheral blood of breast cancer patients. We assume that in cancer, more inducible  $T_{regs}$  that include a major subset of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells rather than

CD4<sup>+</sup> CD25<sup>+</sup>FoxP3<sup>+</sup>  $T_{regs}$  are potentially capable of HA-mediated adhesion and therefore more effective migration to tumor sites and maintenance of immune suppression [32].

In healthy donors, the ability to bind HA distinguished CD4<sup>+</sup>FoxP3<sup>+</sup>  $T_{regs}$  with elevated expression of CTLA-4 after activation. The inhibitory mechanism of CTLA-4 is mediated through competition with the co-stimulatory molecule CD28, which, together with TCR, is necessary for T cell activation [33]. Massive aberrant activation and expansion of conventional T cells has been observed in transgenic mice lacking CTLA-4 expression in  $T_{regs}$  [34]. Apparently, under normal physiological conditions CTLA-4 on regulatory T cells dampens pathological naïve T cell activation and its elevated expression on HA<sup>+</sup>  $T_{regs}$  implies a specific role of this subset in control of periphery tolerance in tissues.

Further analysis showed that CD39 is more highly expressed by HA<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>  $T_{regs}$  in breast cancer than in healthy subjects. Catalytic inactivation and conversion of extracellular ATP by CD39 is one of the key anti-inflammatory mechanisms of  $T_{regs}$  [35]. CD39 drives the sequential hydrolysis of both adenosine triphosphate and adenosine diphosphate to adenosine monophosphate, while CD73 further hydrolyses it to adenosine, a nucleoside that exhibits direct immunosuppressive effects [36,37]. A significant increase of CD39 expression on  $T_{regs}$  in cancer patients, which is strongly associated with tumor progression, has been reported earlier [24,38]. Here, we demonstrate that particularly the frequency of CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup>  $T_{regs}$  that are able to adhere to HA is increased in the periphery in breast cancer patients. It is unclear if the binding of HA promotes expression of CD39 on  $T_{regs}$  in breast cancer patients, but the obtained results suggest that accumulation of CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup>  $T_{regs}$  in the tumor environment reported earlier may be mediated by HA [38-40].

Here we show a new approach for the isolation and investigation of  $T_{regs}$ , which can discriminate a new subset of  $T_{regs}$  potentially capable of adhesion to extracellular matrix. The approach allowed us to formulate several key notions concerning HA-binding  $T_{regs}$ . First, the normal immune system generates  $T_{regs}$  that intensively bind immobilized high molecular weight HA. The ability to bind HA discriminates  $T_{regs}$  with elevated suppressive activity and increased percentage of CTLA-4-expressing cells after activation *in vitro*. The reason for maintenance of such HA-binding  $T_{regs}$  under normal physiological conditions is not clear; they can represent an important subpopulation of previously activated circulating  $T_{regs}$  that may be involved into mechanisms of peripheral tolerance to auto-antigens. Second, the subset of HA-binding  $T_{regs}$  is increased in breast cancer and characterized by augmented expression of ectonucleotidase CD39. The increased proportion of HA-binding  $T_{regs}$  in circulation may reflect the upregulated activity of  $T_{regs}$  in breast cancer. Furthermore, HA-mediated rolling may be involved in infiltration of CD39<sup>+</sup>  $T_{regs}$  into the tumor stroma and accumulation there. These findings, however, need to be proved in a larger cohort of patients in different phases of the disease to elucidate whether strategies targeting HA-mediated migration of  $T_{regs}$  may have therapeutic value.

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