American Journal of Immunology 1 (4): 143-147, 2005
ISSN 1553-619X

Association Between Circulating Early Endothelial Progenitors and CD4+CD25+ Regulatory T Cells: A Possible Cross-talk between Immunity and Angiogenesis?

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Abstract: Regulatory T-cells (Treg) are a recently defined subset of CD4+ cells that can suppress inflammation and induce tolerance. Phenotypically, T-regs are characterized by a high level of expression of the IL-2 receptor alpha chain, CD25. Endothelial progenitor cells (EPCs) can transform into mature endothelial cells and promote vessel formation by inducing postnatal angiogenesis and vasculogenesis. Herein, we tested the hypothesis that an association exists between circulating EPC and Tregs that could potentially allude to cross talk between immunity and angiogenesis. Peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation from 28 subjects. Circulating number of EPCs at various developmental stages (CD133+CD34+, CD133+VEGFR2+, CD34+VEGFR2+), total CD4+ and Treg CD4+CD25^{high}) numbers were determined by FACS analysis. We found a positive correlation between early progenitor cell (CD133+CD34+) number and Tregs, but no correlation between differentiated EPCs and Tregs, or between CD4+ and any of the EPCs sampled. Early EPCs (CD133+CD34+) did not correlate with CD34+/KDR or with CD133/KDR cells. Circulating numbers of early but not 'mature' EPC correlate with Tregs but not CD4 numbers. This finding may suggest a novel role for Tregs in promoting EPC recruitment or delaying EPC maturation.

Key words: EPC, HSC, Tregs, angiogenesis

INTRODUCTION

Regulatory T-cells (Treg) are a subset of CD4+ cells that can suppress inflammation and induce tolerance, thereby modulating adaptive immune responses[1]. Some T cells with suppressor activity are part of a unique lineage of $CD4^+$ T cells that are "naturally occurring" and are present in the thymus and peripheral lymphoid tissues of mice and humans[1]. Other types of suppressor T cells can be recovered from $CD4^+$ T cells by specific experimental manipulations *in vitro*[2].

Naturally occurring Tregs were identified by a high level of expression of IL-2Rα chain CD25[3]. IL-2R is also expressed by activated nonregulatory T cells, but at low levels[4]. In addition, Tregs express also the forkhead/winged helix transcription factor-Foxp3, which acts as a silencer of cytokine gene promoters and programs the development and function of these cells. Mutation in the Foxp3 gene, in humans and mice, causes absence of Tregs and an autoimmune disease known as IPEX, characterized by autoimmune endocrinopathy, early onset of type I diabetes, thyroiditis and in some cases, severe atopy and food allergy[5].

CD4+CD25+ Tregs inhibit the proliferation and expansion of naïve T cells both *in vitro* and *in vivo*[2]. Suppressor cytokines, such as interleukin-4 (IL-4), IL-10 and transforming growth factor- β (TGF-β) and cell-contact-dependent mechanisms might play a role in the suppression of autoimmune phenomena *in vivo*, as shown in animal models of gastritis, thyroiditis, inflammatory bowel disease and type I diabetes mellitus[6].

Endothelial progenitor cells (EPCs) are a scarce population of cells that can mobilize to the vasculature, proliferate and differentiate into mature endothelial cells and thus play an important role in neoangiogenesis after tissue ischemia has occurred[7-9]. EPCs originate from hemapoietic stem cells (HSC) expressing CD34 or the more immature marker protein CD133. As they mature, EPCs lose the CD133 and acquire vascular endothelial growth factor receptor VEGFR2[10]. VEGF and stromal cell-derived factor-1 (SDF-1) produced in ischemic environments, appear to have important roles in mobilization and trafficking of EPCs. Additional cytokines shown to mobilize EPC from the bone marrow to the peripheral circulation include G-CSF, GM-CFS and erythropoietin[11].

Several observations have prompted us to investigate a possible relationship between EPCs and T cells: *Stabile et al.*[12] have found that CD4+ cells enhance collateral vascular development in acute

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hindlimb ischemia model in mice by inducing monocyte-macrophages recruitment to the ischemic muscle (which in turn trigger the development of collaterals through the synthesis of arteriogenic cytokines such as VEGF); *Mor et al.*[13] have found that activated T cells from rats synthesize and secrete VEGF and could potentially enhance angiogenesis. Since EPCs are key effectors involved in angiogenesis, we investigated whether T cells and/or Tregs associate with EPC at different levels of maturation.

MATERIALS AND METHODS

Study subjects: We studied a total of 28 subjects, 18 of whom underwent coronary angiography demonstrating no signficant coronary artery disease. Institutional ethics committee approved the study and informed consent was obtained from all patients.

