Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circulation 2007;116;1671-1682; originally published online Oct 1, 2007;
DOI: 10.1161/CIRCULATIONAHA.107.694778
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Identification of a Novel Role of T Cells in Postnatal Vasculogenesis

Characterization of Endothelial Progenitor Cell Colonies

Jin Hur, PhD*; Han-Mo Yang, MD*; Chang-Hwan Yoon, MD*; Choon-Soo Lee, MS; Kyung-Woo Park, MD; Ji-Hyun Kim, MS; Tae-Youn Kim, MS; Ju-Young Kim, MS; Hyun-Jae Kang, MD; In-Ho Chae, MD; Byung-Hee Oh, MD; Young-Bae Park, MD; Hyo-Soo Kim, MD

Background—The colony number of early endothelial progenitor cells (EPCs) has been used as a quantitative indicator of the number of EPCs in the blood or as a biological marker of cardiovascular diseases. In the present study, we found a subset of T cells that were localized at the center of the EPC colony and played a pivotal role in colony formation and differentiation of early EPCs.

Methods and Results—We found that $CD3^+CD31^+CXCR4^+$ T cells (referred to as angiogenic T cells in the present study) constituted the center of EPC colonies during cultures of human peripheral blood mononuclear cells. These angiogenic T cells were required for colony formation and differentiation of early EPCs. They secreted high levels of angiogenic cytokines such as vascular endothelial growth factor, interleukin-8, and matrix metalloproteinases. Angiogenic T cells showed superior angiogenic potential to the other subset of T cells in the experiments with regard to Matrigel tube formation, adhesion, transendothelial migration, and collagen invasion assay, mainly through the stromal cell–derived factor 1/CXCR-4 axis. Furthermore, angiogenic T cells enhanced endothelial cell proliferation and function. In vivo study showed that angiogenic T cells play an important role in the process of vessel formation. Clinical study showed that the level of angiogenic T cells in the peripheral blood was well correlated with EPC colony numbers and had inverse relationships with age and the number of risk factors for coronary artery disease.

Conclusions—These findings suggest that angiogenic T cells could be a potential therapeutic target for ischemic cardiovascular diseases. **(*Circulation*. 2007;116:1671-1682.)**

Key Words: lymphocytes ■ cells ■ angiogenesis ■ cytokines

In 1997, Asahara and colleagues[1] demonstrated that $CD34^+$ hematopoietic progenitor cells from peripheral blood mononuclear cells can differentiate into endothelial lineage cells. Since then, many studies have shown that these cells, termed endothelial progenitor cells (EPCs), play an important role in neovascularization.[2,3] In particular, peripheral blood mononuclear cells cultured in vitro usually form EPC colonies that are called colony-forming unit endothelial cells (CFU-ECs or CFU-EPCs). They consist of a central cluster of round cells and multiple spindle-shaped cells (which are regarded as early EPCs) that emanate from this central cluster.[4–6] Hill and colleagues[4] showed that the number of CFU-EPCs, which was regarded as a quantitative variable for EPCs in the blood, might be a surrogate biological marker for cardiovascular risk and vascular function; however, we and others have shown that early EPCs are a heterogeneous group of cells that most likely enhance angiogenesis by secreting cytokines such as vascular endothelial growth factor (VEGF), interleukin (IL)-8, and matrix metalloproteinase (MMP)-9.[7–11]

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To date, no studies have been conducted that have characterized the heterogeneous cells that compose the early EPC colony and the role of cells other than EPCs in postnatal vasculogenesis. It is well known that hematopoietic cells support proliferation and differentiation of endothelial cells and EPCs during both embryonic vascular development and

Received February 5, 2007; accepted July 27, 2007.

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The online-only Data Supplement, consisting of Methods and figures, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.694778/DC1.

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DOI: 10.1161/CIRCULATIONAHA.107.694778

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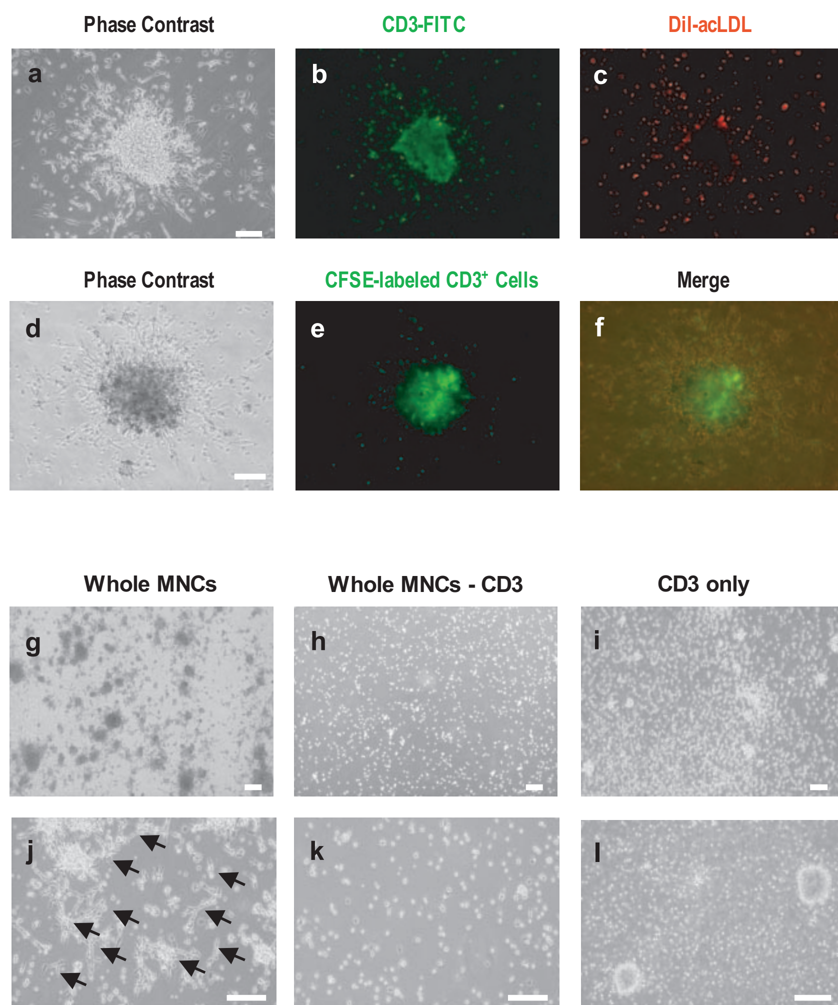

Figure 1. Identification of CD3+ cells localized at the central part of human EPC colonies. A through c, When we stained EPC colonies (a) with various types of surface markers of hematopoietic cells, the central part of EPC colonies was stained mainly with anti-CD3 FITC antibody (b). The remaining part, which shows spindle-shaped cells, was stained with DiI-acLDL (c). Scale bar, 100 μm. d through f, After 7 days of coculture of CD3-depleted MNCs and CFSE-labeled CD3+ cells, CD3+ cells were located at the center of the EPC colony. Scale bar, 100 μm. g through i, After 7 days of culture, the whole-MNC group showed clusters with spindle-shaped EPCs (g), whereas the CD3-depleted MNC group showed neither clusters nor spindle-shaped EPCs (h). On day 10, spindle-shaped EPCs started to appear in the CD3-depleted MNC group (data not shown). CD3+ cells showed only cell clusters (i).
j through l, High magnification of Figure 1g, 1h, and 1i, respectively. Arrow indicates spindle-shaped cells. Scale bar, 200 μm.

angiogenesis.[12] Considering these facts, we hypothesized that the center of EPC colonies may be composed of other hematopoietic cells.

In the present study, we report for the first time that the central cluster of EPC colonies is mainly composed of a specific subpopulation of T cells, $CD3^+CD31^+CXCR4^+$ T cells. We also studied the cell biological characteristics of these T cells and their role in regulating early EPC differentiation and endothelial cell function. Next, we examined the role of angiogenic T cells in in vivo models. Finally, we investigated the clinical implication of these T cells by analyzing the correlation between the level of these T cells and cardiovascular risk factors.

Methods

All materials and methods used for experimental procedures can be found in the online-only Data Supplement.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

The Central Cell Cluster of EPC Colonies Is Mainly Composed of T Cells

On the basis of our hypothesis that a certain type of hematopoietic cell may form the center of EPC colonies, we stained the EPC colony with various hematopoietic cell surface markers (Figure I, online-only Data Supplement). Among these, the strongest positive marker was CD3, a well-known surface marker for T cells (Figure 1a through 1c). The area negative for CD3 was composed mainly of spindle-shaped cells, which were positive for 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate–labeled acetylated low-density lipoprotein (DiI-acLDL), indicating early EPCs. To confirm that the central cluster of EPC colonies is composed of $CD3^+$ cells, we performed coculture of CD3-depleted mononuclear cells (MNCs) and CFSE-labeled $CD3^+$ cells. After 7 days of coculture, we found that $CD3^+$ cells were located at the center of EPC colonies (Figure 1d through 1f).

Next, whole MNCs were divided into 3 groups and were cultivated as follows: intact whole MNCs, CD3-depleted MNCs, and $CD3^+$ T-cell group. On day 7, the whole-MNC group showed cell clusters with sprouting spindle-shaped EPCs (Figure 1g and 1j), but the CD3-depleted MNC group did not (Figure 1h and 1k), which suggests that CD3 cells are required for optimal differentiation of EPCs. On day 10, spindle-shaped cells started to appear in the CD3-depleted MNC group. Interestingly, the $CD3^+$ cell group showed nonadherent round cell clusters but not spindle-shaped EPCs during the entire culture period (Figure 1i through 1l), which indicates that CD3 cells did not directly differentiate into early EPCs.

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$CD3^+CD31^+$ T Cells but Not $CD3^+CD31^-$ T Cells Constitute the Central Cell Cluster of EPC Colonies

To characterize these $CD3^+$ cells in more detail, we performed fluorescence-activated cell sorter analysis and characterized these cells using various markers at multiple time points (Figure 2a). In the case of CD34, CD133, the kinase insert domain-containing receptor (KDR), and vascular endothelial cadherin (VE-cadherin), the fraction of cells that were positive for these markers was so small that it would be unlikely that these cells would make up the entire center of EPC colonies; however, the $CD31^+$ fraction was relatively high and was maintained during culture. On the basis of these results, we stained the colonies again and found that the central clusters of EPC colonies were positive for CD31 and for CD4 and CD8 (Figure 2b).

