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, Tregs 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 CD3+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].

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-I (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-CSF 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...
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 significant 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°C, 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 assessed by several different analyses of all the data, which yielded similar results.

**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
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 correlations 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+KDR[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
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\textsuperscript{[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 interfere 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 analyzing 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 conclusion, 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.

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REFERENCES

1. Sakaguchi, S., 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu. Rev. Immunol., 22: 531-62.
2. O’Garra, A. and P. Vieira, 2004. Regulatory T cells and mechanisms of immune system control. Nat Med., 10: 801-5.
3. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger and G. Schuler, 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J. Exp. Med., 193: 1303-10.
4. Baecher-Allan, C., V. Viglietta and D.A. Hafler, 2004. Human CD4+CD25+ regulatory T cells. Semin. Immunol., 16: 89-98.
5. Wildin, R.S., S. Smyk-Pearson and A.H. Filipovich, 2002. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J.Med.Genet., 39: 537-45.
6. Shevach, E.M., 2002. CD4+ CD25+ suppressor T cells: more questions than answers. Natl. Rev. Immunol., 9: 389-400.
7. Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman and J.M. Isner, 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science, 275: 964-7.
8. Takahashi, T., C. Kalka, H. Masuda, D. Chen, M. Silver, M. Kearney, M. Magner, J.M. Isner and T. Asahara, 1999. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat. Med., 5: 434-8.
9. Rafii, S. and D. Lyden, 2003. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med., 9: 702-12.
10. Urbich, C. and S. Dimmeler, 2004. Endothelial progenitor cells functional characterization. Trends Cardiovasc. Med., 14: 318-22.
11. Aicher, A., A.M. Zeiher and S. Dimmeler, 2005. Mobilizing endothelial progenitor cells. Hypertension, 45: 321-5.
12. Stabile, E., M.S. Burnett, C. Watkins, T. Kinnaird, A. Bachis, A. la Sala, J.M. Miller, M. Shou, S.E. Epstein and S. Fuchs, 2003. Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. Circulation, 108: 205-10.
13. Mor, F., F.J. Quintana and I.R. Cohen, 2004. Angiogenesis-inflammation cross-talk: vascular endothelial growth factor is secreted by activated T cells and induces Th1 polarization. J. Immunol., 172: 4618-23.
14. Vasa, M., S. Fichtlscherer, A. Aicher, K. Adler, C. Urbich, H. Martin, A.M. Zeiher and S. Dimmeler, 2001. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ. Res., 89: E1-7.
15. Adams, V., K. Lenk, A. Linke, D. Lenz, S. Erbs, M. Sandri, A. Tarnok, S. Gielen, F. Emmrich, G. Schuler and R. Hambrecht, 2004. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. Arterioscler. Thromb. Vasc. Biol., 24: 684-90.
16. Baecher-Allan, C., E. Wolf and D.A. Hafler, 2005. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells. Clin. Immunol., 115: 10-8.
17. Mottonen, M., J. Heikkinen, L. Mustonen, P. Isomaki, R. Luukkainen and O. Lassila, 2005. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clin. Exp. Immunol., 140: 360-7.
18. Couffinhal, T., M. Silver, M. Kearney, A. Sullivan, B. Witzenlichler, M. Magner, B. Annex, K. Peters and J.M. Isner, 1999. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE-/- mice. Circulation, 99: 3188-98.
19. Hill, J.M., G. Zalos, J.P. Halcox, W.H. Schenke, M.A. Waclawiw, A.A. Quyyumi and T. Finkel, 2003. Circulating endothelial progenitor cells, vascular function and cardiovascular risk. N. Engl. J. Med., 348: 593-600.
20. George, J., I. Herz, E. Goldstein, S. Abashidze, V. Deutch, A. Finkelstein, Y. Michowitz, H. Miller and G. Keren, 2003. Number and adhesive properties of circulating endothelial progenitor cells in patients with in-stent restenosis. Arterioscler. Thromb. Vasc. Biol., 23: e57-60.
21. Heeschen, C., A. Aicher, R. Lehmann, S. Fichtlscherer, M. Vasa, C. Urbich, C. Mildner-Rihm, H. Martin, A.M. Zeiher and S. Dimmeler, 2003. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood, 102:1340-6.
22. Werner, N., S. Kosiol, T. Schiegk, P. Ahlers, K. Walenta, A. Link, M. Bohm and G. Nickenig, 2005. Circulating endothelial progenitor cells and cardiovascular outcomes. N. Engl. J. Med., 353: 999-1007.
23. Scheubel, R.J., H. Zorn, R.E. Silber, O. Kuss, H. Morawietz, J. Holtz and A. Simm, 2003. Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. J. Am. Coll. Cardiol., 42: 2073-80.
24. Peichev, M., A.J. Naiyer, D. Pereira, Z. Zhu, W.J. Lane, M. Williams, M.C. Oz, D.J. Hicklin, L. Witte, M.A. Moore and S. Rafii, 2000. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood, 95: 952-8.
25. George, J., H. Shmilovich, A. Abashidze, V. Deutsch, H. Miller and G. Keren, 2006. Comparative analysis of methods for assessment of circulating endothelial progenitor cells. Tissue Eng, 12: 331-5.