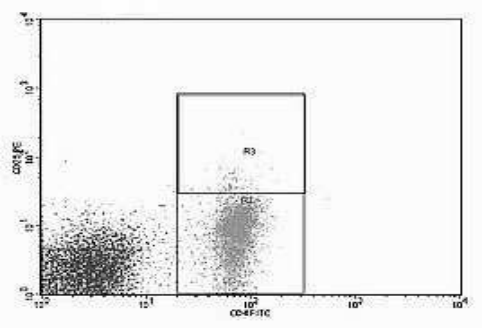
Preparation of blood samples: Peripheral blood mononuclear cells were isolated from 30 ml of freshly drawn heparinized blood using Isopaque-Ficoll (Amersham Biosciences, Buckinghamshire, United Kingdom) gradient centrifugation.

EPC and Treg phenotyping by flow cytometry: The number of circulating EPC was assessed by FACS analysis by staining 5 million cells for 3 color-FACS analysis employing the following monoclonal antibodies: fluorescein isothicyanate (FITC)-anti-CD34 (IQ products), allophycocyanin (APC)-anti VEGF-receptor 2 (KDR, R&D systems) and phycoerythrin (PE)-anti-CD133 (R&D systems).

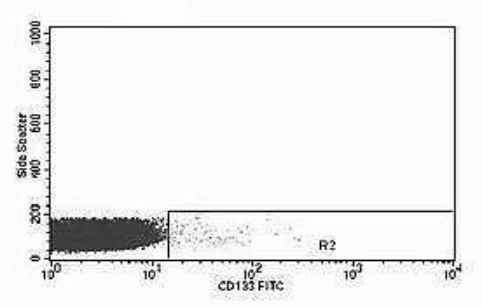
The number of circulating Treg cells was assessed by FACS analysis by staining 2 million cells for 2 color-FACS analysis employing the following monoclonal antibodies: FITC-anti-CD4 (e-Bioscience; RPA-T4) and PE-anti-CD25 (e-Bioscience; BC96).

To analyze cell surface molecule expression, peripheral blood mononuclear cells were washed with phosphate-buffered saline (PBS), 2% fetal calf serum (FCS) and stained for 30 min at 4^0C, after which they were washed again. Cell fluorescence was measured immediately after staining and data were analyzed with the use of the CellQuest software (FACSCalibur, Becton Dickinson). EPC-gated analysis included at least 100000 events and each Treg analysis included at least 10000 events. In this study, units of EPCs represent the percentage of respectively marked cells out of those in the lymphocyte gate; units of all CD4+ cells represent the percentage of CD4+ cells out of those in the lymphocyte gate and units of Tregs represent the percentage of CD4+CD25^{high} cells out of the CD4+ gate. The method of EPC and Treg FACS plot gating and analysis is illustrated in Fig. 1 and is based on previously described methods[14-17].

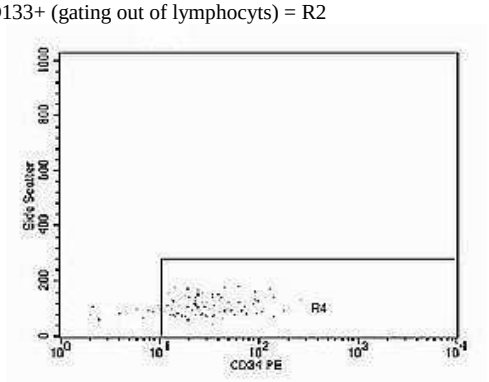
Reproducibility was assesssed by several different analyses of all the data, which yielded similar results.



A. Treg : CD4+CD25^{high} (gating out of lymphocytes) = R3



B. CD133+ (gating out of lymphocyts) = R2



C. Early EPC/HSC: CD133+CD34+ (gating out of R2) = R4

Fig. 1: Representative FACS analyses of Tregs and EPCs, Fig. A shows the gating of the CD4+CD25 ^{high} population, which characterizes Tregs (marked as R3), out of the CD4+ population (marked as R1) after lymphocyte gate selection (not shown), Fig. B illustrates the CD133+ gating (marked as R2) out of the lymphocyte gate, Fig. C shows the subsequent gating of the CD34+ population out of R2 (marked as R4). R4 are therefore CD34+CD133+ cells, representing early EPC/HSC cells

Statistical analysis: As distribution of values was non-gaussian, correlation between groups were assayed employing the Spearman's test. Level of significance was set at 0.05. Results represent mean±SEM unless otherwise specified in the text.

