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VEGF and Angiopoietins Promote Inflammatory Cell Recruitment and Mature Blood Vessel Formation in Murine Sponge/Matrigel Model

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ABSTRACT

A key feature in the induction of pathological angiogenesis is that inflammation precedes and accompanies the formation of neovessels as evidenced by increased vascular permeability and the recruitment of inflammatory cells. Previously, we and other groups have shown that selected growth factors, namely vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2) do not only promote angiogenesis, but can also induce inflammatory response. Herein, given a pro-inflammatory environment, we addressed the individual capacity of VEGF and angiopoietins to promote the formation of mature neovessels and to identify the different types of inflammatory cells accompanying the angiogenic process over time. Sterilized polyvinyl alcohol (PVA) sponges soaked in growth factor-depleted Matrigel mixed with PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) were subcutaneously inserted into anesthetized mice. Sponges were removed at day 4, 7, 14, or 21 post-procedure for histological, immunohistological (IHC), and flow cytometry analyses. As compared to PBS-treated sponges, the three growth factors promoted the recruitment of inflammatory cells, mainly neutrophils and macrophages, and to a lesser extent, T- and B-cells. In addition, they were more potent and more rapid in the recruitment of endothelial cells (ECs) and in the formation and maturation (ensheating of smooth muscle cells around ECs) of neovessels. Thus, the autocrine/paracrine interaction among the different inflammatory cells in combination with VEGF, Ang1, or Ang2 provides a suitable microenvironment for the formation and maturation of blood vessels. J. Cell. Biochem. 116: 45–57, 2015.

KEY WORDS: VEGF; ANGIOPOIETINS; ANGIOGENESIS; NEOVESSEL MATURATION; INFLAMMATORY CELLS

The ramification of novel blood vessels from pre-existing vascular network, termed angiogenesis, is a coordinated sequence of cellular events consisting of sprouting, endothelial cell (EC) proliferation, directed migration of ECs, EC tube formation, and perivascular stabilization [Carmeliet and Jain, 2011]. Such multistep process is tightly regulated through the maintenance of a balance between soluble pro-angiogenic (stimulatory) and anti-angiogenic (inhibitory) factors [Liekens et al., 2001; Noonan et al., 2008]. A local perturbation of this equilibrium can result in either excessive or insufficient angiogenesis leading to a variety of diseases. With the identification of several pro-angiogenic molecules, potential therapeutic interference with vessel formation is being studied as promising tool for clinical applications [Griffioen and Molema, 2000]. For instance, while therapeutic inhibition of

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angiogenesis may be beneficial in diseases associated to excessive neovessel growth (e.g., solid tumor, rheumatoid arthritis, diabetic retinopathy, atherosclerosis, and psoriasis) [Hanahan and Folkman, 1996], stimulation of angiogenesis may be beneficial in conditions associated with insufficient formation of new vasculature (e.g., tissue damage after reperfusion of ischemic tissue and cardiac failure) [de Muinck and Simons, 2004].

Vascular endothelial growth factor (VEGF) is a well-studied growth factor that effectively promotes neovessel sprouting and growth in the initial phase of angiogenesis [de Muinck and Simons, 2004; Carmeliet and Jain, 2011]. Upon discovery, its high angiogenic potential arose the hypothesis that VEGF monotherapy may be sufficient to promote therapeutic angiogenesis. However, in both pre-clinical and clinical testing, although VEGF monotherapy was successful in promoting the formation of blood vessels, they lacked vascular basement membrane and/or the ensheathing of α-smooth muscle actin (α-SMA)-positive pericytes and smooth muscle cells (SMCs), leading to the regression of newly formed vessels [Dor et al., 2002; Henry et al., 2003; de Muinck and Simons, 2004]. Thus, VEGF-orientated clinical trials did not support the expected beneficial outcome in patients [Simons et al., 2000; Stewart et al., 2009]. The discovery of a novel class of EC-specific ligands termed angiopoietins (Ang1 and Ang2) showed their capacity through the activation of Tie2 receptor to modulate the maturation and stabilization of newly formed vessels. For instance, while Ang1 in the late phase of angiogenesis plays an important role in promoting vascular maturation and contributing to enhance the integrity of EC barrier, Ang2 is identified to have the capacity to destabilize pre-existing vessels prior to VEGF-induced angiogenesis [Davis et al., 1996; Maisonpierre et al., 1997; Thurston et al., 2000]. Furthermore, Ang1 has also been demonstrated to have the capacity to promote in vivo angiogenesis and both Ang1 and Ang2 have the potential to increase EC migration and sprouting under certain experimental conditions [Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001]. Nonetheless, the angiopoietins themselves exert low mitogenic or proliferative activity on ECs [Davis et al., 1996], suggesting that VEGF and angiopoietins exhibit distinct and overlapping expression patterns which collaborate to regulate the different stages of physiological angiogenesis. Hence, a single pro-angiogenic factor may not be sufficient and effective in orchestrating all stages of the angiogenic process and a combination of pro-angiogenic mediators (e.g., growth factors with cytokines) may be required in the formation of stable blood vessels. In agreement with such premise, the emerging relationship between leukocyte infiltration and angiogenesis attracted a lot of attention over the last years.

Proliferating tissue in rheumatoid arthritis, psoriasis, and solid tumors per se, contains an abundance of inflammatory cells (neutrophils, monocytes/macrophages, and dendritic cells) that promote pathological angiogenesis either directly and/or indirectly leading to the creation of a highly vascularized granulation tissue [Costa et al., 2007]. The angiogenic events, in these pathologies, further support the inflammatory response, creating a vicious cycle. In accordance with these observations, clinical trial reports referring to coronary angiogenesis suggested that inflammation is an important stimulus in the induction of the angiogenic cascade [Simons et al., 2000] and very little angiogenesis takes place in the absence of inflammation [Jones et al., 1999]. We, in parallel with other groups, have demonstrated that VEGF and angiopoietins, in addition to being angiogenic factors, are also potent inflammatory regulators; once again indicating the necessity of inflammation in the accompaniment of angiogenesis [Maliba et al., 2008; Neagoe et al., 2009; Dumas et al., 2012; Neagoe et al., 2012]. During the last years, we have shown that Ang1 and Ang2, acting on Tie2 receptor, are capable of promoting the synthesis of platelet activating factor (PAF), a potent pro-inflammatory mediator, in both ECs and neutrophils. Upon its synthesis, PAF promotes neutrophil upregulation of β_2-integrin complex (CD11b/CD18) contributing to neutrophil adhesion and their migration onto activated ECs [Lemieux et al., 2005; Maliba et al., 2006]. In addition, we have reported the capacity of Ang1 to promote the synthesis and release of IL-1 and IL-8 [Dumas et al., 2012; Haddad and Sirois, 2014] which are both involved during inflammation and angiogenesis [Voronov et al., 2007; Qazi et al., 2011]. Nonetheless, the exact link between inflammation and angiogenesis such as the type and the temporal role of the recruited inflammatory cells during angiogenesis remains unanswered.

Various in vivo models using biomaterials (e.g., polyvinyl alcohol sponges) and/or Matrigel have been used extensively to analyze the angiogenic capacity of growth factors, cytokines, chemokines, and non-protein mediators in a number of different hosts [Norrby, 2006]. Yet, many of these studies either did not look at the maturation of blood vessels, a crucial event in the stabilization of nascent blood vessels, or the newly formed vessels were identified to be immature (lacking the ensheathing of SMCs). Hence, in the current study, we utilized a novel variant of the sponge/Matrigel angiogenic model such that the pro-inflammatory sponges were pre-incubated in growth factor depleted Matrigel containing the tested growth factor prior to subcutaneous implantation into wild type mice in order to: (1) assess the individual pro-angiogenic capacity of VEGF, Ang1, and Ang2 to promote the formation and the maturation of neovessels; and (2) to identify the different inflammatory cells accompanying angiogenesis in a spatio-temporal manner.