To isolate $CD3^+CD31^+$ T cells, we performed multistep magnetic activated cell sorting (Figure II, online-only Data Supplement). After CD3 selection with magnetic activated cell sorting, we separated $CD3^+$ cells into 2 groups using CD31 antibody and magnetic beads: $CD3^+CD31^+$ double-positive (DP) T cells and $CD3^+CD31^-$ single-positive (SP) T cells. When we performed confocal microscopy, CD31 was evenly expressed on the surface of a DP cell but not on the surface of an SP cell (Figure III, online-only Data Supplement).

After isolation, we compared DP cells with SP cells. When we cultivated these DP and SP cells separately, DP cells started to form multiple round cell clusters, whereas SP did not form any clusters (Figure 2c). To determine whether DP cells could incorporate into the preformed EPC clusters, we added CFSE-labeled DP or SP cells onto whole MNCs that were already cultivated. After 1 day, DP cells incorporated into the center of preformed EPC colonies, whereas SP cells did not (Figure 2d). In another experiment in which CFSE-labeled DP cells were cocultured with DP-depleted entire MNCs, DP cells were located at the center of EPC colonies on day 7 (Figure 2e).

$CD3^+CD31^+$ T Cells Are Required for EPC Colony Formation and Early EPC Differentiation

To assess the essential role of DP cells in EPC colony formation, we compared colony formation between intact whole MNCs and DP-depleted whole MNCs. After 5 days of culture, in the whole-MNC group, we observed colonies with spindle-shaped EPCs; however, the DP-depleted group did not show any colony or spindle-shaped EPCs (Figure 3a). Interestingly, when we added DP cells to DP-depleted MNCs, we observed the formation of EPC colonies, which suggests that DP cells can rescue the EPC cluster-forming capability (data not shown). On day 10, spindle-shaped EPCs appeared in DP-depleted MNCs. Their number, however, was significantly fewer than that in the whole-MNC group, which suggests that DP cells are necessary for early EPC differentiation.

The time of first appearance of spindle-shaped EPCs was significantly delayed in the DP-depleted group compared with the whole-MNC group by 5 days (Figure 3b). Moreover, levels of KDR and VE-cadherin, differentiation markers of EPCs, were significantly decreased in DP-depleted MNCs (Figure 3c and 3d). Matrigel tube formation assay with human umbilical vein endothelial cells (HUVECs) showed that the DP-depleted group lacked functional differentiation compared with the whole-MNC group (Figure 3e). These results suggest that DP cells play a very important role in early EPC differentiation. From this point on, we refer to DP cells as "angiogenic T cells."

$CD3^+CD31^+$ T Cells Show Better Proangiogenic Characteristics Than $CD3^+CD31^-$ T Cells

We compared the immunologic and angiogenic features of DP cells with those of SP cells. The range of percentage of DP cells among T cells was from 33% to 61%, and the mean value was ≈46% in normal volunteers (n=8). Forty-four percent of these DP cells were $CD4^+$, and 46% were $CD8^+$. Because stromal cell–derived factor 1 (SDF-1) has an important role in the homing and migration of hematopoietic stem cells into the ischemic region,[13] we compared the expression of CXCR4, a receptor for SDF-1. Most DP cells expressed CXCR4, whereas only a small portion of SP cells expressed CXCR4 (DP versus SP, 97.6% versus 37.6%).

Levels of various proangiogenic cytokines in the culture supernatants were compared between DP and SP cells. Whereas SP cells secreted minimal levels of cytokines, DP cells secreted much higher levels of proangiogenic cytokines such as VEGF, IL-8, IL-17, and granulocyte colony–stimulating factor (Figure 4a). Moreover, DP cells secreted higher levels of MMP-9, which is known to play an important role in angiogenesis, than did SP cells, whereas DP and SP cells equally secreted MMP-2 (Figure 4b).[14,15]

Next, we investigated functional differences between DP and SP cells in their interaction with endothelial cells, such as capillary tube formation, adhesion, transendothelial migration, and collagen invasion. Tube formation assay on GFR growth factor reduced Matrigel matrix showed that DP cells incorporated with HUVECs and helped HUVECs form significantly more complete capillary tubes than SP cells (Figure 4c and 4d). Adhesion assay showed that DP cells had a higher capacity for adhesion on HUVECs than SP cells (Figure 4e). In a transendothelial migration assay, DP cells migrated more than SP cells toward SDF-1 in the lower chamber. Blocking of CXCR4 with a neutralizing antibody significantly decreased the extent of migration, which suggests that transendothelial migration of DP cells depends on the SDF-1/CXCR4 axis (Figure 4f).

To assess both transendothelial migration and invasion capacity, we performed a modified vertical collagen gel assay (Figure IVa, online-only Data Supplement). Confocal microscopy showed DP cells transmigrating through the HUVEC monolayer (Figure IVb, online-only Data Supplement). DP cells showed a greater collagen invasion capacity toward SDF-1 than SP cells (Figure IVc, online-only Data Supplement), which was neutralized by blocking antibody against CXCR4 (Figure IVd, online-only Data Supplement).

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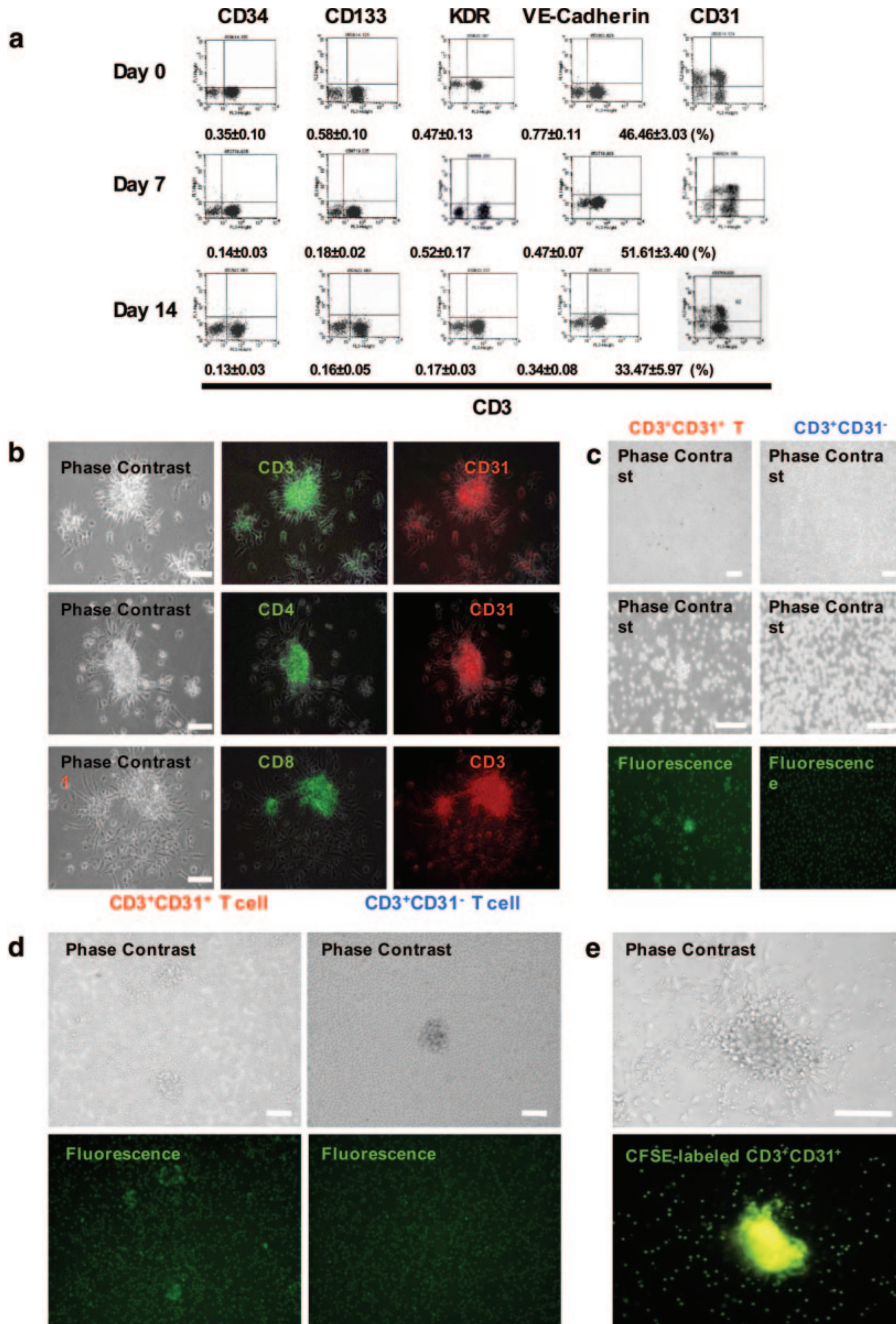

Figure 2. $CD3^+CD31^+$ DP T cells, but not $CD3^+CD31^-$ SP T cells, constitute the central part of human EPC colonies. a, Serial changes in each surface marker in $CD3^+$ T cells when MNCs were cultivated in endothelial cell growth media. Except for CD31, the fraction of other marker-positive cells was so small that these cells would be unlikely to form the entire center of EPC colonies. The values in the graph represent the percentage of each type of marker-positive cells in each gated $CD3^+$ T-cell subset. The horizontal axis shows CD3 positivity, and the vertical axis shows the positivity of each marker. Data are presented as mean±SEM (n=8). b, Immunofluorescence staining of CD3, CD4, CD8 (green), and CD31 (red) in EPC colonies. The center of the EPC cluster was stained with CD4, CD8, and CD31. $CD31^+$ area appeared more diffuse than $CD3^+$ area due to the surrounding EPCs, which were also positive for CD31. Scale bar, 100 μm. c through e, To confirm that $CD3^+CD31^+$ DP T cells localize at the center of the EPC colony, we performed 3 different

