The role of the innate and adaptive immune system on vascular remodeling

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Chapter 5

The role of CD27-CD70-mediated T cell co-stimulation in vasculogenesis, arteriogenesis and angiogenesis.

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Abstract

Background

T cells have a distinctive role in neovascularization, which consists of arteriogenesis and angiogenesis under pathological conditions and vasculogenesis under physiological conditions. However, the role of co-stimulation in T cell activation in neovascularization has yet to be established. The aim of this study was to investigate the role T cell co-stimulation and inhibition in angiogenesis, arteriogenesis and vasculogenesis.

Methods and Results

Hind limb ischemia was induced by double ligation of the left femoral artery in mice and blood flow recovery was measured with Laser Doppler Perfusion Imaging in control, CD70$^{-/-}$, CD80/86$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA4$^{+/-}$ mice. Blood flow recovery was significantly impaired in mice lacking CD70 compared to control mice, but was similar in CD80/86$^{-/-}$, CTLA4$^{+/-}$ and control mice. Mice lacking CD70 showed impaired vasculogenesis, since the number of pre-existing collaterals was reduced as observed in the pia mater compared to control mice. *In vitro* an impaired capability of vascular smooth muscle cells (VSMC) to activate T cells was observed in VSMC lacking CD70. Furthermore, CD70$^{-/-}$, CD80/86$^{-/-}$ and CD70/80/86$^{-/-}$ mice showed reduced angiogenesis in the soleus muscle 10 days after ligation. Arteriogenesis was also decreased in CD70$^{-/-}$ compared to control mice 10 and 28 days after surgery.

Conclusions

The present study is the first to describe an important role for T cell activation via co-stimulation in angiogenesis, arteriogenesis and vasculogenesis, where the CD27-CD70 T cell co-stimulation pathway appears to be the most important co-stimulation pathway in pre-existing collateral formation and post-ischemic blood flow recovery, by arteriogenesis and angiogenesis.

Introduction

Peripheral arterial disease (PAD) is characterized by the formation of atherosclerotic plaques in lower extremities and is a major cause of morbidity and mortality[1, 2]. The body can restore the blood flow to ischemic tissues by initiating neovascularization, which is a similar mechanism that occurs in patients after myocardial infarction. Neovascularization consists of angiogenesis and arteriogenesis under pathological conditions, such as PAD or myocardial infarction, and vasculogenesis under physiological conditions. Angiogenesis is the process of sprouting of new capillaries from pre-existing microvasculature, which is due to hypoxia and occurs mainly far distal to the occlusion[3]. Arteriogenesis initiates by inflammation, shear stress and circumferential stretch on the vascular wall, which causes inactive pre-existing arterioles, formed by vasculogenesis, to mature into functional collateral arteries, which occurs mainly nearby the occlusion[4, 5,6]. Vasculogenesis is the formation of new blood vessels during embryogenesis through differentiation of angioblasts into endothelial cells followed by the recruitment of vascular smooth muscle cells (VSMC), which can shape new blood vessels[7]. In PAD patients, for collateral artery formation a proper vascular bed of pre-existing arterioles is essential. These pre-exiting arterioles are formed by vasculogenesis. Therefore, this is an important process in PAD. The maturation of pre-existing collateral arteries by arteriogenesis, together with the angiogenetic sprouting of new capillaries, can restore blood flow towards ischemic tissues[8, 9].

We and others have shown a specific role of CD4+ T cells in arteriogenesis by using a hind limb ischemia (HLI) model[10, 11]. CD4+ T cells have the capacity to attract macrophages and monocytes to the site of occlusion, which in turn triggers arteriogenesis through the release of inflammatory cytokines. Various studies showed increased release of VEGF by hypoxic cells triggered through inflammatory cytokines, indicating a possible role of CD4+ T cells in angiogenesis as well[12, 13]. CD8+ T cells also contribute to the early phase of arteriogenesis and recruit CD4+ mononuclear cells through the expression of IL-16[14]. Others also suggest a role for CD8+ T cells in angiogenesis[15]. A previous study further showed that T cells play an important role in vasculogenesis[16]. However, it is still unknown what the activation mechanism of T cells in vasculogenesis, angiogenesis and arteriogenesis is.

For T cell activation, different T cell co-stimulation and inhibitory pathways are described. Co-stimulatory molecules of the B7 family, such as CD80 and CD86, were the first described and are the most well-known and studied molecules[17]. CD28 is a co-stimulation receptor expressed constitutively on the cell-surface of T cells, which

interacts with both CD80/CD86 proteins present on antigen presenting cells (APC)[18] and promotes T cell activation and proliferation [19]. As a counteracting system, CTLA4 is an inhibitory receptor on T cells, which down regulates the immune response by binding to CD80/86 with a higher affinity than CD28[17]. CD27 is a second co-stimulation receptor located constitutively on the surface of T cells, which interacts with CD70 proteins on APC to activate T cell[20]. In contrast to their receptors, co-stimulatory ligands CD80, CD86, and CD70 are transiently up regulated upon activation. The signaling pathway of CD27 in T cells is different compared to CD28 and CD27 promotes T cell survival via up regulation of anti-apoptotic factors[21, 22].

The CD28-CD80/86 T cell co-stimulation pathway and CD28-CTLA4 T cell inhibitory pathway were shown to regulate the development of both native atherosclerosis[23, 24] as well as post-interventional accelerated atherosclerosis[25]. But this pathway was also shown to be involved in other vascular diseases[26], graft arterial disease[27,28,29] and inflammatory diseases such as rheumatoid arthritis[30]. The CD27-CD70 T cell co-stimulation pathway is less investigated, however, immune activation via the CD27-CD70 T cell co-stimulation pathway showed to protect against atherosclerosis[31]. In the current study we aimed to elucidate the role of the CD27-CD70 and CD28-CD80/86 T cell co-stimulation pathway, and CD28-CTLA4 T cell inhibitory pathway in post-ischaemic neovascularization and vasculogenesis. By visualising pre-existing collaterals in the pia mater of $CD70^{-/-}$, $CD80/86^{-/-}$ and $CD70/80/86^{-/-}$ mice we observed a particular effect of CD27-CD70-mediated T cell co-stimulation on vasculogenesis. Furthermore, CD27-CD70-mediated T cell co-stimulation was also important for optimal blood flow recovery, angiogenesis and arteriogenesis.

Materials and methods

Mice

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All experiments were approved by the committee on animal welfare of the Leiden University Medical Center (Leiden, the Netherlands). For the experiments 10 to 18 week old male mice were used. C57BL/6 mice were purchased from Harlan Laboratories. $CD70^{-/-}$[32], $CD80/86^{-/-}$ [33] and $CTLA4^{+/-}$ mice were bred in house to the obtained C57BL/6 background[34]. $CD70/80/86^{-/-}$[34] mice were generated by crossing $CD70^{-/-}$ with $CD80/86^{-/-}$ mice. Ovalbumin-specific T cell receptor (TCR) transgenic OT-I mice[35] were obtained from The Jackson Laboratory. Mice were fed a chow diet ad libitum.

Murine HLI model

Mice were anesthetized with an intraperitoneal injection of midazolam (8 mg/kg, Roche Diagnostics), medetomidine (0.4 mg/kg; Orion), and fentanyl (0.08 mg/kg; Janssen Pharmaceuticals). Unilateral HLI was induced by electrocoagulation of the left common femoral artery proximal to the superficial epigastric artery and proximal to the bifurcation of the popliteal and saphenous artery (double ligation model)[36]. After surgery, anaesthesia was antagonised with flumazenil (0.7 mg/kg, Fresenius Kabi). Buprenorphine (0.1 mg/kg, MSD Animal Health) was given after surgery to relieve pain in a fixed regime and when circumstances required this was repeated. Mice were sacrificed 10 days after surgery (n=46) or 28 days after surgery (n=48) to investigate neovascularization in time.

Laser Doppler perfusion

Blood flow recovery was measured in the paw of the mice with the use of Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments). Blood flow was measured before and directly after ligation and at day 3, 7, 10, 13, 21 and 28. Before LDPI, mice were anaesthetised with an intraperitoneal injection of midazolam (8 mg/kg) and medetomidine (0.4 mg/kg). After LDPI, anaesthesia was antagonised by subcutaneous injection of flumazenil (0.7mg/kg). Paw perfusion was expressed as a ratio of left (ischemic) to right (non-ischemic) paw.