RESULTS AND DISCUSSION

Mean age of the 28 subjects studied was 49±27 years. Fifty-five percent of the subjects had hypertension, 20% had diabetes, 25% were current

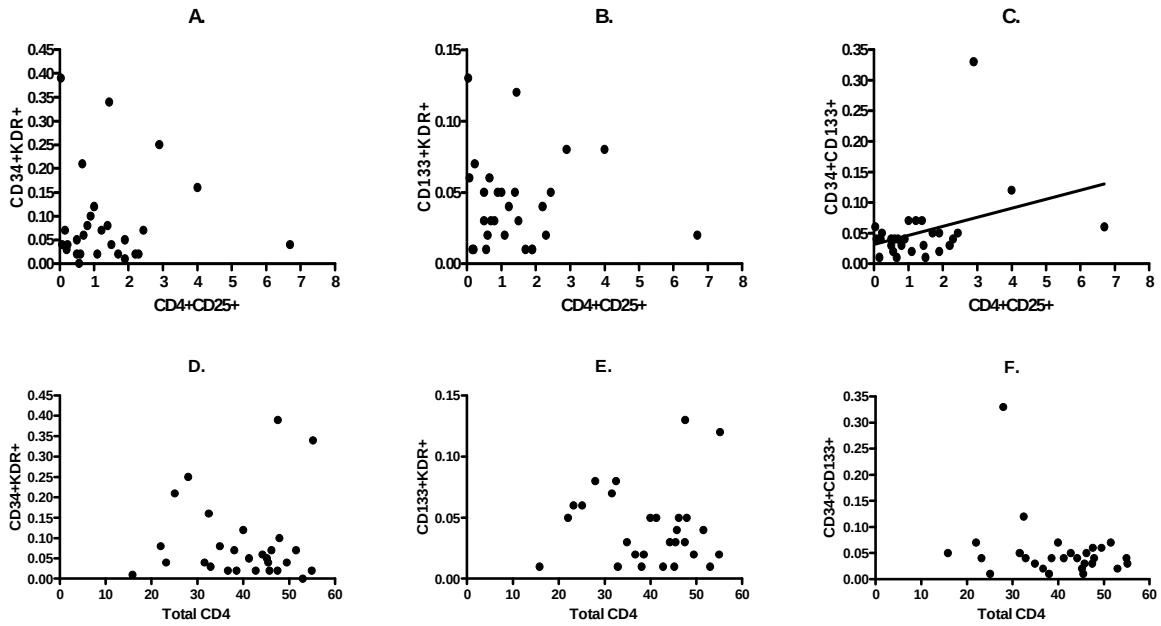


Fig. 2: Tregs correlation versus the various EPC/HSC populations . Units of EPCs represent the percentage of respective cells out of those in the lymphocyte gate, units of Tregs represent the percentage of CD4+CD25^{high} cells out of the CD4+ gate and units of CD4+ cells represent the percentage of CD4+ cells out of those in the lymphocyte gate, Fig. A shows Tregs vs CD34+KDR+, Fig. B shows Tregs vs CD133+KDR+, Fig. C shows Tregs vs CD34+CD133+, Fig. D shows Tregs vs CD34+KDR+, Fig. E shows Tregs vs CD133+KDR+, Fig. F shows Tregs vs CD34+CD133+

smokers and 60% had hyperlipidemia. 40% of the patients were taking beta-blockers, 10% calcium blockers, 45% ACE inhibitors, 55% aspirin, 25% nitrates, none diuretics and 25% statins.

A typical Treg cell FACS analysis out of the lymphocyte gate is shown in Fig. 1A and a typical EPC FACS analysis out of the lymphocyte gate is shown in Fig. 1B.

We have demonstrated a significant positive correlation ($r=0.45$; $p<0.01$) between early EPC/hematopoietic stem cells (HSC) CD34+CD133+ and naturally occurring Tregs CD4+CD25^{high}, as shown in Fig. 2C. This correlation was restricted to this particular immature cells, as more mature EPCs (CD34+VEGFR2+ or CD133+VEGFR2+) failed to show any statistically significant correlation with Treg numbers. Furthermore, this positive correlation pertained solely to the Treg population and not to the entire CD4+ population. Fig. 2D-2F show that the various EPC populations including the CD34+CD133+ cells do not correlate with total CD4+ cells.

In addition, we plotted the corrrelations between the various EPC populations analysed in Fig. 3. As this figure shows, CD34+133+ do not correlate with CD34+KDR or CD133+KDR but the latter two do correlate significantly ($r=0.87$; $p<0.0001$).

Several investigators have shown that T cells are necessary for the development of collateral blood vessels in ischemia models[12,18]. The most likely mechanism contributing to the collateral-enhancing effects of CD4+ T cells appears to reside in their ability to enhance VEGF blood levels. This effect is achieved either by direct production and secretion of VEGF[13,18], or indirectly, by inducing monocyte-macrophage accumulation in the ischemic muscle, which in turn secrete a broad array of cytokines and growth factors, which facilitate collateral development[12].