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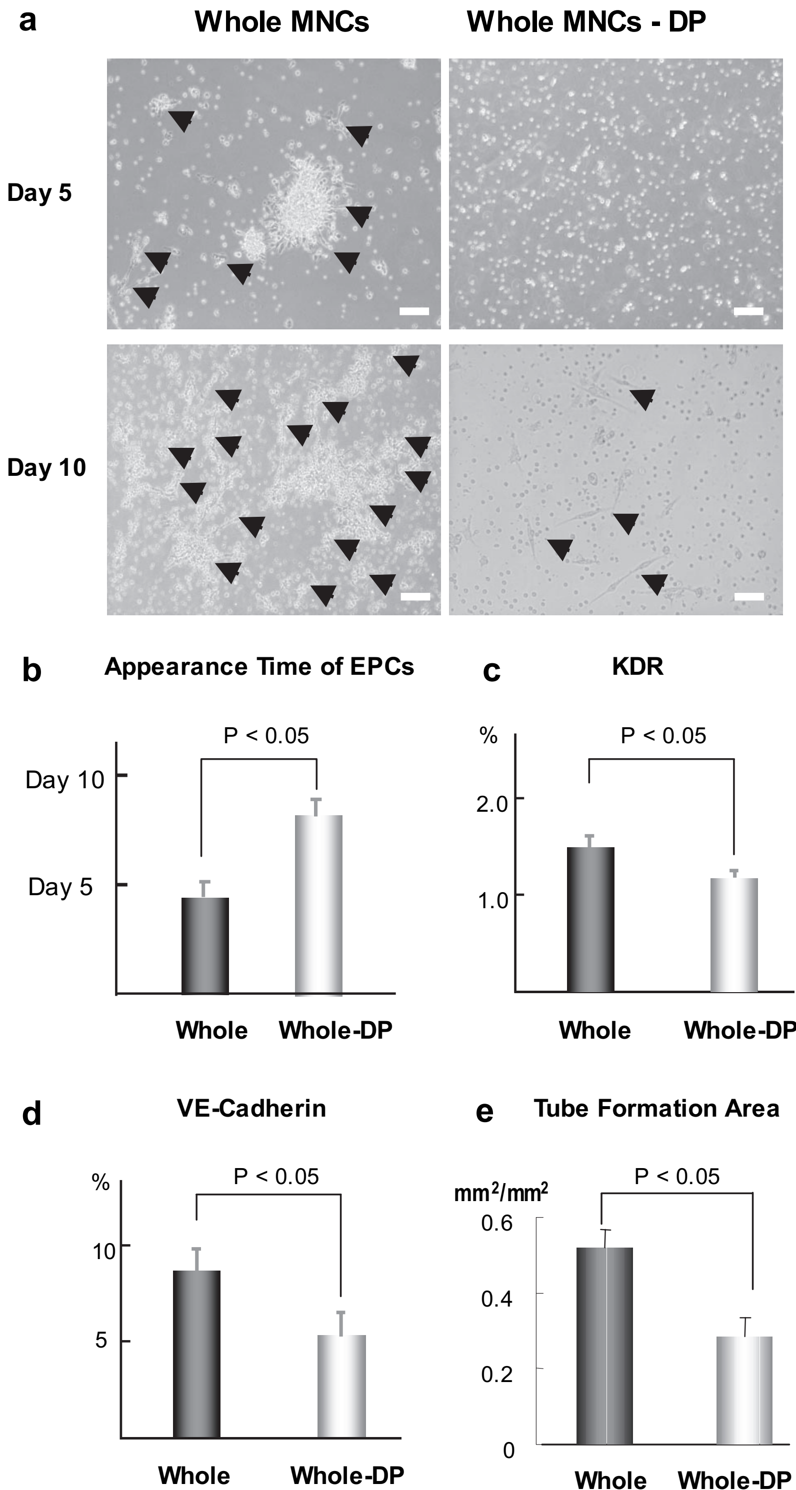

Figure 3. Effects of $CD3^+CD31^+$ (DP) T cells on EPC differentiation. a, We compared DP-depleted whole MNCs with intact whole MNCs with the same cell number. After 5 days of culture, in the whole-MNC group, we observed a colony with spindle-shaped EPCs; however, in the DP-depleted group, we did not see any colony with spindle-shaped EPCs (upper panel). On day 10 of cultivation, in the whole-MNC group, we saw more spindle-shaped cells. In the DP-depleted group, we saw fewer spindle-shaped cells (lower panel). Arrows indicate spindle-shaped EPCs. Scale bar, 100 μm. b, To quantify this, we examined time to first appearance of spindle-shaped EPCs. The appearance of spindle-shaped cells was delayed in DP-depleted MNCs by 5 days compared with intact whole MNCs. Data are presented as mean±SEM (n=5). c and d, In fluorescence-activated cell sorter analysis of surface markers such as KDR (c) and VE-cadherin (d), which are known to be differentiation markers of EPCs, the level of such surface molecules decreased in DP-depleted MNCs compared with whole MNCs. Data are presented as mean±SEM (n=5). e, Tube formation assay using coculture with HUVECs showed that depletion of DP cells decreased the ability of tube formation compared with the whole MNCs. Data are presented as mean±SEM (n=5).

Figure 2 (Continued). experiments. c, Comparison between $CD3^+CD31^+$ DP cells and $CD3^+CD31^-$ SP cells. Areas in upper panels are magnified in middle panels. When we cultivated these CFSE-labeled DP and SP cells separately in endothelial cell growth media, DP cells started to form multiple round cell clusters, whereas SP cells did not form any clusters. Lower panels are fluorescence photomicrographs of middle panels that show CFSE-labeled cells (green). Scale bar, 200 μm. d, One day after the addition of CFSE-labeled DP or SP cells to whole MNCs already cultured, DP cells could incorporate into the center of the preformed EPC colony, but SP cells could not. Lower panels show fluorescence photomicrographs of upper panels. Scale bar, 100 μm. e, After coculture with DP cell-depleted whole MNCs, CFSE-labeled DP cells were located at the center of EPC colonies on day 7. Scale bar, 100 μm. From these results, we concluded that $CD3^+CD31^+$ DP cells form the center of EPC colonies among $CD3^+$ cells.

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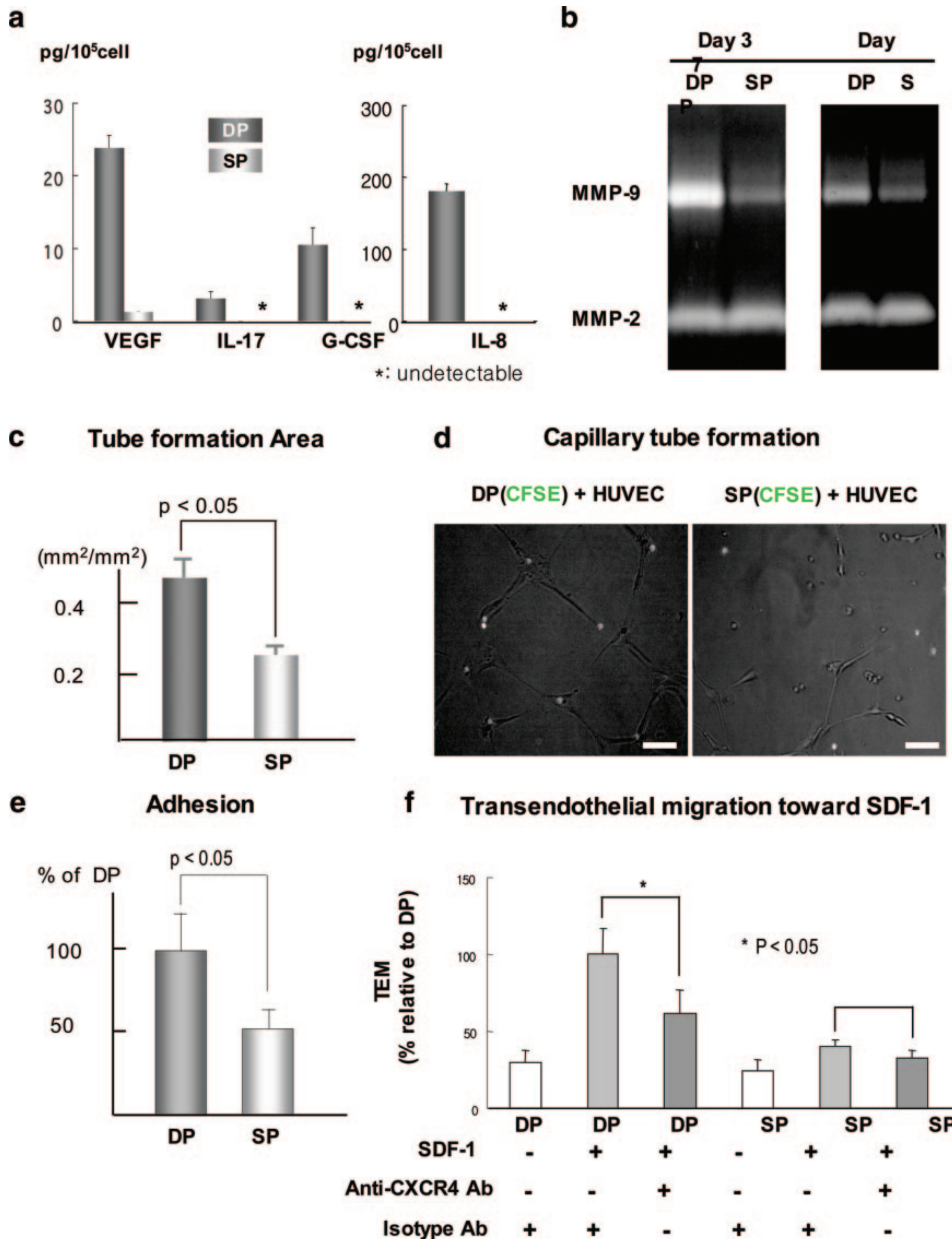

Figure 4. Comparison between DP cells and SP cells. a, Cytokine level of IL-8, VEGF, IL-17, and granulocyte colony–stimulating factor (G-CSF) secreted from either DP cells or SP cells. Whereas SP cells secreted the minimal level of cytokines, DP cells secreted a much higher level of proangiogenic cytokines. b, Moreover, gelatin zymography showed that DP cells expressed an increased level of MMP-9, which is known to play an important role in angiogenesis. c and d, When we performed a tube formation assay on Matrigel with the coculture of either DP cells or SP cells (both are labeled with CFSE) and HUVECs, DP cells showed significantly more complete tube formation with HUVECs than SP cells (c). Interestingly, DP cells could not incorporate into the tube itself but attached to the tube formed by HUVECs (d). Scale bar, 50 μm. e, Adhesion assay showed that DP cells had greater adhesion capacity than SP cells. Data are presented as mean±SEM (n=5). f, Result of transendothelial migration (TEM) assay. DP cells in the upper chamber showed more migration through the endothelial monolayer toward SDF-1 in the lower chamber. Overall, DP cells showed a greater capability for transendothelial migration than SP cells. When we blocked CXCR4 using blocking antibody (Ab), the extent of migration decreased, which suggests that transendothelial migration of DP cells depends on the SDF-1/CXCR4 axis.

