LEUKOCYTES AND PERFUSION RECOVERY

AFTER ARTERIAL OCCLUSION

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LEUKOCYTES AND PERFUSION RECOVERY

AFTER ARTERIAL OCCLUSION

LEUKOCYTEN EN PERFUSIE HERSTEL

NA ARTERIËLE OCCLUSIE

met een samenvatting in het Nederlands

Promotoren: Prof.dr. D.P.V. de Kleijn
Prof.dr. G. Pasterkamp

Co-promotor: Dr. I.E. Höfer

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INTRODUCTION AND OUTLINE

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Cardiovascular diseases

Cardiovascular disease is a major cause of disability and premature death throughout the world and contributes substantially to the escalating costs of health care. The underlying pathology is atherosclerosis, which develops over many years. Acute coronary and cerebrovascular events frequently occur suddenly, and are often fatal before medical care can be given.

Of an estimated 58 million deaths globally from all causes in 2005, cardiovascular disease (CVD) accounted for 30%. This proportion is equal to that due to infectious diseases, nutritional deficiencies, and maternal and perinatal conditions combined[1]. It is important to recognize that a substantial proportion of these deaths (46%) were of people under 70 years of age, in the more productive period of life; in addition, 79% of the disease burden attributed to cardiovascular disease is in this age group[2].

Arterial occlusion

In cardiovascular-patients the underlying problem is often the occlusion of a major artery. As a result, blood flow is obstructed and the tissue distal to the occlusion is deprived from both nutrients and oxygen. The underlying cause for arterial occlusion is often atherosclerosis; a chronic inflammatory process occurring in the vascular wall leading to plaque formation[3]. Plaque formation can result in gradual narrowing of the vascular lumen or the plaque can rupture resulting in acute occlusion of the artery. Rupture prone or vulnerable plaques contain copious amount of fat and inflammatory cells and have been associated with cardiovascular outcome[4].

Depending on the vascular territory involved a vascular occlusion can be life threatening. For example, an occlusion in one of the carotid or coronary arteries will have devastating effects as both the brain and the heart have a high and constant need for the supply of oxygen and nutrients. In such cases, the blood supply has to be restored rapidly to avoid a stroke or myocardial infarction. This is done via an interventional procedure that instantly removes the occlusion but which is not without risk for complications. However, as an alternative some studies have reported an improvement in cardiac function as a result from exercise due to vascular growth leading to increased perfusion of the areas at risk[5].

This natural capacity of the body to restore blood flow after arterial occlusion could be a powerful tool to help patients suffering from Coronary Artery Disease (CAD) or Peripheral Artery Disease (PAD) especially in non-acute situations. Therapeutic stimulation of vascular growth in CAD or PAD patients during gradual occlusion could potentially prevent loss of tissue perfusion. The research described in this thesis contributes to our knowledge on this process of vascular growth. Furthermore, it identifies the peptidase CD26 as a potential therapeutic target to increase vascular growth.

Vascular growth

In the human body there are three major forms of vascular growth, namely vasculogenesis, angiogenesis and arteriogenesis[6]. Vasculogenesis, the *de novo* formation of blood vessels mainly occurs during embryonic growth and is virtually absent in the adult human. Angiogenesis occurs frequently in humans and involves the sprouting of new blood vessels from pre-existing vessels. Angiogenesis is an ischemia driven process and plays a role in wound healing and tumor growth. In contrast, arteriogenesis is an ischemia independent process and involves the remodeling of anastomoses (pre-existent arteriolar connections) into larger collateral arteries (figure 1). These pre-existing arteriolar connections are present at birth and have been identified in humans in the heart as well as the peripheral circulation[7]. Of the two forms of vascular growth occurring in adult humans, arteriogenesis is the process most capable of restoring blood flow and tissue perfusion after arterial occlusion[8]. Although many capillaries are formed during angiogenesis, their small lumen results in a relatively low amount of blood flow while the larger lumen of collateral arteries is capable of transporting larger amounts of blood. This difference between angiogenesis and arteriogenesis is derived from Poiseuille's law which states that flow increases with the fourth power over the radius of the vessel[9-11]. The remodeling of a pre-existing anastomose into a functional collateral artery starts with the phenotypical change of the endothelial and smooth muscle cells into synthetic and proliferative cells. Subsequently, part of the existing extracellular matrix of the vessel is broken down to provide space for the proliferating cells of the growing vessel. Finally, the newly formed smooth muscle cells are ordered in circular layers and a new extracellular matrix is formed to stabilize the larger vessel[12].

Onset of arteriogenesis

In contrast to angiogenesis, arteriogenesis is not driven by an ischemic stimulus, arteriogenesis often occurs without the presence of local ischemia. This was nicely shown using a hind limb model in which ischemia induced angiogenesis occurred in the lower hind limb while arteriogenesis occurred in the non-ischemic upper hind limb[13].

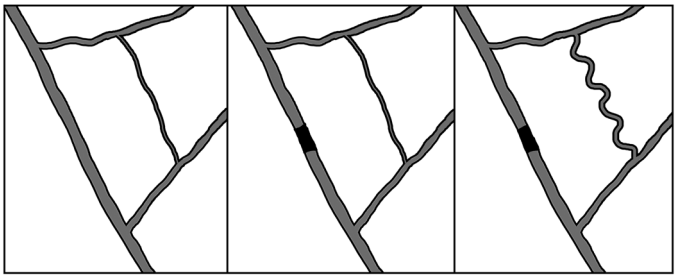

Figure 1 | Development of a collateral artery
Small interconnecting anastomoses are present from birth on (first panel). Upon arterial occlusion of a major artery (second panel) blood starts to flow through the anastomose and vessel remodeling commences. Upon vessel remodeling a large collateral artery has been formed with its typical cork-screw like appearance (third panel).

The stimulus for arteriogenesis is believed to be of a more mechanical origin. When an artery becomes occluded, the blood pressure distal to the occlusion drops. As a result, a pressure gradient is formed over the existing anastomose which results in the onset of blood flow through the anastomose. This flow of blood is one the most important stimuli for arteriogenesis as it increases the shear stress on the endothelium of the collateral artery as well as cyclic stretch on the vessel wall[14,15]. A cation channel named Trpv4 has recently been reported to be involved in this process[16]. The increase in shear stress activates the endothelium and induces the expression of chemokines and adhesion molecules[17,18]. Endothelial activation thus becomes the start of an inflammatory like response which involves the attraction of several subsets of circulating leukocytes.
When an endothelial cell becomes activated the expression of adhesion molecules is up regulated. These adhesion molecules belong to either of three gene families: the selectins, the integrins or the immunoglobulin family. Receptors for these adhesion molecules are expressed on the circulating leukocytes. Leukocyte extravasation occurs in three different steps namely leukocyte rolling, leukocyte adhesion and leukocyte penetration of the endothelial cell layer. Adhesion molecules are involved in all of these three steps[19].

Inflammatory forces in arteriogenesis

The activation of inflammatory pathways in circulating leukocytes is of crucial importance for arteriogenesis. A recent study has shown that signaling via the inflammatory Toll-like-Receptors 2 and 4 is crucial in perfusion recovery[20]. In addition, TLR signaling in bone marrow derived cells was solely responsible for the observed effect. Of the various leukocyte subsets which are involved in arteriogenesis, monocytes were the first to be linked to arteriogenesis. As early as 1976 monocytes were detected near growing collaterals[21] and numerous studies afterwards have shown their importance. Blood monocyte concentration is directly related to perfusion recovery[22,23] and transfusion of monocytes after arterial occlusion increases perfusion recovery[24]. This relationship between monocyte number and collateral growth has also been established in humans[25]. In support of these findings, mice which lack factors involved in either the attraction or extravasation of monocytes show the same results[26-28]. Once monocytes have left the blood stream they differentiate into macrophages. These macrophages subsequently secrete various cytokines and/or chemokines which stimulate collateral vessel growth and the attraction of more monocytes and/or lymphocytes. Some of these cytokines such as MCP-1 and TNF-alpha have been tested in animal models and improved perfusion recovery[28-30]. In addition to monocytes, various subsets of lymphocytes have also been shown to be important in arteriogenesis. Studies into the role of lymphocytes have mainly focused on T-lymphocytes of which the various subsets interact to stimulate arteriogenesis[31-34].

Circulating vs. residential cells

In arteriogenesis both local residential cells as well as circulating cells are important. Residential cells such as smooth muscle cells and endothelial cells provide the new cells which are necessary to enlarge vessel diameter[35]. Using chimeric mice in which the bone marrow derived cells were EGFP labeled it was clearly shown that circulating cells do not become part of the new vessel structure[36]. However, circulating cells are of crucial importance in perfusion recovery as shown in many studies including some which have used chimeric mice[20,37,38]. Furthermore, they are an excellent therapeutic target as they are easily accessible via the bloodstream. However, many questions on the exact role of circulating cells remain to be elucidated. For example, the precise mechanisms by which the different subsets of circulating cells interact to orchestrate their role in arteriogenesis remain unknown. Further basic research on the role of leukocytes and leukocytes derived factors in arteriogenesis is therefore necessary. In addition, circulating cells are also involved in the progress of atherosclerosis. Identifying factors involved in arteriogenesis which, upon therapeutical targeting, do not aggravate the atherosclerotic disease will therefore be of crucial importance.

Enhancing arteriogenesis

Enhancing collateral artery growth to speed up perfusion recovery bears great potential for cardiovascular patients. In the past decade, many studies have therefore been conducted in search of pro-arteriogenic factors[39]. Factors such as MCP-1[40], GM-CSF[41], FGF-2[42], VEGF[43] and LPS[44] were shown to increase perfusion recovery in various animal models. However, many of these pro-arteriogenic factors have serious unwanted side-effects. As both arteriogenesis and atherosclerosis are enhanced by endothelial activation and leukocyte extravasation, factors enhancing arteriogenesis might enhance atherosclerosis as well. MCP-1 for example stimulates arteriogenesis but in atherosclerosis prone mice it also stimulated plaque progression[45]. This stimulation of plaque progression by pro-arteriogenic factors has been named the Janus phenomenon[46].

Some pro-arteriogenic factors have been tested in clinical trials successfully[47,48]. However, when these experiments were repeated in larger randomized trials the positive effect on collateral growth was no longer observed[49,50] and one trial even reported serious side-effects[51].

Perspectives

Given the above described Janus phenomenon, translation to the clinic of pro-arteriogenic factors remains problematic. In addition, many of the animal models are conducted in young and healthy animals while the majority of the cardiovascular patients is older and suffers from co-morbidities such as diabetes and atherosclerosis. Ideally, age and co-morbidities should therefore be incorporated into animal models that are used to test

pro-arteriogenic factors. In addition, there is a large discrepancy in perfusion recovery based on genetic traits. The number of pre-existing collaterals as well as the collateral growth rate differs for example between BalbC and C57Bl6/J mice[52]. These genetic differences are also likely in humans. In addition, acute arterial occlusion is often used in animal models while in humans collaterals grow more gradually during occlusion development. This gradual occlusion should be incorporated in animal models and recently such an animal model has been developed[53].

Targeted drug delivery could also have great potential as it would enable the promotion of arteriogenesis without affecting plaque progression. Such a strategy could include selected receptors on nanoparticles which 'home' to the site of arteriogenesis. Alternatively, larger microspheres can be infused which subsequently become lodged in the microvasculature. Microbubbles can also be used which could be triggered to collapse at the desired site using externally applied ultrasound. Overall, targeted drug delivery is a promising tool to circumvent many of the problems often related to the application of arteriogenic factors such as a short half life of the drugs or severe systemic side-effects when used in high concentrations.

In addition, further research should shed more light on the mechanisms involved in arteriogenesis, both locally and systemically. Especially factors that are unique for arteriogenesis and which do not stimulate atherosclerosis, could be promising therapeutic targets.

Outline of this thesis

This thesis focusses on the role that circulating leukocytes play in collateral artery growth for perfusion recovery. In **chapter 2** systemic changes in leukocyte subsets after arterial occlusion are documented which point to an orchestrated leukocytic response after arterial occlusion. In chapters 3 and 4 we focus on the role of the peptidase CD26. In **chapter 3** we show that inhibition of CD26 improves perfusion recovery in atherosclerosis prone ApoE -/- mice without detrimental effects on atherosclerosis. In **chapter 4** we show that high CD26 levels in human plaques are correlated to a more vulnerable plaque phenotype. In chapters 5 and 6 we focus on previously unknown factors involved in arteriogenesis. **Chapter 5** establishes a role for platelets in perfusion recovery. Platelet depleted mice display decreased vascular remodeling and macrophage number resulting in decreased perfusion recovery. **Chapter 6** shows that the chemokine CXCL10 is involved perfusion recovery as it was decreased in CXCL10 -/- mice. Using chimeric mice we show that this decrease in perfusion recovery can be attributed to the loss of CXCL10 in leukocytes.

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2

ARTERIAL OCCLUSION INDUCES SYSTEMIC CHANGES IN LEUKOCYTE COMPOSITION

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Abstract

Aim

Lack of tissue perfusion due to arterial occlusion can result in mortality and morbidity. In response to local tissue ischemia, extravasation of leukocytes into the region at risk is initiated to facilitate matrix remodeling and subsequent perfusion recovery. However, it is unknown if local tissue ischemia also induces a more generalized response of leukocyte trafficking and compartmentalization. This study was designed to gain insight into the temporal changes in circulating and bone marrow derived leukocyte fractions following peripheral arterial occlusion in mice.

Design

Mouse peripheral blood and bone marrow samples were collected at baseline and subsequently at day 1, 2, 3, 4 and 7 after femoral artery ligation. Leukocyte and bone marrow cell subsets were quantified using flow cytometry.

Results

After arterial occlusion, peripheral blood leukocyte numbers did not vary significantly over time. However, significant intrinsic temporal changes in cell numbers were observed for monocytes, lymphocytes, neutrophils and their subsets with fluctuations of >50%. Granulocytes, for example, showed an initial upregulation, while monocytes and lymphocytes numbers initially decreased. These variations in the circulation were largely preceded by changes in the corresponding bone marrow lineages. Progenitor cells of the myeloid and lymphoid lineage in the bone marrow were up regulated after the decrease in the numbers of their progeny in the peripheral blood.

Conclusions

Local arterial occlusion results in an orchestrated systemic response of leukocyte trafficking. This response substantiates the pivotal role of leukocytes as mediators of processes leading to perfusion recovery and tissue remodelling.

Introduction

Mortality and morbidity in cardiovascular patients is often caused by a lack of tissue perfusion due to the development of an arterial occlusion. Perfusion recovery by medical or interventional treatment is widely performed to save patients' lives. In addition, acute and chronic ischemia also initiates a natural response to restore tissue perfusion via collateral artery growth (arteriogenesis). During arteriogenesis, circulating leukocytes play and important role as they are required for the creation of an inflammation like environment to provide the space for the growing vessel and the induction of cellular proliferation.[1-3]

The pivotal role of monocytes in arteriogenesis has been examined and confirmed in pre-clinical and clinical studies.[4-9] Hence, monocyte attraction and activation provide powerful tools to enhance adaptive vascular growth and the resulting perfusion recovery.[8,10-13] In addition to monocytes, lymphocytes, especially T-lymphocytes, have been described to be involved in arteriogenesis. Helper and killer T-cells act in concert to enhance arteriogenesis and their loss impairs perfusion recovery.[14-16] Also natural killer cells (NK cells) are involved in arteriogenesis as mice deficient for or depleted of NK cells show a reduced perfusion restoration.[17] Previous studies have confirmed the critical role of circulating cells in perfusion recovery and established a correlation between monocyte count and collateralization.[4,7,18,19] Most studies on arteriogenesis have focused on local extravasation and the mechanistic role of the circulating leukocyte that invades the tissue area at risk. Our aim was to gather knowledge about the temporal changes in number and subset composition of leukocytes and their progenitors in blood and bone marrow following local tissue ischemia to further improve our understanding of the role of circulating cells in the systemic response to tissue ischemia.

Material and Methods

The present study was approved by the university animal experimental committee following the *Guide for the Care and Use of Laboratory Animals published by The* US *National Institute of Health* (NIH Publication No. 85-23, revised 1996). C57Bl/6J mice underwent permanent unilateral femoral artery ligation as described previously.[20] Blood and bone marrow was collected at baseline and 1, 2, 3, 4 or 7 days after ligation.

During follow up different antibody panels were used to discriminate leukocyte and bone marrow subpopulations using flow cytometry (supplemental figure 1 and supplemental tables 1& 2). The definitions used for the different subsets can be found in the online supplement. For the calculation of absolute cell numbers, fluorescent beads were added as reference to each tube.

Bioplex

Chemokine and cytokine levels in plasma were determined using the Bioplex assay from Biorad per manufacturers instructions.

Statistics

Results are expressed as mean ± Standard Error of the Mean (SEM). One-Way ANOVA tests were performed in SPSS with a posthoc Bonferroni correction to determine the significance of differences between time-points. P-values <0.05 were considered significant.

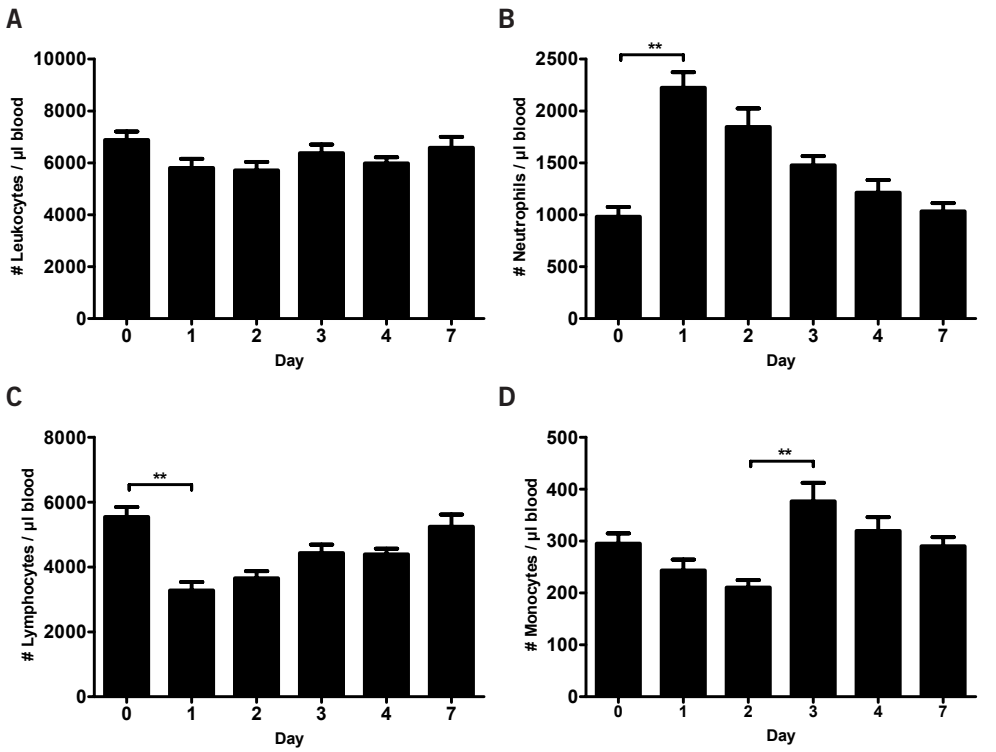

Figure 1 | Leukocyte counts in the peripheral blood
Quantification of total leukocyte (A), neutrophil (B), lymphocyte (C) and monocyte (D) number after ligation of the femoral artery. Error bars indicate SEM. **p< 0.001.

Results

All animals survived surgery and the subsequent measurements. Total leukocyte number in peripheral blood did not vary significantly over time after femoral artery ligation (figure 1A). In contrast, leukocyte subsets showed major variations in number over time as depicted in figures 1B-D, but returned to baseline levels at day 7. The number of circulating neutrophils, being the first responders to any damage, showed a significant increase at day 1 (227% of baseline) (figure 1B). Subsequently, neutrophil numbers decreased slowly over time resulting in a return to baseline levels at day 7. Lymphocyte numbers showed a pattern opposite to that of neutrophils (figure 1C). A strong significant decrease at day 1 (59% of baseline) was followed by a restoration of lymphocyte numbers to baseline levels at day 7. Monocytes showed a more intricate pattern: an initial decrease was followed by a strong and sudden significant increase from day 2 to day 3 (from 71% of baseline at day 2 to 127% of baseline at day 3) (figure 1D). Afterwards, monocyte numbers declined again, returning to baseline levels at day 7. These changes were consistent for the mice that were studied.

Furthermore, various subtypes of these major leukocyte subsets were identified to assess if and which subtypes deviated from the aforementioned patterns. T-lymphocytes ($CD3^+$) showed a pattern comparable to that of the whole lymphocyte population (figure 2A). The helper ($CD4^+$) and killer ($CD8^+$) T-cells did not deviate from the pattern of the whole T-lymphocyte population and as a result their ratio remained stable ($CD4^+/CD8^+ = 1.5$). Killer T-cells were the only cell population that differed significantly from baseline at day 7 (figure 2B-C). NK cells showed a comparable but less pronounced pattern as the whole lymphocyte population, with the exception of a strong significant decrease at day 4 (figure 2D).

In summary, leukocyte subsets displayed distinct temporal patterns after femoral artery occlusion. However, all subsets returned to baseline values within one week, with the exception of killer T-cells, supporting the notion that involvement of circulating cells in arteriogenesis is most prominent in the early phases.