After the last LDPI measurement at day 10 or day 28, analgesic fentanyl (0.08 mg/kg) was administered subcutaneously and mice were sacrificed via cervical dislocation. Blood, lymph nodes, spleen and femur and tibia (to isolate bone marrow macrophages) were harvested for FACS analysis. The adductor and soleus muscles were harvested for (immuno)histochemical analysis. Adductor muscles were fixed in 4% formaldehyde and embedded in paraffin, soleus muscles were snap frozen on dry ice and stored at -80°C.

Immunohistochemistry

Cross sections of 6 µm were made throughout the embedded adductor muscle. Adductor muscle sections were stained with alpha smooth muscle cell actin (aSMActin, DAKO) to visualize vascular smooth muscle cells (VSMC). Stained slides were photographed (20x magnification) with microscope photography software (Axiovision, Zeiss) and analysed with ImageJ (FIJI) by counting the number of arterioles and measuring the diameters of each arteriole with a visible lumen to determine arteriogenesis. Arterioles were divided in two groups; diameter $<20\ \mu m^2$ and $>20\ \mu m^2$. Fresh-frozen soleus muscles were cross sectioned in 6 µm slices with a cryostat and sections were fixated in acetone. To visualise endothelial cells, soleus muscle sections

were stained with CD31 (BD Pharmingen). Stained slides were photographed (20x magnification) with microscope photography software. The number of endothelial cells was quantified with ImageJ, which was used to determine angiogenesis levels.

Immunofluorescent double staining was performed to identify the presence of T cells in close proximity of capillaries in the soleus muscle. Soleus muscle samples were stained for CD31 (Abcam) and CD3 (AbD Serotec). Slides were covered with Invitrogen Diamond Antifade Mountant (Invitrogen) with 4′,6-diamidino-2-phenylindole (DAPI).

Pre-existing collateral density in pial circulation

Pre-existing collateral density was determined in pial circulation of the pia mater. Pre-existing collateral density in the cerebral pial circulation is representative for collateral density in skeletal muscle[37]. Mice were anesthetized with midazolam (8 mg/kg), medetomidine (0.4 mg/kg) and fentanyl (0.08 mg/kg) via intraperitoneal injection and heparinized via intramuscular injection, after onset of anaesthesia. The thoracic aorta was cannulated retrograde and the circulation was maximally dilated by perfusion with sodium-nitroprusside (30 µg/ml) and papaverine (40 µg/ml) in PBS at approximately 100 mm Hg prior to vascular casting. After craniotomy Yellow Microfil (Flow Tech Inc.) was infused under a stereomicroscope. The dorsal cerebral circulation was fixed with topical application of 4% paraformaldehyde (PFA) to prevent any degradation in vessel dimensions after Microfil injection. The brains were fixed overnight in 4% PFA and were subsequently incubated in Evans Blue (2 µg/ml in 4% PFA) for several days to improve contrast for visualization of the vasculature. Digital images were acquired of the dorsal brain surface and processed with ImageJ software (NIH). Collateral density was calculated by counting the total number of pial collaterals between the anterior cerebral artery (ACA)-middle cerebral artery (MCA) and MCA-posterior cerebral artery (PCA) as described elsewhere[38-41] divided by the dorsal surface area of the cerebral hemispheres. Hemispheres that sustained damage, were incompletely filled, or were otherwise uncountable, were excluded from analysis.

Cell culture

VSMCs were acquired by isolating VSMCs from mice aortas. VSMCs were cultured in medium containing DMEM Glutamax with 20% FCS (Gibco® by Life Technologies), non-essential amino acids (MEM 100x, Gibco® by Thermofisher) and 100 U/mL Penicillin/streptomycin (Gibco® by Life Technologies). T cells were isolated from the spleens of OT-I mice. In total 50.000 VSMCs per well in a 24-wells plate were added with SIINFEKL peptide(0.3ug/ml, the CD8 OT-I T cell epitope of chicken ovalbumin) for 4 hours, after which 250.000 OT-I T cells were added and cells were stimulated

with 100ng/ml Lipopolysaccharide from Escherichia coli K-235 (LPS) purchased from Sigma-Aldrich (MO). Supernatant was removed after 24 hours after which IFN-ɣ concentrations were measured using ELISA assays (BD Biosciences).

Flow cytometry

Flow cytometry was performed on spleen, lymph nodes, bone marrow macrophages (BMM) and blood of 6 mice per group sacrificed after 10 and 28 days. BMM were acquired by isolating monocytes from femur and tibia bone marrow. Single-cell suspensions were prepared from spleens, lymph nodes and BMM by mincing the tissue through a 70 um cell strainer (BD Biosciences). Cells were washed with 10 ml IMDM Glutamax with 8% FCS and 100 U/mL Penicillin/streptomycin. Erythrocytes were lysed in red blood cell lysis buffer (hypotonic ammonium chloride buffer). Conjugated monoclonal antibodies to mouse CD3 (V500), CD62L (Allophycocyanin [APC]), CD8 (Alexa Fluor 700), KLRG1 (PE-Cy7), CD4 (Brilliant Violet 605/Qdol605) were purchased from eBioscience or BD Biosciences. Dead cells were excluded by positivity for 7-aminoactinomycinD (7-AAD) (Invitrogen). Flow cytometric acquisition was performed on a BD LSR II flow cytometer (BD Biosciences). Data were analysed using FlowJo V10.1 software.

Statistical analysis

All data are presented as mean SEM. In statistics software GraphPad Prism 7.0, statistical analyses on parametric data were performed by using a 2-tailed Student's t-test to compare individual groups, Mann-Whitney test was used for nonparametric data. A 1-way ANOVA was performed on parametric data comparing more than 2 groups and a Kruskal-Wallis test was performed on nonparametric data. P value of <0.05 was considered significant.

Results

Differential impact of co-stimulation pathways on T cell activation in lymphoid organs and blood. Initially, we determined if co-stimulation has a differential impact in lymphoid organs and blood, by analyzing the T cell activation levels in blood, bone marrow, lymph node and spleen of control CD80/86$^{-/-}$, CD70$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA4$^{+/-}$ mice. The phenotypical markers KLRG1+ CD62L- were used to determine the percentage of activated CD4+ or CD8+ T cells in each compartment. CD4+ and CD8+ T cell activation in bone marrow, lymph node and spleen of CD80/86$^{-/-}$ and CD70/80/86$^{-/-}$ mice was significantly decreased. In CD70$^{-/-}$ mice, CD4+ T cells in the bone marrow and CD8+ T cells in the lymph nodes and spleen showed significantly

decreased T cell activation compared to control (figure S1 and S2), indicating a more important role of the CD28-CD80/86 co-stimulation pathway compared to the CD27-CD70 co-stimulation pathway in lymphoid organs. This was also demonstrated by a trend towards increased T cell activation in lymphoid organs, blood and bone marrow in both CD4+ and CD8+ T cells of CTLA4$^{+/-}$ mice compared to control mice. However, CD80/86$^{-/-}$, CD70$^{-/-}$ and CD70/80/86$^{-/-}$, all showed no difference on T cell activation in blood. Together, these results demonstrate a differential effect of co-stimulatory pathways on T cell activation in bone marrow and lymphoid organs compared to the blood circulation.

Impact of CD27-CD70-mediated T cell co-stimulation on post-ischaemic blood flow recovery.

The above descibed results confirm that co-stimulatory pathways have distinct effects in lymphoid organs and blood, but whether such differential effects also occur in peripheral (non-lymphoid) tissues such as blood vessels or the formation thereof remains to be elucidated. Here we aimed to address the (differential) role of co-stimulation in neovascularization. First, we studied post-ischaemic blood flow recovery by analysing paw perfusion in control, CD80/86$^{-/-}$, CD70$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA-4$^{+/-}$ mice before ligation of the femoral artery and serially after surgery until sacrifice of the mice after 28 days. Paw perfusion was decreased directly after surgery and control mice showed 74% blood flow recovery in 28 days after surgery with a small drop in recovery between 7 and 13 days (figure 1a). CD80/86$^{-/-}$ and CTLA4$^{+/-}$ mice showed a similar blood flow recovery pattern in the paw as control mice, indicating that the CD28-CD80/86 T cell co-stimulation pathway is not a major co-stimulatory pathway in blood flow recovery. Blood flow recovery in CD70$^{-/-}$ mice ($p=0.03$) and CD70/80/86$^{-/-}$ ($p=0.01$) was significantly impaired 28 days after surgery. We observed a decreased blood flow recovery in CD70$^{-/-}$ mice in time with significantly lower paw perfusion ratios at all time points (except 13 days after surgery) compared to control mice. In CD70/80/86$^{-/-}$ mice, blood flow recovery was also impaired in time with lower paw perfusion ratios 3 days ($p=0.006$), 7 days ($p=0.002$) and 10 days ($p=0.004$) after surgery compared to control mice. With the comparable blood flow recovery of control mice and CD80/86$^{-/-}$ mice, and the reduced recovery in CD70$^{-/-}$ mice compared to control mice, we conclude that the impaired blood flow recovery of CD70/80/86$^{-/-}$ mice is most likely caused by the lack of CD70 co-stimulation.