MATERIALS AND METHODS

MICE

C57BL/6 and BALB/c mice, 10–11 weeks old, were purchased from Charles River Laboratories (Montréal, Canada), *CD115*$^{gfp/+}$, and *Zbtb46*$^{gfp/+}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal experiments were approved by the ethical animal care committees of the Montreal Heart Institute and Institut de Recherches Cliniques de Montréal.

SPONGE PREPARATION AND IMPLANTATION

Sterilized polyvinyl alcohol (PVA) sponges (6 mm diameter ×2 mm width) were soaked in 200 μl of growth factor depleted Matrigel (BD Biosciences, Mississauga, Canada) containing PBS or 200 ng of VEGF (PeproTech, Rocky Hill, NJ), Ang1, or Ang2 (R&D Systems, Minneapolis, MN) for 20 min at 4°C. Subsequently, the sponges were incubated for 20 min at 37°C prior to implantation. Under

anesthesia with 2% isoflurane USP, two sponges treated with the same growth factors were inserted subcutaneously through two 1 cm orthogonal incisions in the dorsa of the animals. The incisions were then clipped for closure, and the mice were subcutaneously injected with an analgesic agent (0.1 ml of Anaphen; 1 mg/ml). The mice were sacrificed under anaesthesia at day 4, 7, 14, or 21 post-procedure.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY ANALYSES

The harvested sponge implants were fixed in 10% formalin PBS-buffered solution, embedded into paraffin blocks and sectioned sagittally (6 μm thick). The sections were stained with Masson's trichrome reagent for a global overview of cellular invasion in the implants. Immunohistological stainings were performed using the avidin-biotin complex for the validation of angiogenesis and inflammatory cells infiltration as previously described [Marchand et al., 2002; Lemieux et al., 2005]. The primary antisera used in this study were: ECs specific goat anti-mouse CD31 (Santa Cruz Biotechnology Inc., CA), SMCs specific mouse anti-mouse α-SMA (Sigma–Aldrich, Steinheim, Germany; clone 1A4), neutrophils specific rabbit anti-mouse myeloperoxydase (MPO) (Thermo Scientific, Rockford, IL), and macrophages specific rat anti-mouse F4/80 (BioLegend, San Diego, CA; clone BM8).

To assess the maturation of neovessels, a sequential double immunohistochemistry (IHC) staining was performed. The sponge sections underwent first round of IHC using the primary antisera anti-CD31 and host specific biotinylated secondary antibody. Peroxidase was developed by the DAB substrate. The tissues underwent a second round of IHC protocol with the primary antisera anti-α-SMA and host specific biotinylated secondary antibody. α-SMA expression was detected in turquoise using Vina green chromogen (Biocare Medical Inc., Concord, CA).

MICROSCOPY AND QUANTIFICATION

Images were collected using a brightfield microscope and were analyzed using Image-Pro Plus software. Images of selected regions of highest positive signal were acquired under 200× magnification of each stained section (endothelial cells, neutrophils, macrophages, and smooth muscle cells). These selected regions were then quantified using the color segmentation method. Thresholds were empirically set to select pixels by analyzing a test set of 10 images per batch of staining. The selected pixels represented the expression of the stained cell. These empirically determined thresholds were recorded in a macro and were applied to all images that were analyzed. The number of pixels counted by the macro was recorded in mm^2. The Matrigel area was measured using Image Pro's calibrated area measurement tool in mm^2. The percent occupancy of studied cells in the Matrigel from each sponge was calculated by taking the mean of: (area of counted pixels (mm^2)/area of Matrigel (mm^2)) × 100 of five randomly selected images per sponge. The mean microvessel density was expressed as the absolute number of microvessels counted/area of Matrigel (mm^2). The cross-sectional area occupied by these blood vessels was also simultaneously measured. The vessel maturation index was measured as: ((number of α-SMA-positive vessels/number of CD31-positive vessels) × 100).

SPONGE SINGLE CELL PREPARATION AND FLOW CYTOMETRY ANALYSIS

Single cell suspensions were isolated from sponges and spleen as previously described [Choi et al., 2011]. Briefly, the sponges and corresponding spleens were isolated from C57BL/6 mice, minced and incubated for 60 min at 37°C in an enzyme mixture. Following the blockage of Fc receptors using culture supernatant of 2.4G2 hybridoma, the cells were stained with fluorophore-conjugated antibodies. The stained cells were acquired using LSR Fortessa (Becton Dickinson, Mississauga, Canada) and were analyzed using FlowJo (Tree Star Inc., Ashland, OR). The monoclonal antibodies used in both flow cytometry analysis and FACS were anti-mouse CD45, CD64, CD3, CD19, Ly6G (clone: 1A8), MHCII, CD11c, and corresponding isotype controls were purchased from BioLegend.

ANALYSIS OF PHAGOCYTOSIS

Sponge and splenic $CD45^+CD11c^+MHCII^+$ cells isolated from C57BL/6 mice, were incubated with 0.00134% of 0.50 μm Fluoresbrite® YG Microspheres (Polysciences, Inc., Warrington, PA) for 30 min at 37°C. The cells were then labeled with monoclonal antibodies against CD45, CD11c, MHCII, and CD19 and analyzed by flow cytometry.

MIXED LEUKOCYTE REACTIONS

Sponge and splenic $CD45^+$ cells were FACS (Beckman Coulter MoFlo, Mississauga, Canada) sorted into $CD11c^+MHCII^+$ cell population and $CD11c^-MHCII^-Ly6G^+$ neutrophils from C57BL/6 mice. For proliferative analysis, splenic T-cells were isolated from BALB/c mice by excluding $B220^+$, $F4/80^+$, $CD49b^+$, and $I\text{-}Ab^+$ cells using anti-rat IgG Dynabeads (Invitrogen, Burlington, Canada). These allogenic T-cells were subsequently labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE) and were combined with isolated stimulator cells (splenic $CD11c^+MHCII^+$ cells, sponge $CD11c^+MHCII^+$ cells, and neutrophils; stimulator: T-cell ratio of 1:10) in microtest wells at 5,000 of stimulator to 50,000 T-cells/well. Four days later, T-cell proliferation was evaluated by CFSE dilution in flow cytometry.

STATISTICAL ANALYSIS

Results are presented as the mean ± SEM and all comparisons were made between each conditions at corresponding days by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. Differences were considered significant at *P*-values less than 0.05.

RESULTS

VEGF AND ANGIOPOIETINS PROMOTE BLOOD VESSEL FORMATION

Previous studies have demonstrated that VEGF and angiopoietins play precise, complementary, and coordinated roles in angiogenesis. In the present study, we wanted to assess the individual pro-angiogenic activities of VEGF and angiopoietins in a novel variant of the sponge/Matrigel angiogenic model. To monitor vascularization and to examine the angiogenic microenvironment in the sponges, we performed histological analysis using Masson's trichrome staining of the sponges at different time points from day 4 to day 21. Sponges

containing PBS followed the time-course of the host foreign body response in terms of cellular infiltration and neovessels formation (Fig. 1A and C). In contrast, sponges loaded with VEGF, Ang1, or Ang2 (200 ng/200 μl) elicited a robust invasion of various cell types into the Matrigel giving rise to a highly vascularized matrix by day 7 (Fig. 1B and D–F).