During arteriogenesis peripheral blood mononuclear cells locally extravasate from the bloodstream to modulate collateral development. However, prior to extravasation, changes in cytokine or chemokine plasma levels could modulate leukocyte responsiveness. Furthermore, cytokines or chemokines are capable of mediating a more general systemic response. The plasma levels of different cytokines and chemokines were determined over time. As shown in figure 3, plasma levels of interferon-γ (INF-γ) and interleukin 6 (IL-6) which are known for their pro-inflammatory roles[21,22] are upregulated directly after femoral artery ligation. Various other interleukins known for their pro-inflammatory role were upregulated direct or shortly after femoral artery ligation (supplemental figure 2).

More importantly however, various cytokines which are known to be important for the upregulation of specific bone marrow subsets in response to local inflammation were also identified (figure 4).

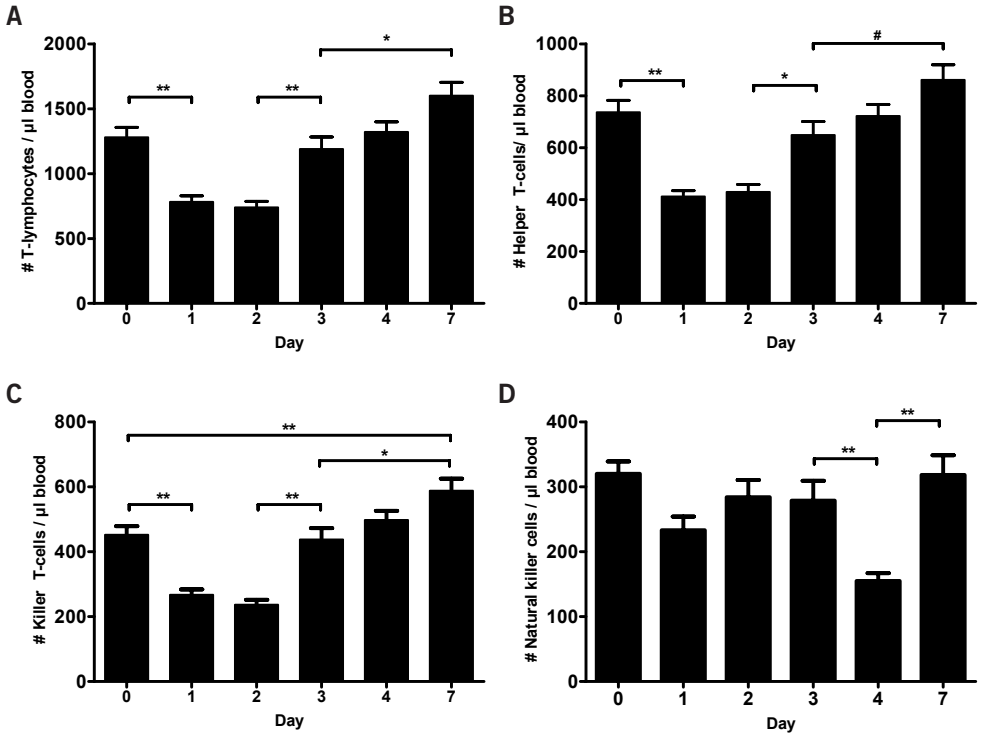

Figure 2 | Lymphocyte subset counts in the peripheral blood
Quantification of T-lymphocyte (A), helper T-cell (B), killer T-cell (C) and natural killer cell (D) number after ligation of the femoral artery. Error bars indicate SEM. **p< 0.001.

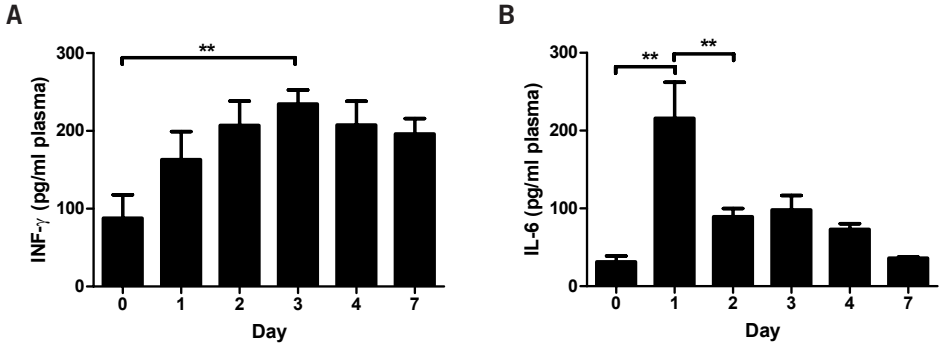

Figure 3 | Cytokine levels in plasma
Quantification of INF-γ and interleukin 6 levels after ligation of the femoral artery. Error bars indicate SEM. **p< 0.001.

Interleukin 3 (IL-3), known to stimulate the myeloid lineage of the bone marrow[23], was increased almost 2-fold towards day 2 as were other factors known to stimulate the myeloid lineage such as granulocyte colony stimulating factor (G-CSF) and macrophage colony stimulating factor (M-CSF)[24]. Several chemokines regulating the attraction of leukocytes such as CXCL-1, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) were also assessed. With the exception of CXCL-1, a neutrophil chemoattractant[25], these chemokines did not show any significant differences in their plasma levels (supplemental figure 3).

As peripheral blood mononuclear cells originate from the bone marrow, the number of stem/progenitor cells present in the bone marrow was also assessed. The different lineages in the bone marrow can be divided into a myeloid and a lymphoid lineage. The myeloid lineage gives rise to erythrocytes, monocytes and granulocytes while the lymphoid lineage gives rise to all lymphocytes. An overview of cell types and markers used for discrimination is depicted in supplemental figure 1.
CFU-GM numbers dropped significantly shortly after femoral artery ligation and remained at that level with the exception of day 2 which showed a significant increase (figure 5A).

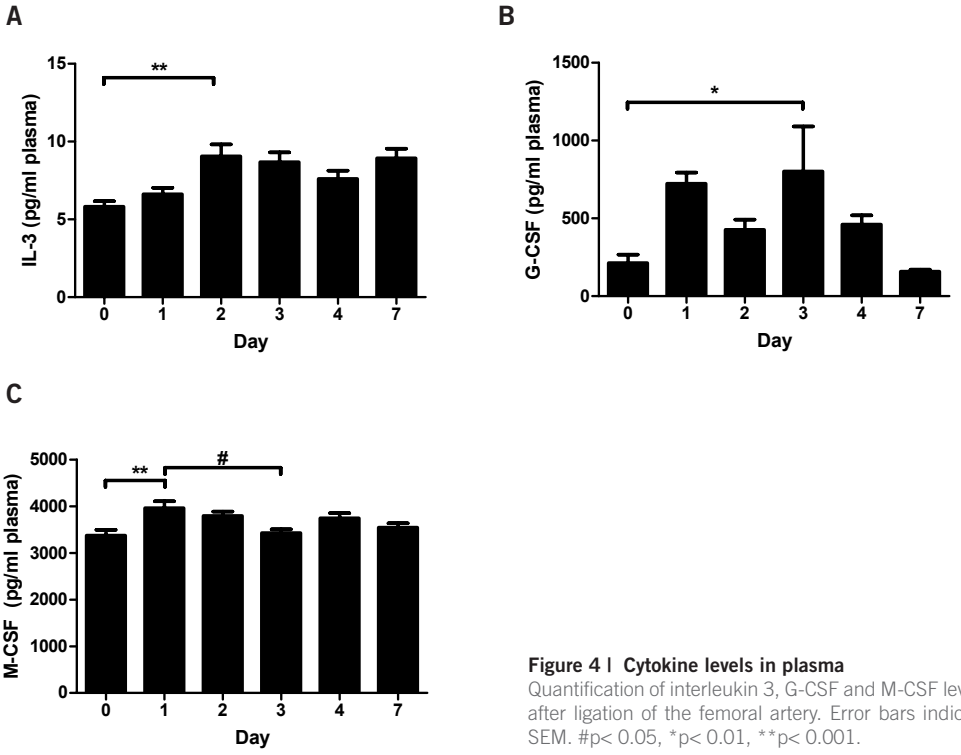

Figure 4 | Cytokine levels in plasma
Quantification of interleukin 3, G-CSF and M-CSF levels after ligation of the femoral artery. Error bars indicate SEM. #p< 0.05, *p< 0.01, **p< 0.001.

CFU-GM give rise to CFU-G and CFU-M, the latter being the stem cell of the monocytic lineage. CFU-M numbers increased significantly after femoral artery ligation with a peak at day 2 and a return to baseline levels by day 7 (figure 5B). Promonocyte numbers decreased markedly and significantly at day 1.

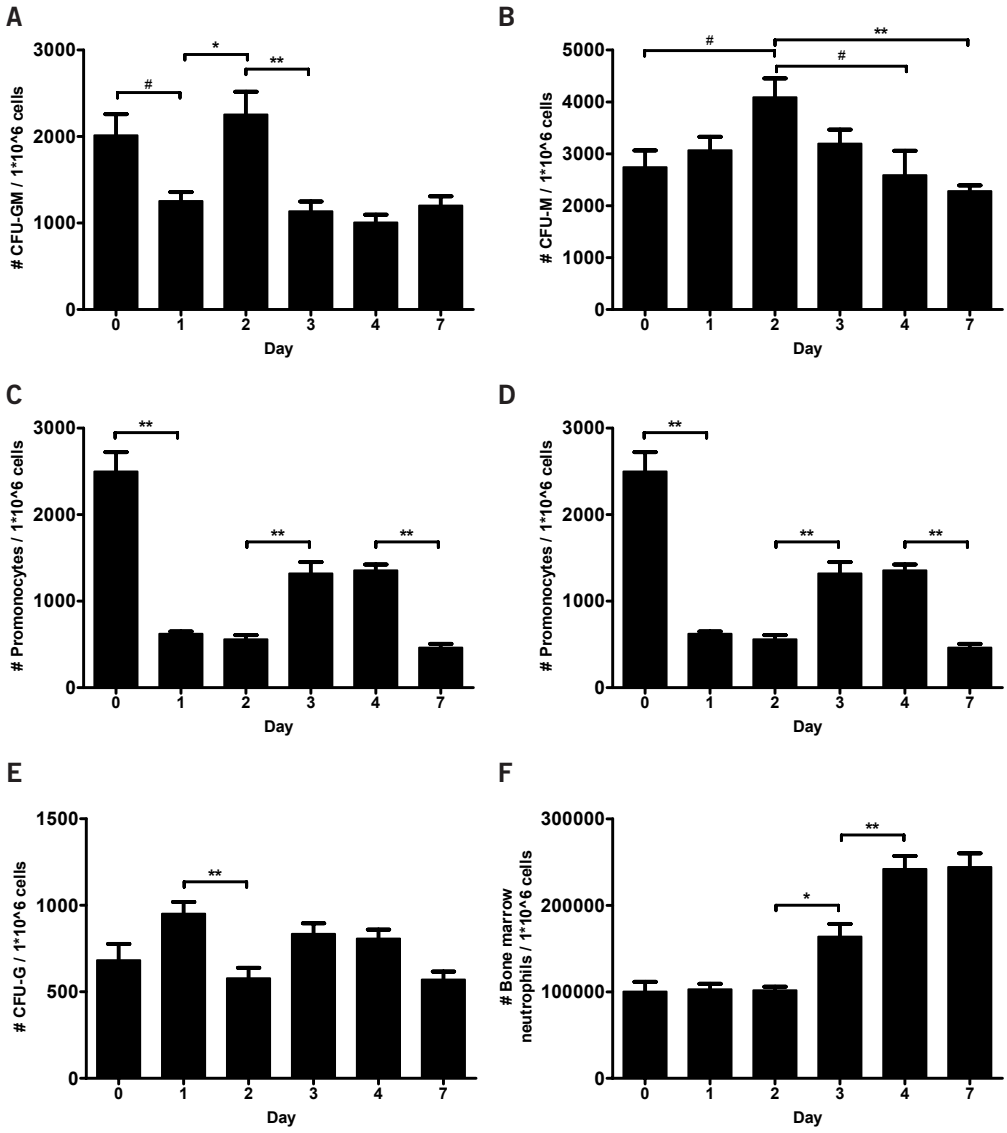

Figure 5 | Bone marrow cell counts in the bone marrow
Quantification of CFU-GM (A), CFU-M (B), promonocytes (C), bone marrow monocytes (D), CFU-G (E) and bone marrow neutrophils after ligation of the femoral artery. Error bars indicate SEM. #p< 0.05, *p< 0.01, **p< 0.001.

Subsequently their numbers remained low, rising again at day 3, one day later than the CFU-M numbers did (figure 5C). The monocyte pattern in bone marrow was again intricate. A first dramatic significant decrease in monocyte numbers at day 1 was followed by a stabilization towards baseline levels throughout days 3 and 4, followed by another significant reduction at day 7 (figure 5D). As mentioned above, CFU-GM also give rise to CFU-G, the stem cell of the granulocyte lineage. CFU-G numbers increased after femoral artery ligation until day 7 when CFU-G numbers returned to baseline. However, a single drop was observed at day 2, when CFU-G numbers returned to baseline levels for that day only (figure 5E). Neutrophil numbers did not rise until day 3 after which their numbers more than doubled until day 7 (figure 5F). The lymphoid stem cell was strongly and significantly up regulated from day 1 to day 2. After that, the number of lymphoid stem cells steadily declined (supplemental figure 4).

Discussion

The importance of peripheral blood cells in the pathogenesis of tissue degeneration and repair following tissue ischemia is well established. Prospective clinical studies have also revealed a predictive value for the number of peripheral blood monocyte numbers for the outcome after myocardial infarction.[26] These studies indicate that PBMC number and composition can be of functional importance in perfusion recovery and that knowledge about these processes might lead to new mechanistic insights.

To the best of our knowledge, the current study is the first to describe the time course of peripheral blood leukocyte composition in response to an arterial occlusion. On first sight, the changes in total leukocyte number are small and insignificant. However, a closer look at leukocyte subpopulations reveals an intrinsic pattern with major temporal differences within, as well as between subsets (supplemental figure 5). After 7 days all but killer T-cell numbers had returned to baseline levels, supporting our hypothesis that the most marked changes observable in peripheral blood occur in the early phase of arteriogenesis. Further involvement of leukocytes in the remodeling process is probably mainly orchestrated 'on site' from that point forward.

During inflammatory processes, the initial accumulation of neutrophils usually is followed by attraction of monocytes/macrophages and lymphocytes. In contrast to neutrophils, T-cell numbers show an initial decrease in the peripheral blood, which could indicate an increased extravasation of these cells during the first few days after femoral artery ligation. This is in line with previous reports where $CD4^+$ and $CD8^+$ T-cells were found to accumulate in the perivascular space of growing collateral arteries.[16,17] However, the previously described $CD8^+$ T-cell dependent recruitment of $CD4^+$ T-cells via IL-16[16] seems to occur almost simultaneously as their ratio in the peripheral blood remains

unaltered over time. Further studies on IL16 plasma levels and assessment of $CD4^+$ and $CD8^+$ T-cells presence in hind limb muscle will have to shed more light on the exact interplay between these different leukocyte populations in the peri-collateral tissue. The return of circulating T-cell numbers to baseline levels at day 3 could indicate a crosstalk between hind limb and bone marrow to replenish the migrated cells in the peripheral blood. The presence of such a crosstalk is supported by the observation that the number of lymphoid stem cells is significantly up regulated at day 2, directly before the number of circulating T-lymphocytes rises again at day 3 (supplemental figure 5). NK cells show a more or less comparable pattern to the T-cells. However, in contrast to the T-cells, NK cell numbers significantly decrease at day 4. This decrease is intriguing and we speculate that this could indicate a highly functional role for NK cells around day 4, concurring with previous reports showing the most prominent effect of NK cell depletion between day 4 and 7 after femoral artery occlusion.[17]

As mentioned before, monocytes are beneficial for arteriogenesis and several studies have investigated their role.[4,10,27-30] Peripheral blood monocytes display an intrinsic pattern with an initial decrease followed by a strong up regulation at day 3 and a subsequent return to baseline. This initial decrease in the peripheral blood is remarkable as there is a coinciding release of monocytes from the bone marrow which should theoretically result in an increase of 50-100 monocytes per µl in the peripheral blood. A possible explanation could be that the extravasation of monocytes from the peripheral blood into the perivascular space or e.g. the spleen is so extensive that it completely masks the release of monocytes from the bone marrow. In addition, the myeloid lineage of the bone marrow is extensively stimulated via several pathways (IL-3 and M-CSF) after femoral artery ligation as monocytes and macrophages are important mediators of collateral growth. This release of growth factors results in a subsequent and temporally corresponding upregulation in myeloid progenitor number in the bone marrow leading to replenishment of both monocytes and granulocytes, further supporting the existence of a direct link between arterial occlusion and proliferation and maturation of bone marrow cells and their derivatives (dotted line supplemental figure 5).

At first glance, the pattern of the granulocytic lineage in the bone marrow does not seem to correspond to the neutrophil pattern in the peripheral blood (supplemental figure 5). In the circulation, a single but dramatic increase in neutrophil number occurs at day 1. In contrast, the number of neutrophils in the bone marrow remains stable until day 2 after arterial occlusion. These findings possibly point to a source other than the bone marrow, e.g. the spleen, responsible for the major release of neutrophils into the circulation. Another intriguing observation is the more than 2-fold increase in bone marrow neutrophil numbers during day 3-7. The preceding rise in G-CSF plasma levels and CFU-G numbers corresponds well to this observation and would provide a possible explanation. However, a clear reason for replenishment of neutrophils in the bone

marrow seems to be lacking as bone marrow neutrophil numbers never drop below baseline. One explanation could be that the observed increase in bone marrow neutrophil number is not only due to newly formed bone marrow neutrophils in the bone marrow, but is also the result of blood neutrophil clearance from the circulation by the bone marrow.[31,32] The increase in neutrophil number in the bone marrow might thus indicate a functional role of the bone marrow in clearing neutrophils from the circulation after arterial occlusion. At the same time, bone marrow neutrophil replenishment by the CFU-G probably occurs as indicated by their rise in cell number, but might involve more intricate signals than mere neutrophil numbers in the bone marrow.

Our data indicate a clear link between the occurrence of vascular occlusion, the number of specific leukocyte subsets in the blood and the corresponding up regulation of progenitor cells in and/or release of differentiated cells from the bone marrow. Such a link requires tight regulation and communication between different organs and compartments in the body. Most likely, signaling occurs via the blood, e.g. by local upregulation of colony stimulating factors in the collateral vessels or the downstream tissue and their transport to the bone marrow where it results in CFU proliferation.[33]

Although this study only describes changes observed in otherwise healthy mice, the scale and intrinsicity of these changes indicate that there might be a relationship with actual perfusion recovery. Especially as other confounding processes, such as tissue necrosis and muscle atrophy, are virtually absent after femoral artery occlusion in C57Bl6/J mice.[34] Previous studies on perfusion recovery have mainly focused on the local compartment; the important role that already extravasated cells play. Our data however shows that changes also occur over time within the source of these extravasated cells, implicating a more extensive role of the blood and bone marrow compartments than just mere “cell providers”. Additional studies on temporal changes in these cells are required to further elucidate the functional consequences they might have.

Further research has to be performed on the likely role of the spleen in vascular growth after arterial occlusion as the spleen was recently shown to be involved in the storage and release of monocytes involved in the inflammatory response after myocardial infarction.[35] The lack of information on splenic monocytes in our study is a limitation as it is likely that splenic monocytes also play a role in perfusion recovery after arterial occlusion.

This study provides valuable insights into an orchestrated and tightly regulated systemic reaction to arterial occlusion, reflected by the changes in circulating cell numbers. Previous studies have shown critical involvement of peripheral blood leukocytes in arteriogenesis and angiogenesis and we here establish a temporal dimension to these earlier findings. The major changes observed in this study and their temporal distribution strongly support the circulating cell as a carrier of information about processes elsewhere in the body.

Acknowledgements
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Author contribution
R.H. designed the study, performed experiments, collected and analyzed data and contributed to writing the paper. D.G. performed experiments and collected data. P.B. performed experiments and collected data. G.P. contributed to writing the paper. I.E.H. designed the study, performed experiments, collected data and contributed to writing the paper.

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Supplemental

Arterial occlusion induces systemic changes in leukocyte composition

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Material and Methods

Animal procedures

The present study was approved by the university animal experimental committee following the *Guide for the Care and Use of Laboratory Animals published by The* US *National Institute of Health* (NIH Publication No. 85-23, revised 1996). Eighty C57Bl/6J mice (Harlan, The Netherlands) were divided into 8 groups (n=10 each) and underwent permanent unilateral femoral artery ligation on day 0. From each mouse, blood was collected by puncture of the vascular plexus on the cheek (n=20 per time point) during follow-up and by cardiac aspiration before sacrifice (n=10 per time point). At each time point (baseline and 1, 2, 3, 4 or 7 days after femoral artery ligation) one group (n=10) was sacrificed and blood, bone marrow and hind limb muscles were collected. During cheek puncture a limited amount of blood (100-120 µl) was collected in order to exclude any effects of this intervention on circulating cell composition, thus limiting the number of antibody panels which could be tested after cheek puncture. Blood was collected in heparinized tubes and kept on ice until further processing. For each panel (supplemental table 1), 50 µl of blood was used. During follow up and after sacrifice two antibody panels were used to discriminate leukocyte subpopulations which were analyzed by flow cytometry (FC500, Beckman Coulter, Miami, FL, USA).