Since paw perfusion was rapidly recovering in the first 10 days after surgery (figure 1a), we performed a second paw perfusion experiment in which the mice were sacrificed 10 days after surgery. Blood flow recovery in CD80/86$^{-/-}$ and CTLA-4$^{+/-}$ mice was comparable to control mice (figure 1b). However, CD70$^{-/-}$ mice showed

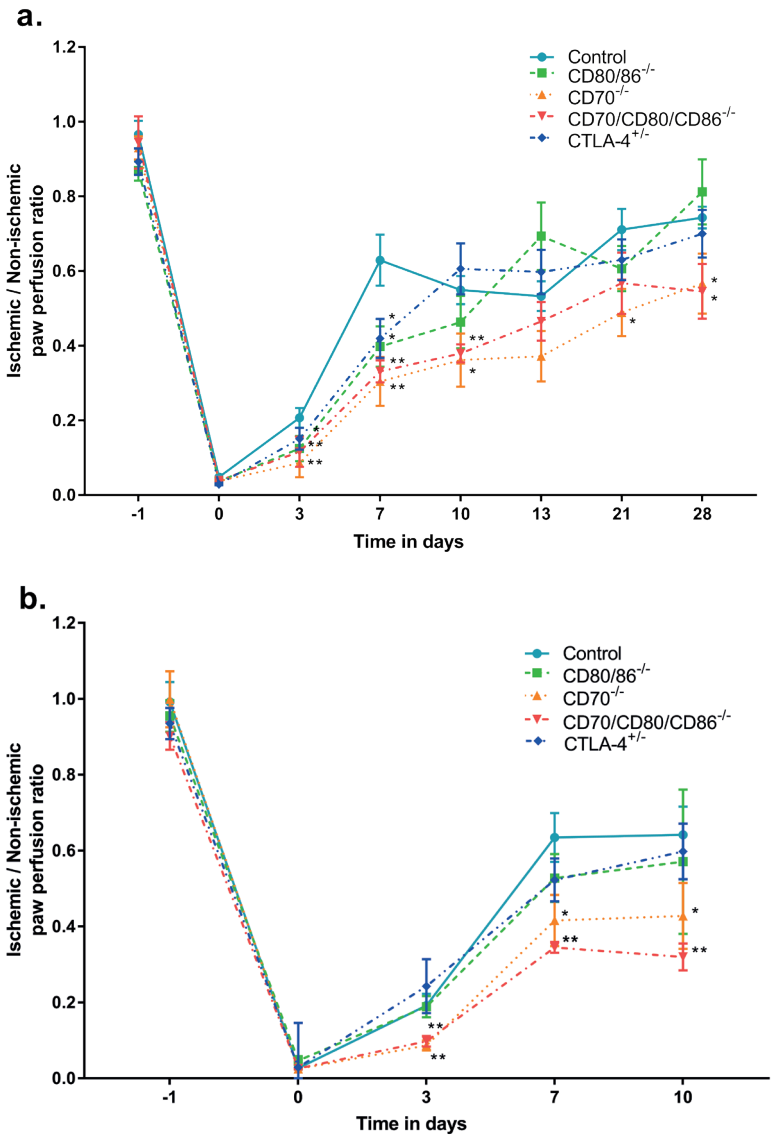

Figure 1. Blood flow recovery after induction of hind limb ischemia. **a.** Paw perfusion was measured before and after surgery and 3, 7, 10, 13, 21 and 28 days after surgery. Control mice (n=11, light blue), CD80/86$^{-/-}$ mice (n=11, green), CD70$^{-/-}$ mice (n=7, orange), CD70/80/86$^{-/-}$ mice (n=8, red) and in CTLA-4$^{+/-}$ mice (n=11, dark blue) were sacrificed after 28 days. **b.** Paw perfusion was measured before and after surgery and 3, 7 and 10 days after surgery. Control mice (n=9), CD80/86$^{-/-}$ mice (n=10), CD70$^{-/-}$ mice (n=11), CD70/80/86$^{-/-}$ mice (n=10) and in CTLA-4$^{+/-}$ mice (n=6) were sacrificed after 10 days. Paw perfusion is expressed as a ratio of left (ischemic) to right (non-ischemic) paw perfusion. Data is presented as mean SEM; * $p<0.05$; ** $p<0.01$.

significantly impaired blood flow recovery after 3 days (p=0.002), 7 days (p=0.04) and 10 days (p=0.01) compared to control mice. CD70/80/86$^{-/-}$ mice also showed impaired blood flow recovery after 3 days (p=0.0095), 7 days (p=0.003) and 10 days (p=0.002) after surgery compared to control mice. This confirms that the CD27-CD70 T cell co-stimulation pathway has an important role in blood flow recovery.

Pre-existing collateral formation is affected by CD27-CD70 T cell co-stimulation.

To determine if T cell co-stimulation affects collateral vasculogenesis, pre-existing collateral density was determined in pial circulation of the pia mater of CD70$^{-/-}$, CD80/86$^{-/-}$, CD70/80/86$^{-/-}$ and control mice. CD80/86$^{-/-}$ mice showed a similar pre-existing collateral density compared to control mice (figure 2a). However, compared to control mice a decrease in pre-existing collateral density was observed in CD70$^{-/-}$ (p=0.04) and CD70/80/86$^{-/-}$ mice (p=0.04) (figure 2b). This decreased formation of pre-existing collaterals, indicates an important role for the CD27-CD70 T cell co-stimulation pathway in vasculogenesis and collateral development.

T cell activation via vascular smooth muscle cells is mediated by CD27-CD70 T cell co-stimulation.

VSMC are essential in vascular remodeling and may also act as APCs in T cell activation. To determine the role of VSMC co-stimulation in vitro, we used control, CD80/86$^{-/-}$, CD70$^{-/-}$ and CD70/80/86$^{-/-}$ VSMC. We added OT-I T cells (recognizing the MHC class I SIINFEKL epitope of chicken ovalbumin) and LPS and measured the IFNy concentration in the supernatant, as measure of T cell activation, after 24 hours. CD70$^{-/-}$ (p=0.002) and CD70/80/86$^{-/-}$ (p=0.001) VSMCs showed a decreased T cell activation since the IFNy concentration in the supernatant was significantly lower compared to control VSMCs (figure S3). CD80/86$^{-/-}$ VSMC showed no differences in T cell activation compared to control VSMC. Indicating that the CD27-CD70 pathway, rather than the CD28-CD80/86 pathway, might play an important role in T cell activation via VSMCs.

Decreased angiogenesis in soleus muscles of CD70$^{-/-}$ mice.