Whereas some investigators refer to colony forming units as measures for estimating EPC numbers[19,20], others employ flow cytometry for assessment of circulating cells positive for either CD34/KDR[21,22], CD34/133[23] or CD34/CD133/KDR[24]. Given this lack of standardization of EPC methodology, we assessed separately the correlation of several EPC accepted phenotypes (CD34+CD133+, CD133+VEGFR2+, CD34+VEGFR2+) with Tregs, defined by their CD4+CD25^{high} phenotype. We have recently demonstrated that the population of EPCs most likely to associate with circulating VEGF levels is the CD34+KDR and no correlation was evident between these cells and colony forming unit or CD34+CD133+ numbers.

We have found a positive correlation between frequency of early EPCs (CD34+CD133+) and Tregs . This association was found with Tregs and not with total CD4 cell numbers. This relationship might indicate that Tregs deliver signals that either promote EPC recruitment or alternatively ameliorate EPC maturation and thus could play an important role in

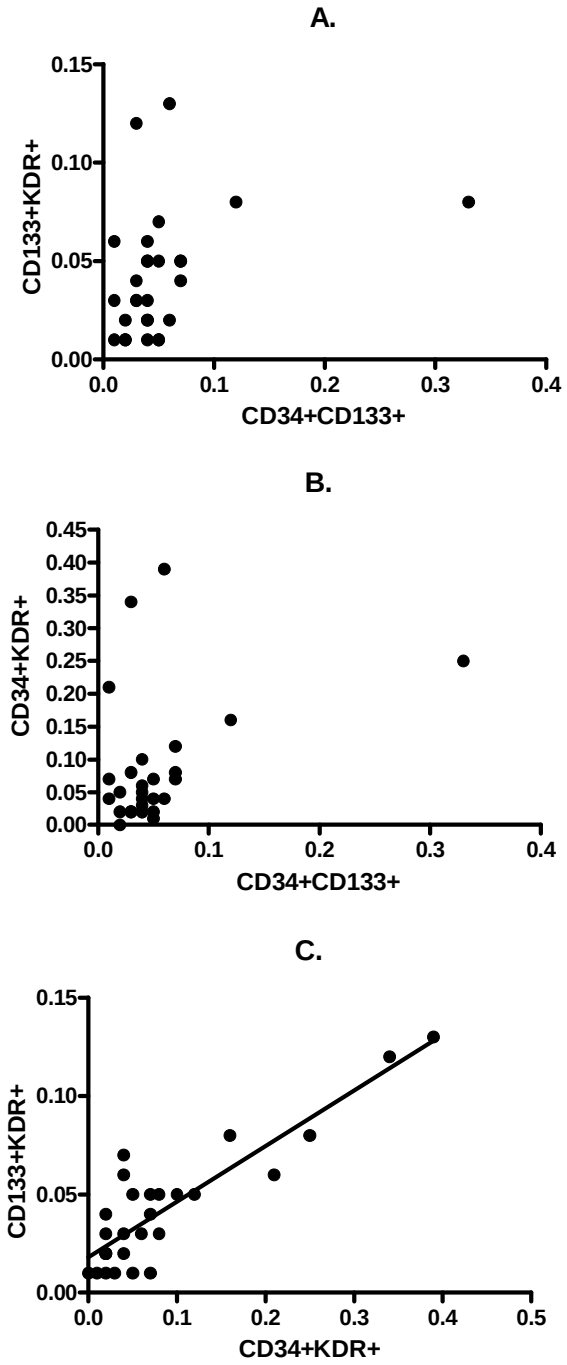


Fig. 3: Inter-correlation of the various EPC populations. Units of EPCs represent the percentage of respective cells out of those in the lymphocyte gate, Fig. A shows CD34+CD133+ vs CD133+KDR+, Fig. B shows CD34+CD133+ vs CD34+KDR+ and Fig. C shows CD34+KDR+ vs CD133+KDR+

modulating angiogenesis. Interestingly, 'mature' EPCs, characterized by CD34+VEGFR2+ or CD133+VEGFR2+ phenotype, were not found to correlate with Treg numbers. The finding that CD34+133+ do not correlate with CD34/KDR or CD133/KDR but the latter two do correlate very significantly, as shown in Fig. 3, is consistent with our recent report[25] and raises the possibility that this early cell does not necessarily transform into mature EPC. Alternatively, signals derived from systemically secreted cytokines/chemokines could intefere with the maturation of CD34+CD133+ into CD34+KDR and subsequently, to mature endothelial cells.

Our study is limited by the relatively small number of subjects and by the difficulties analysing FACS readings of Treg and EPCs given the lack of standardization with respect to both methodologies. Therefore, this association should be further corroborated and defined by various techniques in larger clinical populations and in animal models.

In conlusion, this study demonstrates that a cross-talk could exist between regulatory T cells and endothelial progenitors, a finding that should prompt further research into the complex mechanisms that influence post-natal angiogenesis and vasculogenesis.

*Supported in part by Grants from the Israeli Science Foundation and the Chief Scientist of Israel (JG)

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