Based on endothelial cell-specific CD31 IHC detection (Fig. 2A; upper left insert), for a more comprehensive analysis, sponges containing PBS, VEGF, Ang1, or Ang2 showed marginal amount of EC recruitment by day 4 (Fig. 2A). However, in the presence of any one of the tested growth factors, this effect became significant by day 7 and 14 with an increase of ≈3–5 fold as compared to PBS-treated sponges. At day 21, the percentage of CD31 expression in PBS-treated sponges became comparable to VEGF and Ang2 treated sponges, whereas Ang1 continuously maintained its capacity to recruit ECs. These recruited ECs took their neovessel structure (lumen formation) by day 7 in presence of VEGF, Ang1, or Ang2 (Fig. 2B). Once formed by day 7, the microvessel density remained stable,

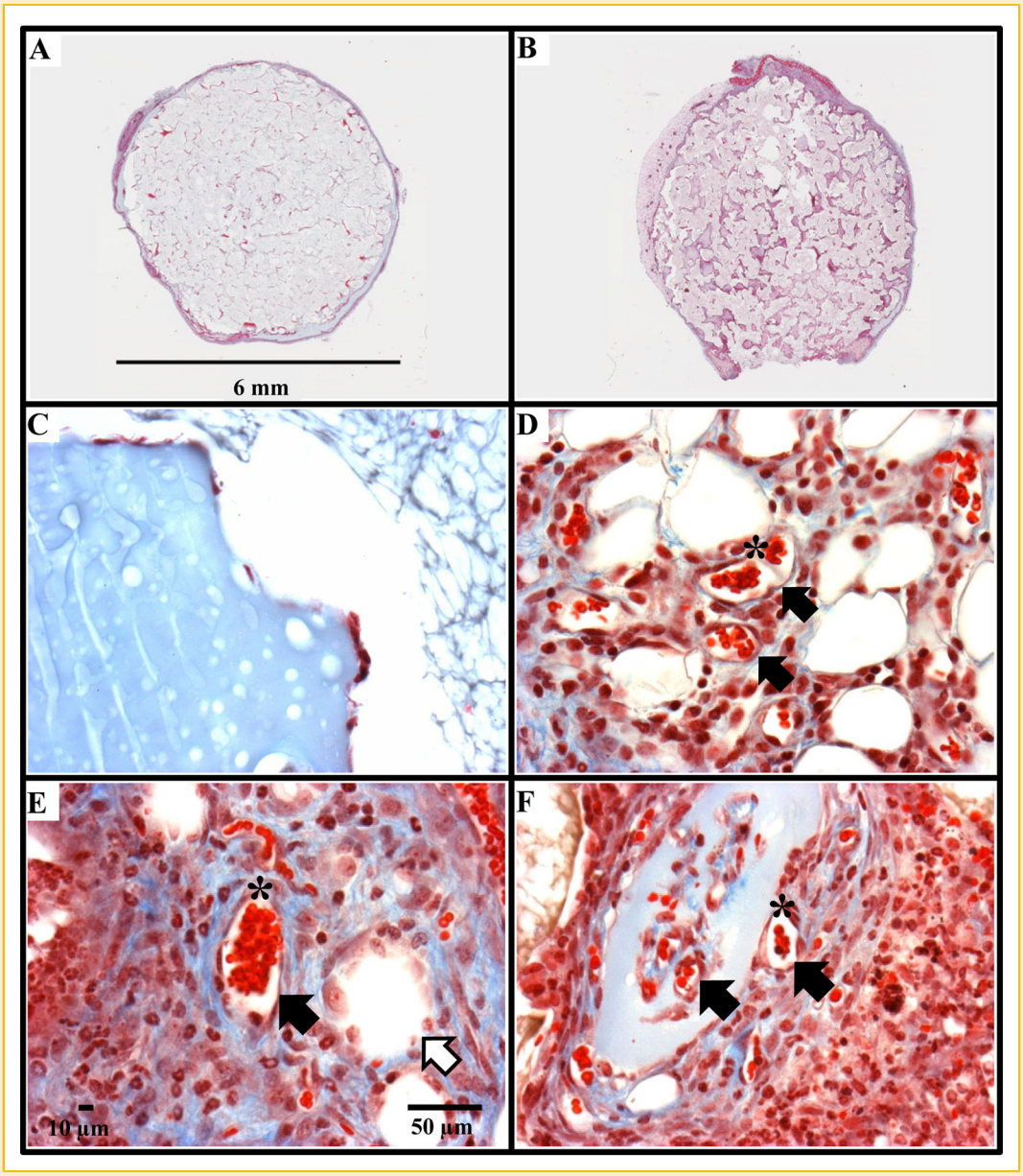

Fig. 1. Pro-angiogenic and inflammatory activities of VEGF and angiopoietins in mice. The images illustrate representative scans (A and B) and representative histological sections (Masson's trichrome staining, 400× magnification) of PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) harvested at day 7 (C–F). Treatment with VEGF, Ang1, or Ang2 promoted a marked recruitment of numerous inflammatory cells, endothelial cells, and mural cells in the region of Matrigel within the sponges and the formation of neovessels (black arrow) containing circulating red blood cells (star), indicative of functional blood vessels (B and D–F). Neovessel formation from endothelial cells (lumen formation) lacking circulating red blood cells was also observed (white arrow). In contrast, PBS treated sponges showed less cellular accumulation and no blood vessel formation by day 7 (A and C).