After sacrificing the animals, the femurs and tibias were excised for bone marrow collection (n=10). Bone marrow was isolated and collected by flushing the bones with phosphate buffered saline. The bone marrow suspension was filtered using a 70 µm filter after which cells were spun down. Subsequently cells were counted (Countess automated cell counter, Invitrogen, Paisley, UK) and for each antibody panel (n=4) $1*10^6$ cells were stained (Supplemental figure 1 and supplemental table 2) and analyzed by flow cytometry. For the calculation of absolute cell numbers, fluorescent beads (Countbright absolute counting beads, Invitrogen, Paisley, UK) were added as reference to each tube.

Definition of leukocyte subsets

Subsets of peripheral blood circulating cells were quantified as follows: monocytes were selected based upon $F4/80^+$, $CD11a^+$, $CD11b^+$ staining and their scatter properties. $CD11a^+$

cells in the appropriate scatter region and negative for monocyte markers were considered lymphocytes. T-lymphocytes were identified as $CD3^+$, helper T-cells as $CD3^+$/ $CD4^+$, and killer T-cells as $CD3^+$/ $CD8^+$. NK cells were defined as $CD1d^+$ cells within the lymphocyte gate. Neutrophils were identified based on their forward/sideward scatter profile and $Ly6g^+$ staining.

Bone marrow cell populations were defined as follows: Granulocyte/macrophage colony forming units (CFU-GM) were defined as $CD13^+$, $CD34^+$ and $CD54^+$. Macrophage colony forming units (CFU-M) were defined as $CD13^+$, $CD14^-$ and $CD38^+$. Promonocytes were defined as $CD14^+$ and $CD11b^-$, bone marrow monocytes were defined as $CD14^+$ and $CD11b^+$. Granulocyte colony forming units (CFU-G) were defined as $CD13^+$, $CD34^-$ and $CD54^+$. Bone marrow neutrophils were defined as $Ly6g^+$. Lymphoid stem cells were defined as $CD38^+$ and $CD90^+$. Megakaryocytes were defined as $CD41^+$.

Bioplex

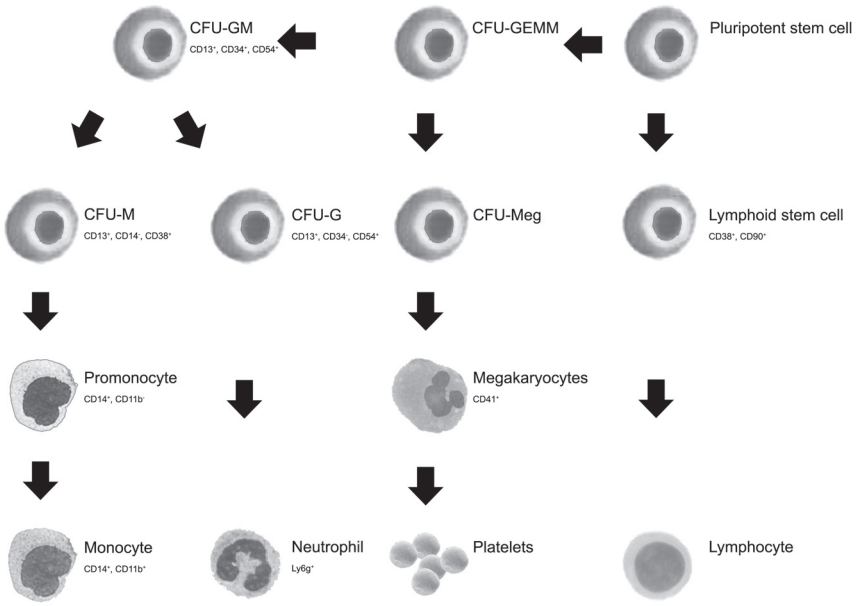
Chemokine and cytokine levels in plasma were determined using the Bioplex assay from Biorad. Plasma samples were obtained from blood samples collected after sacrifice. Blood was spun down for 15 min at 3000 G and stored at -80 °C until further use. Plasma samples were diluted 4x after which chemokines and cytokine levels were determined using the bioplex kit.

Statistics

Results are expressed as mean ± Standard Error of the Mean (SEM). One-Way ANOVA tests were performed in SPSS with a posthoc Bonferroni correction to determine the significance of differences between time-points. P-values <0.05 were considered significant.

Supplemental table 1 | Peripheral blood antibodies
List of antibodies used in this study to discriminate leukocyte subsets. Amount of antibody stated is µl/100 µl PBS which was added to 50 µl of blood

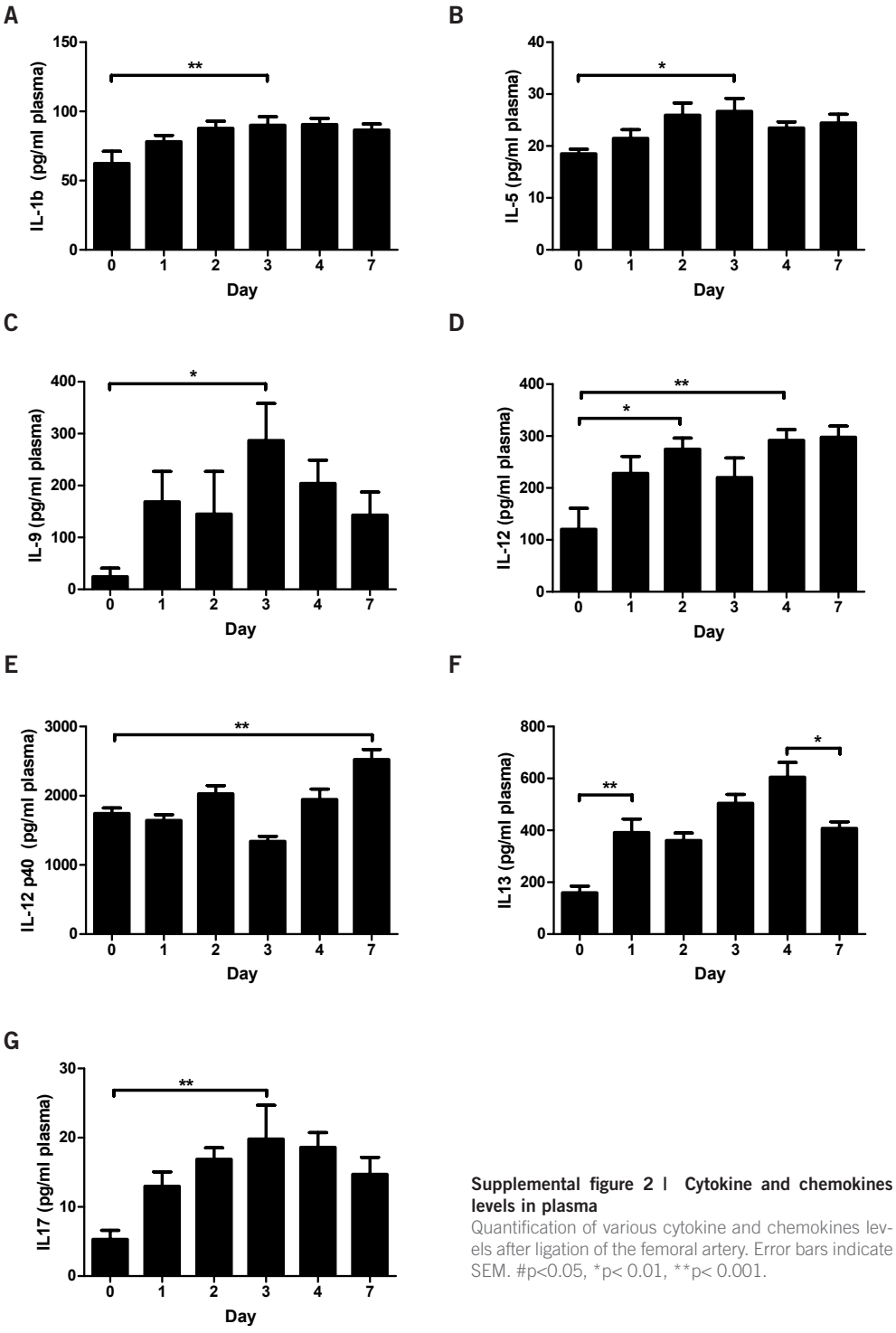
Marker	Panel	Amount	Conjugate	Host-target	Company
CD11a	1	5 µl	RPE	Rat anti Mouse	AbD Serotec
CD11b	1	0.5 µl	PE-Cy7	Rat anti Mouse	eBiosciences
CD3	2	0,5 µl	FITC	Hamster anti Mouse	eBiosciences
CD4	2	1,25 µl	PE/Texas red	Rat anti Mouse	Invitrogen
CD8	2	1,25 µl	PE/Cy7	Rat anti Mouse	AbD Serotec
F4/80	1	0.25 µl	Alexa 647	Rat anti Mouse	AbD Serotec
Ly6g	2	0,25 µl	APC	Rat anti Mouse	R&D Systems
CD1d	2	0.5 µl	PE	Rat anti Mouse	Biolegend



Supplemental figure 1 | Overview of the (stem) cells and their markers assessed in the bone marrow
Schematic overview of the different lineages present in the bone marrow including the markers used to differentiate between the different markers celltypes. Celltypes for which no panel of markers is mentioned in this overview were not assessed.

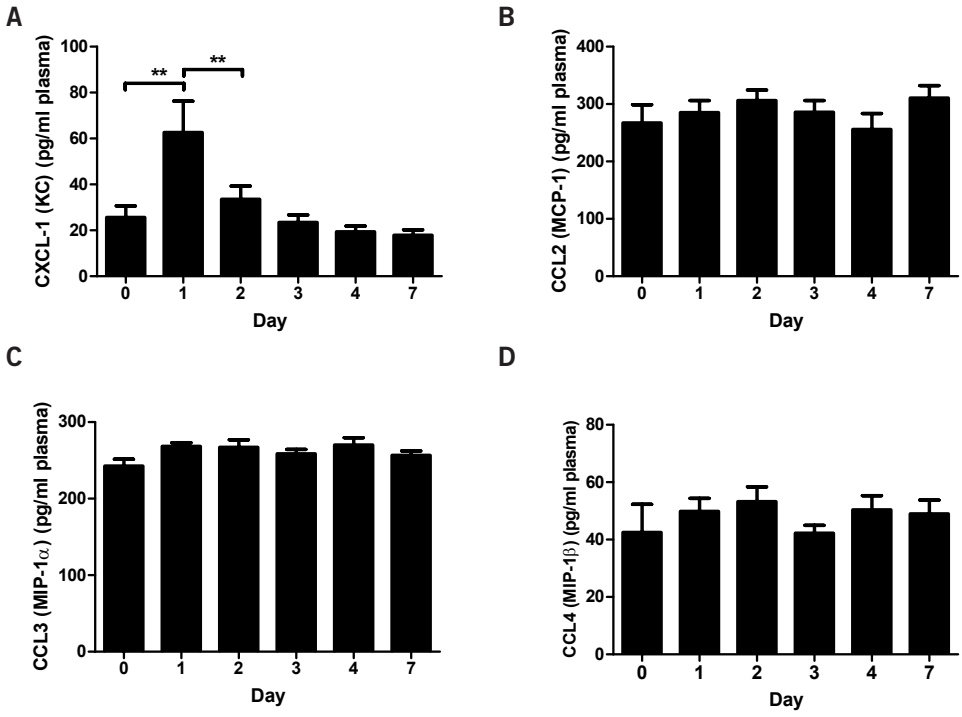
Supplemental table 2 | Bone marrow antibodies
List of antibodies used in this study to discriminate bone marrow subsets. Amount of antibody stated is µl/100 µl PBS which was added to $1*10^6$ cells.

Marker	Panel	Amount	Conjugate	Host-target	Company
CD13	1 & 2	10 µl	Alexa 647	Rat anti Mouse	AbD Serotec
CD11b	4	0.1 µl	PE-Cy7	Rat anti Mouse	eBiosciences
CD14	1 & 4	10 µl	FITC	Rat anti Mouse	BD Biosciences
CD34	2	10 µl	FITC	Rat anti Mouse	AbD Serotec
CD38	1 & 3	0.1 µl	Alexa 700	Rat anti Mouse	eBiosciences
CD41	2	0.25 µl	PE/Cy7	Rat anti Mouse	eBiosciences
CD54	2	10 µl	PE	Hamster anti Mouse	BD Biosciences
CD90	3	0.5 µl	Alexa 488	Mouse anti Rat	AbD Serotec
Ly6g	4	0.5 µl	APC	Rat anti Mouse	R&D Systems

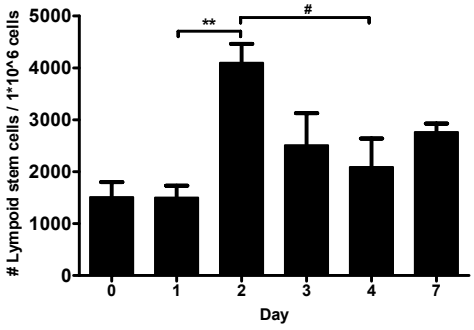


Supplemental figure 2 | Cytokine and chemokines levels in plasma

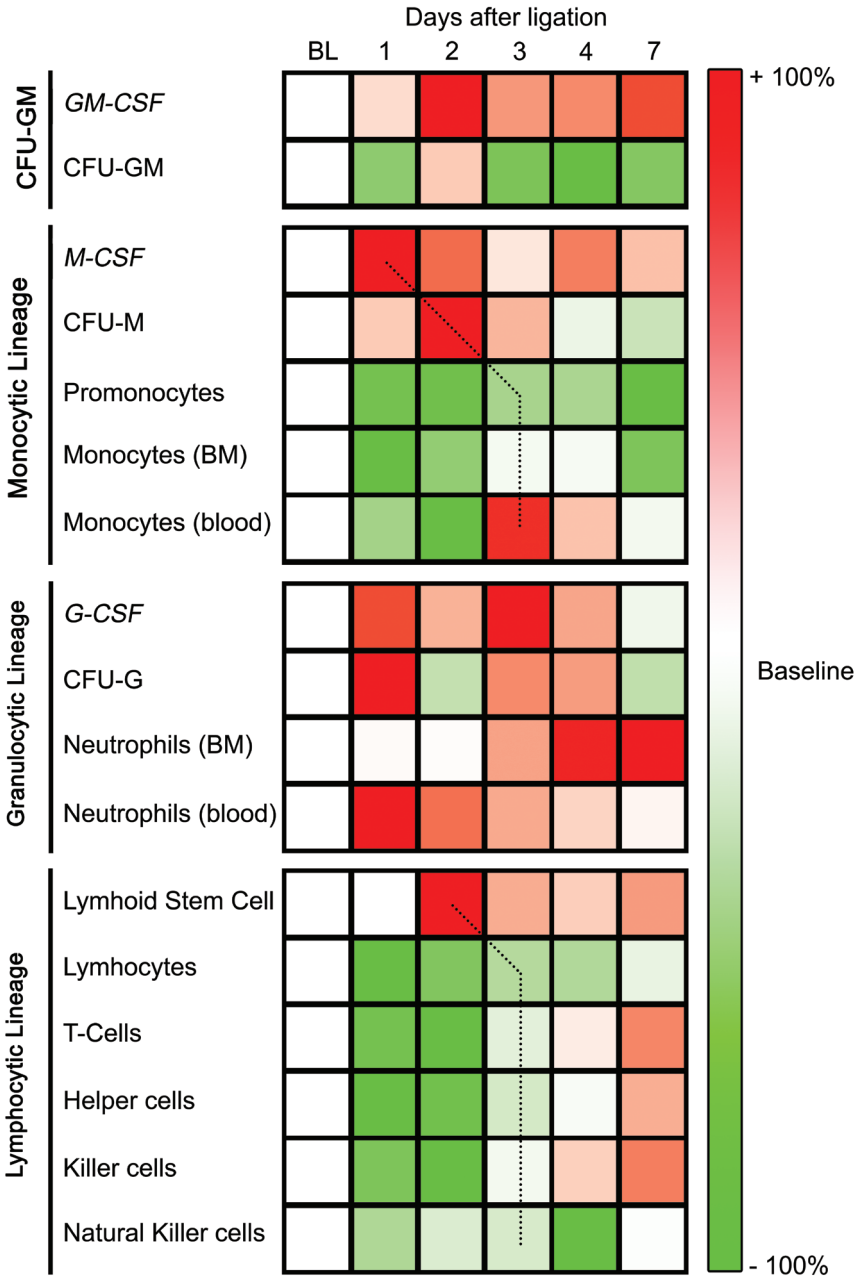
Quantification of various cytokine and chemokines levels after ligation of the femoral artery. Error bars indicate SEM. #p<0.05, *p< 0.01, **p< 0.001.



Supplemental figure 3 | Cytokine and chemokines levels in plasma.
Quantification of various cytokine and chemokines levels after ligation of the femoral artery. Error bars indicate SEM. **p< 0.001.



Supplemental figure 4 | Lymphoid stem cells in the bone marrow
Quantification of lymphoid stem cells after ligation of the femoral artery. Error bars indicate SEM. #p<0.05, **p< 0.001.



Supplemental figure 5 | Relative changes in cytokine levels and leukocyte subsets over time
Changes in cytokine levels and leukocyte numbers are depicted relative to baseline. The biggest quantitative change in cytokine level or cell number per subset was set at a 100% and all other changes were calculated as percentage of that change. Dotted lines indicate a chronologic sequence in peaks in cytokine levels and/or cell numbers within a lineage.

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3

CD26 INHIBITION ENHANCES PERFUSION RECOVERY IN APOE-/- MICE

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Abstract

Objective

The adaptive growth of blood vessels is important to prevent tissue loss following arterial occlusion. Extravasation of monocytes is essential for this process. The peptidase CD26 targets SDF-1 alpha, a chemokine regulating monocyte trafficking. We hypothesized that blocking SDF-1 alpha inactivation, using a commercially available CD26 inhibitor, accelerates perfusion recovery without detrimental side effects on plaque stability.

Methods and Results

Atherosclerosis prone ApoE-/- mice underwent femoral artery ligation and received a CD26 inhibitor or placebo. CD26 inhibition increased short term (7 days) perfusion recovery after both single and daily doses compared to placebo, 36%±2 ($p=0.017$) and 39%±2 ($p=0.008$) vs. 29%±3 respectively. Long term (56 days) perfusion recovery increased after daily treatment compared to placebo 83%±3 vs. 60%±2, ($p<0.001$). CD26 inhibition did not result in increased atherosclerotic plaque instability or inflammatory cell infiltration.

CD26 inhibition increased macrophage number around growing collaterals, SDF-1 alpha plasma levels and monocyte expression of the activation marker CD11b and the SDF-1 alpha receptor CXCR-4.

Conclusions

CD26 inhibition enhanced perfusion recovery following arterial occlusion via attenuated SDF-1 alpha inactivation and increased monocyte activation. There was no observable aggravation of atherosclerosis and CD26 inhibition could therefore offer a novel approach for therapeutic arteriogenesis in patients.

Introduction

Cardiovascular disease and its resulting mortality and morbidity is often associated with arterial narrowing or occlusion and subsequent decrease in tissue perfusion. In response to this decrease in arterial conductance, collateral arteries that can compensate for the occluded artery without therapeutic intervention are recruited and start growing, a process referred to as arteriogenesis. However, this natural recruitment is a slow process and often insufficient to rescue the ischemic tissue. Enhancement of arteriogenesis could be an attractive alternative therapeutic solution for patients suffering from vascular occlusive disease that do not adequately benefit from the current therapeutic options. Arteriogenesis is driven by an increase in shear stress as a result of the increased flow in the pre-existing collaterals following the pressure drop distal to the arterial occlusion or narrowing.[1] In response to the increase in shear stress the endothelium becomes activated which results in expression of pro-inflammatory cytokines and adhesion molecules. This leads to the attraction and extravasation of peripheral blood mononuclear cells (PBMC), that are important mediators of collateral artery formation.[2-4] Previous experimental studies have proven that enhancement of PBMC extravasation results in enhanced perfusion recovery and many pro-arteriogenic factors, such as MCP-1, GM-CSF, G-CSF, TGF-beta and FGF2, have been identified.[5] The systemic administration of these cytokines, however, might result in unwanted side effects such as increased plaque instability, as observed after administration of MCP-1.[6,7]

Stromal cell-derived factor-1 alpha (SDF-1 alpha), also known as CXCL-12, is a chemokine involved in the migration of PBMC and signals by binding to the CXC chemokine receptor-4 (CXCR-4).[8,9] Previous studies, utilizing a murine model for hind limb ischemia, have shown that expression of SDF-1 alpha is up regulated after arterial occlusion in the arterioles and surrounding muscle tissue with a peak around day 3.[10] At the same time, SDF-1 alpha plasma levels increase, resulting in a chemotactic gradient[10] and cell activation.[11] Nevertheless, this process is short lived as SDF-1 alpha is quickly cleaved by dipeptidyl-peptidase IV, also known as CD26, resulting in the loss of its signaling capabilities.[12] Until now, it is unknown if preventing SDF-1 alpha degradation improves perfusion after occlusion and if this can influence monocyte homing by providing a chemotactic gradient and cell activation.

We hypothesize that inhibition of CD26 results in an increase in SDF-1 alpha plasma levels thereby improving PBMC homing and extravasation and subsequent perfusion recovery. CD26 inhibitors are used in the clinic for glycemic control in diabetic patients and it was proven safe and feasible in randomized clinical studies.[13,14] In addition, CD26 inhibition in mice has shown to improve homing of mononuclear cells to the ischemic myocardium[15] and to reduce infarct size and increase cardiac function.[16,17]

Using a murine hind limb model, the effect of CD26 inhibition on perfusion recovery

after arterial occlusion was investigated. We assessed the effect of CD26 inhibition on SDF-1 alpha protein levels and on the extravasation, activation and migration of monocytes. In addition, possible side effects of CD26 inhibition with regard to atherosclerotic plaque stability were also investigated. This study identifies inhibition of CD26 as a potential therapy to enhance perfusion recovery after arterial occlusion.