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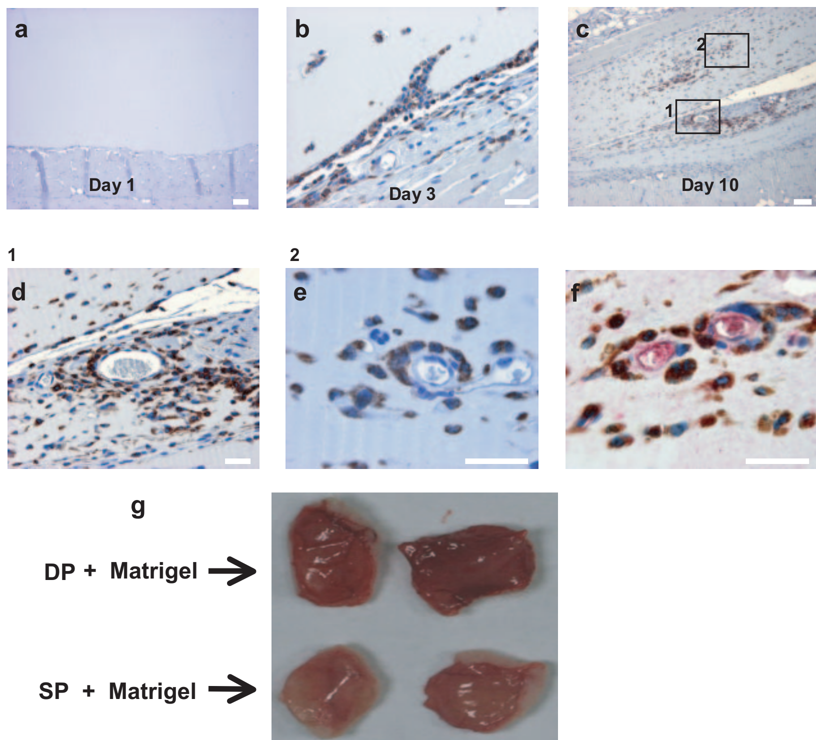

Figure 5. In vivo angiogenic capacity of angiogenic T cells. We first performed Matrigel plug assay in vivo by injecting a mixture of Matrigel and SDF-1 into subcutaneous tissue of normal BALB/c mouse. a through c, Serial representative figures of each time point are shown. a, On day 1, no cell infiltration was present. Scale bar, 100 μm. b, On day 3, some MNCs infiltrated into the Matrigel plug. Immunohistochemical staining for CD3 (brown) showed that among these cells, some $CD3^+$ cells were present. Scale bar, 50 μm. c, On day 10, in the Matrigel plug, many cells and some vessel-like structures were present. Scale bar, 100 μm. d, Higher magnification of area in (c) showed that $CD3^+$ cells (brown) surrounded the vessel-like structures. Scale bar, 50 μm. e, Another higher magnification of a vessel-like structure. $CD3^+$ cells (brown) surrounded the vessel-like structure. On the luminal side of endothelial cells, some cells like red blood cells were present. Scale bar, 50 μm. f, Double-immunohistochemical staining in an adjacent section of the same tissue shown in (e) revealed that $CD3^+$ cells (brown) were also stained with reddish CD31. Endothelium-lining area was also stained with reddish CD31. These results suggest that the cells surrounding the vessel-like structure might be $CD3^+CD31^+$ angiogenic T cells. Scale bar, 50 μm. g, After mixing Matrigel and DP or SP cells, we injected Matrigel plug into subcutaneous tissue of nude mouse. DP-injected plugs show more reddish gross appearance than SP-injected plugs.

$CD3^+CD31^+$ T Cells Enhance Proliferation, Migration, and Capillary Tube Formation of Endothelial Cells

Neovascularization includes not only recruitment of EPCs (vasculogenesis) but also proliferation of preexisting endothelial cells (angiogenesis). To evaluate the effects of cytokines secreted by DP and SP cells on proliferation of endothelial cells, we performed a WST-1 assay using conditioned medium of both cells. We found that DP-conditioned media significantly increased proliferation compared with SP-conditioned media (Figure Va, online-only Data Supplement). When we added a mixture of neutralizing antibodies against IL-8 and VEGF, this proliferation decreased significantly. In addition, DP-conditioned media enhanced the migration of endothelial cells and capillary tube formation more than SP-conditioned media, and this result was attenuated by neutralizing antibodies against IL-8 and VEGF (Figure Vb and Vc, online-only Data Supplement).

Angiogenic T Cells Participate in New Vessel Formation In Vivo

To investigate the role of angiogenic T cells in angiogenesis in vivo, we performed Matrigel plug assay by injecting a mixture of Matrigel and SDF-1 into the subcutaneous tissue of normal BALB/c mice. On day 3, a few MNCs infiltrated into the Matrigel plug (Figure 5b), which included T cells as indicated by brown $CD3^+$ cells. On day 10, the number of infiltrating MNCs increased with new vessel-like structures (Figure 5c), and with meticulous observation, it could be seen that $CD3^+$ cells surrounded the vessel-like structures (Figure 5d and 5e). Double immunohistochemistry showed that the $CD3^+$ cells were also positive for CD31 (Figure 5f), which suggests that these DP angiogenic T cells participate in new vessel formation in vivo. To compare the angiogenic capacity of DP versus SP cells in vivo, we performed another experiment in which we introduced either DP or SP cells in Matrigel plug into nude mice. Plugs with DP cells showed more new vessel formation than those with SP cells, as shown by their more vascularized and reddish gross appearance (Figure 5g).

Angiogenic T Cells Can Restore the Impaired Angiogenic Capacity of Nude Mice

To examine whether angiogenic T cells can rescue the impaired angiogenic potential of nude mice, we tested these cells in a hind limb ischemia model. Mice were randomly assigned to 1 of 4 groups: media-injected normal BALB/c mice, media-injected nude mice, DP-injected nude mice, and SP-injected nude mice. The group of nude mice showed much-delayed recovery of blood flow in ischemic hind limb compared with normal BALB/c mice (Figure 6a and 6b). Nude mice receiving DP cells systemically showed a higher recovery of blood flow than those receiving SP cells or media. In addition, capillary density increased markedly in DP-injected mice compared with SP- or media-injected mice (Figure 6c). In contrast, transplantation of SP cells could not improve the recovery of blood flow compared with control nude mice injected with media. When we evaluated the tissue of ischemic hind limb in DP-injected mice, we found that the level of SDF-1 in the perivascular area increased and that CFSE-labeled DP cells were located in areas where SDF-1

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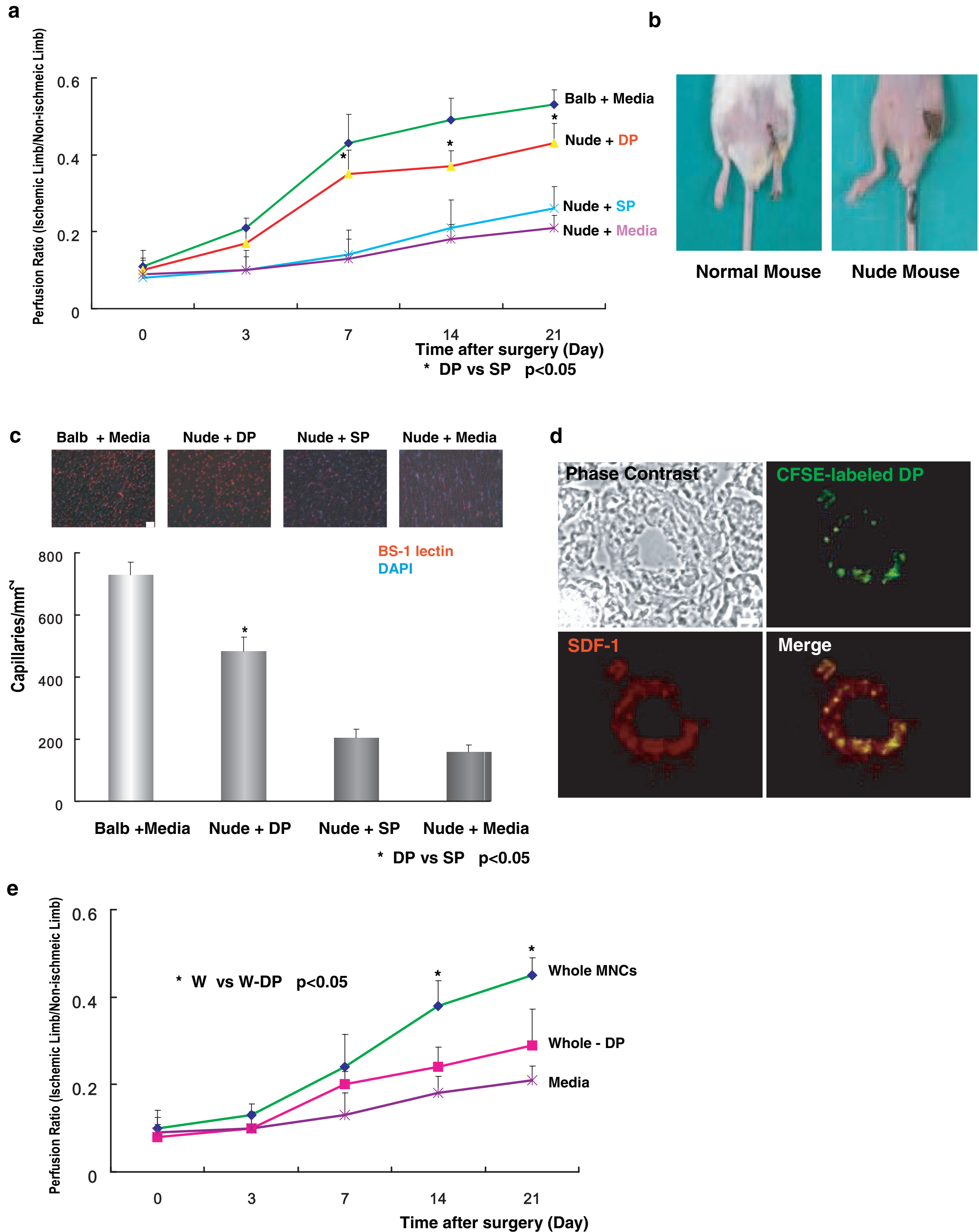