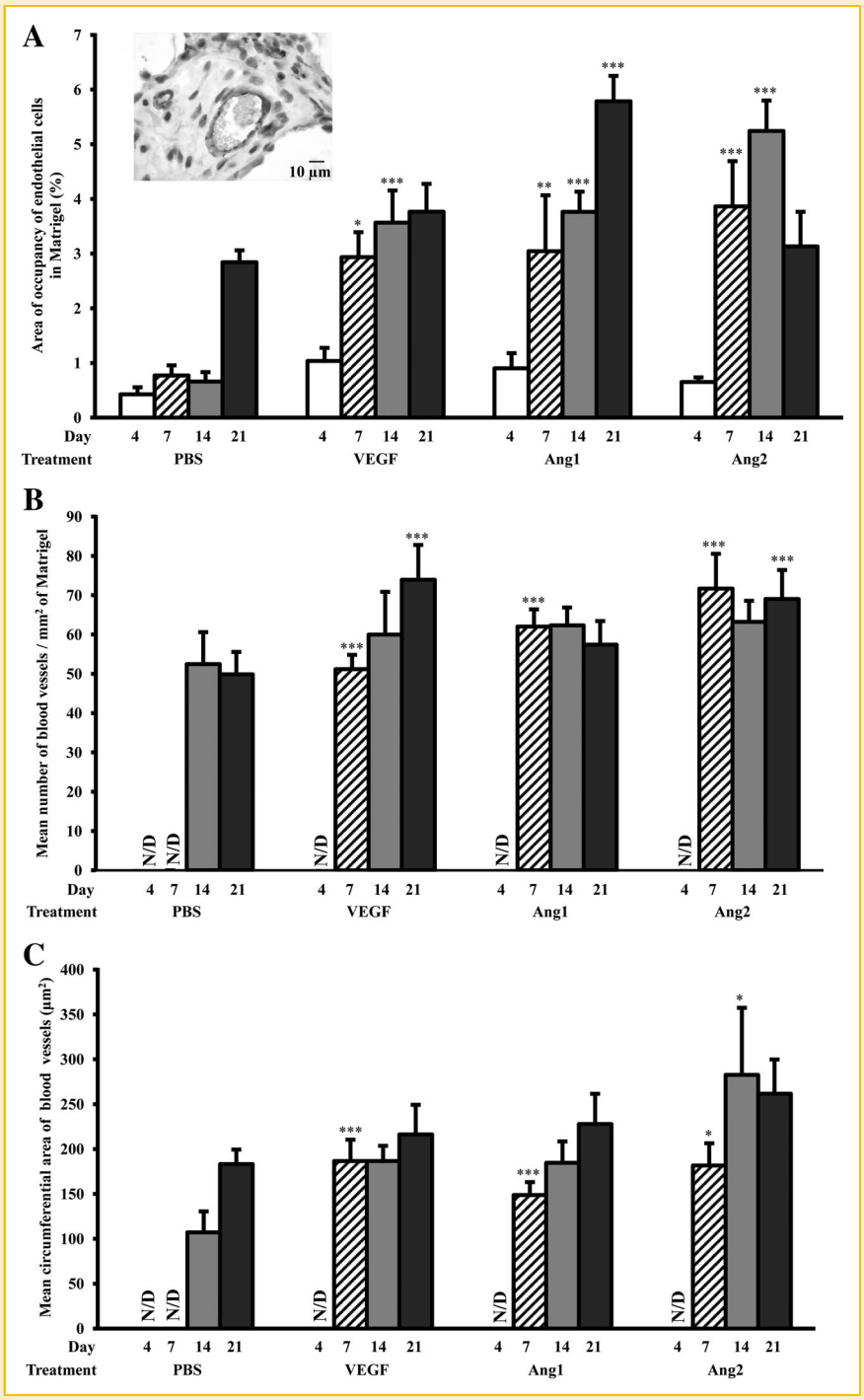

Fig. 2. Effect of VEGF and angiopoietins on angiogenesis in a time-dependent manner. PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) were removed from the animals at day 4, 7, 14, or 21. Subsequently, IHC staining against endothelial cell specific CD31 protein was performed in order to assess the recruitment of endothelial cells (IHC insert; 1000×; A), microvessel density (B) and the average cross-sectional area occupied by the vessels (C) in the Matrigel. Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to PBS-treatment at corresponding days, N/D: not detectable.

ranging from 50 to 70 vessels/mm^2 of Matrigel. Although delayed in time, the microvessel density under PBS-treatment became comparable (≈50 vessels/mm^2 of Matrigel) to the growth factor-treated sponges by day 14 (Fig. 2B). Finally, in presence of Ang2 the average cross-sectional area occupied by the neovessels formed by day 14 to 21 (>250 μm^2) was greater than the area of vessels formed in presence of VEGF or Ang1 (≈150–225 μm^2). However, under PBS treatment, the primary vessels formed by day 14, were smaller

(≈100 μm^2) but underwent remodeling and nearly doubled by day 21 (Fig. 2C).

VEGF AND ANGIOPOIETINS PROMOTE BLOOD VESSEL MATURATION

Vessel maturation is critical in angiogenesis, as the stability of an induced vasculature is dependent on the mural cell association to prevent vessel regression [Bergers and Song, 2005]. We thus, wanted to elucidate the temporal sequel of VEGF and angiopoietins mediated maturation of neovessels given a pro-inflammatory environment. The recruitment of SMCs was detected based on α-SMA protein expression by day 7 in all tested conditions (Fig. 3A). Yet, treatment with VEGF and angiopoietins individually triggered a more rapid and pronounced recruitment of SMCs, producing a ≈10–12-fold increase as compared to PBS-treated group. By day 14, the number of SMCs detected under growth factor stimulation plateaued, while the venue of SMCs under PBS treatment caught up yet remaining ≈2.5–3-fold lower to what was mediated by VEGF and the angiopoietins. We also observed that by day 21, the area covered by SMCs in presence of VEGF or Ang1 was maintained whereas it partially declined under PBS or Ang2 treatment (Fig.3A).

To assess whether the SMCs remained sparse into the Matrigel and/or associated with neovessels, we performed double IHC staining against CD31 and α-SMA proteins. We observed a common inflection point by day 7 in presence of the different growth factors, favoring the formation of neovessels, the migration of SMCs, and the surrounding of SMCs around the neovessels as compared to PBS-treated sponges (Fig.3B). Sponges harvested at day 4 under all of the tested treatments only supported the recruitment of ECs and not of SMCs (Fig.3A). By day 14 and 21, the maturing blood vessels were covered with multiple layers of SMCs for all conditions (Fig.3B). However, although the number of neovessels surrounded by at least a single layer of SMCs by day 7 was ≈60–70% and reached up to 80% by day 14 or 21 under growth factor treatments, it plateaued to about 40% by day 14 in PBS-treated sponges (Fig.3C).

VEGF AND ANGIOPOIETINS MEDIATED ANGIOGENESIS IS ACCOMPANIED BY INFLAMMATORY CELLS

Inflammatory cells, namely neutrophils and monocytes/macrophages participate in the angiogenic process through the secretion of pro- and anti-inflammatory cytokines by controlling EC activation, migration, and proliferation [El et al., 2000; Lingen, 2001; Voronov et al., 2003]. Using anti-MPO antibody (Fig. 4A; upper right insert), we observed significant recruitment of neutrophils by day 7 in presence of VEGF, which peaked by day 14 covering about ≈2% of total surface area, and then faded away by day 21. In addition, VEGF was more potent as compared to the angiopoietins to promote the recruitment of neutrophils by day 14. Ang2 showed a significant peak (≈1%) in neutrophil recruitment by day 7 and its potency decreased gradually over time. On the other hand, Ang1 showed a mild effect on neutrophil recruitment as compared to PBS-treated