Materials and Methods

Animal procedures

The present study was performed after having obtained appropriate institutional approvals. It *conforms to the Guide for the Care and Use of Laboratory Animals published by The US National Institute of Health* (NIH Publication No. 85-23, revised 1996). Twenty wild type (WT) C57Bl/6J (Harlan) and one hundred and one atherosclerosis-prone ApoE deficient (-/-) mice were divided into different groups and underwent permanent unilateral femoral artery ligation in the right leg and a sham operation in the left leg as described before.[18] During the operation mice were anesthetized using inhalation of Isoflurane and analgesized using buprenorfine (0.15 mg/kg). Wild type mice were 12 weeks old at the time of intervention while ApoE-/- mice were 6 months old at the time of intervention to ensure an adequate representation of the desired phenotype. Mice were treated orally with Januvia (Merck & Co) containing Sitagliptin (CD26 inhibitor and active compound of Januvia) dissolved in water or with water alone. Final concentration of Sitagliptin was 40 mg/kg for each mouse. Solutions were given to each mouse by pipetting the solution directly into the mouth of the mice. Mice were closely monitored to ensure adequate uptake of the drug. Mice were given water, a single dose of the CD26 inhibitor 3 days after arterial occlusion or daily doses from day 3 onward until termination. Day 3 was chosen as previous studies have shown that SDF-1 alpha levels decline starting at day 3 after ligation.[10]

For blood and tissue collection WT mice were anesthetized and analgesized with 75 mg/kg ketamine and 1 mg/kg medetomidine. Blood was collected by cardiac puncture using heparinized needles and kept on ice until further processing. Mice were sacrificed and hind limb tissue samples were collected and snap frozen using liquid nitrogen and stored at - 80 °C until further processing.

To assess macrophage extravasation around the growing vessels and stability of plaques in ApoE-/- mice after CD26 inhibition, mice were anesthetized and analgesized with 75 mg/kg ketamine and 1 mg/kg medetomidine. A needle was inserted into the left ventricle of the heart and mice were flushed with 5% Nitro-glycerine/NaCl after which they were perfusion fixed using 5% Nitro-glycerine/Formaldehyde and tissue was collected.

Hemodynamic measurements

Perfusion restoration after femoral artery occlusion was assessed as previously described.[18] In short, the abdominal aorta was cannulated and differently labeled fluorescent microspheres were injected at different pressures during maximal vasodilatation with adenosine. Afterwards, hind limb tissue samples were harvested, weighed, digested and the microspheres were counted using flow cytometry (FC500, Beckman Coulter). Perfusion restoration was calculated and expressed as percentage perfusion in the occluded hind limb vs. the sham operated contra lateral hind limb.

Immunohistochemistry

Presence of macrophages was assessed in paraffin embedded adductor tissue of ApoE-/- mice. Macrophages were identified by staining for Mac-3 (BD-Pharmingen, 553322). Macrophages accumulating in the peri-vascular tissue were counted in at least 6 tissue sections per animal and expressed as the number of Mac-3 positive cells/vessel. Plaque composition was assessed in formalin perfusion fixed aorta's (n=8 per group) of 8 month old ApoE-/- mice. After fixation the heart and aorta were excised and embedded in paraffin. The part of the aorta at the level of the heart valves was stained using a Picrosirius Red staining in combination with a Hematoxylin Eosin staining to assess plaque size and collagen density. Plaques were also stained using antibodies against αSMA (Sigma, F3777), CD3 (Dako, A0452) and Mac-3 (BD-Pharmingen, 553322).

SDF-1 alpha ELISA

Protein was isolated from WT adductor muscle using a 40mM TRIS solution containing protease inhibitors (Roche) and total protein levels were determined using the BCA protein assay (Pierce). SDF-1 alpha levels were determined in both plasma (diluted 1:1 in 50% Fetal Bovine Serum(FBS)/PBS) and TRIS protein samples using a Duoset ELISA (R&D systems, DY460) according to manufacturer's instructions.

Flow cytometry measurements

WT whole blood was stained with the appropriate antibodies (supplemental table 1) and after incubation fluorescent beads (Countbright absolute counting beads, Invitrogen) were added as a reference to calculate absolute cell numbers. Erythrocyte lyses was performed and samples were analyzed by flow cytometry (FC500, Beckman Coulter).

Migration experiments

Whole blood was collected from ten ApoE-/- mice by cardiac puncture using heparinized needles. PBMC were isolated using Ficoll-PaqueTM (Greiner BioOne), according to manufacturer's protocol and stored in liquid nitrogen in RMPI 1640 + Glutamax (Gibco, 61870) supplemented with 10% Fetal Bovine Serum (FBS) and 10% dimethylsulfoxide

(DMSO, Sigma-Aldrich). On the day of the assay, cells were thawed in RPMI (10% FBS) and left to recover for 1 h at 37 °C in an incubator (5% CO2.)
For CD26 inhibition treatment, the cell suspension was supplemented with a 50 mM solution of Januvia (Merck & Co) in water, resulting in a final concentration of 1 mM Januvia. A placebo treatment was performed by addition of sterile water. Subsequently, the cells were allowed to incubate for 15 minutes at room temperature before starting the migration assay. Samples of both cell suspensions were kept to determine the absolute number of cells which was loaded into the wells.
RPMI 1640 containing 20% FBS was added to the lower well of a polycarbonated Transwell system with 5 µm pores (Corning, 3421). Subsequently, ±100,000 PBMC were added to the upper well, after which half of the wells was supplemented with 100 ng/ml SDF-1 alpha (R&D Systems, 460-SD). Cells were allowed to migrate for 5 hours at 37 °C and 5% CO2 after which top wells were removed carefully and the migrated cells in the lower well were resuspended in 2 mM EDTA/PBS. Cells were stained for CD3, CD11b and F4/80 (supplemental table 2) to identify the number of migrated monocytes (CD3-, CD11b+ and F4/80+) by flow cytometry (FC500, Beckman Coulter). For the calculation of absolute cell numbers, fluorescent beads (Countbright absolute counting beads, Invitrogen) were added as reference to each tube, after which the number of migrated monocytes was calculated as the number of monocytes that had migrated per 10,000 monocytes loaded in the upper well.

Statistical tests
Data are presented as mean ± Standard Error of the Mean. Differences were assessed using a Mann Whitney U test. Values of $p \geq 0.05$ were considered significant.

Results

Perfusion recovery after arterial occlusion
To determine the effect of CD26 inhibition on perfusion recovery, 6 month old ApoE-/- mice were treated either with a single dose of the CD26 inhibitor 3 days after arterial occlusion or daily from day 3 onward. Perfusion recovery was measured seven days after arterial occlusion and was increased in both treatment groups compared to placebo. A single dose of the CD26 inhibitor resulted in an increase in perfusion recovery from 29% ±3 in untreated mice (N=7) to 36% ±2 (p=0.017) in treated mice (N=9). Daily treatment (N=10) increased perfusion recovery to 39% ±2 (p=0.008) (Figure 1A). Perfusion recovery was also determined at 56 days after hind limb ligation. Single treatment (N=9) with CD26 inhibitor did not result in an increase in perfusion recovery at 56 days compared to untreated mice (N=11)(64% ±2 vs. 60% ±2, p=0.210). Daily treatment (N=13) from day

3 until day 56, however, significantly increased perfusion recovery when compared to untreated mice (83% ± 3 vs. 60% ±2, p<0.001) (Figure 1B).

Monocyte/macrophage extravasation

We investigated if CD26 inhibition increases macrophage extravasation around the collateral vessels in the adductor muscle. ApoE-/- mice were treated with a single dose of the CD26 inhibitor or placebo at 3 days after ligation and hind limb tissue was harvested the next day. The number of macrophages per vessel was higher after treatment with the CD26 inhibitor (N=10) compared to placebo (N=8), 7.3 ±0.1 vs. 6.0 ±0.3 (p=0.005) (Figure 2).

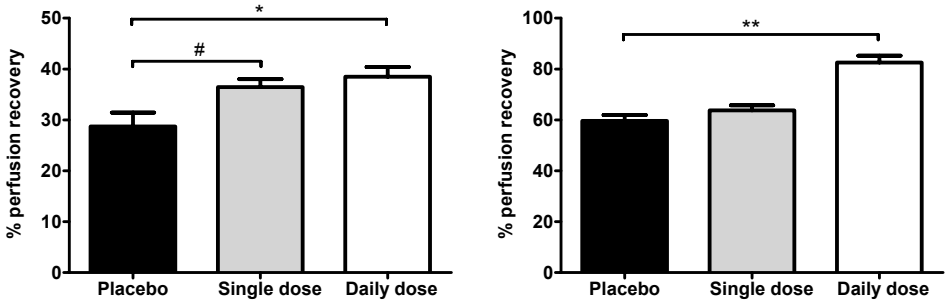

Figure 1 | Perfusion recovery after CD26 inhibition

Perfusion recovery was measured in ApoE-/- mice 7 days (A) or 56 days (B) after arterial occlusion using a combination of microspheres and flow cytometry. Mice were divided into three groups: placebo (black bar) and single dose (grey bar) or daily dose treatment (white bar) with the CD26 inhibitor. Perfusion restoration was calculated and expressed as percentage perfusion in the occluded hind limb vs. the sham operated contra lateral hind limb. Error bars indicate SEM. #p< 0.05, *p< 0.01, **p< 0.001.

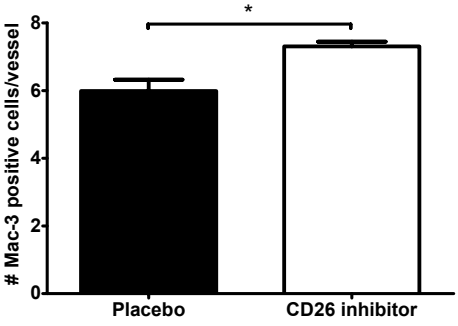

Figure 2 | Quantification of macrophages around growing collaterals

Three days after femoral artery ligation mice were treated with the CD26 inhibitor or a placebo. Presence of macrophages was assessed in paraffin embedded adductor tissue of ApoE-/- mice by staining for Mac-3 (BD-Pharmingen, 553322). Macrophage number is expressed as the number of Mac-3 positive cells/vessel. Error bars indicate SEM. *p< 0.005.

CD26 inhibition and plaque stability

To determine whether CD26 inhibition affected plaque composition and stability, plaques of ApoE-/- mice were investigated (N=8 per group). Mice were operated when they were six months old and sacrificed two months later. Plaque size increased after a single dose of the CD26 inhibitor compared to placebo (0.70 ±0.11mm^2 vs. 0.50 ±0.07mm^2, p=0.015). However, two months lasting daily doses of the CD26 inhibitor did not significantly increase plaque size compared to placebo (0.64 ±0.08 mm^2 vs. 0.50 ±0.07mm^2, p=0.208)(Figure 3).

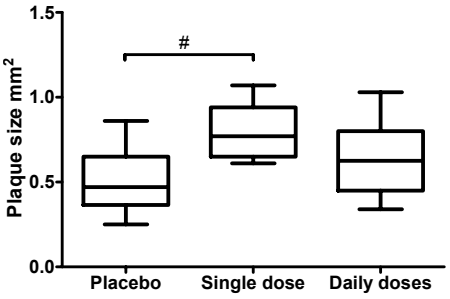

Figure 3 | Plaque size after CD26 inhibition
The effect of both single and daily CD26 inhibition on aortic plaque size was assessed using a Hematoxylin Eosin staining 56 days after femoral artery ligation. Error bars indicate SEM. #p< 0.05.

To assess plaque stability, the effect of CD26 inhibition on collagen density and alpha Smooth Muscle Actin (αSMA) content was determined. Collagen density did not differ significantly between groups but was lower after a single treatment with the CD26 inhibitor compared to placebo (7.3 ±2.6 AU vs. 11.2 ±2.5 AU, p=0.083). Daily treatment resulted in a collagen density of 8.2 ±1.8 AU compared to 11.2 ±2.5 AU for the placebo (p=0.294; Supplemental figure 1A). The percentage of plaque area positive for αSMA did not differ significantly between groups (single dose vs. placebo, 9.3 ±2.3 % vs. 8.1 ±1.5 %, p=0.886, daily doses vs. placebo 6.3 ±1.4 % vs. 8.1 ±1.5 %, p=0.519) (Supplemental figure 1B).

To determine whether CD26 inhibition increased inflammatory cell infiltration in atherosclerotic plaques, plaques were stained for the presence of macrophages (Mac-3+) and T-lymphocytes (CD3$^+$). The percentage of plaque surface area positive for the staining was quantified. Neither a single nor a daily treatment with the CD26 inhibitor increased macrophage plaque infiltration (single vs. placebo, 34.5 ±3.5 % vs. 26.7 ±6.9 %, p=0.165, daily vs. placebo, 27.4 ±3.5 % vs. 26.7 ±6.9 %, p=0.643)(Figure 4A&C). T-lymphocyte plaque infiltration was also not affected by either a single or daily treatment with the CD26 inhibitor (single vs. placebo, 10.9 ±1.6 % vs. 9.9 ±0.9 %, p=0.862, daily vs. placebo, 10.8 ±1.4 % vs. 9.9 ±0.9 %, p=0.848)(Figure 4B&D).

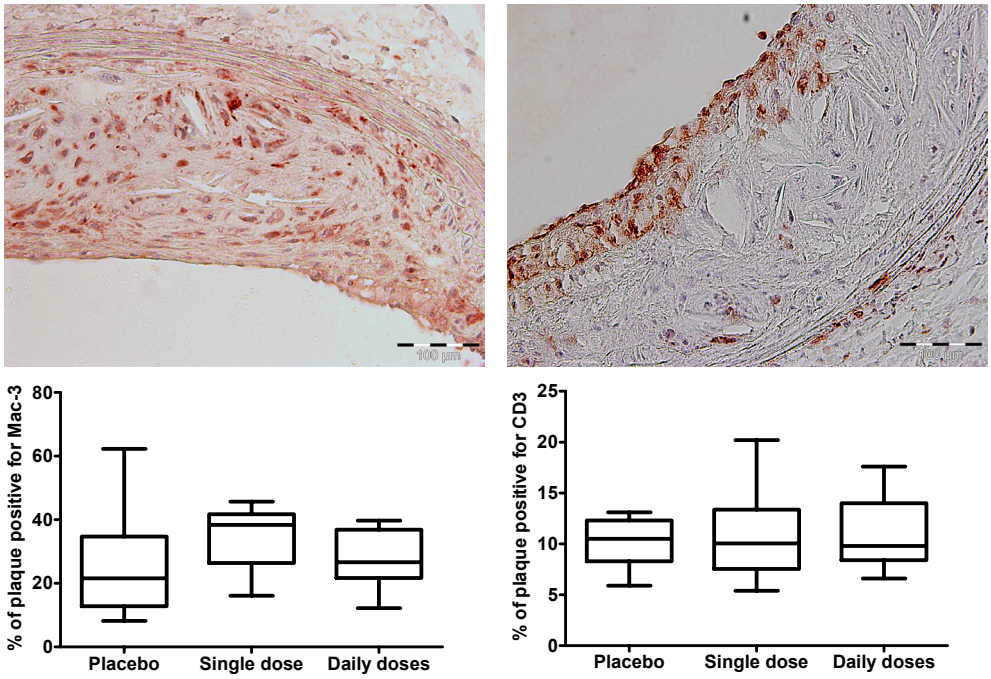

Figure 4 | Effect of CD26 inhibition on plaque infiltration by inflammatory cells
The effect of both single and daily CD26 inhibition on the presence of macrophages (Mac-3+) and T-lymphocytes ($CD3^+$) in aortic plaques was assessed using immunohistochemistry 56 days after femoral artery ligation in ApoE-/- mice. Representative pictures are shown for both the Mac-3 (A) and CD3 (B) staining. The percentage of plaque area positive for Mac-3 (C) and CD3 (D) was subsequently quantified.

SDF-1 alpha levels

As an increase in SDF-1 alpha levels could be responsible for the increase in macrophage influx observed in the hind limb sections, SDF-1 alpha levels in both adductor muscle tissue and plasma were determined one day after the start of CD26 inhibitor treatment in WT mice (N= 10 vs. 10). SDF-1 alpha levels in the adductor tissue of CD26 inhibitor treated mice did not significantly differ from placebo treated mice (2922 ±237 pg/ml vs. 4070 ±677 pg/ml, p=0.257)(Figure 5A). SDF-1 alpha levels in the plasma, however, were significantly higher in CD26 inhibitor treated mice vs. untreated mice (2295 ±117 pg/ml vs. 1732 ±105 pg/ml, p=0.006)(Figure 5B).

The effect of CD26 inhibition on circulating monocytes

Having established that SDF-1 alpha plasma levels were higher following CD26 inhibitor treatment, we assessed whether there was an effect on circulating monocyte number and/or activation. One day after CD26 inhibitor treatment, circulating monocyte number, expression of the SDF-1 alpha receptor CXCR-4 and CD11b expression as a marker for

monocyte activation were assessed in WT mice (N= 10 vs. 10). CD26 inhibitor treatment resulted in a 44% lower peripheral blood monocyte number compared to placebo (180 ±21 vs. 322 ±27 monocytes/µl blood, p<0.001), while concomitantly monocyte CXCR-4

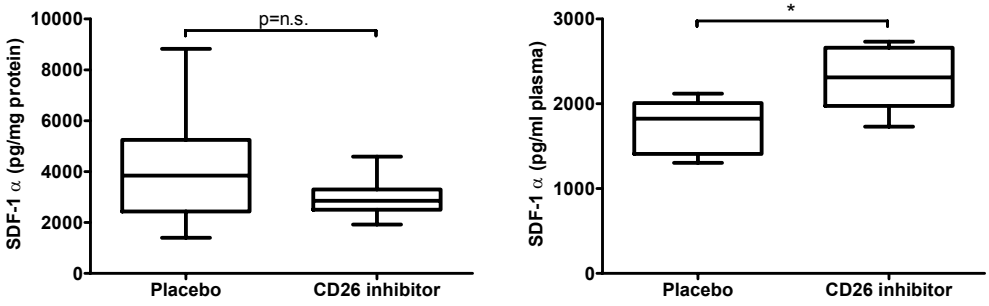

Figure 5 | The influence of CD26 inhibition on SDF-1 alpha levels
Three days after femoral artery ligation mice were treated with the CD26 inhibitor or a placebo. Mice were terminated the next day and SDF-1 alpha levels were determined in both the adductor muscle (A) and plasma (B). Error bars indicate SEM. *p< 0.01.

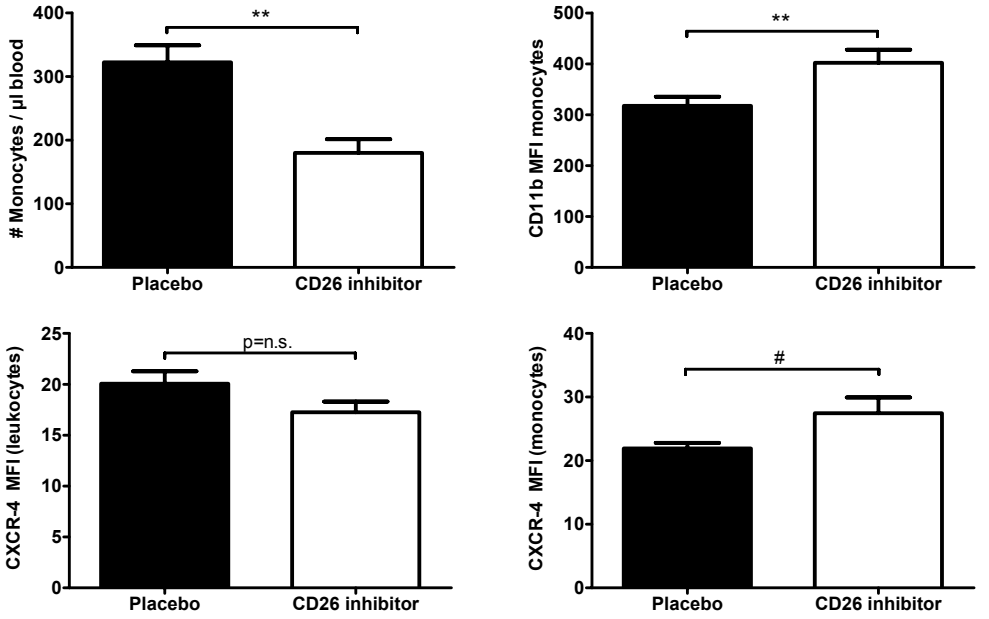

Figure 6 | The effect of CD26 inhibition on peripheral blood monocyte number, activation and CXCR4 expression
Three days after femoral artery ligation mice were treated with the CD26 inhibitor or a placebo. Mice were terminated the next day and monocyte number (A) and expression of the activation marker CD11b (B) were determined. CXCR-4 expression was measured on circulating monocytes (C) and leukocytes (D). Error bars indicate SEM. #p< 0.05, **p< 0.001.

and CD11b expression were up regulated compared to placebo (MFI 28 ±2.5 vs. 22 ±0.9, p=0.029 and MFI 402 ±26 vs. 317 ±18, p<0.001) (Figure 6A-C). CXCR-4 expression for the whole leukocyte population did not differ after CD26 inhibitor treatment compared to placebo (MFI 17 ±1.0 vs. 20 ±1.2, p=0.134)(Figure 6D). T-lymphocyte number also declined significantly, without up regulation of the activation marker CD11a on these cells (Data not shown).