Figure 6. In vivo angiogenic capacity of angiogenic T cells. a, Quantitative analysis of perfusion recovery by laser Doppler perfusion analysis. Hindlimb ischemia was created in each group, and 1×10^6 CFSE-labeled cells were delivered systemically by intracardiac injection. Media-injected nude mice showed much-delayed recovery of blood flow compared with normal BALB/c mice. Nude mice receiving DP cells showed a significant improvement in blood flow recovery to ischemic hindlimb compared with nude mice injected with SP cells or media. In contrast, SP injection in nude mice showed little effect on recovery compared with media-injected nude mice. n=10 for each group. These results suggest that, in contrast to SP cells, angiogenic T cells can restore the impaired angiogenic

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was strongly expressed (Figure 6d). To confirm the in vivo angiogenic potential of angiogenic T cells, we compared angiogenic potential between whole MNCs and DP cell-depleted MNCs after 7 days' culture. The capability for blood flow recovery by whole MNCs was significantly impaired by depletion of angiogenic T cells from MNCs (Figure 6e), which suggests that the depletion of angiogenic T cells impairs angiogenic potential in vivo.

Angiogenic T Cells Are Correlated With the Number of CFU-EPCs and Are Inversely Correlated With Cardiovascular Risk Factors

To evaluate the clinical correlation of angiogenic T cells with CFU-EPCs, we counted the number of angiogenic T cells and the number of CFU-EPCs simultaneously in 40 healthy volunteers. As the level of angiogenic T cells in blood increased, the number of cultured CFU-EPCs increased (Figure 7a). Interestingly, an inverse correlation existed between angiogenic T cells and age (Figure 7b).

To evaluate the clinical relevance of angiogenic T cells to cardiovascular risk, we analyzed the relationship between cardiovascular risk factors and the percentage of angiogenic T cells in peripheral blood MNCs in 58 subjects, including patients who underwent coronary angiography. We found that the percentage of angiogenic T cells decreased as Framingham risk scores increased (Figure 7c). In patients with many risk factors, however, the relationship was not evident.

Discussion

The present study demonstrated that human $CD3^+CD31^+$ $CXCR4^+$ T cells constitute the central cluster of EPC colonies. This subset of T cells, termed "angiogenic T cells," was critical in the formation of EPC colonies. Furthermore, angiogenic T cells enhanced the differentiation of early EPCs and potentiated the function of both early EPCs and endothelial cells by secreting angiogenic cytokines. Moreover, in vivo studies showed that angiogenic T cells play an important role in the process of new vessel formation. In addition, the number of angiogenic T cells in blood was correlated with the number of EPC colonies cultured from human peripheral blood and was significantly influenced by donor's age and cardiovascular risk factors.

T Cells Localized at the Center of the EPC Colony

Asahara et al[1] described that EPC colonies were composed of round cells clustered at the center and sprouting spindle-shaped cells at the periphery. Moreover, the EPC colony or CFU-EPCs recently have been used as a quantitative variable for EPCs in peripheral blood and have been regarded as a cardiovascular prognostic factor.[4,5] Considering the growing importance of the CFU-EPC in cardiovascular research, we investigated the characteristics of the EPC colony in detail. We expected that the central cluster might contain hematopoietic lineage cells, and we found that the central round cells mainly consisted of a specific subset of T cells ($CD3^+CD31^+CXCR4^+$). Furthermore, we proved that this subset of T cells was necessary for the formation of an EPC colony by depleting or adding these T cells during EPC culture, which suggested an important role of T cells in early EPC differentiation.

A Novel Role of T Cells in Early EPC Differentiation and Angiogenesis

Stabile et al[16,17] recently reported that T cells regulated angiogenesis in vivo. After they induced hind limb ischemia in mice, they observed that $CD4^+$ and $CD8^+$ T cells infiltrated into the ischemic tissue. They suggested that VEGF and macrophage recruitment controlled by T cells was the mechanism by which T cells affect angiogenesis. We evaluated the role of T cells not only in early EPC differentiation during culture but also in endothelial function in vitro for the first time in the present study. These results implied that the subset of T cells might be important regulators in postnatal vasculogenesis and angiogenesis, not just incidental bystanders.

Theoretically, we can divide the process of neovascularization into multiple steps, such as EPC mobilization, its homing to the ischemic zone via adhesion, transendothelial migration and invasion, and then formation of new vessels by EPCs and preexisting endothelial cells.[18] From the results of the present study, we can postulate that angiogenic T cells expressing high levels of CXCR4 home to areas of ischemia where SDF-1 level is high. Then, these angiogenic T cells may easily attach to the endothelial cells, transmigrate through the endothelial junction using CD31, and then invade the ischemic tissue using MMP-9. Angiogenic T cells are supposed to facilitate early EPC differentiation and stimulate the local resident endothelial cells by secreting angiogenic cytokines such as VEGF and IL-8. Angiogenic T cells could enhance neovascularization by orchestrating all these processes together (Figure VI, online-only Data Supplement).

$CD31^+$ Was a Specific Marker for T Cells With Vasculogenic Potential

We demonstrated that $CD31^+$ T cells showed better angiogenic capacity than other subsets of T cells. CD31 (also known as platelet/endothelial cell adhesion molecule-1, or PECAM-1) is not a specific marker of endothelial cells. It is also expressed on leukocytes and platelets[19] and is known to modulate leukocyte transmigration and angiogenesis.[20,21]

Figure 6 (Continued). capacity of nude mice. b, Representative pictures of each group. Left picture shows intact hindlimb in normal BALB/c mouse, whereas right picture shows amputated hindlimb of nude mouse. c, Consistent with the results of blood flow recovery in each group, capillary density was markedly increased in DP-injected mice compared with either SP-injected or media-injected mice. Scale bar, 100 μm. d, The tissue of ischemic hindlimb of DP-injected nude mice showed that the level of SDF-1 (red) was high in the perivascular area where CFSE-labeled DP cells (green) were located. Scale bar, 50 μm. e, We compared angiogenic potential between whole MNCs (W) and DP cell-depleted MNCs (W-DP) after 7 days' culture. The capability of blood flow recovery by whole MNCs was significantly impaired by depletion of angiogenic T cells from MNCs, which suggests that depletion of angiogenic T cells impairs angiogenic potential in vivo. n=10 for each group.

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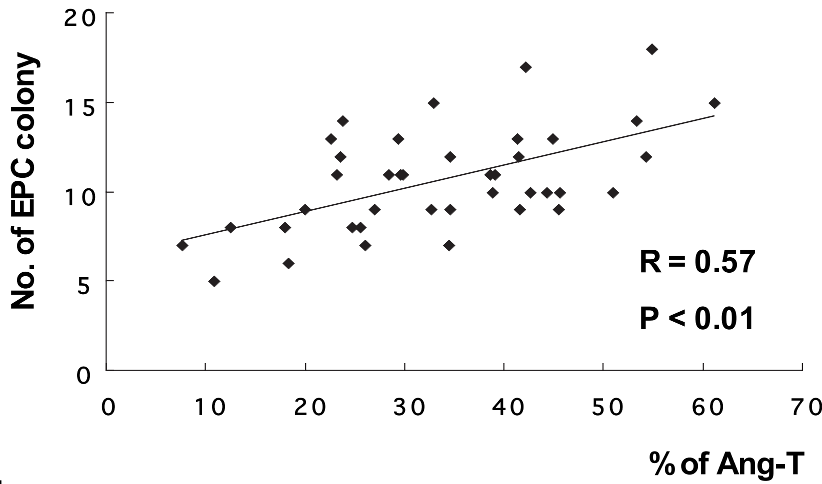
a



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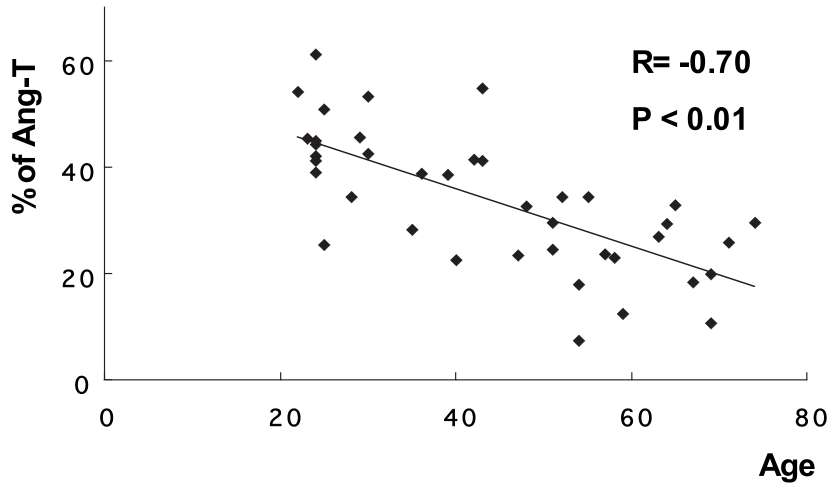