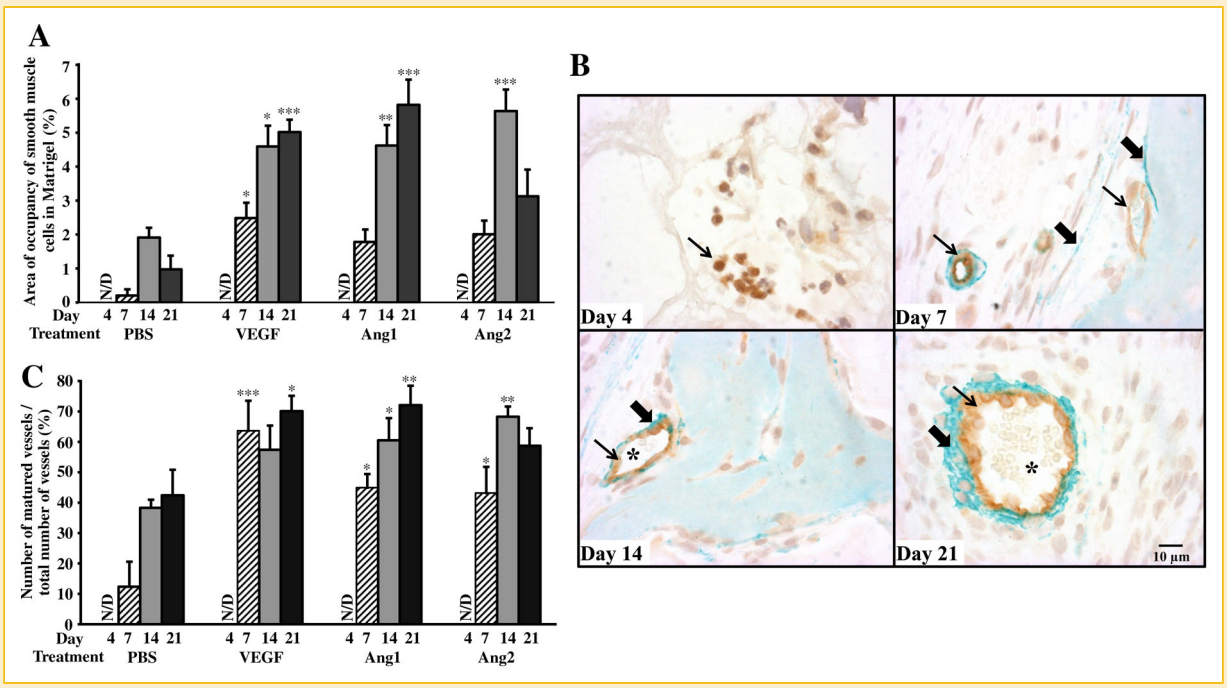

Fig. 3. VEGF and angiopoietins mediated SMC migration and neovascular maturation in the sponges. PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) were removed from the animals at day 4, 7, 14, or 21. Subsequently, IHC staining against α-SMA was performed in order to assess the venue of SMCs (A). The 4 panels represent immunohistogical snapshots illustrating the temporal evolution of mature blood vessels in the sponges in presence of the studied growth factors (Ang1) at days 4, 7, 14, and 21. Endothelial cells were stained with anti-CD31 (brown staining; thin arrow) and SMCs were stained with anti-α-SMA (turquoise staining; thick arrow) (1000× magnification). The neovessels were not only ensheathed by SMCs, but also contained red blood cells (star) (B). The percentage of mature blood vessels was quantified as the number of neovessels surrounded by SMCs over the total number of blood vessels (C). Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to PBS-treatment at corresponding days, N/D: not detectable.

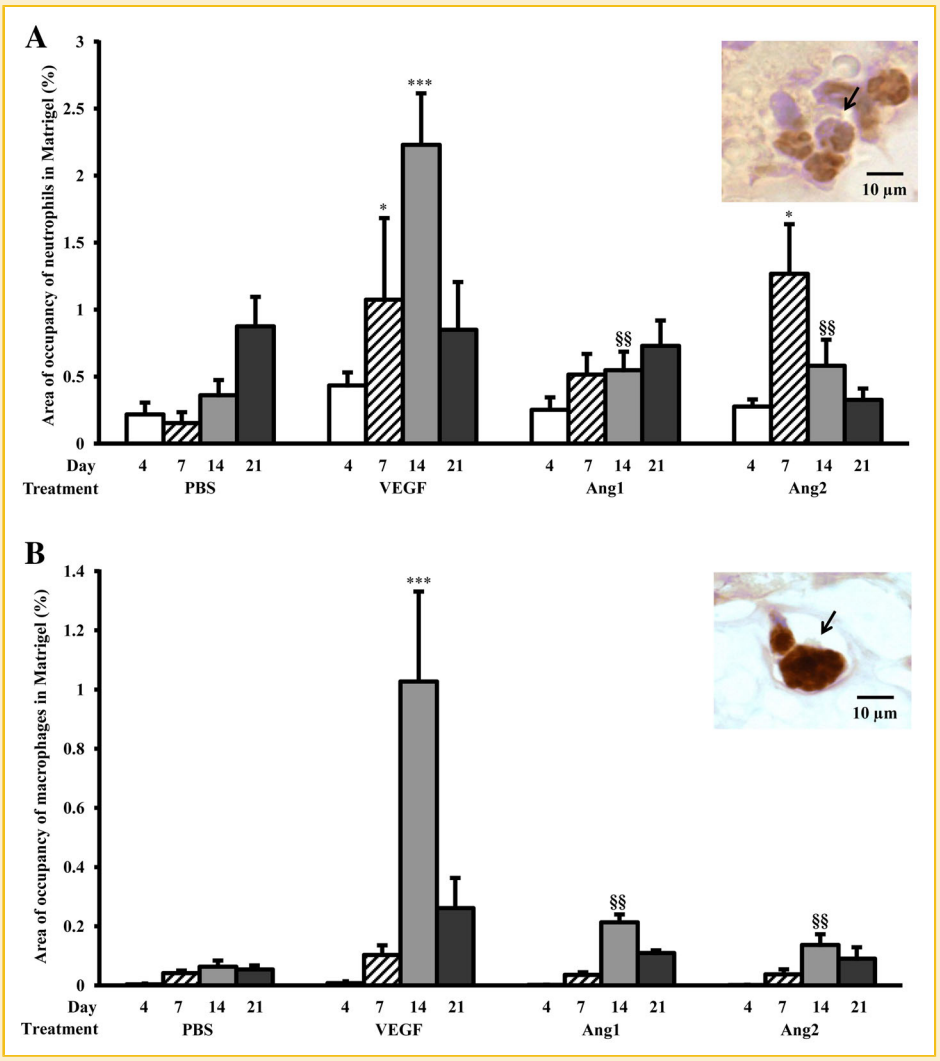

Fig. 4. Identification of VEGF and angiopoietins mediated inflammatory cells influx in the sponges. Neutrophil (A) and macrophage (B) accumulation in the sponge implants were measured as MPO (IHC insert; 1000×; A) and F4/80 (IHC insert; 1000X; B) expression, respectively, in the Matrigel region of the sponges. VEGF, Ang1, and Ang2 mediated inflammatory cells (neutrophil and macrophage) recruitment was temporal-dependent with different potency. Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to PBS-treatment, $^{\S\S}P < 0.01$ as compared to VEGF-treatment at corresponding days, N/D: not detectable.

sponges (Fig. 4A). We looked at the recruitment of monocytes/macrophages based on F4/80 selective protein detection (Fig. 4B; upper right insert). The three growth factors individually tended to have a peak recruitment of macrophages by day 14, which was massive and significant under VEGF treatment (≈1%) as compared to PBS and angiopoietins-treated animals (Fig. 4B).

CHARACTERIZATION OF INFLAMMATORY AND IMMUNE CELLS IN SPONGES BY FACS ANALYSES

To delineate the different leukocyte subsets recruited in the sponges, we utilized multicolor flow cytometry procedure on single cell preparation from sponges. $CD45^{+}$ leukocytes in the sponges were primarily comprised of $CD11c^{+}MHCII^{+}$ cells, $CD11c^{-}MHCII^{-}Ly6G^{+}$ cells (neutrophils), $CD11c^{-}MHCII^{-}CD3^{+}$ cells (T-cells), and $CD11c^{-}MHCII^{+}CD19^{+}$ cells (B-cells) (Fig. 5A). The fate of $CD11c^{+}MHCII^{+}$ cells at this point remained to be investigated. Previous studies demonstrated that dentritic cells (DCs) constitutively express the hematopoietic markers CD45, CD11c, and MHCII in lymphoid tissues such as spleen and lymph nodes. Nonetheless, this marker expression profile on its own is not sufficient to define classical DCs (cDCs) in nonlymphoid tissues. In fact, high and similar levels of CD11c and MHCII expression have been observed in both cDCs and in macrophages [Gautier et al., 2012]. Thus, we performed marker analyses, genetic, and functional studies to specifically identify the $CD11c^{+}MHCII^{+}$ cell population in the sponges as cDCs and/or macrophages. Recently, *Zbtb46* was identified as a selectively expressed transcription factor by cDCs but not by monocytes, macrophages and other lymphoid and myeloid lineages (e.g., neutrophils, T-cells and B-cells) [Satpathy et al., 2012]. Therefore, we harvested sponge cells from $Zbtb46^{+/+}$ (WT) and $Zbtb46^{gfp/+}$ mice