Migration and CD26 inhibition

To determine the role of CD26 inhibition in ApoE -/- PBMC migration, *in vitro* migration experiments were performed in which SDF-1 alpha was used to activate ApoE -/- PBMCs with or without the addition of a CD26 inhibitor (N=6 per experimental condition).
Treatment of ApoE -/- PBMC with CD26 inhibitor alone did not have an effect on the number of migrated monocytes when compared to monocytes that did not receive the CD26 inhibitor, (548 ±65 monocytes/10,000 monocytes loaded vs. 485 ±36 monocytes/10,000 monocytes loaded, p=0.522). Treatment with the CD26 inhibitor in combination with activation by 100 ng/ml SDF-1 alpha in the upper well resulted in an increase in monocyte migration compared to monocytes that received only SDF-1 alpha (805 ±95 monocytes/10,000 monocytes loaded vs. 506 ±55 monocytes/10,000 monocytes loaded, p=0.037)(Figure 7).

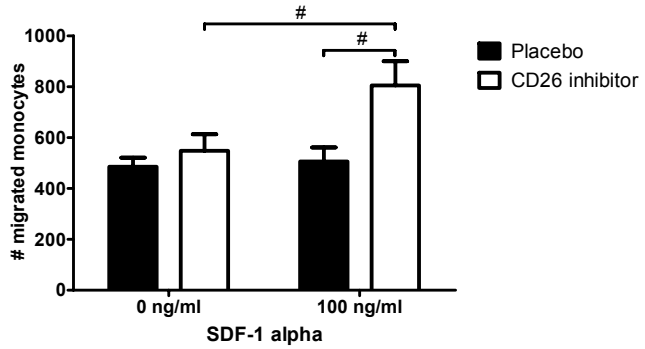

Figure 7 | The role of CD26 in monocyte migration
The role of CD26 and monocyte activation by SDF-1 alpha in monocyte migration was assessed using a transwell migration assay based on a serum gradient (10% to 20% FBS). ApoE -/-PBMC were treated with the CD26 inhibitor (white bars) or a placebo (black bars) and where appropriate 100 ng/ml SDF-1 alpha was added to cell suspension in the upper well of the transwell system. Error bars indicate SEM. #p< 0.05.

Discussion

The initial treatment for patients suffering from occlusive peripheral artery disease is based on the stimulation of collateral artery growth by (supervised) training. However, the success of treatment is limited and there is currently no drug available that could improve therapy outcome by enhancing natural perfusion recovery.[19] In this study we investigated the effect of CD26 inhibition on perfusion recovery as CD26 inhibition has been proven to be both clinically safe and feasible.[13,14]

We show for the first time that inhibition of the peptidase CD26 increases perfusion recovery after arterial occlusion both on the short and the long term. Our data indicates that CD26 plays a role in both the early and later stages of perfusion recovery and that its inhibition, even at later stages, is beneficial. Whether this effect is solely due to enhanced preservation of SDF-1 alpha levels, without an effect of other CD26 substrates, cannot be established from this study. GLP-1 for example, which is also a CD26 substrate, has been shown to be cardio-protective in ischemia reperfusion models. However, the half-life of a substrate for a given enzyme at a given concentration is expressed in the selectivity constant. With regard to CD26, the selectivity constant is highest for SDF-1 alpha, being 25 fold higher compared to GLP-1, which indicates that CD26 has a higher affinity for SDF-1 alpha than GLP-1.[20] Together with the other data presented in this study it is therefore highly suggestive that the effect of CD26 inhibition on perfusion recovery is mediated via SDF-1 alpha.

SDF-1 alpha levels in the muscle did not change in response to arterial occlusion. In contrast, plasma SDF-1 alpha levels increased, as well as expression of CXCR-4 and the activation marker CD11b on circulating monocytes. CD26 inhibition also enhanced macrophage number around the growing collaterals. The increased perfusion recovery and extravasation of macrophages after CD26 inhibition is in line with earlier studies in which enhanced monocyte extravasation was also associated with increased perfusion recovery.[21] Several studies have shown that inhibition or depletion of CD26 is associated with enhanced (stem) cell homing, which has been attributed to enhanced chemotaxis of SDF-1 alpha[15,17,22] although a direct role for CD26 in cell activation has been described as well.[23] As we did not observe a difference in SDF-1 alpha levels in the hind limb tissue at the site of collateral artery growth, which would be the source for a chemotactic gradient, we investigated whether improved monocyte activation could be responsible for the observed increase in monocyte migration.

To study this, we conducted *in vitro* migration experiments in which we used SDF-1 alpha to activate the cells that were loaded in the upper chamber of a transwell assay. Without SDF-1 alpha present, CD26 inhibition did not result in an enhanced migration of monocytes. In the presence of SDF-1 alpha, however, CD26 inhibition improved monocyte migration when compared to placebo treatment.

Having shown that there is no difference in muscle SDF-1 alpha levels and that CD26 inhibition results in high SDF-1 alpha levels in plasma, more activated monocytes and stimulated monocyte migration, we conclude that the improved extravasation of monocytes after CD26 inhibition is due to an enhanced activation of these cells by SDF-1 alpha rather than a stronger chemotactic gradient.

These findings are in agreement with previous studies where increased SDF-1 alpha signaling through the CXCR-4 receptor resulted in activation of various integrins such as LFA-1, VLA-4 and VLA-5 which are involved in leukocyte activation and extravasation.[11] SDF-1 alpha signaling also induced rapid leukocyte arrest in *in vitro* experiments.[24] Furthermore, SDF-1 alpha signaling results in activation of NF-KB[25], a known pro-inflammatory transcription factor who's crucial role in perfusion recovery is mainly mediated via its expression in circulating cells.[26] The use of two different strains is a limitation of this study. However, ApoE -/- mice have a C57Bl6/J background and therefore share many similarities and common mechanisms with its wild type strain, such as the involvement of SDF-1 alpha in plaque formation.[27,28]

Increased collateral formation is often associated with increased plaque formation and plaque instability. [6] As a consequence, many strategies to enhance collateral formation have not made it to a clinically applicable therapy. To assess the effect of CD26 inhibition on plaque formation, we analyzed plaque composition in atherosclerosis prone ApoE-/- mice after both single and daily treatment. Although plaque size increased significantly after a single dose with the CD26 inhibitor, daily doses did not have an effect. More importantly however, plaque stability did not seem to be affected by CD26 inhibition as we observed no change in collagen density and alpha smooth muscle actin levels. Furthermore, we found no differences in the infiltration of macrophages and T-lymphocytes into plaques as a result of CD26 inhibition. Although the group sizes were small, these results indicate that CD26 inhibition can enhance perfusion restoration without detrimental side effects on atherogenesis.

The findings on macrophage plaque infiltration appear contra-intuitive with the higher influx of monocytes around the collateral arteries as a result of increased systemic monocyte activation. Although our study does not provide a conclusive explanation for the observed differences, we speculate that diversity in other factors than leukocytes might underlie this discrepancy in leukocyte extravasation. Differential biochemical composition and subsequent activation of the endothelium of collateral arteries and aorta might contribute to a difference in leukocyte extravasation.[29] Furthermore, given the large number of cytokines involved in leukocyte extravasation, a likely existing variance in cytokine expression between plaques and growing collaterals could also provide a possible explanation.[30]

Furthermore, enhanced SDF-1 alpha levels as a result of CD26 inhibition might directly contribute to plaque stability. It has been shown that loss of SDF-1 alpha signaling,

although it plays a crucial role in the initiation of plaque formation[28], is detrimental in later stages of atherosclerosis.[31] In addition, low SDF-1 alpha plasma levels are associated with unstable angina[32] and patients that suffer from unstable angina or myocardial infarction have a higher prevalence of CD26 positive cells in the atherosclerotic plaque.[33] These studies might also explain the fact that single treatment increases plaque size while daily treatment did not. We speculate that the underlying mechanism might be related to the fact that single treatment occurred at an early single time point while daily treatment occurred for a longer period. As SDF-1 alpha signaling is crucial in plaque formation[28], a single early increase in SDF-1 alpha levels might therefore be detrimental compared to daily treatment as daily treatment could be beneficial due to a prolonged increase in SDF-1 alpha levels.

In summary, this study shows for the first time that CD26 inhibition increases perfusion recovery after arterial occlusion. CD26 inhibition treatment results in increased SDF-1 alpha plasma levels and enhances activation and subsequent extravasation of the circulating monocyte. As we observed no effect of CD26 inhibition on the influx of inflammatory cells in atherosclerotic plaques or plaque instability, systemic CD26 inhibition might be used as a possible non-invasive intervention method to enhance perfusion recovery after arterial occlusion in peripheral artery disease.

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Disclosures

The authors have nothing to disclose.

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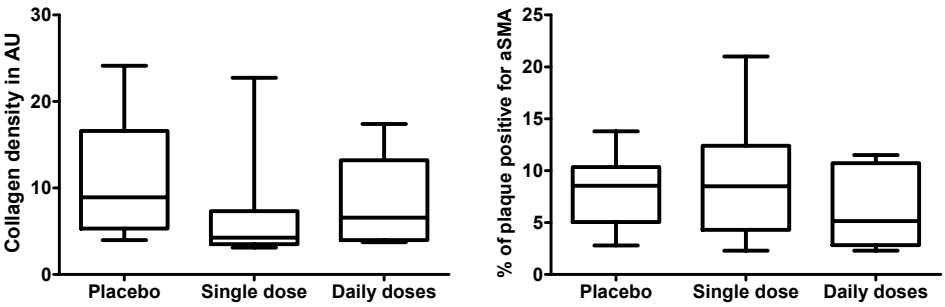
Supplemental



Supplemental figure 1 | Effect of CD26 inhibition on plaque size and stability
The effect of both single and daily CD26 inhibition on plaque stability was assessed 56 days after ligation in ApoE-/- mice. Collagen density (A) was assessed using a Picrosirius Red and Hematoxylin Eosin staining. In addition, plaques were stained for the presence of αSMA which was subsequently quantified as the percentage of plaque area positive for αSMA (B). Error bars indicate SEM.

Supplemental table 1 | Peripheral blood antibodies
List of antibodies used in this study to discriminate leukocyte subsets. For all experiments antibodies were diluted in 100 µl PBS which was added to 50 µl of whole blood

Marker	Clone	Host-target	Company
CD11b	M1/70	Rat anti Mouse	AbD Serotec
CXCR-4	247506	Rat anti Mouse	R&D Systems
F4/80	CI:A3-1	Rat anti Mouse	AbD Serotec

Supplemental table 2 | Migration assay antibodies
List of antibodies used in this study to discriminate monocytes after cell migration. For all experiments antibodies were diluted in 100 µl PBS which was added to the cells which had migrated to the lower well.

Marker	Clone	Host-target	Company
CD11b	M1/70	Rat anti Mouse	AbD Serotec
CD3	500A2	Golden Syrian Hamster anti Mouse	eBioscience
F4/80	CI:A3-1	Rat anti Mouse	AbD Serotec

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4

HIGH CD26 LEVELS ARE ASSOCIATED WITH HIGH MACROPHAGE NUMBERS AND A LARGE LIPID CORE IN HUMAN ATHEROSCLEROTIC PLAQUES

In preparation

Abstract

Objective

CD26 was recently shown to be involved in the development of atherosclerosis in two murine studies. Our objective was to assess CD26 content in human plaques and to correlate these findings to plaque histology including macrophage content.

Methods

Patients were selected from the Athero Express Biobank and CD26 plaque content was measured using a Luminex setup. These results were correlated to plaque histology and macrophage, smooth muscle cell and lipid core content.

Results

CD26 content correlated to plaque phenotype and is higher in plaques with a more unstable phenotype. A high CD26 content was also related to high macrophage numbers, a large lipid core and low smooth muscle cells numbers.

Conclusion

Our study shows that there is a relationship between a higher CD26 plaque content and the presence of a more vulnerable plaque phenotype in humans and supports a detrimental role for CD26 in the development of atherosclerosis.

Introduction

Atherosclerosis and its resulting mortality and morbidity remain a worldwide major health problem. In the past decade, however, our understanding of the various mechanisms leading to plaque formation and progression has increased. Different types of plaques have been identified: from a relatively stable plaque to the vulnerable plaque which is prone to rupture[1]. In addition, our understanding of the relationship between the local plaque and the systemic disease atherosclerosis has also increased. We now know that the local plaque can contain information that is predictive for the systemic outcome[2]. CD26, also known as Dipeptidyl-Peptidase IV, has been the subject of extensive research in the field of diabetes as it is involved in the breakdown of incretins which are important in glucose homeostasis[3]. Inhibition of CD26 has already been achieved successfully in the clinic to treat patients with diabetes[4,5]. Recently CD26 was shown to play a role in two murine models of atherosclerosis as CD26 inhibition resulted in a decrease in plaque size and macrophage infiltration[6,7]. In addition, an earlier study found that the expression of activation markers, including CD26, was increased on T-cells in patients with unstable angina[8]. However, the relationship between CD26 and human atherosclerosis remains unclear. Given the results from earlier studies we hypothesized that CD26 plaque content is higher in plaques with an unstable phenotype. To test our hypothesis and to establish a possible role for CD26 in plaque progression in humans we assessed CD26 content in human carotid plaques and correlated these findings to plaque histology including macrophage content.

Methods

Patient inclusion

All samples were retrieved from the Athero Express databank for which the patient inclusion has been described before[9]. In short, plaques obtained during carotid endarterectomy are processed following a standard protocol and patient follow up is documented. Patient characteristics are depicted in table 1.

Plaque phenotyping

Paraffin embedded plaque sections were stained as described before using Haematoxylin-Eosin staining for analysis of fat content, CD68 for macrophages and α actin for smooth muscle cells[9].

Table 1 | Patient characteristics

Characteristics	Patients (n=180)
Gender	120 male / 60 female
Age, mean ±SD (range)	69.6 ±9.4 (49-90)
Smoker (n)	58
Diabetics (n)	42

Luminex

Carotid plaque samples were collected, processed and stored at -80 °C in a 40 mM TRIS buffer as previously described[10]. Samples were retrieved from the -80 °C and diluted 1:1 in Roche buffer. Sample CD26 content was measured using the Bio-Plex 200 system (Biorad) and a Biotin labeled anti-hDPPIV antibody (R&D Systems, cat #BAF1180). A standard curve was created using recombinant hDPPIV (R&D Systems, cat #1180-SE-010).

Statistics

Statistical analysis was performed with SPSS version 15.0 (SPSS Inc). Connections between variables were analyzed using a bivariate analysis and pearson correlations. Whiskers in boxplots depict the 5-95 percentile. Correction for co-factors (gender, age at time of surgery, smoking and diabetes mellitus) was performed using binary logistic regression. To correct for co-factors the semi-quantitative measurements of macrophage and smooth muscle cell content were binned into "no/minor" and "moderate/heavy" groups. For lipid core content the results were binned into <40% or ≥40% lipid core plaque content. Odds ratios are depicted with a confidence interval of 95%. P-values < 0.05 were considered significant.

Results

CD26 content is higher in atheromatous plaques

Plaques were divided into three phenotypic classes: fibrous, fibroatheromatous and atheromatous in which a fibrous plaque represents a more stable plaque and an atheromatous plaque the more vulnerable plaque. CD26 content correlates to plaque phenotype and is higher in plaques with a more unstable phenotype (pearson correlation = 0.261, $p<0.0001$) (Figure 1A).

CD26 content is associated with macrophage and smooth muscle cell content

Macrophage plaque content was assessed using a CD68 antibody. CD68 staining was semi-quantitatively scored using a four-category system: no, minor, moderate or heavy staining. CD68 staining was found to be positively correlated to CD26 content (pearson correlation = 0.337, $p<0.0001$) (Figure 1B). These results were validated by computer measurements which determined the percentage of area positive for CD68 relative to the total image (pearson correlation = 0.271, $p<0.0001$). Using a regression model we established that CD26 content was correlated to macrophage content independent of gender, age at time of surgery, smoking and diabetes (odds ratio=1.136, 95% confidence interval 1.048 – 1.232, corrected p=0.002).

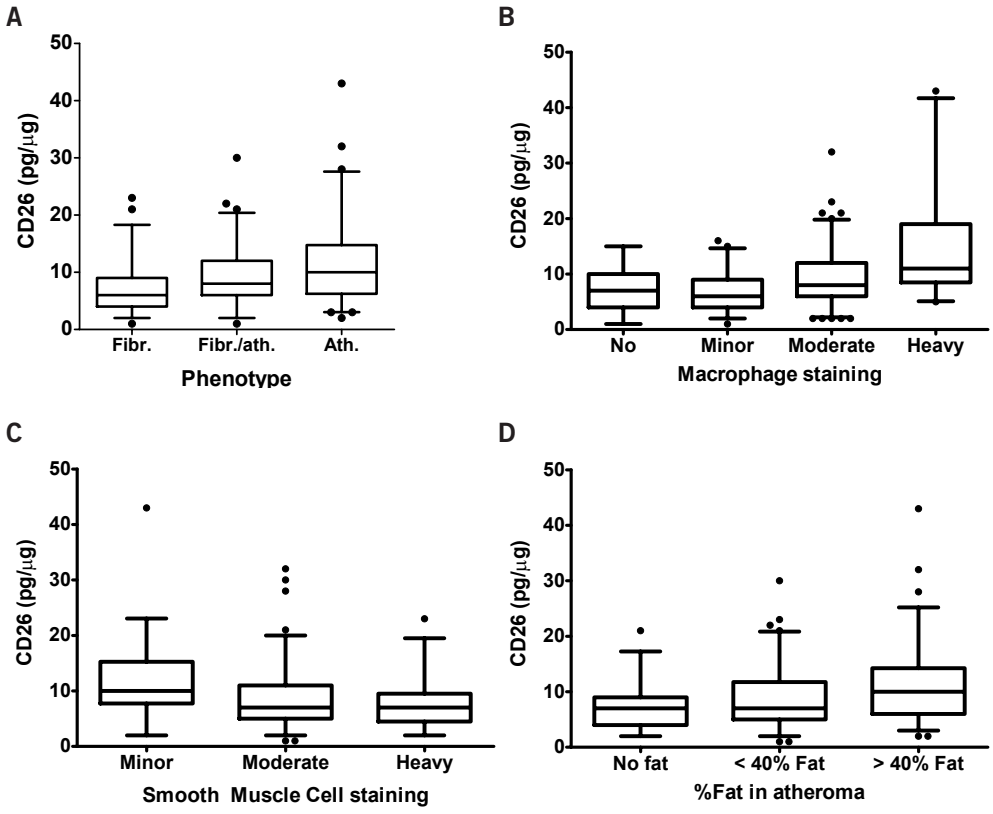

Figure 1 | CD26 plaque content is correlated to plaque characteristics
CD26 content was determined and correlated to A) overall plaque phenotype, B) macrophage staining, C) Smooth Muscle Cell staining and D) %Fat in atheroma.

Smooth muscle cell plaque content was assessed using an alpha Smooth Muscle Actin antibody (αSMA). αSMA was semi-quantitatively scored using a four-category system: no, minor, moderate or heavy staining. αSMA staining was found to be negatively correlated to CD26 content (pearson correlation = -0.183, p=0.014) (Figure 1C). These results were validated by computer measurements which determined the percentage of area positive for αSMA relative to the total image (pearson correlation = -0.170, p<0.022). Using a regression model we established that CD26 content was correlated to αSMA plaque content independent of gender, age at time of surgery, smoking and diabetes (odds ratio=0.942, 95% confidence interval 0.890 – 0.996, corrected p=0.037).

CD26 content is associated with plaque lipid core size

As a large lipid core is associated with a more vulnerable plaque phenotype we assessed lipid core size. Lipid core size was scored using a three-category system: no lipid core,

lipid core size <40% or lipid core size >40%. Lipid core size was found to be positively correlated to CD26 content (pearson correlation = 0.215, p=0.004) (Figure 1D). Using a regression model we established that CD26 content was correlated to lipid core size independent of gender, age at time of surgery, smoking and diabetes (odds ratio=1.082, 95% confidence interval 1.019 – 1.149, corrected p=0.010).

Discussion

Recently, two studies have established a role for CD26 in atherosclerosis using atherosclerotic prone mice[6,7]. Their results showed that the administration of a specific CD26 inhibitor was able to decrease both plaque size and macrophage influx. To facilitate the translation of these results to humans we used the Athero Express Biobank to correlate CD26 plaque content to plaque phenotype and macrophage influx. We found that a higher CD26 content is related to a more vulnerable plaque phenotype which is more atheromatous, has more macrophages and less smooth muscle cells.

Several characteristics are associated with a more vulnerable plaque phenotype such as a higher macrophage content, lower smooth muscle cell content, higher fat content, neovascularization and remodeling. Of these, three could be related to CD26 content and these showed that a higher CD26 content is related to a more vulnerable plaque phenotype. Plaques with a higher CD26 content have more fat, less smooth muscle cells and a higher infiltration of macrophages. We did not find any relationship between neovascularization or plaque remodeling with CD26 content. These results are in line with the aforementioned murine studies which respectively showed a decreased plaque size and macrophage infiltration after CD26 inhibition in LDLR-/- mice[6] and a decrease in plaque size in diabetic ApoE-/- mice7. They both identify the function of CD26 in monocytes as a potential mechanism of action[6,7].

Our study cannot confirm or contradict the mechanistic role of CD26 in plaque progression nor identify the source of CD26 in plaques. Further research will have to elucidate whether CD26 is actively involved in the mechanisms leading to a vulnerable plaque phenotype. In addition, clinical trials have started which will elucidate the effect of CD26 inhibition on cardiovascular safety and outcome[11].