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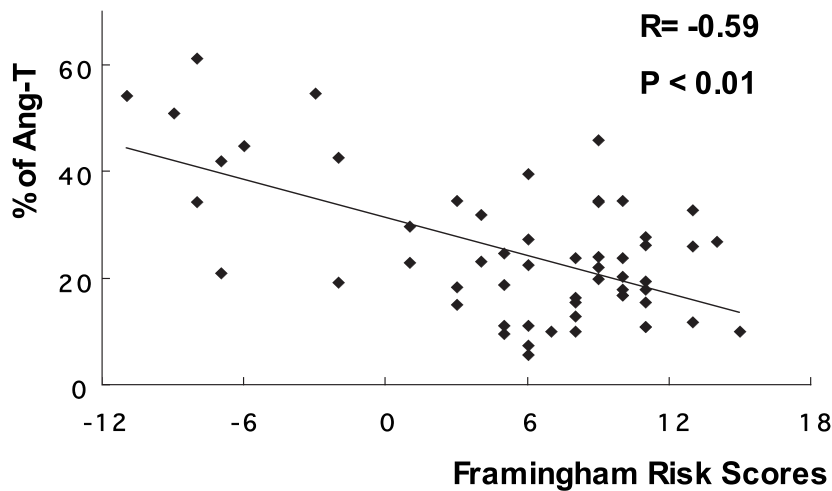

Figure 7. Clinical relevance of angiogenic T cells. a, Association between angiogenic T cells and EPC colonies. After isolating MNCs from 40 normal volunteers, we examined the relation between angiogenic T cells and the number of EPC colonies after 7 days of culture. As the level of angiogenic T cells increased, the number of CFU-EPCs increased. b, Association between angiogenic T cells (Ang-T) and age. An inverse relationship existed between angiogenic T cells and age in normal volunteers. n=40. c, Relation between angiogenic T cells and cardiovascular risk. As Framingham risk score increased, the percentage of angiogenic T cells in the peripheral blood decreased in patients with coronary artery disease. The percentage of angiogenic T cells indicates the fraction of both $CD3^{+}$ and $CD31^{+}$ cells in the lymphogate for fluorescence-activated cell sorter analysis. n=58.

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Taken together, these facts suggest that $CD31^+$ T cells may easily interact with or pass through endothelial cells via CD31-mediated interaction in contrast to $CD31^-$ T cells. Moreover, in the process of passage, the intracellular signal pathway of these angiogenic T cells might be activated by CD31 and thus show an enhanced angiogenic effect.[22]

Angiogenic T Cells as a Potential Biomarker in Cardiovascular Disease

Hill et al[4] previously showed an inverse relationship between the number of EPC colonies and cardiovascular risk factors. In the present study, we found that angiogenic T cells in peripheral blood were well correlated with EPC colonies and appeared to reflect the number of EPC colonies. Furthermore, we found an inverse relationship of angiogenic T cells with age and cardiovascular risk factors, although patients with multiple risk factors showed a lesser correlation. Therefore, angiogenic T cells may be used as a new biological marker for cardiovascular disease and endothelial function.

The CFU-EPC in vitro assay is the standard method to quantitate the number of early EPCs in the circulating blood. However, colony formation in vitro is influenced by angiogenic T cells; therefore, in future studies, a CFU-EPC assay should be performed and evaluated in association with angiogenic T cells.

Other Considerations

One question that can be raised is by which mechanism angiogenic T cells facilitate early EPC differentiation. We could consider 2 mechanisms: One is a paracrine effect by proangiogenic cytokines, and the other is cell-to-cell direct interaction between angiogenic T cells and EPCs. The present study showed that angiogenic T cells produce multiple proangiogenic cytokines. During EPC culture, we routinely observed the phenomenon that spindle-shaped EPCs first emerged only around the EPC colonies where a high-concentration state of angiogenic cytokines by angiogenic T cells could be formed first.

These angiogenic T cells might have other roles in different situations, such as atherosclerosis. For example, Caligiuri et al[23,24] showed that $CD31^+$ T cells play an important regulatory role in the pathogenesis of plaque thrombosis and the development of aortic aneurysm.

In conclusion, the present study reports for the first time the presence of a novel subpopulation of T cells that constitute the central cell cluster of EPC colonies and that can enhance EPC differentiation and angiogenesis, resulting in neovascularization in vivo. Considering the clinical relevance of angiogenic T cells, it may be of interest to investigate the feasibility of these cells as an important surrogate marker of cardiovascular risk factors and as a therapeutic target for patients with ischemic heart disease.

Sources of Funding

The present study was supported by grants from the program of National Research Laboratory for Cardiovascular Stem Cells, KOSEF (M10600000230-06J0000-23010), and the Stem Cell Research Center (SC3150), Republic of Korea.

Disclosures

None.

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CLINICAL PERSPECTIVE

It is known that the number of endothelial progenitor cell colonies is related not only to the level of endothelial progenitor cells but also to cardiovascular prognosis. The present study demonstrated that the level of angiogenic T cells correlated significantly with the number of EPC colonies. Moreover, angiogenic T cells showed a significant inverse correlation with age and cardiovascular risk factors. Therefore, angiogenic T cells could be used as a novel putative biological marker for cardiovascular disease and vascular function. In addition, it would be very helpful to increase the number of angiogenic T cells locally in the ischemic region in patients with ischemic heart disease. There might be other roles for angiogenic T cells. In terms of stem cell biology, angiogenic T cells could facilitate differentiation of other stem cells by secretion of cytokines or by direct cell-to-cell interaction. In addition, recent studies have shown that T cells might be very important in the initiation and development of tumors such as breast cancers, colorectal cancers, and ovarian cancers through the stromal cell–derived factor 1/CXCR4 axis. Because angiogenic T cells may participate in this tumor angiogenesis, angiogenic T cells could be good therapeutic targets for cancer therapy. Given these facts, further studies of angiogenic T cells could have a considerable clinical impact.

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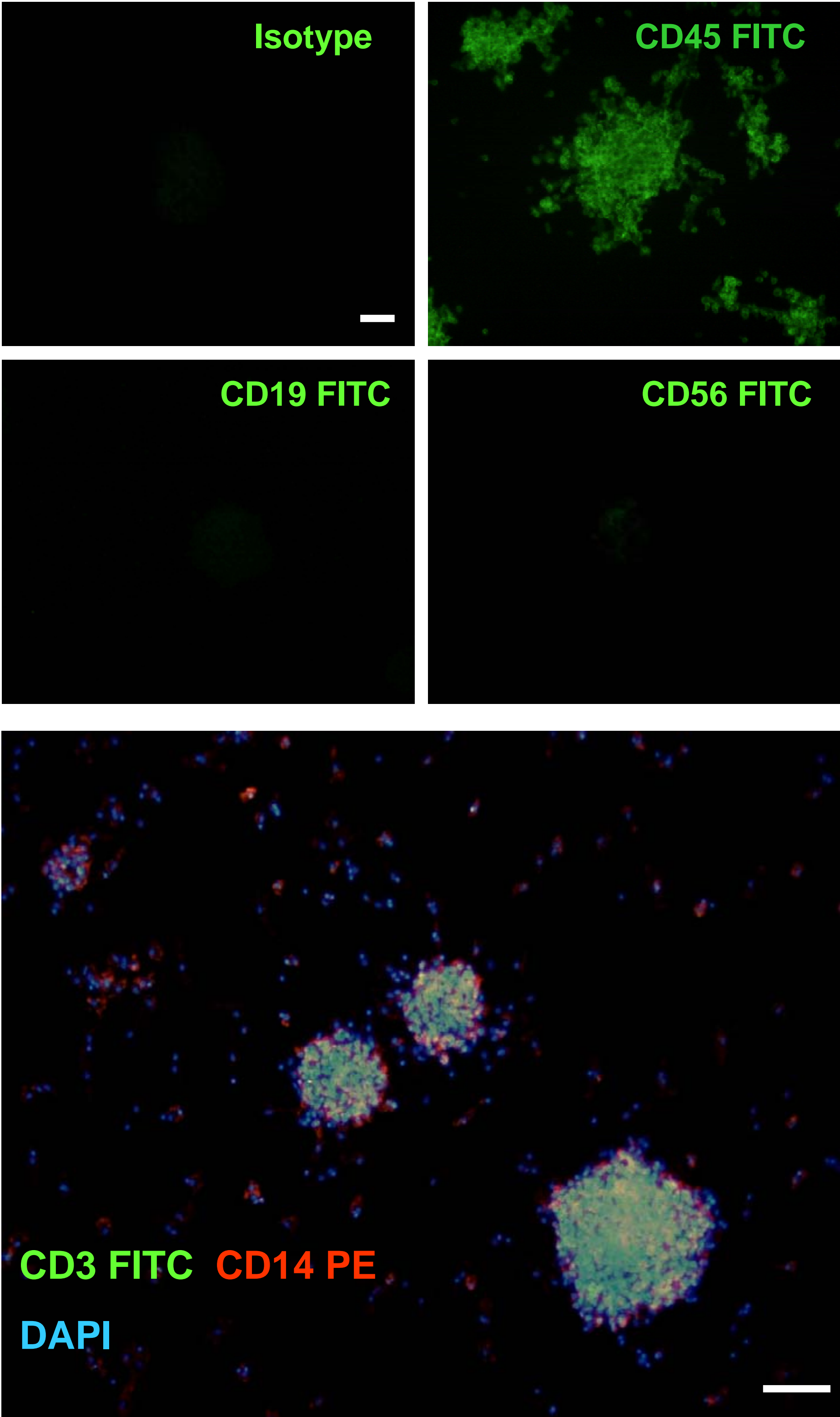
Supplemental Figure 1