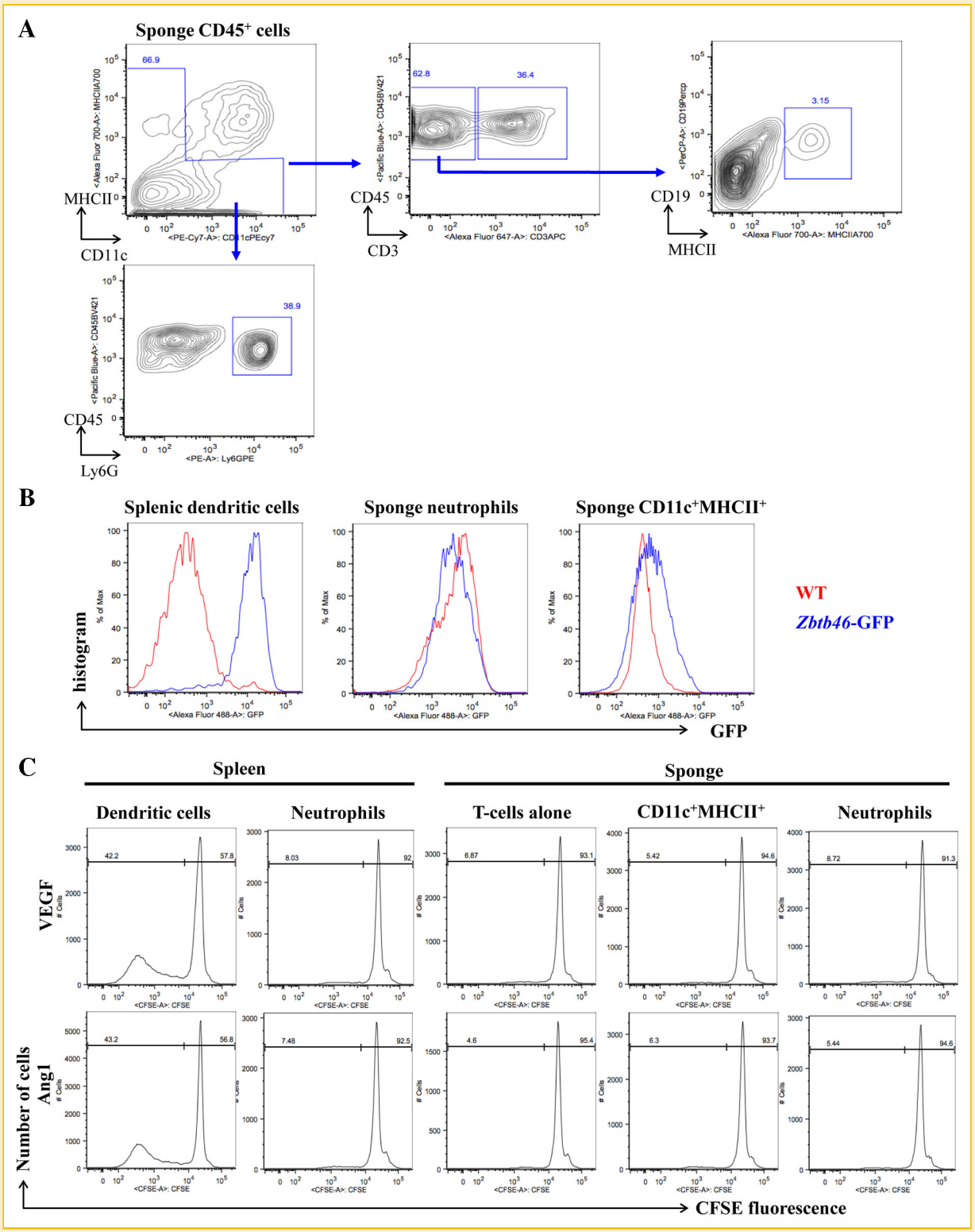

Fig. 5. $CD11c^{+}MHCII^{+}$ cells recruited in the sponges do not possess DC-characteristics. Single cell suspensions from sponges harvested from C57BL/6 mice were examined for surface expression of indicated markers. The data illustrates the expression profile of CD11c and MHCII, CD3 (T-cells), CD19 (B-cells), and Ly6G (neutrophils) within $CD45^{+}$ gated cell population (A). Representative histogram of GFP expression of $CD11c^{+}MHCII^{+}$ cells in spleen ($n = 2$), VEGF-treated sponges and neutrophils ($n = 4$) harvested from *Zbtb46*$^{+/+}$ (WT) and *Zbtb46*$^{gfp/+}$ mice (B). FACS purified $CD11c^{+}MHCII^{+}$ cells isolated from sponges (treated with VEGF or Ang1) and spleens along with neutrophils retrieved from C57BL/6 mice were co-cultured with T-cells purified from BALB/c mice ($CD3^{+}$CFSE-labled T-cells) in MLR. CFSE levels were analyzed four days later. Proliferation of allogenic T-cells results in a reduction of CFSE fluorescence intensity ($n = 4$; C).

and analyzed for GFP expression. As anticipated, $CD45^+$ cell population containing splenic DCs were GFP^+ while neutrophils recruited in the sponges were devoid of GFP expression. In contrast to splenic DCs, $CD11c^+MHCII^+$ cell population isolated from the sponges lacked expression of GFP (Fig. 5B). Next, FACS-sorted $CD11c^+MHCII^+$ cells from sponges and spleens along with neutrophils were tested for their ability to stimulate allogenic T-cells. Only splenic DCs were strong stimulators of T-cell proliferation (Fig. 5C). T-cells alone, neutrophils from sponges and spleen and $CD11c^+MHCII^+$ cells from sponges did not induce allogenic T-cell proliferation. These results confirm that $CD11c^+MHCII^+$ cells in the sponges do not possess DCs functional characteristics.

In contrast, $CD11c^+MHCII^+$ cells from PBS, VEGF, Ang1, and Ang2-treated sponges were positive for F4/80 marker while the neutrophils from the corresponding sponges were negative (Fig. 6A). Although in the past, F4/80 served as a reliable marker of