In conclusion, our study has shown a relationship between high CD26 content and the presence of a more vulnerable plaque phenotype. Our findings are in line with the recent murine studies and support a detrimental role for CD26 in the development of atherosclerosis.

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5

PLATELETS CONTRIBUTE TO PERFUSION RECOVERY AFTER HIND LIMB ISCHEMIA

Submitted

Abstract

Objective

Vascular occlusion and its resulting mortality and morbidity remain a major health problem. After vascular occlusion, tissue perfusion can be restored via a natural process of vascular remodeling named arteriogenesis which depends on the influx of monocytes. The role of platelets in perfusion recovery is still unexplored. Platelets, however, are described to be involved in leukocyte adhesion and the objective of this study was to determine their role in perfusion recovery

Methods and Results

Using a murine hind limb model, we investigated the effect of platelet depletion on perfusion recovery using a CD41 antibody. Platelet depletion decreased perfusion recovery in mice 7 days after ligation of the femoral artery (p=0.005). Platelet depletion resulted in a decrease in alpha smooth muscle actin vessel wall positive area (p=0.009) and macrophage number in and around collateral vessels (p=0.035). E*x vivo* experiments in a flow chamber model using blood from platelet-depleted mice showed that leukocyte rolling was abolished after platelet depletion.

Conclusions

Platelets are involved in perfusion recovery after hind limb ligation probably via a role in monocyte extravasation leading to limited vessel remodeling.

Introduction

Cardiovascular disease (CVD) is often a consequence of vascular occlusion. The high morbidity and mortality rates of CVD are still a major health problem. The human body is capable of redirecting blood flow after arterial occlusion to maintain the distribution of nutrients and oxygen to distal tissue. This process of adaptive vascular growth is named arteriogenesis and the resulting increase in blood flow can be sufficient to completely restore perfusion to the distal tissue [1]. In recent years it has become evident that arteriogenesis is an inflammatory process involving bone marrow derived cells which locally extravasate from the blood stream. Arteriogenesis is initiated by an increase in shear stress in pre-existing vascular anastomoses due to initiation of blood flow after a drop in distal blood pressure. A subsequent up regulation in the expression of adhesion molecules results in the attraction of circulating leukocytes which are crucial for further vessel growth and remodeling of the peri-vascular space [2]. Monocytes have shown to be important contributors to arteriogenesis in many preclinical and clinical studies [3-8], but also T-lymphocytes and NK cells play distinctive roles in perfusion recovery [9-11]. The role of leukocytes is mediated via immune system components such as Toll-Like Receptor-2, Toll-Like Receptor-4 and Nuclear-Factor-Kappa-B p50, as studies using chimeric mice have shown that the expression of these components on bone marrow derived cells is crucial for perfusion recovery [12,13].

In the past decades it has become increasingly clear that the functions of platelets are much more abundant than mere coagulation. Platelets have now been implicated in many inflammatory processes by facilitating the adhesion and rolling of leukocytes to the vascular endothelium but also more directly by releasing pro-inflammatory factors from their granules [14]. Recently, platelets were also found to mediate leukocyte influx and MMP expression following myocardial infarction [15].

Based on the role for platelets in leukocyte adhesion and rolling and the established importance of macrophage influx during perfusion recovery, we hypothesized that platelets are involved in perfusion recovery after arterial occlusion.

In this study, we investigated the role of platelets by generating platelet-depleted mice using a CD41 antibody and subsequently measured perfusion recovery after femoral artery ligation as well as macrophage influx and vascular wall dimensions.

Materials and Methods

Animal procedures

The present study was approved by the university animal experimental committee following the Guide for the Care and Use of Laboratory Animals published by The US

National Institute of Health (NIH Publication No. 85-23, revised 1996). Eighty-six C57Bl6/J mice underwent permanent unilateral femoral artery ligation at the age of 10-12 weeks as described previously [16]. Before the operation procedure mice were anesthetized with dormicum (5 mg/kg) and medetomidine (0.15 mg/kg) and analgesized with fentanyl (0.02 mg/kg) via an intra-peritoneal injection. After operation anesthesia was antagonized by a subcutaneous injection of anexate (1 mg/kg) and antisedan (5 mg/kg). After the operation mice received two injections of buprenorphine (0.15 mg/kg, 8 hour intervals). Paw perfusion was assessed using Laser Doppler (Moor instruments) before and after the operation procedure. In addition, paw perfusion was assessed 4 and 7 days after ligation but no fentanyl was used during these procedures. Perfusion recovery was calculated and expressed as percentage perfusion in the occluded hind limb vs. the contra lateral hind limb.

To achieve platelet depletion, mice were given an i.p. injection (100 µg) with a rat-anti-mouse CD41 antibody (Clone: MWReg30, eBioscience, 16-0411). CD41 is also known as glycoprotein IIb or integrin alpha IIb and is involved in stable platelet adhesion to matrix proteins, aggregation and thrombus formation [17]. Unless stated otherwise, mice received an antibody injection the day before operation and 3 days after operation as platelets started to re-appear 4 days after injection (supplemental figure 2A). To assess the effect of platelet depletion on perfusion recovery from day 4 until day 7 mice were injected with the CD41 antibody at day 4 directly after Laser Doppler measurements. In ±10% of the mice, injection with the antibody failed to result in platelet depletion. Therefore, blood was collected the day after antibody injection by cheek puncture to confirm platelet depletion by flow cytometry (example supplemental figure 2B).

Mice were terminated 4 or 7 days after femoral artery ligation for blood and tissue collection. Mice were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg). Blood was collected by cardiac puncture using heparinized needles. Subsequently a needle was inserted into the left ventricle of the heart and mice were flushed with 5% nitro-glycerine/NaCl after which they were perfusion fixed using 5% nitro-glycerine/formaldehyde and tissue was collected.

Flow cytometry measurements

To confirm platelet depletion, 5 µl blood was added to 50 µl 1 M HEPES buffer (Gibco, 15630) containing 0.1 µg CD41-PE antibody CD41-PE (eBioscience, 12-0411, clone: MWReg30) and incubated in the dark for 30 minutes. Samples were fixed using 1 ml of fixation buffer containing 0.2% formaldehyde and 0.9% NaCl and subsequently measured using flow cytometry. Platelet content was assessed based on the forward/sideward scatter of events and CD41 positivity. In addition, several leukocyte subsets were assessed for CD41 positivity using antibodies for CD3-FITC (eBioscience, 11-0033, 0.25 µg, clone: 500A2), CD41-PE (Ebioscience, 12-0411, clone: MWReg30, 0.1 µg), F4/80-A647 (AbD

Serotec, MCA497A647, 0.125 µg, clone: Cl:A3-1) and Ly6g-A700 (BD Pharmingen, 561236, 0.5 µg, clone: 1A8).

Immunohistochemistry

Presence of macrophages was assessed in paraffin embedded adductor tissue. Macrophages were identified by staining for Mac-3 (BD-Pharmingen, 553322). Sections were counterstained using a hematoxylin eosin staining. To assess alpha Smooth Muscle Actin (αSMA) positive area, paraffin embedded adductor tissue samples were stained with a FITC-labeled αSMA antibody (Sigma, F3777). Nuclei were counterstained with hoechst. Sections were automatically analyzed using Cell^P software (Olympus).

Vessel size measurements

Vessel size measurements were performed in hematoxylin eosin stained paraffin embedded sections of the adductor muscle obtained 7 days after ligation. Maximal vasodilatation was assured by adding a vasodilator (nitro-glycerine) during perfusion fixation. Measurements were performed using Cell^P software. The length of the endothelial lining of the vessels was designated the inner perimeter. The length of the outer border of the vessel wall bordering the peri-vascular space was determined and designated the outer perimeter. Please see supplemental figure 1 for a graphic designations of these perimeters.

Calculations

Inner and outer vessel perimeter were measured using Cell^P software (see above). From this, the inner and outer diameter were calculated using the following formula: diameter = perimeter/π. Vessel wall thickness was calculated using the following formula: (outer diameter – inner diameter) / 2. Vessel wall area was calculated using the following formula: (π x outer radius^2) – (π x inner radius^2). Vessel lumen area was calculated as follows: inner radius^2 x π.

Flow chamber model

Four control and four platelet-depleted C57Bl6/J mice were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg). Blood was drawn via cardiac puncture and collected in 10% sodium citrate (3.2% w/v) and 100U/ml hirudin. Glass coverslips cleaned with chromic-sulfuric acid (Sigma) and dH2O were placed on a laminar-flow perfusion chamber with a channel height of 100µm and width of 1mm. The channel was coated with 100µg/ml fibrinogen (Enzyme Research Laboratories) for 1.5 hours at room temperature and subsequently blocked in 1% HSA overnight at 4˚C. The perfusion chamber was placed under an inverted microscope (Zeiss observer Z.1, Carl Zeiss) and a syringe pump (Harvard Apparatus) was used to perfuse the blood at a shear rate of

100 sec-1 over the coverslip. Blood was perfused for a minimum of 45 minutes to study platelet adhesion and monocyte rolling. Movies and snapshots were recorded using differential interference contrast (DIC) microscopy using a 40x/1.25 oil EC-plan Neofluar objective (Carl Zeiss). All images were analyzed with AxioVision software (Release 4.6, Carl Zeiss) and ImageJ software (Release 1.41).

Statistical tests

Data are presented as mean ± Standard Error of the Mean (SEM). Differences were assessed using a Mann Whitney U test. Values of $p \leq 0.05$ were considered significant.

Results

Platelet depletion efficiency and side effects

Platelet depletion was achieved using a monoclonal murine CD41 specific antibody which was injected intraperitoneally. Therefore, platelet depletion was confirmed in every single mouse the day after antibody administration using flow cytometry (supplemental figure 2). About 30% of the platelet-depleted mice died after a second i.p. injection of antibody or anesthesia due to increased susceptibility to peritoneal bleeding. All other mice which survived throughout the procedure were closely monitored but did not display any side effects as a result of platelet depletion.

The effect of platelet depletion on perfusion recovery

Mice were treated with the CD41 antibody the day before femoral artery ligation and again 3 days after operation to maintain platelet depletion throughout the 7 day period. Perfusion of the hind limb was assessed using Laser Doppler before ligation and directly, 4 days and 7 days after ligation. Normal non-depleted mice display an increase in perfusion recovery throughout the 7 day period (figure 1A). Platelet depletion significantly decreased perfusion recovery 7 days after ligation (depleted vs. control, n= 13 vs. 17, 64% ±8 vs. 93% ±5, p=0.005), while there was no difference in perfusion recovery 4 days after ligation (depleted vs. control, n=13 vs. 17, 68% ±5 vs. 66% ±5, p =0.558) (figure 1A).
To assess the time point at which platelet depletion affects perfusion recovery, platelets were depleted either at day 0 or at day 4 (figure 1B). Platelet depletion at day 0 did not affect perfusion recovery on day 4 (day 0 depletion vs. control, n=13 vs. 17, 73% ±5 vs. 66% ±5, p=0.368) or day 7 (day 0 depletion vs. control, n=13 vs. 17, 96% ±4 vs. 93% ±5, p=0.983) when compared to untreated mice. Platelet depletion at day 4 resulted in a lower perfusion recovery on day 7 compared to control (day 4 depletion vs. control, n=13 vs. 17, 64% ±8 vs. 93% ±5, p=0.005).

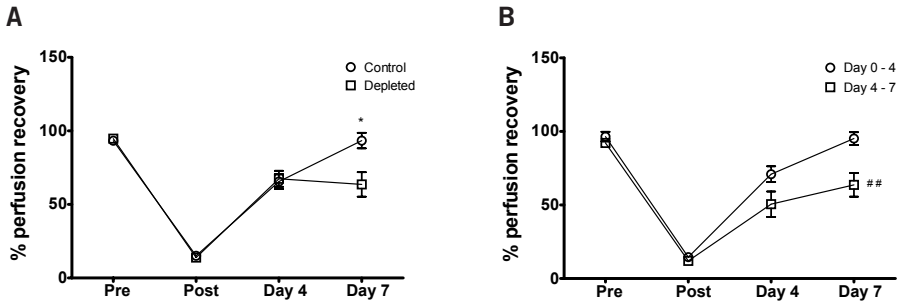

Figure 1 | The effect of platelet depletion on perfusion recovery
A) Perfusion recovery in control (circles, n=17) and platelet-depleted mice (squares, n=13). B) Perfusion recovery in mice depleted of platelets at day 0 (circles, n=13) or at day 4 (squares, n=13). Error bars indicate SEM, ** p=0.005 for control vs. depleted mice, # # p=0.005 for day 4 until day 7 platelet-depleted mice vs. control mice.

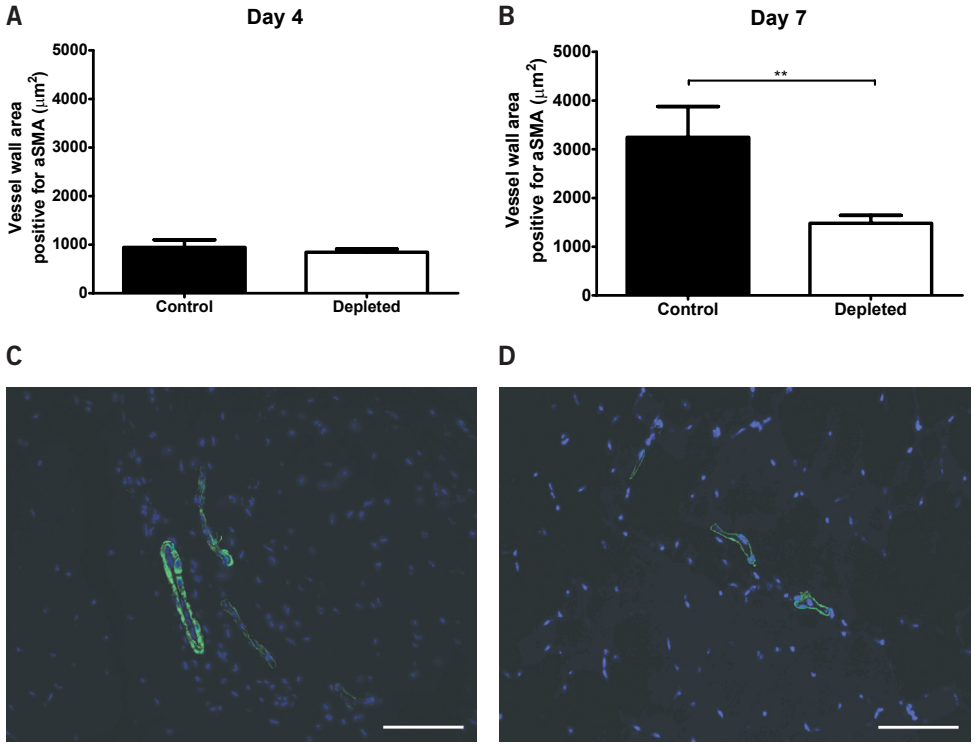

Figure 2 | αSMA positive area in the vessel wall after platelet depletion
αSMA positive area in the vessel wall was assessed both 4 (A) and 7 (B) days after ligation. C) Representative picture of an αSMA staining 7 days after ligation in a control mouse and D) a platelet-depleted mouse. Error bars indicate SEM, ** p=0.009. Scale bars indicate 100 μm.

Table 1 | Collateral vessel dimensions

	Control	Depleted	p value
Outer perimeter (µm)	251 ±28	171 ±13	0.048
Inner perimeter (µm)	188 ±25	106 ±11	0.025
Outer diameter (µm)	80 ±8	55 ±4	0.048
Inner diameter (µm)	60 ±8	34 ±3	0.025
Vessel lumen area (µm^2)	12394 ±2901	3806 ±777	0.025
Vessel wall thickness (µm)	10.1 ±0.6	10.5 ±0.7	0.608
Vessel wall area (µm^2)	2298 ±386	1478 ±185	0.142

Inner and outer perimeter of collateral vessels was measured in paraffin embedded sections of adductor muscle obtained 7 days after ligation. From the inner and outer perimeter measurements values for diameter and vessel area were derived to illustrate the increase in blood flow as a result of increased vessel perimeter.

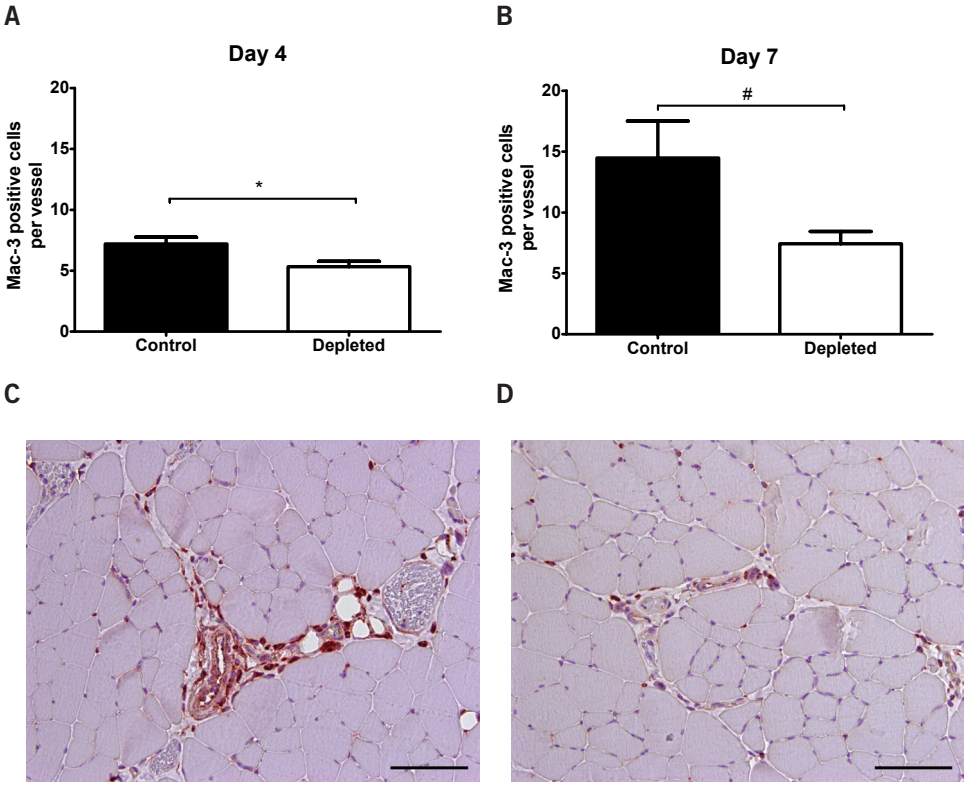

Figure 3 | Macrophage number after platelet depletion
Macrophage number around growing collaterals was assessed both 4 (A) and 7(B) days after ligation. C) Representative picture of a MAC-3 staining 7 days after ligation in a control mouse and D) a platelet-depleted mouse. Error bars indicate SEM, * p=0.018, # p=0.035. Scale bars indicate 100 µm.

The effect of platelet depletion on vessel wall remodeling and vessel size

As vessel wall remodeling is important in order to maintain an increase in vessel size, we assessed αSMA area in the vessel wall as a marker for smooth muscle cells (figure 2) in the adductor muscle. αSMA positive area in platelet-depleted mice did not differ 4 days after ligation when compared to control mice (depleted vs. control, n=8 vs. 10, 860 μm^2 ±74 vs. 945 μm^2 ±156, p=0.859). αSMA positive area was, however, significantly lower in platelet-depleted mice 7 days after ligation compared to control (depleted vs. control, n=7 vs. 7, 1480 μm^2 ±165 vs. 3247 μm^2 ±631, p=0.009) while vessel wall thickness or surface did not differ between groups (table 1).

To further validate these findings we assessed collateral vessel dimensions in the adductor muscle in both platelet-depleted and control mice 7 days after ligation. Vessel inner and outer perimeter were measured in sections of adductor muscle obtained and fixated during maximal vasodilation. Vessel inner perimeter was smaller in platelet-depleted mice compared to control mice (depleted vs. control, n= 7 vs. 7, 106 ±11 vs.

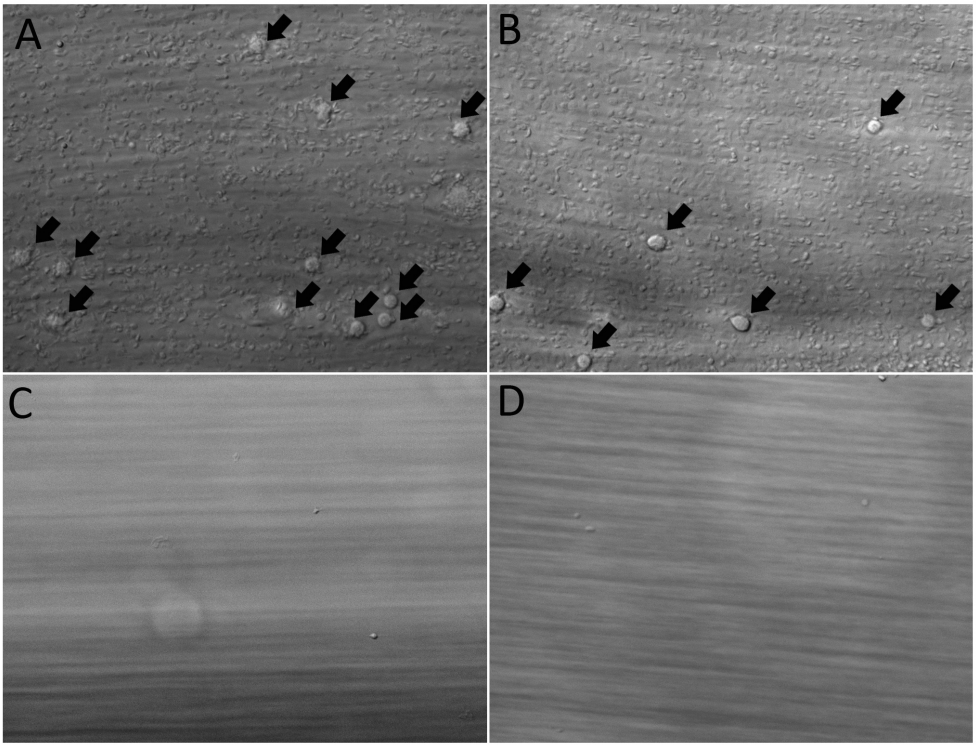

Figure 4 | Leukocyte rolling

The effect of platelet depletion on leukocyte rolling was assessed using a flow chamber assay (n= 4 vs. 4). Representative images are shown of control mice (A & B) and platelet-depleted mice (C & D). Arrows indicate rolling leukocytes.