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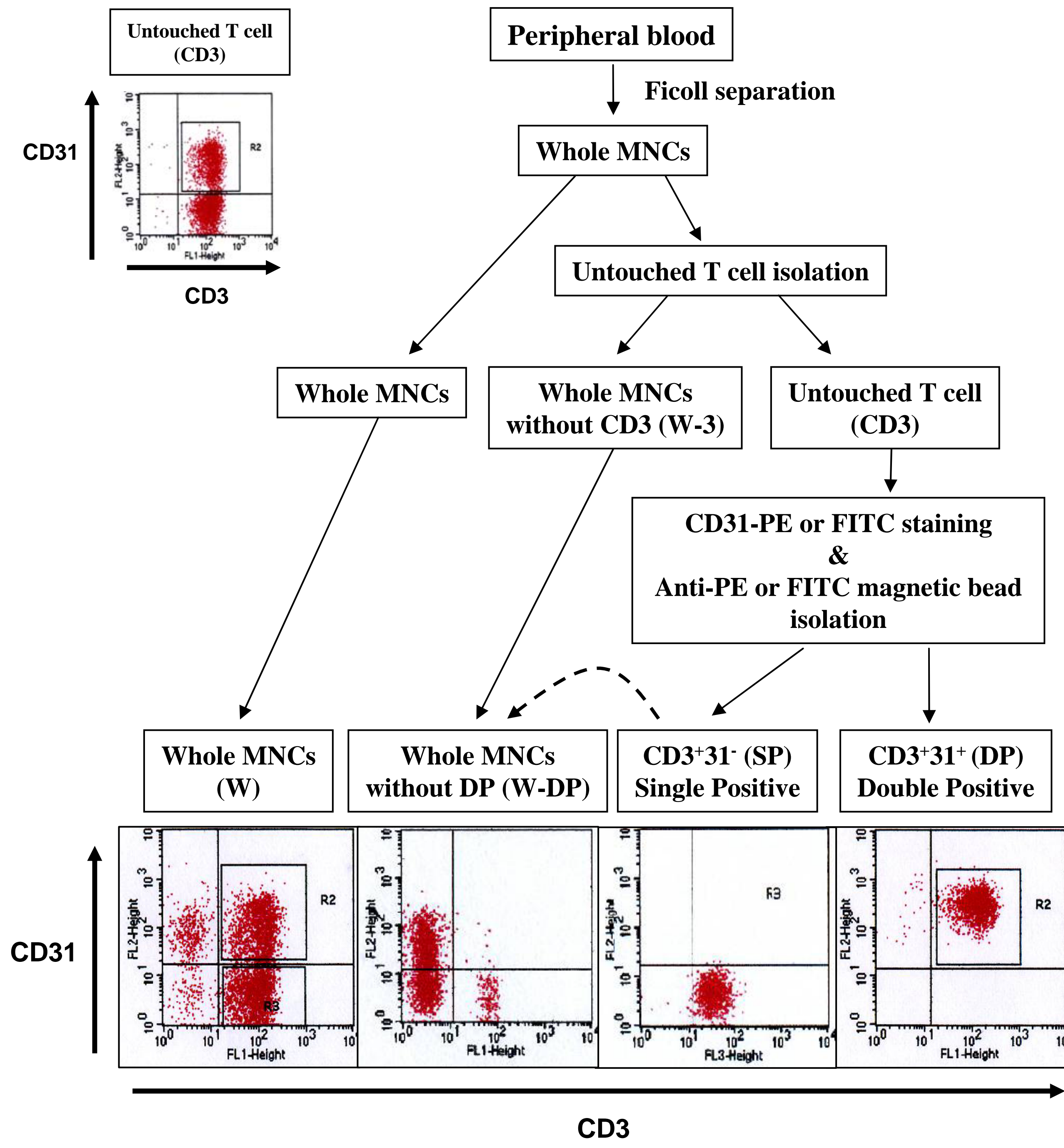
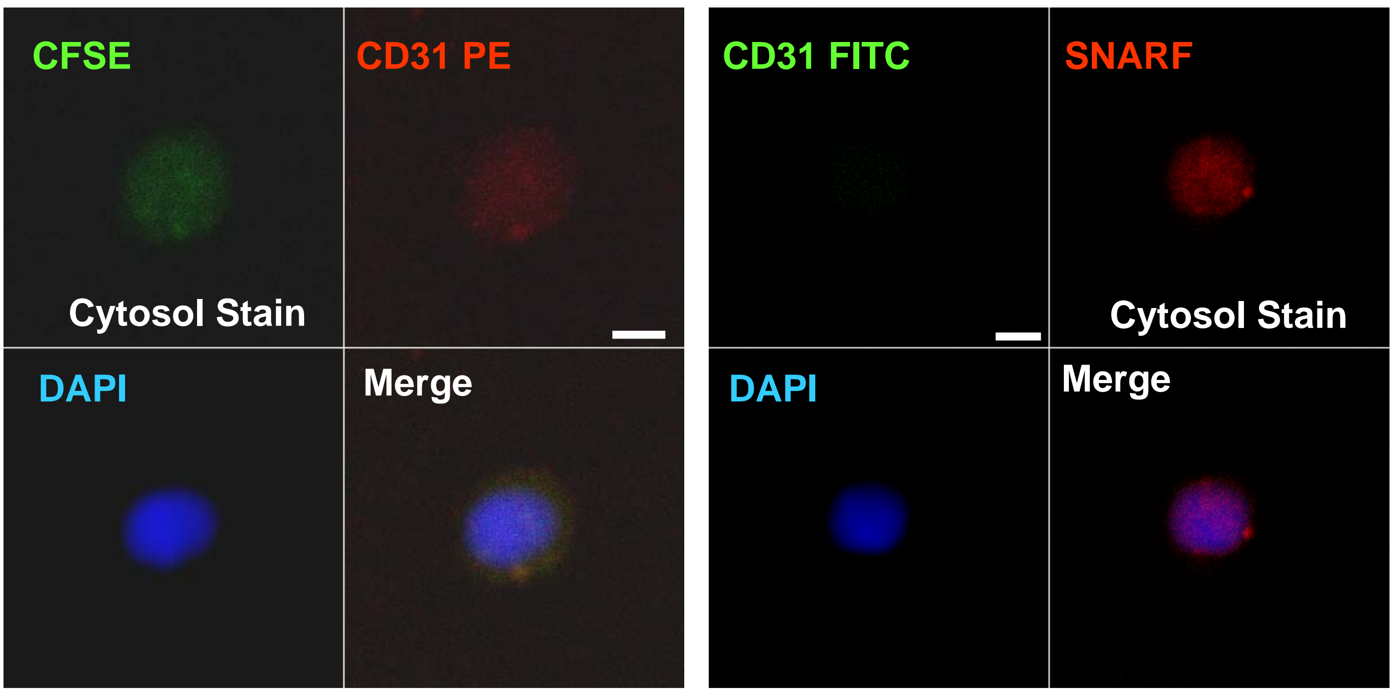
Supplemental Figure 2



Supplemental Figure 3

$CD3^{+}CD31^{+}$ T cell

$CD3^{+}CD31^{-}$ T cell



CFSE (green) : cytosol staining for DP cells

SNARF (red) : cytosol staining for SP cells

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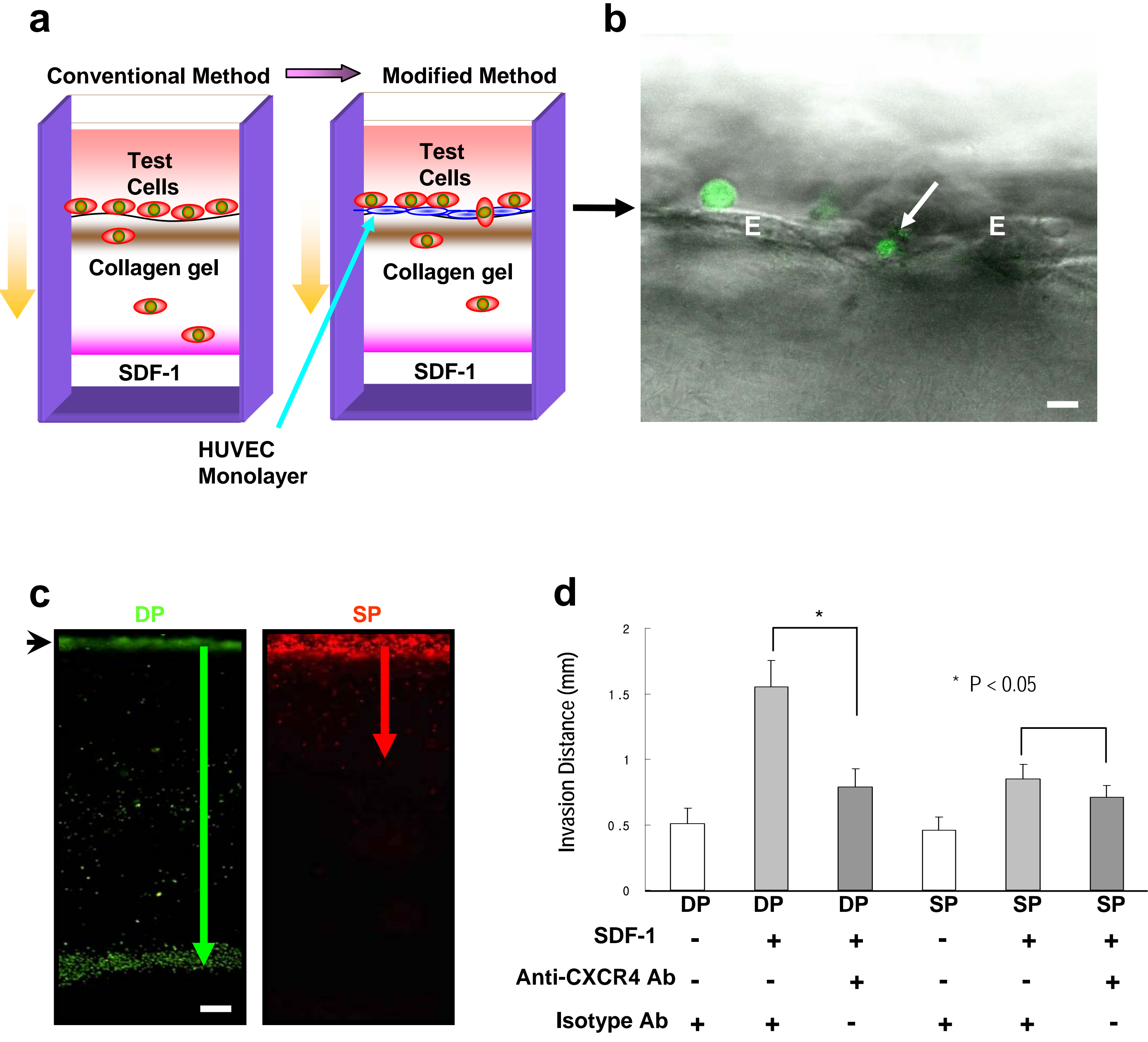
Supplemental Figure 4