To demonstrate the presence of T cells in the proximity of capillaries in soleus muscles, a double staining was performed for CD31 and CD3 in soleus muscles of mice sacrificed at 28 days after HLI. We here show the presence of T cells (CD3+ cells) in the soleus muscle around the capillaries (CD31+ cells), suggesting a contribution of T cells in angiogenesis (figure 3a). Angiogenetic capillary formation was determined by measuring the number of CD31 positive cells in soleus muscles (typical example of CD31 IHC staining shown in figure 3b). In mice lacking either CD70 (p=0.04), CD80/86 (p=0.02) or both (p=0.008), angiogenesis was significantly decreased compared

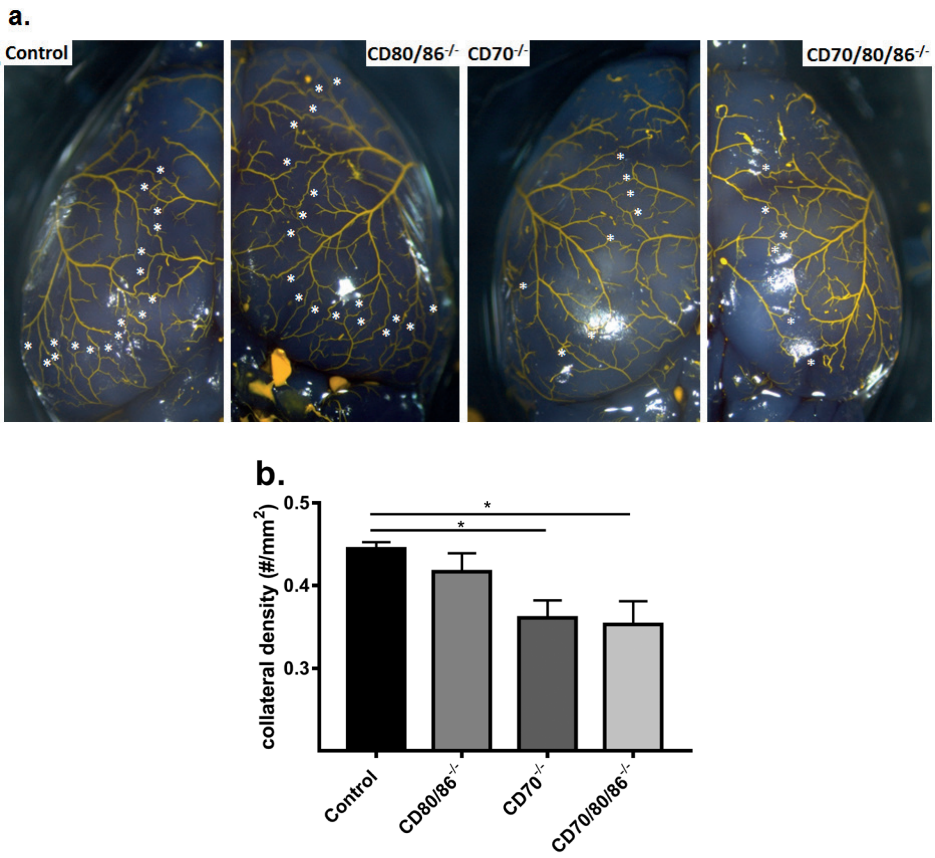

Figure 2. Pre-existing collateral density in pial circulation. **a.** Representative image of pre-existing collaterals indicated by white stars (*) in pial circulation in control, CD80/86$^{-/-}$, CD70/80/86$^{-/-}$ and CD70$^{-/-}$ mice. **b.** Total number of pre-existing collaterals per mm^2 in pial circulation is shown in control mice (n=5), CD80/86$^{-/-}$ mice (n=7), CD70$^{-/-}$ mice (n=7), CD70/80/86$^{-/-}$ mice (n=5). Data are calculated as mean SEM; * $p<0.05$.

to control mice 10 days after surgery. CTLA4$^{+/-}$ mice did not show differences in angiogenesis (figure 3c). In CD70$^{-/-}$ mice, angiogenesis was still impaired after 28 days (p=0.04) compared to control mice, but CD80/86$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA-4$^{+/-}$ mice showed no difference compared to control mice after 28 days (figure 3d). Comparison of angiogenetic capillary formation after 10 and 28 days showed increased angiogenesis in CD80/86$^{-/-}$ (p=0.04), CD70/80/86$^{-/-}$ (p=0.0005) and CTLA-4$^{+/-}$ (p=0.03) mice in time and angiogenesis levels and showed no longer differences compared to control mice after 28 days, where angiogenesis in CD70$^{-/-}$ mice did not increase in time (figure 3e). These results suggest an important role for the CD27-CD70 T cell co-stimulation pathway in angiogenesis.

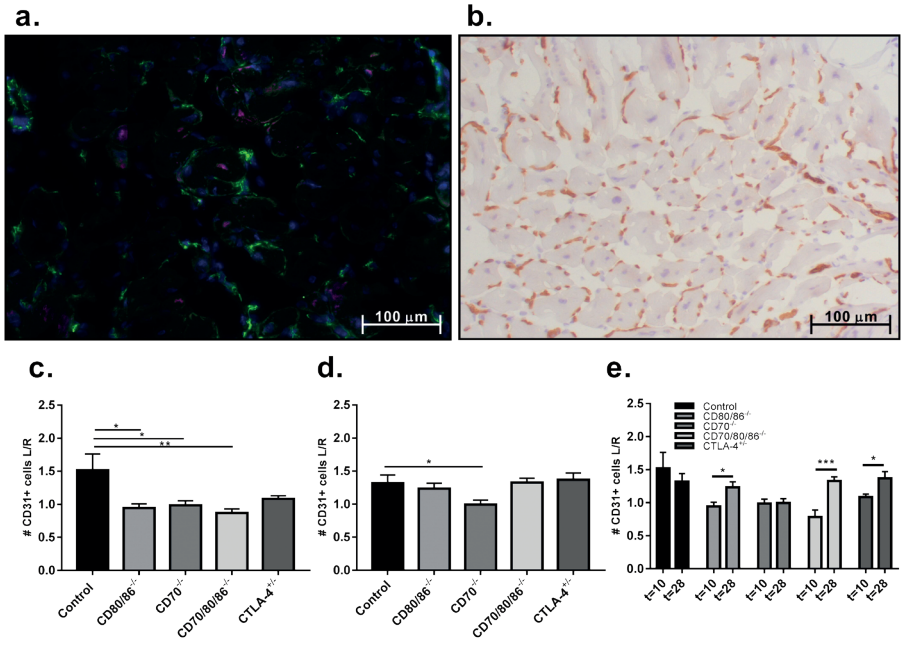

Figure 3. Angiogenesis in soleus muscles. **a.** Representative image of CD31 (green) and CD3 (magenta) immunofluorescent double staining in soleus muscles is shown with DAPI (blue). **b.** representative image of CD31 staining in soleus muscle of a control mice is shown which was used for quantification (20x magnification) **c.** Quantification as L/R (left ischemic/right non-ischemic) ratio of the number of CD31 positive cells in soleus muscles is shown in control mice (n=9), CD80/86$^{-/-}$ mice (n=10), CD70$^{-/-}$ mice (n=11), CD70/80/86$^{-/-}$ mice (n=10) and CTLA-4$^{+/-}$ mice (n=6) 10 days after surgery, **d.** and 28 days after surgery of control mice (n=11), CD80/86$^{-/-}$ mice (n=11), CD70$^{-/-}$ mice (n=7), CD70/80/86$^{-/-}$ mice (n=8) and CTLA-4$^{+/-}$ mice (n=11) and in **e.** both 10 and 28 days after surgery. Data are calculated as the ratio of L/R and presented as mean SEM; * p<0.05; ** p<0.01, ***p<0.001.

Decreased arteriogenesis in adductor muscles of CD70$^{-/-}$ mice.

Arteriogenesis was determined by counting the number of collateral arterioles and measuring the diameter of collateral arterioles of smooth muscle cell (aSMActin) stained adductor muscles of mice sacrificed 10 and 28 days after surgery (typical example of aSMActin staining is shown in figure 4a) and is shown as a ratio of treated compared to untreated adductor muscle. Total number of collateral arterioles in the left paw after arterial ligation was significantly lower in CD70$^{-/-}$ mice 10 days (p=0.02) and 28 days (p=0.03) after arterial ligation compared to control mice (figure 4b and 4c), indicating decreased pre-existing collaterals in CD70$^{-/-}$ mice. No differences in number of collateral arterioles were observed in CD80/86$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA-4$^{+/-}$ mice compared to control mice 10 and 28 days after surgery.