188 ±25, p=0.025) as well as the outer perimeter (depleted vs. control, n= 7 vs. 7, 171 ±13 vs. 251 ±28, p=0.048). To illustrate the effect of these differences in vessel size on blood flow, we used the inner perimeter value to calculate both vessel diameter and lumen area (table 1). This shows a more then 40% decrease in inner diameter and a nearly 70% reduction in lumen area in platelet-depleted animals at 7 days after ligation.

Macrophage number after platelet depletion

As macrophages are important contributors to vessel remodeling and perfusion recovery, we assessed macrophage numbers around the growing collateral vessels (figure 3). Macrophage number was decreased in platelet-depleted mice compared to control mice 4 days after ligation (depleted vs. control, n=9 vs. 10, 5.2 ± 0.4 vs. 7.0 ±0.5, p=0.018). Although macrophage number increased in both groups towards day 7, the increase was much higher in the control group resulting in an even bigger difference in macrophage number on day 7 (depleted vs. control, n=7 vs. 7, 7.4 ±1.0 vs. 14.5 ±3.0, p=0.035). The increase in macrophage number from day 4 until day 7 was significant in control mice (day 4 vs. day 7, n=10 vs. 7, 7.0 ±0.5 vs. 14.5 ±3.0, p=0.013) while the increase in macrophage number in platelet-depleted mice was not significant (day 4 vs. day 7, 9 vs. 7, 5.2 ±0.4 vs. 7.4 ±1.0, p=0.101).

Leukocyte rolling

As leukocyte rolling and adhesion on the vessel wall are important steps prior to leukocyte extravasation, we investigated the effect of platelet depletion on leukocyte rolling in a flow chamber model (depleted vs. control, n= 4 vs. 4). Rolling leukocytes were observed for all of the blood samples from the control mice (Figures 4a and 4b). Leukocyte rolling was not observed at all in any of the blood samples taken from platelet-depleted mice (figures 4c and 4d). For each of the blood samples the number of rolling leukocytes was quantified by counting the number of cells which passed through the field of view of the microscope in 60 seconds confirming that platelet depletion abolishes leukocyte rolling (depleted vs. control, n=4 vs. 4, 0 ±0 cells/min vs. 20.5 ±6.8 cells/min, p=0.014) (table 2).

Table 2 | Quantification of leukocyte rolling

	Control (# cells/min)	Depleted (# cells/min)	p value
Sample 1	40	0	
Sample 2	18	0	
Sample 3	8	0	
Sample 4	16	0	
Mean	20.5 ±6.8	0 ±0	0.014

The number of rolling leukocytes was quantified by manually counting the number of cells which rolled by in a time-frame of one minute.

Discussion

Arteriogenesis is an important process for the restoration of tissue perfusion after arterial occlusion. In recent years it has become clear that perfusion recovery is an inflammatory process involving bone marrow derived cells which locally extravasate from the blood stream [4,12]. Platelets, which are also bone marrow derived, have now been attributed functions in immunity e.g. microbial infection, atherosclerosis, sepsis and rheumatoid arthritis[14]. Moreover, there is cumulating evidence that platelets play a role in atherogenesis[18]. However, their role in arteriogenesis has never been investigated. In this study, we investigate the role of platelets in perfusion recovery using a murine hind limb mouse model with and without platelet depletion. Platelet depletion resulted in reduced perfusion recovery and vessel maturation and was accompanied by a decrease in macrophage number around the growing collaterals.

Platelet depletion resulted in reduction in perfusion recovery at 7 days after arterial occlusion when compared to control mice. Perfusion recovery did not differ between control and platelet-depleted mice at 4 days after arterial occlusion suggesting that platelet depletion only exerts its effects in the later stages of perfusion recovery. Early platelet depletion (at day 0) and late platelet depletion (at day 4) showed that only late platelet depletion reduced perfusion recovery. In addition, vascular dimensions of the collateral vessels were smaller in platelet-depleted mice at 7 days after ligation. This observation strongly points to a role of platelets in the later stages of perfusion recovery. In contrast to the initial phase of perfusion recovery where blood flow is increased due to mere vasodilatation, vessel wall remodeling has to occur during the later stages to further increase vascular flow [1]. The resulting changes in vascular dimensions, such as diameter and lumen area, are of crucial importance for restoration of vascular flow as stated by Poiseuille's law in which flow increases with the 4th power over the radius of the vessel [19-21]. Vascular smooth muscle cells play an important role in vessel remodeling as they provide the structure and vessel plasticity that is required for larger conducting arteries [22-24]. In addition, previous studies have shown that the alpha smooth muscle actin positive area in the vessel wall is higher in mature collateral vessels when compared to growing collateral vessels [25]. Our findings show that the area positive for alpha smooth muscle actin in the vessel wall is reduced in platelet-depleted mice. Together with the decreased perfusion recovery during the later stages, this indicates that vessel wall remodeling is affected by platelet depletion.

In search of a mechanism responsible for this reduced perfusion recovery involving platelets, we focused on macrophages. Macrophages are key players in perfusion recovery[7] as they are a source for various factors such as basic Fibroblast Growth Factor, TNF-alpha and matrix metalloproteinase-12 which contribute to vessel remodeling by extracellular matrix degradation and the stimulation of smooth muscle cell

proliferation[26-28]. In addition, low macrophage numbers around growing collaterals are associated with poor perfusion recovery [29]. We have shown that platelet depletion after femoral artery ligation results in a reduced extravasation of macrophages both at day 4 and day 7 compared to control. Next to this, the normal increase in macrophage number from day 4 until day 7 is absent in platelet-depleted mice which is in line with the observed effect of partial platelet depletion during day 4 until day 7 on perfusion recovery. Although the number of macrophages differs between platelet-depleted and control mice at 4 days, the absolute difference in macrophage number on day 4 is rather small compared to the difference at day 7. For this, we conclude that extravasation of macrophages mostly occurs during the later stages of perfusion recovery when structural remodeling takes place [1]. Previous studies have shown a role for platelets in monocyte extravasation: either directly via platelet/monocyte interactions [30], via the deposition of chemokines by platelets on the activated endothelium [31,32] or via enhanced monocyte adhesiveness due to platelet binding [33]. Furthermore, a recent study using a murine myocardial infarction model has also shown that platelet depletion resulted in decreased leukocyte (CD45+) extravasation after myocardial infarction supporting our results regarding the decreased extravasation of macrophages [15]. To further corroborate these findings, we found similar results regarding platelet-monocyte complexes in the blood stream which are in line with the murine myocardial infarction model [15]: platelet-monocyte complexes were more frequently observed than platelet complexes with other leukocytes and the number of platelet-leukocyte complexes was increased during the period of macrophage extravasation (Supplemental figure 3A). Finally, leukocyte rolling was abolished in blood from platelet-depleted mice, underlining the role of platelets in leukocyte extravasation. Our study, however, cannot exclude a more direct role for platelets, for example by the release of growth factor and/or cytokines.

The identified role for platelets in arteriogenesis could have consequences for the clinical treatment of patients suffering from cardiovascular disease since platelets are involved in both thrombus and plaque formation. Anti-platelet therapy is therefore common in cardiovascular patients to reduce the risk of thrombus formation [34], although these anti-platelet drugs might also affect perfusion recovery. The use of aspirin, for example, leads to decreased monocyte extravasation and reduced perfusion recovery after arterial occlusion [35]. However, the use of drugs more specifically targeting thrombus formation, such as clopidogrel, might circumvent this problem [35].

The beneficial effect of platelets on perfusion recovery might not be limited to mere recruitment of monocytes as many studies have shown that platelets can also modulate leukocyte gene expression and responsiveness prior to extravasation in the bloodstream[36,37]. The binding between monocytes and platelets, for example, has been known to activate NF-κB signaling in monocytes thereby directly affecting chemokine production and secretion [38,39]. Platelet derived chemokines can also induce inflammatory

responses in monocytes directly [40]. Moreover, Thrombosporin-1, which is present in platelet α-granules, has been shown to play a role in hind limb ischemia by modulating macrophage activation state [41]. Further research into these interactions and their results might yield specific pathways by which platelets affect leukocyte trafficking and behavior during collateral formation. The existence of specific pathways in platelets has already been shown for pro-angiogenic and anti-angiogenic factors which are stored in separate α-granules and are specifically released in response to different stimuli [42]. These insights might lead to new therapeutic targets which specifically enhance the positive role of platelets in perfusion recovery without stimulating the role of platelets in the development and progression of atherosclerosis [43,44].

In summary, the results of this study show for the first time that platelets contribute to perfusion recovery after arterial occlusion possibly via a role in monocyte extravasation and subsequent vascular remodeling. This newfound player in perfusion recovery might yield new therapeutic targets to stimulate arteriogenesis in the clinic.

Acknowledgements

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Supplemental

Platelets contribute to perfusion recovery during vessel remodeling

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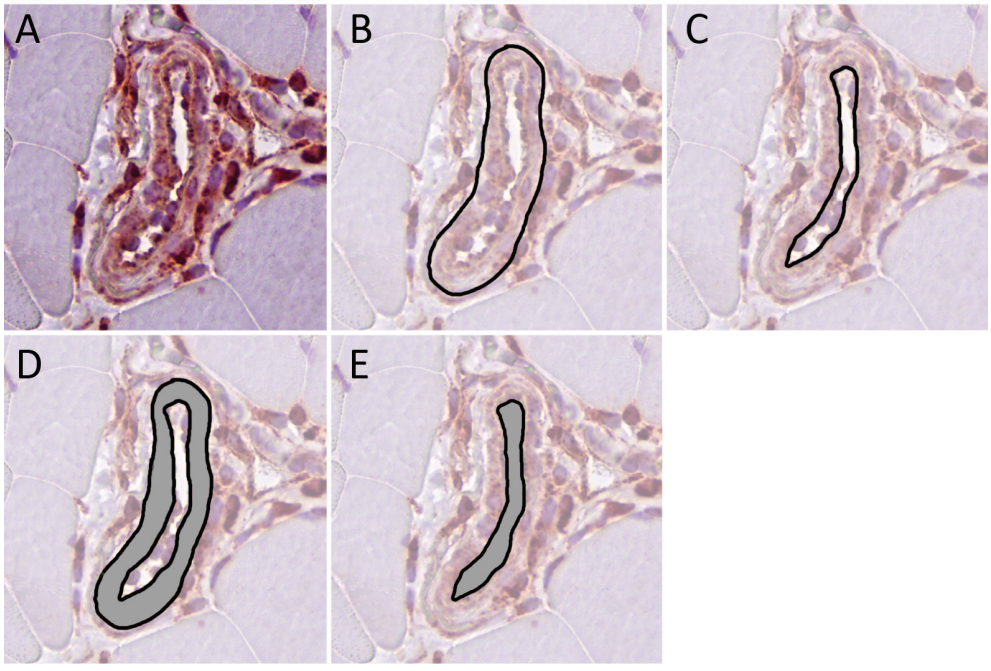
Graphic illustration of collateral vessel dimension measurements

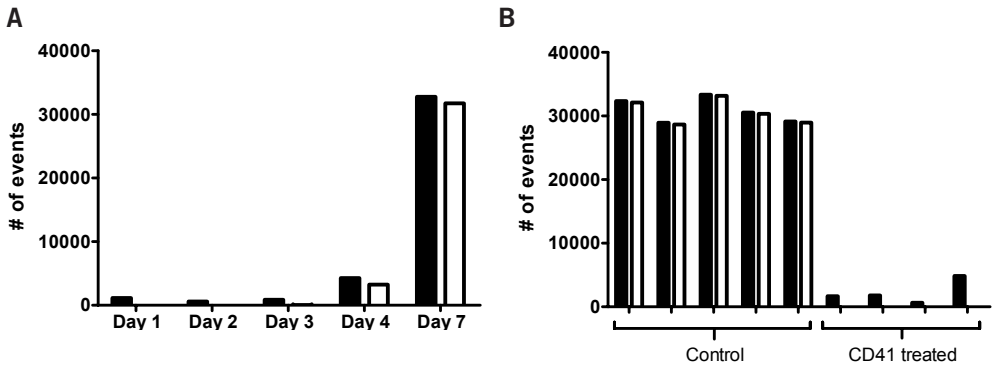
Supplemental figure 1 depicts a graphic illustration of the dimension measurements performed on collateral vessels. The inner and outer perimeter of collateral vessels was determined in HE stained sections of adductor muscle obtained 7 days after ligation. A) collateral vessel, B) Outer perimeter, C) Inner perimeter, D) Vessel wall area and E) Vessel lumen.



Supplemental figure 1 | Graphic illustration of collateral vessel dimension measurements
The inner and outer perimeter of collateral vessels was determined in HE stained sections of adductor muscle obtained 7 days after ligation. A) collateral vessel, B) Outer perimeter, C) Inner perimeter, D) Vessel wall area and E) Vessel lumen.

Platelet content after CD41 antibody administration in mice

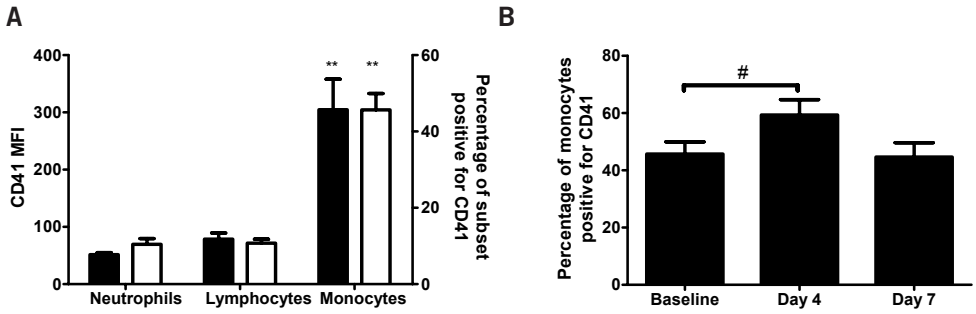
Treatment with the CD41 antibody resulted in a nearly complete depletion of circulating platelets the next day. This depletion lasted for approximately 4 days (supplemental figure 2A). When gating for platelet scatter, the number of events in platelet depleted mice decreased with 93% (CD41 antibody treated n=4 vs. control n=5). In CD41 antibody treated mice, CD41 positive events were absent, while in untreated mice (n=5) 99% of the events within the platelet scatter region were positive for CD41 (supplemental figure 2B).



Supplemental figure 2 | Platelet content after CD41 antibody administration in mice
Platelet number was determined by flow cytometry by gating for events with a forward/sideward scatter characteristic for platelets. Black bars represent all events within this gate. White bars represent CD41 positive events within the same gate. A) After administration of the CD41 antibody at day 0 platelets started to reappear at day 4 and were back at baseline levels at day 7. B) Example of platelet measurements in CD41 treated (n=4) and control mice (n=5) the day after antibody administration.

Interaction between leukocyte subsets and platelets

To identify leukocyte subsets which might interact with platelets in the blood stream we investigated the distribution of platelets on different subsets of peripheral blood mononuclear cells (PBMC) in untreated mice (n=7) without any further intervention. First CD41 expression on the different subsets of PBMC was analyzed (figure 4A, black bars). CD41 expression was found within all subsets, although expression within the monocyte subset was significantly higher when compared to both neutrophils and t-lymphocytes (monocytes vs. neutrophils, MFI 305 ±54 vs. MFI 51 ±4, p=0.001 and monocytes vs. t-lymphocytes, MFI 305 ±54 vs. MFI 78 ±11, p=0.001). These results were in line with the percentage of cells within a subset found to be positive for CD41 (Figure 3A, white bars) as the percentage of monocytes positive for CD41 was significantly higher when compared to both neutrophils and t-lymphocytes (monocytes vs. neutrophils, 46% ±4 vs. 10% ±2, p=0.001 and monocytes vs. t-lymphocytes, 46% ±4 vs. 1% ±1, p=0.001). The percentage of monocytes positive for CD41 increased 4 days after femoral artery ligation when compared to baseline (baseline vs. day 4, n= 7 vs. 9, 46% ±11 vs. 59% ±16, p=0.039).



Supplemental figure 3 | Leukocyte and platelet interaction
A) CD41 expression on subsets of PBMC, indicative for platelet-leukocytes complexes, was analyzed in untreated mice (n=7). Black bars represent the intensity of CD41 expression (MFI), white bars represent the percentage of positive events within a subset. Error bars indicate SEM, ** p=0.001 vs. both neutrophils and lymphocytes. B) Platelet-monocyte complexes in the bloodstream measured using flow cytometry. The number of platelet-monocyte complexes increases during macrophage extravasation. Error bars indicate SEM, # p=0.039.

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6

LEUKOCYTIC CXCL10 IS INVOLVED IN PERFUSION RECOVERY AFTER ARTERIAL OCCLUSION

In preparation

Abstract

Objective

Perfusion recovery after arterial occlusion is crucial for tissue restoration. The TLR-pathway in circulating leukocytes plays a crucial role in this process. CXCL10 is produced by monocytes after TLR activation. This study aims to identify the involvement of CXCL10 in perfusion recovery.

Methods

The role of CXCL10 in perfusion recovery was determined by performing femoral artery ligation in CXCL10 -/- mice. Temporal expression of CXCL10 mRNA in leukocytes was analyzed using Q-PCR. The role of CXCL10 in bone marrow derived cells was assessed using chimeric mice.

Results

After femoral artery occlusion CXCL10 mRNA expression in leukocytes decreased at day 1, 2 4 and 7 days after occlusion, but CXCL10 mRNA levels were back at baseline levels at three days after ligation. CXCL10 -/- mice showed decreased perfusion recovery when compared to WT mice as early as 4 days after ligation (control vs. KO, 81% ±13 vs. 28% ±5, p=0.011). This difference remained visible throughout the observed period up until day 7(control vs. KO, 107% ±8 vs. 57% ±12, p=0.006). Perfusion recovery in chimeric mice was decreased when compared to WT mice (day 4: control vs. chimera, 81% ±13 vs. 44% ±4, p=0.046) (day 7: control vs. chimera, 107% ±8 vs. 61% ±5, p<0.001). These results are comparable to a complete knockout, indicating a role for CXCL10 during perfusion recovery in bone marrow derived cells.

Conclusion

Our results show that CXCL10 is down regulated in circulating leukocytes after femoral artery ligation. CXCL10 expression, in particular in circulating leukocytes, is crucial for perfusion recovery after arterial occlusion.

Introduction

In the modern western world, vascular occlusion and its resulting morbidity and mortality remain a major health problem. Enhancing perfusion recovery after vascular occlusion would therefore be beneficial for many patients. The human body is capable of perfusion restoration via a process of adaptive vascular growth which is named arteriogenesis[1]. However, this process is often to slow to prevent tissue damage. Arteriogenesis involves the remodeling of small pre-existing anestomoses towards larger conduit arteries. Circulating leukocytes, which locally extravasate from the bloodstream, have been shown to be important players in this process. Monocytes / macrophages for example have been studied in many pre-clinical and clinical studies[2-7]. Furthermore, increased attraction and activation of these cells has proven to stimulate perfusion recovery[6,8-11]. In addition, T-lymphocytes have also been attributed important roles in arteriogenesis[12-14], as well as natural killer cells[15]. CXCL10, also known as Interferon-Inducible Protein 10 (IP-10), belongs to the C-X-C chemokine super family and it is up regulated in response to Interferon-gamma(INF-γ)[16]. It is a chemoattractant for monocytes and T-lymphocytes[17] and signals through the CXCR-3 receptor[18]. CXCL10 is secreted by several cell types such as endothelial cells, monocytes, fibroblasts and keratinocytes in response to INF-γ [19] and INF-γ plasma levels increase after arterial occlusion[20]. We have previously shown that arterial occlusion results in an orchestrated systemic response of leukocyte trafficking which resulted in a change in leukocyte number in various subsets[20]. In addition, we have shown that signaling via the TLR-pathway in circulating leukocytes is crucial in perfusion recovery[21,22]. As a follow up to this study, we studied the mRNA expression of various components of the TLR-pathway in leukocytes at different time-points after arterial occlusion. Our results showed modulation of CXCL-10 mRNA levels in circulating cells after arterial occlusion. Involvement of CXCL-10 in perfusion recovery was confirmed using CXCL-10 knockout mice.