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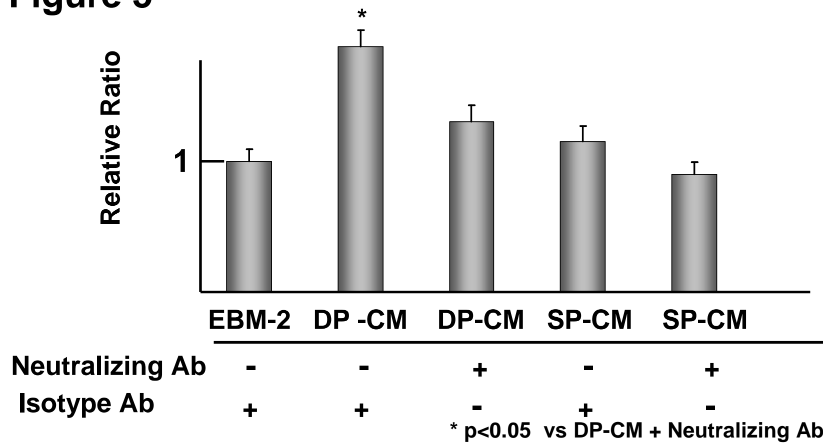
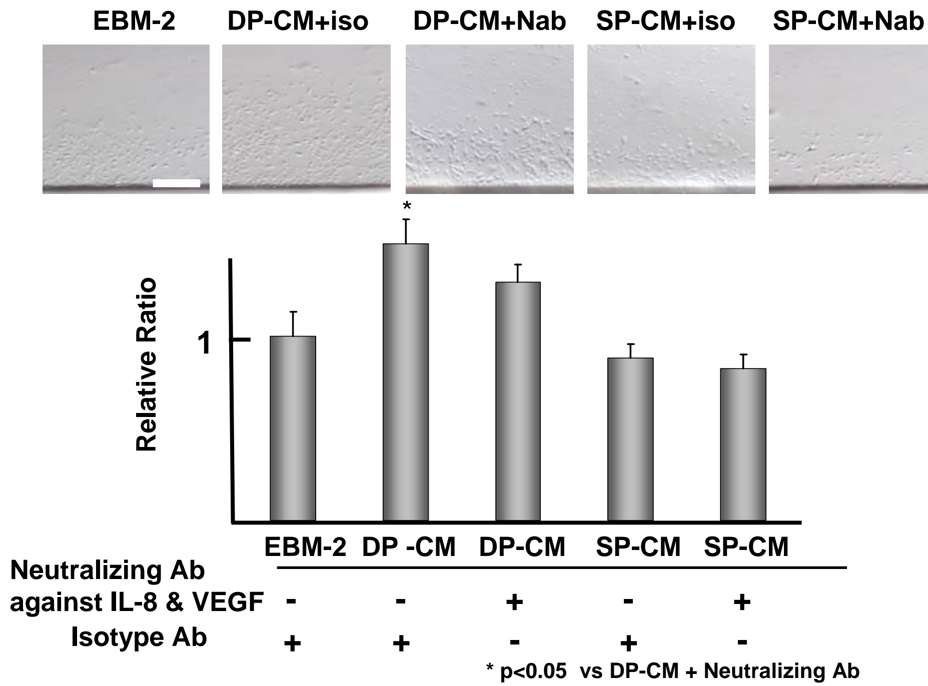
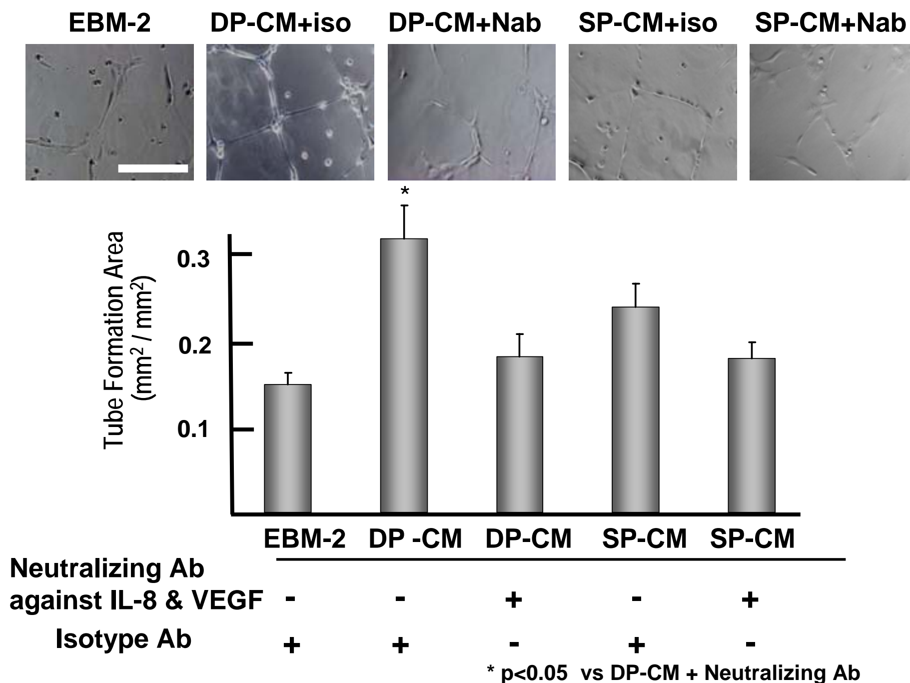
Supplemental Figure 5







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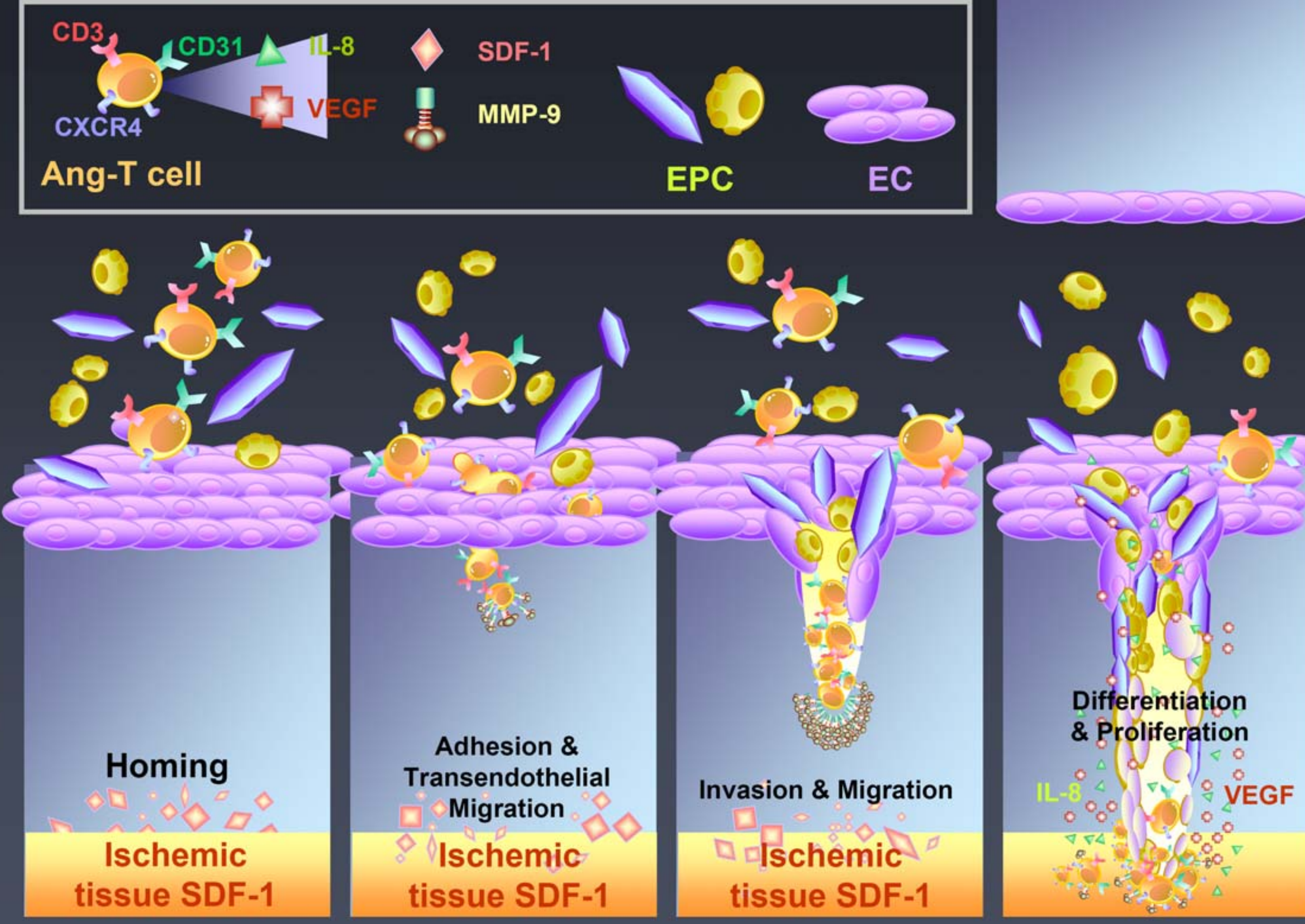

Supplemental Figure 6
CD3
CD31
IL-8
SDF-1
CXCR4
VEGF
MMP-9
Ang-T cell
EPC
EC
Homing
Adhesion &
Transendothelial
Migration
Invasion & Migration
Differentiation
& Proliferation
IL-8
VEGF
Ischemic
tissue SDF-1
Ischemic
tissue SDF-1
Ischemic
tissue SDF-1