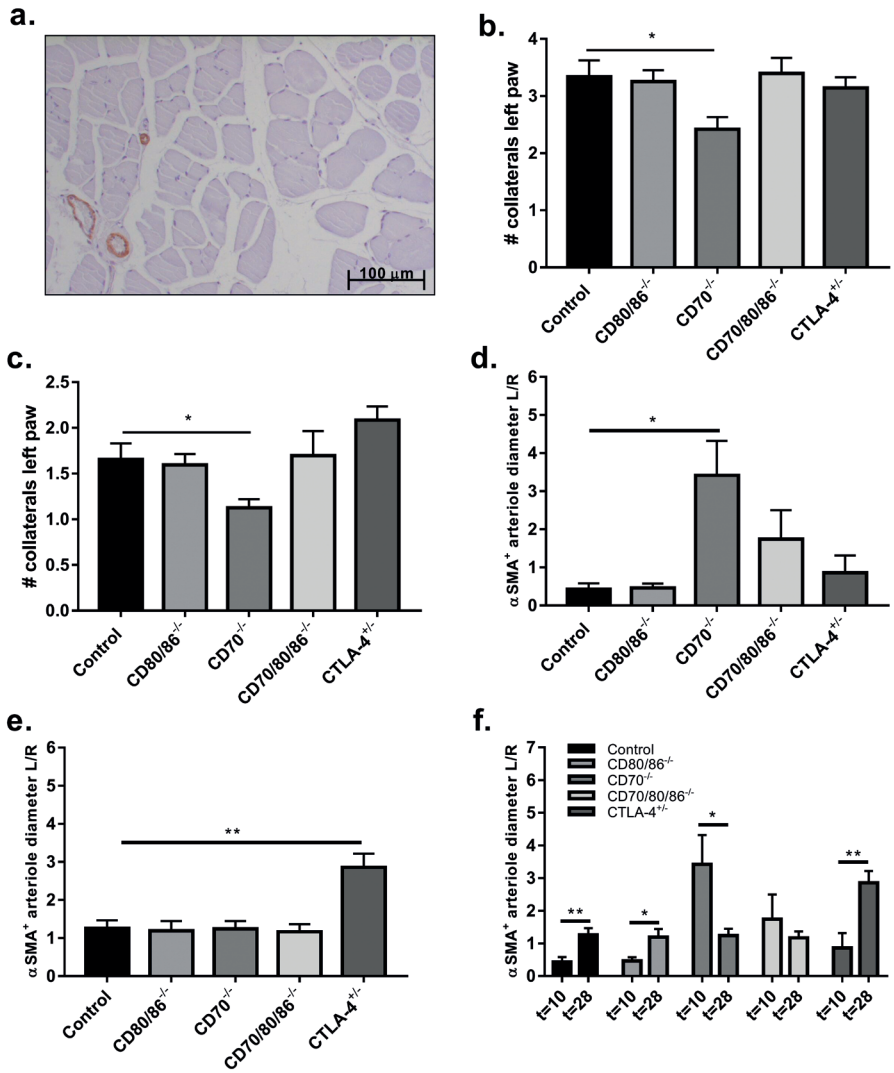

Figure 4. Arteriogenesis in adductor muscles. **a.** Representative image of αSMActin staining in adductor muscle tissue (20x magnification). **b.** Number of collateral arterioles is shown in the left paw 10 days after surgery in control (n=9), CD80/86$^{-/-}$ (n=10), CD70$^{-/-}$ (n=11), CD70/80/86$^{-/-}$ (n=10) and in CTLA-4$^{+/-}$ mice (n=6). **c.** Number of collateral arterioles is shown in the left paw 28 days after surgery in control (n=11), CD80/86$^{-/-}$ (n=11), CD70$^{-/-}$ (n=7), CD70/80/86$^{-/-}$ (n=8) and in CTLA-4$^{+/-}$ mice (n=11). **d.** Diameter of αSMActin positive collateral arterioles, presented as L/R (left ischemic/right non-ischemic) ratio, is shown 10 days after surgery, **e.** 28 days after surgery and **f.** in both 10 and 28 days after surgery. Data are presented as mean SEM; * p<0.05; ** p<0.01, ***p<0.001.

Collateral arterioles diameter L/R ratio was significantly increased 10 days after surgery in CD70$^{-/-}$ mice (p=0.01) compared to control mice and a trend towards an increased diameter of collateral arterioles was observed in CD70/80/86$^{-/-}$ mice (p=0.08) compared to control mice. No differences were observed in CD80/86$^{-/-}$ and CTLA-4$^{+/-}$ adductor muscles compared to control mice (figure 4d). The collateral arterioles were quantified for small (<20um^2) and large (>20um^2) collaterals 10 days after surgery to show an increase in large arterioles. No differences were found in the number of small collaterals (figure S4a). We showed more large collaterals (>20um^2) in CD70$^{-/-}$ mice (p=0.04) compared to control mice (figure S4b).

Collateral arteriole diameter was significantly increased in CTLA-4$^{+/-}$ mice (p=0.009), sacrificed 28 days after surgery (figure 4e). No differences were found in CD70$^{-/-}$, CD80/86$^{-/-}$ and CD70/80/86$^{-/-}$ compared to control mice 28 days after surgery. Numbers of small and large collateral arterioles were higher in CTLA-4$^{+/-}$ mice compared to control mice (figure S4c and S42d). CD70$^{-/-}$ mice did not show an increased number of small or large collateral arterioles 28 days after surgery. Comparison of collateral arterioles diameter L/R ratio after 10 and 28 days showed significantly increased diameter of collateral arterioles in time in control, CD80/86$^{-/-}$ and CTLA4$^{+/-}$ adductor muscles and a decrease in collateral arterioles diameter in CD70$^{-/-}$ adductor muscles. CD70/80/86$^{-/-}$ mice showed no difference in collateral arterioles diameter L/R ratio in time (figure 4f). In conclusion, these results suggest an important role for the CD27-CD70 T cell co-stimulation pathway in arteriogenesis.

Discussion

The current study demonstrates an important role for the CD27-CD70 T cell co-stimulation pathway in angiogenesis, arteriogenesis and vasculogenesis. The CD28-CD80/86 T cell co-stimulation pathway showed to be of great importance in T cell activation in lymphoid organs and bone marrow. Blood flow recovery after induction of HLI showed to be significantly impaired in mice lacking CD70, while in CD80/86$^{-/-}$ mice andCTLA4$^{+/-}$ mice no effect was observed, which indicates a particular important role of the CD27-CD70 T cell co-stimulation pathway in neovascularization. CD70 deficiency resulted in impaired vasculogenesis, as the number of pre-existing collaterals was reduced in the pia mater. This impaired vasculogenesis also affected the skeletal muscle, as the number of pre-existing collaterals was also decreased in the adductor muscle of CD70$^{-/-}$ mice, which led to a severely impaired blood flow recovery after ischemia. Furthermore, mice lacking CD70 or CD80/86 showed reduced angiogenesis in soleus muscles 10 days after ligation. In conclusion, the CD27-

CD70 T cell co-stimulation pathway showed to be most important in pre-existing collateral formation and post-ischemic blood flow recovery, by arteriogenesis and angiogenesis.

This conclusion was substantiated by the fact that the CD28-CD80/86 T cell co-stimulation pathway did not show an effect on either vasculogenesis, arteriogenesis or blood flow recovery. CD80/86$^{-/-}$ mice demonstrated a similar number of pre-existing collaterals as the control mice and both CD80/86$^{-/-}$ mice and CTLA4$^{+/-}$ mice showed the same pattern of blood flow recovery as the control mice after induction of HLI. Since we observed no effect of the CD28-CD80/86 T cell co-stimulation pathway on blood flow recovery, the effect of CD70/80/86$^{-/-}$ mice on blood flow recovery is most likely explained by the lack of CD70 co-stimulation.