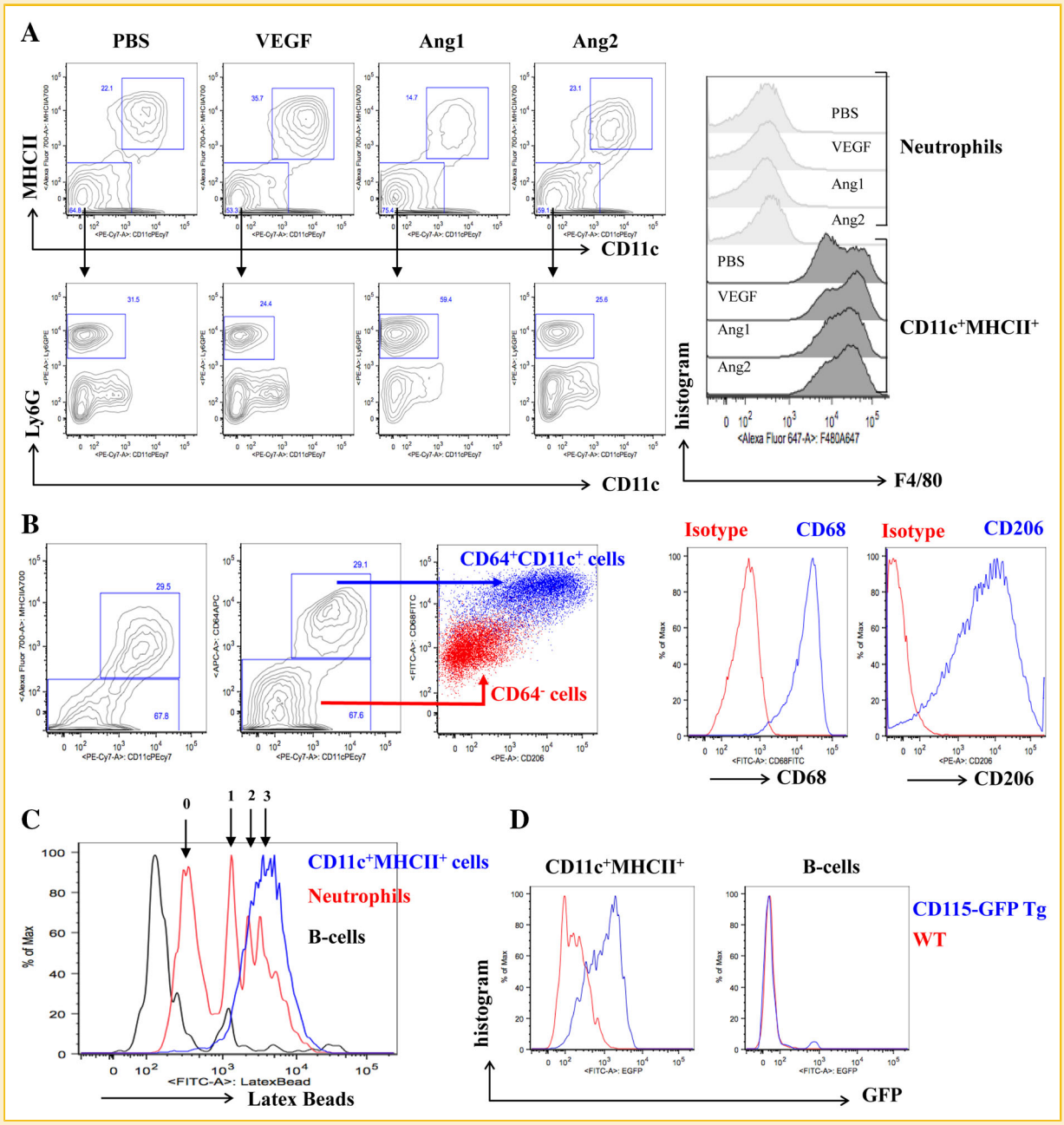

Fig. 6. $CD11c^+MHCII^+$ cells recruited in the sponges are classical and non-classical macrophages. The images illustrate representative histogram (n = 2–4 independent experiments per condition) for F4/80 expression of $CD11c^+MHCII^+$ cells and $CD11c^-MHCII^-$ $Ly6G^+$ neutrophils isolated from sponges pretreated with PBS, VEGF, Ang1, or Ang2 (A). $CD11c^+MHCII^+$ $CD64^+$ cells were stained for the intracellular markers CD68 and CD206 (B). Single cell suspensions isolated from VEGF treated sponge were bathed with 0.5 μm YG microspheres for 30 min at 37°C and the uptake of these microspheres (phagocytosis) were analysed by flow cytometry (C). B-cells, neutrophils, and $CD11c^+MHCII^+$ cells isolated from VEGF treated sponges harvested from *CD115*$^{gfp/+}$ and WT mice were analysed for GFP expression (D).

macrophages, additional analysis of a panel of surface markers is now required to define macrophage population. Recently, the surface marker CD64 expression was identified as a reliable marker of mature tissue macrophages [Gautier et al., 2012]. Interestingly, in our study, we observed that the $CD11c^{+}MHCII^{+}$ cells isolated from the sponges were all $CD64^{+}$. Moreover, they also expressed CD68 and CD206, two additional markers of macrophages [Gautier et al., 2012] (Fig. 6B). To test whether the $CD11c^{+}MHCII^{+}$ cells recruited in the sponges had the phagocytic activity of macrophages, we analyzed their phagocytic capacity by flow cytometry. Neutrophils from the sponges took up 0–2 beads/cell while all sponge $MHCII^{+}CD11c^{+}$ cells were highly phagocytic (>3 beads uptake/cell). B-cells isolated from the sponges served as our negative control and it provided no phagocytic activity (Fig. 6C). To further confirm these isolated cells from the sponges as macrophages, we used transgenic *CD115*$^{gfp/+}$ mice, which express MCSF-1R, a receptor for macrophage-colony stimulating factor [Sasmono et al., 2007]. Indeed, the $CD11c^{+}MHCII^{+}$ cells isolated from the sponges implanted in *CD115*$^{gfp/+}$ mice were GFP positive. However, B-cells (negative control) from the transgenic *CD115*$^{gfp/+}$ and WT mice were GFP negative (Fig. 6D). Taken together, these results demonstrate that the $MHCII^{+}CD11c^{+}$ cells present in the sponges are macrophages and not DCs.

DISCUSSION

Compelling studies have demonstrated the direct participation of neutrophils and monocytes/macrophages in the induction of inflammatory response prior to the initiation of pathological angiogenesis. Indeed, the release of pro-inflammatory cytokines and growth factors provides a suited autocrine/paracrine milieu to fully support blood vessels formation [Schruefer et al., 2005; Aplin et al., 2006; Lin et al., 2006; Gong and Koh, 2010]. As we have previously illustrated the pro-inflammatory activities of VEGF and angiopoietins, we were led to address their capacity to promote inflammatory response associated to in vivo angiogenesis. In the present study, we utilized a novel variant of the murine sponge/Matrigel angiogenic assay to evaluate the sequel of host-derived blood vessel formation and inflammatory cell infiltration into the sponges. Herein, we demonstrate that VEGF, Ang1, and Ang2 individually are highly potent and efficacious in recruiting ECs, SMCs, and inflammatory cells (mainly neutrophils and macrophages, and sparsely T- and B-cells). More importantly, these tested growth factors given individually were not only capable to favor the formation of neovessels but also their maturation as observed by the coordinated ensheating of SMCs around the neovessels and the presence of circulating red blood cells in the vessel lumen. Hence, this study suggests the potential contribution of both inflammatory cells and angiogenic growth factors to fully support blood vessel formation and their maturation.

Recent efforts in clinical trials focus on localized therapy for restoring blood flow in ischemic regions as tissue loss in these patients was localized [Simons et al., 2000]. While growth factor therapy remained a gold standard for the induction of local therapeutic angiogenesis, translating this concept into an effective and safe therapy for patients became a challenge. Presently, biomaterial based approaches is being successfully utilized in animal models to study the capacity of growth factors, cytokines/chemokines, and nonprotein mediators to promote blood vessel formation [Andrade et al., 1997]. One such method is the subcutaneous implantation of PVA sponges in mice, which promotes a robust infiltration of inflammatory cells, providing a pro-inflammatory environment, and giving rise to a highly vascularized sponge matrix. However, due to continuous inflammation, these newly formed vessels were postulated to be fragile, permeable, and dilated with no indication of neovessel maturation (lack of SMCs ensheathing) [Andrade et al., 1997]. A major disadvantage of such matrix implantation is that it induces non-specific inflammatory host response and thus limits to acute studies [Staton et al., 2009]. Later, the Matrigel plug assay became the widely used model for studies involving in vivo testing for angiogenesis, as it provides a natural environment for the formation of neovessels without inducing non-specific immune response [Staton et al., 2009]. Yet, although Matrigel injection containing VEGF in mice successfully promoted the formation of neovessels, the model did not lead to the maturation of the newly formed vessels [Tengood et al., 2010]. As inflammation is an important stimulus for the induction of new vessel growth, we hypothesized that the combination of both these approaches might fulfill the required environment to favor the formation and maturation of neovessels. The classical sponge/Matrigel model, encompassing both the sponge model and the Matrigel assay, requires the subcutaneous injection of Matrigel containing the protein of interest, 20–30 min prior to the surgical introduction of PVA sponges [Akhtar et al., 2002; Norrby, 2006]. This method has been identified to provide variable amount of test compound within the implants and to trigger the fibrotic encapsulation of the sponges [Norrby, 2006]. In our variation of the sponge/Matrigel model, we have soaked PVA sponges into Matrigel containing the tested growth factors prior to the surgical implantation. We observed that our technique was simple, less time consuming, that each sponge implant contained equal volume of the tested growth factors and it did not induce non-specific immune response.