Previous studies showed that the CD28-CD80/86 T cell co-stimulation pathway and CD28-CTLA4 T cell inhibitory pathway regulate the development of native atherosclerosis[23, 24] via reduced T cell activation and proliferation and thus decreased presence of IFNγ producing T cells, and regulatory T cells. Inhibition of the CD28-CD80/86 T cell co-stimulation pathway with abatacept showed beneficial effects on interventional accelerated atherosclerosis, most likely caused by decreased CD4+ T cell activation[25]. These studies are in contrast with our study since we here describe an important role of the CD27-CD70 co-stimulation pathway and not the CD28-CD80/86 T cell co-stimulation pathway in neovascularization. However, these studies were performed in an atherosclerosis model, and not in a HLI model. Furthermore, other T cell co-stimulatory and inhibitory pathways such as PD1, OX40, CD40 and 4-1BB also showed to be involved in vascular diseases[42], although interesting, that is beyond the scope of this study. The CD27-CD70 T cell co-stimulation pathway showed beneficial effect on atherosclerosis, due to monocytes that were susceptible to apoptosis and in that way prevented atherosclerotic plaque formation[31]. This is line with our study where we show a detrimental effect of CD70 deficient mice in neovascularization, suggesting a beneficial effect of CD70 in neovascularization in mice without CD70 deficiency. A particular important role of the CD27-CD70 and not the CD28-CD80/86 T cell co-stimulation pathway in our study could be due to the constitutively expression of CD27 and CD28 on T cells [18, 43, 44], while CD80, CD86, and CD70 are transiently up regulated upon activation on different APCs that contribute to neovascularization e.g. VSMCs, endothelial cells, macrophages or dendritic cells. We here showed that CD80/CD86 did not influence the VSMC functionality and only lack of CD70 resulted in an impaired function of VSMC which can contribute to impaired pre-existing collateral formation and arteriogenesis via decreased attraction of inflammatory cells via VSMCs. Although CD27 and CD28 are constitutively expressed on resting T cells,

CD27 and CD28 expression is lost after differentiation into effector T cells. Transient co-stimulation expression on these cell types might lead to differential functions of co-stimulation. Furthermore, it is shown previously that CD28 promotes T cell proliferation and activation, while CD27 stimulated cell survival. We suggest that the differential functions and expression of individual co-stimulation and inhibitory pathways might lead to differential effects on neovascularization.

This study shows that a combination of all aspects of neovascularization is essential for post-ischemic blood flow recovery. Neovessel formation may be either de novo via vasculogenesis or under pathological conditions via angiogenesis[45, 46]. Together with arteriogenesis, the body can naturally restore a hampered blood flow. In this study, impaired angiogenesis, arteriogenesis and vasculogenesis showed all to be involved in the impaired blood flow recovery in mice lacking CD70, and only an increase in diameter of collateral arterioles was not sufficient to restore blood flow in the paw after ligation of the femoral artery. We expected a decrease in pre-existing collaterals since we also showed an impairment in the ability to activate T cells in $CD70^{-/-}$ and $CD70/80/86^{-/-}$ VSMCs *in vitro* and VSMCs contribute to the development of the pre-existing collaterals via VEGF regulation[47].

However, with an increase in diameter of collateral arterioles, an increase in post-ischemic blood flow recovery could be expected. We here show a decreased post-ischemic blood flow recovery in $CD70^{-/-}$ mice with an increased diameter of collateral arterioles in adductor muscles, which is counterintuitive. This could be explained by the reduced number of collateral arterioles observed in the adductor muscles of the ligated paws in $CD70^{-/}$ mice which can lead to a higher blood pressure in the present collateral arterioles after induction of HLI. This can lead to shear stress and attraction of monocytes and macrophages, which can explain the increased diameter of collateral arterioles in $CD70^{-/-}$ mice 10 days after surgery. The impaired angiogenesis and reduced number of pre-existing collaterals in mice lacking CD70 contributed to a reduced blood flow recovery and only an increased diameter of collateral arterioles in the CD70 deficient mice could not restore blood flow. This indicates that after arterial obstruction, blood flow can only be restored when all components of neovascularization including vasculogenesis, angiogenesis and arteriogenesis, are fully functional.

We here described a different function of co-stimulatory pathways in lymphoid organs, in the systemic circulation and in peripheral tissues. With an important role of the CD28-CD80/86 co-stimulation pathway for T cell activation in lymphoid organs and bone marrow, and the CD27-CD70 co-stimulation pathway in peripheral

tissues after ischemia is induced. Previous studies also showed opposed systemic and peripheral effects of monocytes. An enhanced systemic activation of Ly6C^{hi} monocytes, but a reduced infiltration of Ly6C^{hi} monocytes into peripheral muscle tissue was shown after HLI in RP105 (a TLR4 homologue) deficient mice, which resulted in reduced blood flow recovery in RP105 deficient mice[48]. Another study showed improved post-ischemic blood flow recovery after intravenous infusion of T cell pre-stimulated monocytes. Monocytes were circulating in the blood, but not present in the vessel wall, suggesting a more systemic effect of T cell pre-stimulated monocytes[49]. Furthermore, after HLI, CD4+ T cells were specifically accumulated in adductor muscles regulated via the CCR7-CCL19/CCL21 axis[50]. Which can explain our opposed systemic and peripheral effects of co-stimulation.

In conclusion, we here show an important role for T cell activation via co-stimulation in angiogenesis, arteriogenesis and vasculogenesis, were the CD27-CD70 T cell co-stimulation pathway appears to be the most important T cell co-stimulation factor in pre-existing collateral formation and post-ischemic blood flow recovery, by arteriogenesis and angiogenesis.

References

1. Lu, H. Daugherty, A. Atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2015;35(3):485-91.
2. Fowkes, F. G. Rudan, D. Rudan, I. Aboyans, V. Denenberg, J. O. McDermott, M. M. Norman, P. E. Sampson, U. K. Williams, L. J. Mensah, G. A. Criqui, M. H. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. *Lancet (London, England)*. 2013;382(9901):1329-40.
3. Risau, W. Mechanisms of angiogenesis. *Nature*. 1997;386(6626):671-4.
4. Rizzi, A. Benagiano, V. Ribatti, D. Angiogenesis versus arteriogenesis. *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie*. 2017;58(1):15-9.
5. Bergmann, C. E. Hoefer, I. E. Meder, B. Roth, H. van Royen, N. Breit, S. M. Jost, M. M. Aharinejad, S. Hartmann, S. Buschmann, I. R. Arteriogenesis depends on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. *Journal of leukocyte biology*. 2006;80(1):59-65.
6. Heil, M. Eitenmuller, I. Schmitz-Rixen, T. Schaper, W. Arteriogenesis versus angiogenesis: similarities and differences. *Journal of cellular and molecular medicine*. 2006;10(1):45-55.
7. Risau, W. Flamme, I. Vasculogenesis. *Annual review of cell and developmental biology*. 1995;11:73-91.
8. Peirce, S. M. Skalak, T. C. Microvascular remodeling: a complex continuum spanning angiogenesis to arteriogenesis. *Microcirculation (New York, NY : 1994)*. 2003;10(1):99-111.
9. Semenza, G. L. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. *Journal of cellular biochemistry*. 2007;102(4):840-7.
10. van Weel, V. Toes, R. E. Seghers, L. Deckers, M. M. de Vries, M. R. Eilers, P. H. Sipkens, J. Schepers, A. Eefting, D. van Hinsbergh, V. W. van Bockel, J. H. Quax, P. H. Natural killer cells and CD4+ T-cells modulate collateral artery development. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27(11):2310-8.
11. Stabile, E. Burnett, M. S. Watkins, C. Kinnaird, T. Bachis, A. la Sala, A. Miller, J. M. Shou, M. Epstein, S. E. Fuchs, S. Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. *Circulation*. 2003;108(2):205-10.
12. Rega, G. Kaun, C. Demyanets, S. Pfaffenberger, S. Rychli, K. Hohensinner, P. J. Kastl, S. P. Speidl, W. S. Weiss, T. W. Breuss, J. M. Furnkranz, A. Uhrin, P. Zaujec, J. Zilberfarb, V. Frey, M. Roehle, R. Maurer, G. Huber, K. Wojta, J. Vascular endothelial growth factor is induced by the inflammatory cytokines interleukin-6 and oncostatin m in human adipose tissue in vitro and in murine adipose tissue in vivo. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27(7):1587-95.
13. Melter, M. Reinders, M. E. Sho, M. Pal, S. Geehan, C. Denton, M. D. Mukhopadhyay, D. Briscoe, D. M. Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis in vivo. *Blood*. 2000;96(12):3801-8.
14. Stabile, E., Kinnaird, T., la Sala, A., Hanson, S. K., Watkins, C., Campia, U., Shou, M., Zbinden, S., Fuchs, S., Kornfeld, H., Epstein, S. E., Burnett, M. S. CD8+ T lymphocytes regulate the arteriogenic response to ischemia by infiltrating the site of collateral vessel development and recruiting CD4+ mononuclear cells through the expression of interleukin-16. *Circulation*. 2006;113(1):118-24.
15. Zhou, H. Luo, Y. Mizutani, M. Mizutani, N. Reisfeld, R. A. Xiang, R. T cell-mediated suppression of angiogenesis results in tumor protective immunity. *Blood*. 2005;106(6):2026-32.
16. Hur, J. Yang, H. M. Yoon, C. H. Lee, C. S. Park, K. W. Kim, J. H. Kim, T. Y. Kim, J. Y. Kang, H. J. Chae, I. H. Oh, B. H. Park, Y. B. Kim, H. S. Identification of a novel role of T cells in postnatal vasculogenesis: characterization of endothelial progenitor cell colonies. *Circulation*. 2007;116(15):1671-82.
17. Chen, L. Flies, D. B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature reviews Immunology*. 2013;13(4):227-42.

18. Sharpe, A. H. Freeman, G. J. The B7-CD28 superfamily. *Nature reviews Immunology*. 2002;2(2):116-26.
19. Gerdes, N. Zirlik, A. Co-stimulatory molecules in and beyond co-stimulation - tipping the balance in atherosclerosis? *Thrombosis and haemostasis*. 2011;106(5):804-13.
20. Denoeud, J. Moser, M. Role of CD27/CD70 pathway of activation in immunity and tolerance. *Journal of leukocyte biology*. 2011;89(2):195-203.
21. Watts, T. H. TNF/TNFR family members in costimulation of T cell responses. *Annual review of immunology*. 2005;23:23-68.
22. Denoeud, J. Moser, M. Role of CD27/CD70 pathway of activation in immunity and tolerance. *Journal of leukocyte biology*. 2011;89(2):195-203.
23. Ait-Oufella, H. Salomon, B.L. Potteaux, S. Robertson, A.K. Gourdy, P. Zoll, J. Merval, R. Esposito, B. Cohen, J.L. Fisson, S. Flavell, R.A. Hansson, G.K. Klatzmann, D. Tedgui, A. Mallat, Z. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med*. 2006;12(2):178-80.
24. Buono, C. Pang, H. Uchida, Y. Libby, P. Sharpe, A.H. Lichtman, A.H. B7-1/B7-2 costimulation regulates plaque antigen-specific T-cell responses and atherogenesis in low-density lipoprotein receptor-deficient mice. *Circulation*. 2004;109(16):2009-15.
25. Ewing, M.M. Karper, J.C. Abdul, S. de Jong, R.C. Peters, H.A.B. de Vries, M.R. Redeker, A. Kuiper, J. Toes, R.E. Arens, R. Jukema, J.W. Quax, P.H.A. T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development. *International journal of cardiology*. 2013;168(3):1965-74.
26. Lichtman, A. H. T cell costimulatory and coinhibitory pathways in vascular inflammatory diseases. *Frontiers in physiology*. 2012;3:18.
27. Furukawa, Y. Mandelbrot, D. A. Libby, P. Sharpe, A. H. Mitchell, R. N. Association of B7-1 co-stimulation with the development of graft arterial disease. Studies using mice lacking B7-1, B7-2, or B7-1/B7-2. *The American journal of pathology*. 2000;157(2):473-84.
28. Kim, K. S. Denton, M. D. Chandraker, A. Knoflach, A. Milord, R. Waaga, A. M. Turka, L. A. Russell, M. E. Peach, R. Sayegh, M. H. CD28-B7-mediated T cell costimulation in chronic cardiac allograft rejection: differential role of B7-1 in initiation versus progression of graft arteriosclerosis. *The American journal of pathology*. 2001;158(3):977-86.
29. Mitchell, R. N. Libby, P. Vascular remodeling in transplant vasculopathy. *Circulation research*. 2007;100(7):967-78.
30. Kormendy, D. Hoff, H. Hoff, P. Broker, B. M. Burmester, G. R. Brunner-Weinzierl, M. C. . Impact of the CTLA-4/CD28 axis on the processes of joint inflammation in rheumatoid arthritis. *Arthritis Rheum*. 2013;65(1):81-7.
31. van Olffen, R. W. de Bruin, A. M. Vos, M. Staniszewska, A. D. Hamann, J. van Lier, R. A. de Vries, C. J. Nolte, M. A. CD70-driven chronic immune activation is protective against atherosclerosis. *Journal of innate immunity*. 2010;2(4):344-52.
32. Coquet, J. M. Middendorp, S. van der Horst, G. Kind, J. Veraar, E. A. Xiao, Y. Jacobs, H. Borst, J. The CD27 and CD70 costimulatory pathway inhibits effector function of T helper 17 cells and attenuates associated autoimmunity. *Immunity*. 2013;38(1):53-65.
33. Borriello, F. Sethna, M. P. Boyd, S. D. Schweitzer, A. N. Tivol, E. A. Jacoby, D. Strom, T. B. Simpson, E. M. Freeman, G. J. Sharpe, A. H. . B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity*. 1997;6(3):303-13.
34. Welten, S. P. Redeker, A. Franken, K. L. Oduro, J. D. Ossendorp, F. Cicin-Sain, L. Melief, C. J. Aichele, P. Arens, R. The viral context instructs the redundancy of costimulatory pathways in driving CD8(+) T cell expansion. *eLife*. 2015;4.
35. Welten, S.P. Redeker, A. Franken, K.L. Benedict, C.A. Yagita, H. Wensveen, F.M. Borst, J. Melief, C.J. van Lier, R.A. van Gisbergen, K.P. Arens, R. CD27-CD70 costimulation controls T cell immunity during acute and persistent cytomegalovirus infection. *Journal of virology*. 2013;87(12):6851-65.

36. Hellingman, A. A. Bastiaansen, A. J. de Vries, M. R. Seghers, L. Lijkwan, M. A. Lowik, C. W. Hamming, J. F. Quax, P. H. Variations in surgical procedures for hind limb ischaemia mouse models result in differences in collateral formation. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery*. 2010;40(6):796-803.
37. Wang, S. Zhang, H. Dai, X. Sealock, R. Faber, J. E. Genetic architecture underlying variation in extent and remodeling of the collateral circulation. *Circulation research*. 2010;107(4):558-68.
38. Zhang, H. Prabhakar, P. Sealock, R. Faber, J. E. Wide genetic variation in the native pial collateral circulation is a major determinant of variation in severity of stroke. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2010;30(5):923-34.
39. de Vries, M. R. Peters, E. A. B. Quax, P. H. A. Nossent, A. Y. von Willebrand factor deficiency leads to impaired blood flow recovery after ischaemia in mice. *Thrombosis and haemostasis*. 2017;117(7):1412-9.
40. Chalothorn, D. Clayton, J. A. Zhang, H. Pomp, D. Faber, J. E. Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains. *Physiological genomics*. 2007;30(2):179-91.
41. Bastiaansen, A. J. Ewing, M. M. de Boer, H. C. van der Pouw Kraan, T. C. de Vries, M. R. Peters, E. A. Welten, S. M. Arens, R. Moore, S. M. Faber, J. E. Jukema, J. W. Hamming, J. F. Nossent, A. Y. Quax, P. H. Lysine acetyltransferase PCAF is a key regulator of arteriogenesis. *Arteriosclerosis, thrombosis, and vascular biology*. 2013;33(8):1902-10.
42. Gotsman, I. Sharpe, A. H. Lichtman, A. H. T-cell costimulation and coinhibition in atherosclerosis. *Circulation research*. 2008;103(11):1220-31.
43. Tesselaar, K. Xiao, Y. Arens, R. van Schijndel, G. M. Schuurhuis, D. H. Mebius, R. E. Borst, J. van Lier, R. A. Expression of the murine CD27 ligand CD70 in vitro and in vivo. *Journal of immunology (Baltimore, Md : 1950)*. 2003;170(1):33-40.
44. Borst, J. Hendriks, J. Xiao, Y. CD27 and CD70 in T cell and B cell activation. *Current opinion in immunology*. 2005;17(3):275-81.
45. Kubis, N. Levy, B. I. Vasculogenesis and angiogenesis: molecular and cellular controls. Part 1: growth factors. *Interventional neuroradiology : journal of peritherapeutic neuroradiology, surgical procedures and related neurosciences*. 2003;9(3):227-37.
46. Beck, L., Jr. D'Amore, P. A. Vascular development: cellular and molecular regulation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1997;11(5):365-73.
47. Lucitti, J. L. Mackey, J. K. Morrison, J. C. Haigh, J. J. Adams, R. H. Faber, J. E. Formation of the collateral circulation is regulated by vascular endothelial growth factor-A and a disintegrin and metalloprotease family members 10 and 17. *Circulation research*. 2012;111(12):1539-50.
48. Bastiaansen, A. J. Karper, J. C. Wezel, A. de Boer, H. C. Welten, S. M. de Jong, R. C. Peters, E. A. de Vries, M. R. van Oeveren-Rietdijk, A. M. van Zonneveld, A. J. Hamming, J. F. Nossent, A. Y. Quax, P. H. TLR4 accessory molecule RP105 (CD180) regulates monocyte-driven arteriogenesis in a murine hind limb ischemia model. *PloS one*. 2014;9(6):e99882.
49. Hellingman, A. A. Zwaginga, J. J. van Beem, R. T. Hamming, J. F. Fibbe, W. E. Quax, P. H. Geutskens, S. B. T-cell-pre-stimulated monocytes promote neovascularisation in a murine hind limb ischaemia model. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery*. 2011;41(3):418-28.
50. Nossent, A. Y. Bastiaansen, A. J. Peters, E. A. de Vries, M. R. Aref, Z. Welten, S. M. de Jager, S. C. van der Pouw Kraan, T. C. Quax, P. H. CCR7-CCL19/CCL21 Axis is Essential for Effective Arteriogenesis in a Murine Model of Hindlimb Ischemia. *Journal of the American Heart Association*. 2017;6(3).