We observed an early onset of EC migration in the sponges within the first 4 days and a significant number of blood vessel formation by day 7 under VEGF or angiopoietin stimulation, thus, challenging the classical role of angiopoietins in angiogenesis. Interestingly, the amount of ECs migrated into the sponges kept increasing up to day 14 or 21, while the number of blood vessels once formed by day 7 remained stable, suggesting that the model itself exerts a restrain on the maximal capacity of blood vessel formation even in presence of free ECs. Our data is in line with previous studies reporting the pro-angiogenic and mitogenic activities of VEGF in various in vivo models including the chick chorioallantoic membrane [Plouet et al., 1989], the rabbit cornea [Phillips et al., 1994], and the primate iris [Tolentino et al., 1996]. However, the capacity of angiopoietins to initiate the angiogenic cascade remains controversial. For instance, while some in vivo reports demonstrated that Ang1 alone is unable to induce angiogenesis but can potentiate VEGF mediated angiogenic response [Asahara et al., 1998; Chae et al., 2000], others showed that Ang1 can promote a robust neovascularization in Matrigel implants [Babaei et al., 2003]. The implication of Ang2 in

angiogenesis is tied with VEGF where it promotes destabilization of pre-existing blood vessels in the absence of VEGF [Holash et al., 1999; Lobov et al., 2002]. Yet, other studies reported that Ang2 alone can induce vascular remodeling and angiogenesis in absence of VEGF [Kim et al., 2000b; Mochizuki et al., 2002]. Our study illustrates that the pro-inflammatory environment itself is sufficient to initiate the angiogenic cascade and the addition of the tested growth factors further allows this effect to be more potent and efficient.

Interestingly, we also observed the venue and the ensheathing of SMCs around neovessels by day 7 in presence of the tested growth factors. Indeed, all three growth factors promoted the maturation of blood vessels with equal potency. Although, our result is consistent with the stabilizing effect of Ang1 on vascular endothelium, it is also in contradiction with the proposed role for VEGF and Ang2 during angiogenesis. In fact, VEGF and angiopoietins are incapable of directly activating SMCs. Yet, they can promote the activation of ECs and support the migration of inflammatory cells (e.g., macrophages and neutrophils) which can promote the release of various growth factors and cytokines (e.g., FGF, VEGF, Ang1, interleukins [IL-1β, IL-8 and -10], and CXCL1) [Gaudry et al., 1997; Noonan et al., 2008; Dinarello, 2009; Neagoe et al., 2009]. Ang2 in particular, has been shown to possess pro-inflammatory characteristics on both ECs and neutrophils [Lemieux et al., 2005; Fiedler and Augustin, 2006; Fiedler et al., 2006; Kim and Koh, 2011]. In addition, neutrophils and macrophages can equally trigger the release of numerous metalloproteinases, neutrophil elastase, and reactive oxygen species (ROS), which can facilitate extracellular matrix degradation, favoring the migration and proliferation of ECs and SMCs (reviewed in [van Hinsbergh et al., 2006]). In addition, the presence of neutrophils and macrophages in the sponges at day 7 during the recruitment of SMCs may initiate a paracrine compensation pathway in order to trigger the maturation event. Interestingly, from the histological sections, we observed that the newly formed vessels in presence of VEGF, Ang1, or Ang2 were "functional" based on the presence red blood cells in the neovessels and that they appeared to be non-leaky. Vascular permeability study must be conducted in order to confirm this later statement. However, as not all the neovessels formed in the sponges are necessarily matured at any given time, it is thus, not possible to confirm the absence of vascular leakiness. Furthermore, we also observed that the blood vessels once formed undergo no or marginal diameter remodeling. Ang1 in the past has been identified to play an important role in the reorganization of EC into tubule-like structures during angiogenesis by stimulating the production of proteases. Plasmin and matrix metalloproteases, examples of such proteases, decrease the EC-substratum interaction allowing the ECs to reshape the vessel lumen [Kim et al., 2000a]. However, in our study, upon the formation of neovessels by day 7 (with growth factors), we did not observe additional remodeling over time. This may be due to the rapid maturation of the newly formed vessels taking place simultaneous to blood vessel formation which may prevent further unrestricted enlargement of the growing vessels [Hoeben et al., 2004]. As for the PBS-treated sponges, the delayed recruitment of SMCs may explain the slight remodeling of the area of occupancy that took place between day 14 and 21. Together, VEGF, Ang1, and Ang2 alone are capable of mediating the maturation process in the presence of a pro-inflammatory environment suggesting that inflammation plays a major role in the angiogenic process.

This notion is further strengthened as observed under various pathological conditions. For instance, suppression of inflammatory response by genetic abnormalities, pathophysiological processes, or pharmacotherapy produce adverse effects in the ability of the host to induce new vessel growth [Jones et al., 1999]; hence inflammation, once considered to be a homeostatic response protecting the body from invading pathogens, is now been shown to function as a critical stimulus for neovessel growth. Neutrophils being the most abundant leukocyte in the circulation have been demonstrated to play important roles during pathological angiogenesis. Although, the exact mechanism through which tumor associated neutrophils mediate or modulate angiogenesis has not been fully elucidated, the importance of neutrophils in tumor angiogenesis has been noted from human biopsies [Van den Steen et al., 2000; Nozawa et al., 2006]. Similarly, increased macrophage infiltration in various types of cancer correlates positively with vascularity, tumor stage and malignancy [Torisu et al., 2000; Chen et al., 2003]. Once again the exact function of the macrophages in the tumor environment remains a nuance.

Likewise, although we did not study the exact roles of inflammatory cells in angiogenesis, we observed the presence of neutrophils, macrophages, and sparsely T- and B-cells, in the tissue section by IHC and/or flow cytometry. Surprisingly, the presence of neutrophils, expected to be one of the first cells recruited at the site of inflammation, was still observed at latter time points raising the question whether we have continuous recruitment of neutrophils in sponges or if they have been differentiated to other cell types. Recently, it was suggested that neutrophils could differentiate into neutrophil-DC hybrids with DC-like properties in the setting of experimentally induced inflammatory lesions in mice [Geng et al., 2013; Matsushima et al., 2013]. DCs are professional antigen presenting cells, which reside in peripheral tissues in an immature state. Upon microbial contact and stimulation by inflammatory cytokines, it possesses a unique ability to induce both primary and secondary T- and B-cell responses. It is now clear that DCs express a wide array of pro- and anti-inflammatory mediators that mediate a significant role in those pathophysiological settings characterized by DC activation and angiogenesis [Sozzani et al., 2007]. Thus, we hypothesized that neutrophils may differentiate into neutrophil-DC hybrids in our system. However, $CD11c^{+}MHCII^{+}$ cells, which we initially thought to be DCs, turned out to be neither DCs nor neutrophil-DC hybrid cells. These cells: (1) did not express DC-lineage transcriptional factor Zbtb46; and (2) did not stimulate allogenic T-cells in MLR assay. Instead, they expressed macrophage specific markers including F4/80, CD68, CD206, and CD115/mCSF1R and were highly phagocytic. Hence, our sponge/Matrigel model, apart from T- and B-cells, contains two major inflammatory cell populations: neutrophils and macrophages.

In summary, our murine sponge/Matrigel model in presence of the pro-angiogenic growth factors (VEGF, Ang1, or Ang2) allowed the formation of new vessels and more importantly, it led to their maturation. Moreover, the recruitment of inflammatory cells in the Matrigel by the provided growth factors further accelerated these processes with greater potency. Thus, such pro-inflammatory/

angiogenic model along with the growth factors may provide a suited autocrine/paracrine environment capable of triggering and supporting the formation and maturation of neovessels, illustrating the necessity of inflammation in the creation of mature blood vessels. Further studies will be needed through selected depletion of neutrophils and monocytes/macrophages to delineate the role of these cells in such angiogenic model.

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