Supplemental figures

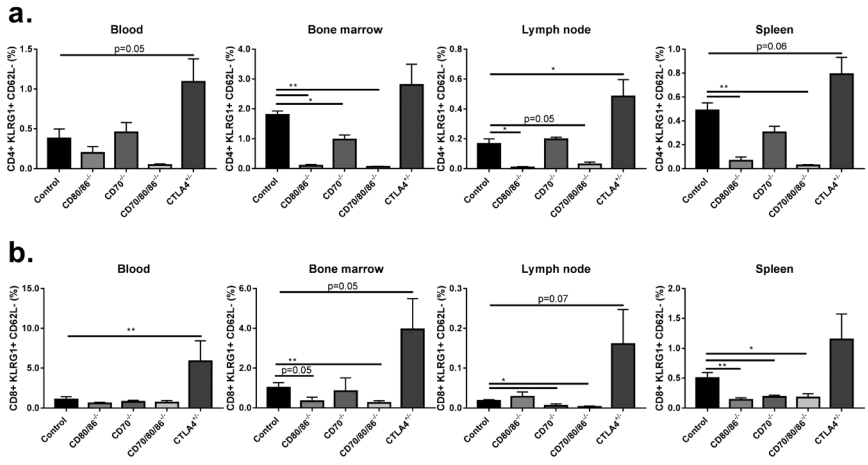

Figure S1. Percentage of KLRG1+ CD62L-, CD4+ or CD8+ T cells. Blood, bone marrow, lymph node and spleen were analysed with FACS of control CD80/86$^{-/-}$, CD70$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA4$^{+/-}$ mice, sacrificed 28 days after surgery. **a.** CD4+ T cells **b.** CD8+ T cells. n=3-6. Data is presented as mean SEM; *p<0.05, **p<0.01

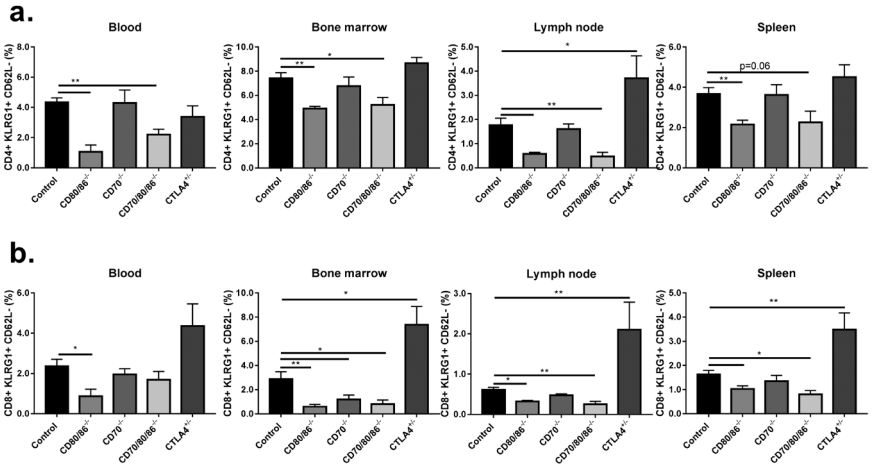

Figure S2 Percentage of KLRG1+ CD62L-, CD4+ or CD8+ T cells. Blood, bone marrow, lymph node and spleen were analysed with FACS of control CD80/86$^{-/-}$, CD70$^{-/-}$, CD70/80/86$^{-/-}$ and CTLA4$^{+/-}$ mice, sacrificed 10 days after surgery. **a.** CD4+ T cells **b.** CD8+ T cells. n=3-6. Data is presented as mean SEM; *p<0.05, **p<0.01

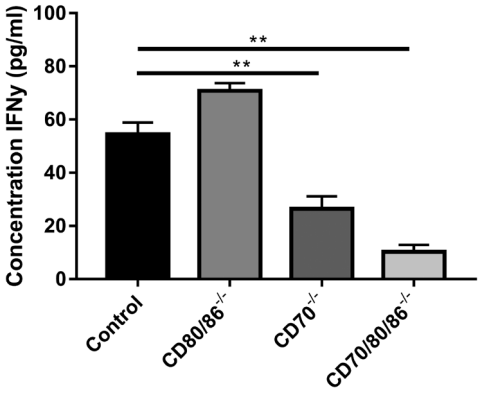

Figure S3. T cell activation via VSMC measured in control, CD80/86$^{-/-}$ CD70$^{-/-}$ and CD70/80/86$^{-/-}$ VSMCs. T cells of an OT-I mouse were added to the cultured VSMCs together with Ova and LPS (100ng/ml). IFNy (in pg/ml) concentration was measured in supernatant of VSMCs after 24 hours. CD80/86$^{-/-}$ CD70$^{-/-}$ and CD70/80/86$^{-/-}$ n=4, control n=10. Data are calculated as mean SEM; **p<0.01

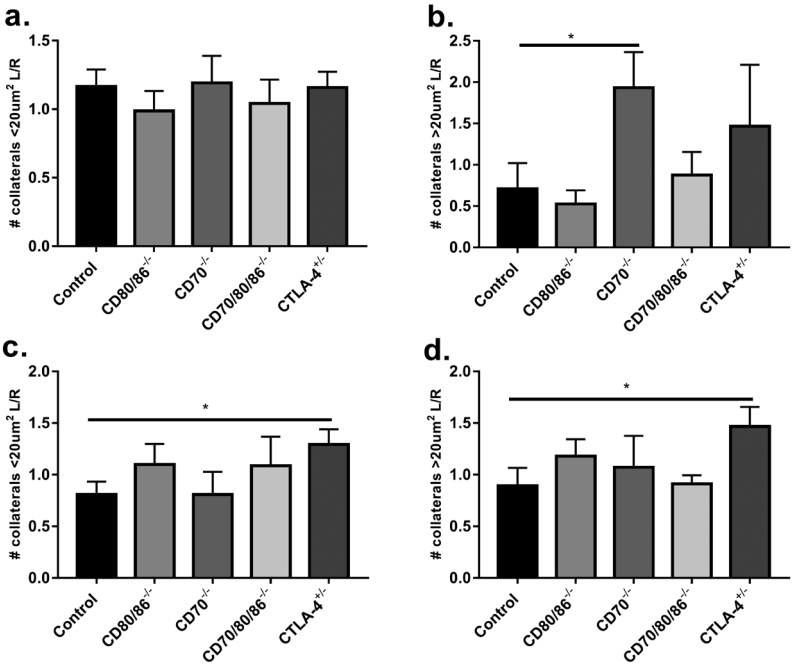

Figure S4. Diameter of αSMActin positive collateral arterioles divided in <20 μm^2 and >20 μm^2. Data presented as L/R (left ischemic/right non-ischemic) ratio. **a.** 10 days after surgery Ischemic/non-ischemic ratio of number of collateral arterioles < 20 μm^2 and **b.** > 20 μm^2 was quantified. **c.** and also 28 days after surgery number of collateral arterioles < 20 μm^2 and **d.** > 20 μm^2 was quantified.