Materials and methods

Quantative RT-PCR

Leukocyte samples were obtained as part of another study which was published before[20]. Leukocytes were isolated by centrifugation at 300 G for 4 minutes and subsequent erythrocyte lysis. Tripure® reagent was added to the remaining leukocytes and total RNA was isolated following manufacturer's instructions. DNAse treatment was performed to remove any genomic DNA using the TURBO™ kit (Ambion Inc.) following manufacturer's instructions. Using the iScript cDNA synthesis kit (Biorad) cDNA was generated.
Clones were generated for P0, RPL27 (household genes) and CXCL10 by performing RT-

PCR (step 1: 2 minutes at 95 °C, step 2: 30 seconds at 94 °C and 45 seconds at a primer specific temperature (40 repeats), step 3: 1 minute at 65 °C). For P0 the following primers were used (forward: GGACCCGAGAAGACCTCCTT, reverse: GCACATCACTCAGAATTTCAATGG) and for RPL27 (forward: CGCCCTCCTTTCCTTTCTGC, reverse: GGTGCCATCGTCAATGTTCTTC). For CXCL10 the primer pair was ordered from SABiosciences (cat# PPM02978D). The resulting products were introduced into XL-1 competent cells and DNA was isolated using a maxi-prep kit (Qiagen, cat # 27361) following manufacturer's instructions. DNA concentration was measured using a Nanodrop N1000 (Nanodrop). Sequencing was performed to assess clone specificity. Quantitative PCR (QPCR) was performed on leukocyte cDNA for all three genes, clones were used to generate a standard curve. Subsequently, CXCL10 levels were normalized against both the P0 and RPL27 levels which resulted in a mean fold change over the household genes.

Animal procedures

The present study was approved by the university animal experimental committee following the Guide for the Care and Use of Laboratory Animals published by The US National Institute of Health (NIH Publication No. 85-23, revised 1996). C57Bl6J mice were ordered from Harlan, the Netherlands. Breeding pairs of the CXCL10 -/- mice were ordered from Jackson Laboratories (strain name: B6.129S4-Cxcl10^{tm1Adl}/J) and kept for breeding at our university. Twenty-five C57Bl6/J mice and nine CXCL10 -/- mice underwent permanent unilateral femoral artery ligation at the age of 10-12 weeks as described previously[23]. Fifteen C57Bl6/J mice had received bone marrow transplantation prior to operation (see below). Before the operation procedure mice were anesthetized with dormicum (5 mg/kg) and medetomidine (0.15 mg/kg) and analgesized with fentanyl (0.02 mg/kg) via an intra-peritoneal injection. After operation anesthesia was antagonized by a subcutaneous injection of anexate (1 mg/kg) and antisedan (5 mg/kg). After the operation mice received two injections of buprenorphine (0.15 mg/kg, 8 hour intervals). Paw perfusion was assessed using Laser Doppler (Moor instruments) before and after the operation procedure. In addition, paw perfusion was assessed 4 and 7 days after ligation but no fentanyl was used during these procedures. Perfusion recovery was calculated and expressed as percentage perfusion in the occluded hind limb vs. the contra lateral hind limb. Mice were terminated after the final perfusion measurement at day 7.

Bone marrow transplantation

Before bone marrow transplantation 15 C57Bl6/J mice were lethally irradiated at the age of 6 weeks with a dose of 700 cGray (1030 monitor units). Bone marrow was isolated from 4 age matched donor CXCL10 -/- mice by flushing the humeral and femoral bone with RPMI 1640 medium (Invitrogen). Each mouse was administered 5 million cells in

250 µl RPMI medium via a tail vein injection. After a recovery period of 6 weeks mice underwent femoral artery ligation as described above and blood was drawn via cheek puncture to check transplantation efficiency. Genomic DNA was isolated automatically using a GeneMole (Mole Genetics) and Mole strips (Mole Genetics, MGK20-100-102). QPCR was performed using primers specific for WT and CXCL10 -/- animals as provided by Jackson Laboratories. In all chimeras less than 15% of the DNA contained the WT gene for CXCL10.

Results

CXCL10 mRNA expression in leukocytes

The expression of CXCL10 mRNA in circulating leukocytes changed after arterial occlusion (figure 1). CXCL10 mRNA could not be detected during the first two days after femoral artery ligation, but levels returned 3 days after femoral artery ligation. CXCL10 mRNA could not be detected during day 4 and 7 after femoral artery ligation.

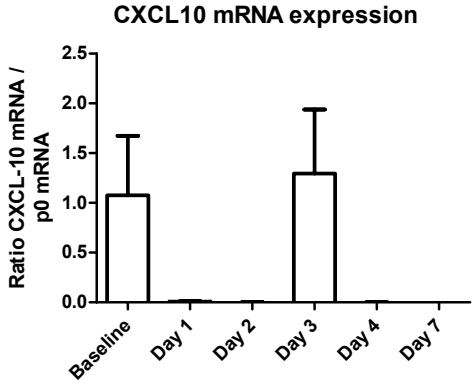

Figure 1 | CXCL10 mRNA expression in leukocytes after femoral artery ligation
CXCL10 mRNA expression was determined in circulating leukocytes after femoral artery ligation. CXCL10 mRNA expression was normalized against two household genes (P0 and RPL27). For each of the days the sample number was 10.

Loss of CXCL10 decreases perfusion recovery

CXCL10 -/- mice show decreased recovery when compared to WT mice. This results in a significant difference at day 4 after ligation (control vs. KO, n=10 vs. 9, 81% ±13 vs. 28% ±5, p=0.011). This difference remains throughout the observation period until day 7 (control vs. KO, n=10 vs. 8, 107% ±8 vs. 57% ±12, p=0.006) (figure 2). To assess whether the effect of CXCL10 is mediated via bone marrow derived cells we performed femoral artery ligation in chimeras which did not express CXCL10 in their bone marrow derived cells. Perfusion recovery was severely decreased in chimeric mice both at day 4 and day 7 when compared to control (day 4: control vs. chimera, n=10 vs. 15, 81% ±13 vs. 44%

±4, p=0.046) (day 7: control vs. chimera, n=10 vs. 15, 107% ±8 vs. 61% ±5, p<0.001) (figure 2). Perfusion recovery was increased on day 4 when compared to total CXCL10 -/- mice (chimera vs. KO, n=15 vs. 9, 44% ±4 vs. 28% ±5, p=0.025), this difference had disappeared on day 7 (chimera vs. KO, n=15 vs. 9, 61% ±5 vs. 57% ±12, p=0.771).

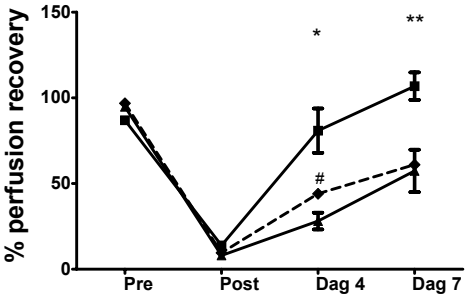

Figure 2 | Loss of CXCL10 decreases perfusion recovery
Perfusion recovery after arterial occlusion was determined in both wildtype (n=10, squares) and CXCL10 -/- mice (n=9, circles). Perfusion recovery was also determined in chimeras which lack the CXCL10 gene in their bone marrow derived cells (n=15, dotted line & triangles). Error bars indicate SEM, * p=0.011 (WT vs KO, day 4), # p=0.046 (WT vs. chimeras, day 4) and ** p<0.001 (WT vs. KO and WT vs. chimeras, day 7).

Discussion

In recent years it has become clear that circulating leukocytes are important in the process of perfusion recovery and the role of the different subsets of bone marrow derived cells has been studied extensively in the past decade[2,13-15]. A next step could be to identify keyproteins that are involved in the mechanisms which are responsible for the interaction between the different subsets. In this study we focused on the TLR-pathway as its activation in circulating leukocytes has shown to be crucial for perfusion recovery[21,22]. We found that mRNA expression for the chemokine CXCL-10 was decreased in circulating leukocytes after arterial occlusion. Its role in perfusion recovery was confirmed using CXCL10 -/- mice.

To identify factors important in perfusion recovery we performed a temporal analysis of the TLR-pathway in circulating leukocytes (data not shown). We found that expression of the mRNA coding for the chemokine CXCL10 was altered after arterial occlusion. To validate these pilot findings we performed Q-PCR on samples collected in an earlier study[20]. These results confirmed a decrease in CXCL10 mRNA expression in leukocytes after arterial occlusion. The decrease in CXCL10 mRNA expression upon arterial occlusion is extensive as CXCL10 mRNA could not be detected in circulating leukocytes on day 1, 2, 4 and 7 after arterial occlusion. At present we cannot explain the major increase in CXCL10 mRNA expression on day 3. However, it might be the result of an increase in monocyte number as it coincides with an increase in the number of circulating monocytes[20]. The apparent total absence of CXCL10 mRNA on days 1, 2, 4 and 7 after

arterial occlusion might be a result of the limitations of QPCR. Peaks in expression can readily be detected, however as CXCL10 mRNA expression is relatively low, a slight decrease can already result in an observed absence of CXCL10 mRNA.

Nevertheless, the absence of CXCL10 mRNA in leukocytes in the peripheral circulation is intriguing. A possible explanation might be that CXCL10 at baseline is only expressed within a subset of cells of which the majority extravasates after arterial occlusion. This could for example involve a subset of monocytes as the increase in CXCL10 mRNA on day 3 coincides with an increase in monocyte number in the peripheral circulation[20]. In addition, there is a possible inverse relationship between protein and mRNA. Expression of mRNA is usually up regulated after protein secretion. As CXCL10 secretion is prone to occur by extravasated cells an increase in CXCL10 mRNA expression will not be detected in the peripheral circulation.

To validate the role of CXCL10 in perfusion recovery we performed femoral artery ligation in CXCL10 -/- mice. We found that perfusion recovery was severely decreased in CXCL10 -/- mice as early as day 4. This difference in perfusion recovery remained visible throughout the observation period until day 7. To assess the role of CXCL10 expression in circulating leukocytes we performed bone marrow transplantation on WT mice which received CXCL10 -/- bone marrow cells. Perfusion recovery in chimeric mice did not differ from CXCL10 -/- mice, indicating that expression of CXCL10 in bone marrow derived cells is of crucial importance in perfusion recovery. Experiments with CXCL10 -/- mice receiving WT bone marrow should be performed to validate these findings.

This study does not reveal any insight into the mechanism how CXCL10 deficiency affects perfusion recovery. Results of previous studies give some clues on the possible mechanisms involved. First of all, the source of CXCL10 during arteriogenesis is of leukocyte origin given the results obtained with chimeric mice in this study. It has been shown that TLR4 activation results in the expression of CXCL10 in murine macrophages[24]. As TLR4 is highly expressed on circulating monocytes and not on T-lymphocytes we hypothesize that during arteriogenesis monocytes are a source for CXCL10[22]. The role for CXCL10 during arteriogenesis could be linked to the attraction of T-cells and/or NK-cells which have also been shown to be important during perfusion recovery[13-15]. CXCL10 is a chemoattractant for these cells and signals via the CXCR3 receptor[17,25,26]. Therefore, a possible role for CXCL10 could be to organize the attraction of lymphocytes and/or NK cells to the site of arteriogenesis. The importance of the CXCR3 receptor during perfusion recovery, which has other ligands such as CXCL9 and CXCL11, was shown in a hind limb model. CXCR3 -/- mice show decreased perfusion recovery and diminished attraction of T-cell which supports our hypothesis[27].

In summary, our study indicates that CXCL10 is involved in perfusion recovery possibly acting through bone marrow derived cells. Additional research on the role of CXCL10 is needed to elucidate the role of the different leukocyte subsets in CXCL10 signaling.

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7

SUMMARY AND DISCUSSION

The main topic of this thesis is arteriogenesis and the role circulating leukocytes play in this process of perfusion recovery. We have used clinically applicable inhibitors to increase leukocyte attraction and extravasation and discovered previously unknown factors which play a role in arteriogenesis.

In **chapter 2** we sought to further elucidate the systemic changes that occur in circulating leukocytes after arterial occlusion. The important role they play in arteriogenesis has been known for a while as well as the fact that peripheral monocyte count is related to the degree of perfusion recovery. However, there was no data available on the temporal patterns in leukocyte and bone marrow cell number after arterial occlusion. We have shown that the total number of leukocytes did not change after arterial occlusion. However, leukocyte number within the different subsets changed dramatically and the emerging temporal patterns differed per leukocyte subset. In addition, the observed changes could be linked to the corresponding progenitor cells in the bone marrow.

In **chapter 3** we used the clinically available CD26 inhibitor Sitaglipin to improve perfusion recovery in atherosclerosis prone ApoE -/- mice. Daily doses of the CD26 inhibitor improved both short term (7 days) and long term (56 days) perfusion recovery. This increase in perfusion recovery coincided with an increase in the number of macrophages around the growing collateral. As previous pro-arteriogenic factors also proved to increase atherosclerosis we assessed plaque stability in ApoE -/- mice during CD26 inhibition. Plaque stability and plaque size were unaffected by CD26 inhibition indicating that CD26 inhibition might be a feasible therapeutic strategy to improve perfusion recovery in the clinic. In addition, recent results have shown that CD26 inhibition deters the progression of atherosclerosis in murine models[1,2].

To improve our understanding of the role that CD26 plays in human plaques we assessed CD26 content in **chapter 4**. We show that a higher CD26 content is correlated to a more atheromatous plaque in contrast to a more fibrous plaque. In addition, a higher CD26 content is related to a high number of macrophages, a larger lipid core and less smooth muscle cells. Our data indicates that CD26 is involved in the progression towards a more vulnerable plaque phenotype. However, based on our studies we cannot draw any conclusions on the mechanisms involved.

In **chapter 5** we explored the role of platelets in perfusion recovery. Platelet depleted mice were generated using a platelet depleting antibody. Platelet depleted mice show decreased perfusion recovery as well as reduced arterial remodeling. Macrophage numbers around growing collaterals were decreased and *ex vivo* models showed that leukocyte rolling was absent in blood from platelet depleted mice. Our results indicate that platelets are involved in the process of monocyte extravasation which is crucial for perfusion recovery.

In **chapter 6** we assessed differences in mRNA expression between leukocyte samples obtained at different time points after perfusion recovery. We assessed mRNA expression

of CXCL10 which is a component of the TLR-pathway and found that its expression was down regulated. Using CXCL10 -/- mice we showed that mice lacking CXCL10 display a decrease in perfusion recovery when compared to WT mice. To elucidate the role of CXCL10 specifically in bone marrow derived cells we generated chimeric mice which lack CXCL10 in their bone marrow derived cells. These mice showed a similar decrease in perfusion recovery indicating that the expression of CXCL10 in leukocytes is of importance during perfusion recovery.

Interpretation of this thesis

Almost all Carotid Artery Disease (CAD) or Peripheral Artery Disease (PAD) patients suffer in some degree from arterial occlusion leading to decreased perfusion in the distal tissue. Currently, most occlusions are removed via a percutaneous intervention which carries a risk for complications. Therefore, researchers have searched for other methods to improve perfusion recovery without the need for an intervention. Most of the research towards such a method has focused on the growth of collateral arteries and the mechanisms involved. The research in this thesis provides new insights into the mechanisms involved and might provide new strategies to improve arteriogenesis in the clinic.

The crucial role of leukocytes during arteriogenesis has been shown in various previous studies. Research described in chapter 2 of this thesis on the systemic changes in leukocyte subsets provides a new temporal dimension to the role these cells play during arteriogenesis. In addition, the broad systemic effect of arterial occlusion on both peripheral and bone marrow cells indicates that there might be additional systemic processes involved which are currently unknown. For example, studies on wound healing have shown that granulocytes, after local extravasation, return to the blood stream to deliver information to the bone marrow [3]. Recent studies on myocardial infarction have discovered a role for a previously unknown reservoir of monocytes in the spleen which were involved in the process of regeneration [4]. Given the important role of leukocytes in arteriogenesis it might be worthwhile to investigate the possible role of these mechanisms in arteriogenesis. In both cases signaling events would have to travel through the blood stream which would present a window for a therapeutic strategy. However, to be successful these therapeutic targets will need to have an unique role in arteriogenesis. Should such a target also stimulate the progression of atherosclerosis, systemic treatment would inevitably result in the progression of atherosclerosis resulting in an aggravation of the underlying disease.

Our studies on the peptidase CD26 are an example of just such a target that has different functions in arteriogenesis and atherosclerosis. Our studies have shown that the use of a CD26 inhibitor in atherosclerosis prone ApoE -/- mice increases perfusion recovery without detrimental effects on atherosclerosis. This work was recently supported by

findings in two studies which studied the effect of a CD26 inhibitor on the development of atherosclerosis [1,2]. These studies both showed that CD26 had no detrimental effect on plaque progression and in some mice would even decrease plaque progression. To provide more insight into the role of CD26 in human plaque progression we measured CD26 content in 180 plaques of the Athero Express database. We found that a high CD26 plaque content was related to a more vulnerable plaque phenotype which is in line with the murine studies on the role of CD26 inhibition. A possible explanation for the differential effects on arteriogenesis and atherosclerosis could involve the different roles of the chemokine SDF-1 alpha which is rapidly broken down by the peptidase CD26 [5]. In our hind limb model we showed that CD26 inhibition resulted in an increase in SDF-1 alpha plasma levels, an increase in monocyte activation and extravasation and improved perfusion recovery. Although SDF-1 alpha plays a role in plaque formation a loss of SDF-1 signaling in later stages of atherosclerosis is detrimental [6]. Additional research on the effect of CD26 inhibition on SDF-1 alpha signaling in later stages of atherosclerosis might well provide a partial answer for the different results of CD26 inhibition in arteriogenesis and atherosclerosis.

Research described in this thesis has also identified previously unknown factors involved in arteriogenesis. During the past decade many studies have shown that platelets are involved in many more processes than mere coagulation. They are fully loaded with several adhesion molecules and various cytokines and chemokines which can be displayed or released upon platelet activation[7]. In particular their role in monocyte extravasation identified them as possibly involved in arteriogenesis[8]. We have generated platelet depleted mice using a platelet targeting antibody and showed that these mice suffer from decreased perfusion recovery after arterial occlusion. As a follow up to our study additional levels of platelet involvement in arteriogenesis could be the subject of investigation. As platelets adhere and stick to extravasating monocytes they could potentially be involved in the release of the various cytokines and/or chemokines in the peri-vascular space. They could furthermore play a role in the systemic changes we observed in chapter 2. Activated platelets are known to alter gene expression in leukocytes and they could thus influence leukocyte response after arterial occlusion[9,10]. Additional research is needed to identify these potential mechanisms and the molecules involved.

Research described in this thesis has further identified a role for the chemokine CXCL10 in arteriogenesis. Leukocytic CXCL10 was found to be of crucial importance in perfusion recovery using chimeric mice which was in line with a previous study on the role of its receptor CXCR-3[11]. Previous research has also shown that CXCL10 is involved in the attraction of T-lymphocytes [12]. CXCL10 might thus be involved in the attraction of T-lymphocytes to the site of arteriogenesis. In addition, CXCL10 might facilitate communication between the different leukocyte subsets during arteriogenesis. Further

insight into these mechanisms is crucial to improve our understanding of arteriogenesis and to find key differences with processes such as atherosclerosis.

Future perspectives

In the past decades many studies have aimed to translate the results from animal studies into clinically applicable therapies. Many of these failed to improve perfusion recovery due to various reasons. First of all, the animal models used do not include many of the co-morbidities observed in the clinic. Patients in need of perfusion recovery often also suffer from other diseases, for example atherosclerosis or diabetes, which can potentially influence many of the mechanisms involved in arteriogenesis. Future research will have to take this into account and include these co-morbidities in the animal model.

In addition, animal models are often based on instant total occlusion of the major artery which is not in line with the circumstances under which collateral arteries develop naturally. On top of that, clinically applied therapeutic stimulation of collateral artery growth after instant total occlusion is improbable. Time is required for the collateral artery to grow but that is something the patient does not have when there is an acute demand for perfusion recovery. Therefore, clinical application of therapeutic arteriogenesis is more likely to occur over time when gradual occlusion of the artery is observed and animal models for such a process are lacking. Recently an animal model of gradual occlusion has been developed in the pig[13] but such a model is costly and requires a higher degree of expertise when compared to the hind limb model in mice.

In short, arteriogenesis remains a promising strategy to improve perfusion recovery in CAD or PAD patients. However, more insight is needed into the mechanisms involved to develop specific therapeutic strategies which are feasible and safe in CAD or PAD patients.

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8

NEDERLANDSE SAMENVATTING

DANKWOORD

CURRICULUM VITAE

LIST OF PUBLICATIONS

Nederlandse samenvatting

Eén van de kenmerken van een goede wetenschapper is dat hij/zij in staat is de essentie van zijn/haar onderzoek ook aan het brede publiek uit te leggen. Deze Nederlandse samenvatting is dan ook nadrukkelijk vanuit deze gedachte geschreven. Ik wil collega onderzoekers om begrip vragen wanneer ik in deze tekst zaken wat simpel voorstel of wat kort door een bocht ga omwille van de leesbaarheid. Ik hoop dat de overige lezers met deze samenvatting een idee krijgen van de kern van mijn onderzoek en de kleine puzzelstukjes die nodig zijn om het geheel te kunnen overzien. Investeren in wetenschappelijk onderzoek is en blijft van groot belang voor onze maatschappij, maar het is naar mijn mening de plicht van de onderzoeker om de verkregen kennis ook voor het brede publiek beschikbaar te maken, zodat ook zij wetenschappelijk onderzoek op waarde kunnen schatten.

Verstopte bloedvaten (atherosclerose) zijn een groeiend probleem in de Westerse Wereld. Zij zijn vaak de onderliggende oorzaak van bekende en vaak dodelijke fenomenen zoals een hartinfarct of beroerte. Dit proefschrift beschrijft mijn onderzoek naar de vaatgroei die optreedt wanneer een bloedvat verstopt raakt. Dit groeiproces wordt ook wel arteriogenese of collateraal vorming genoemd. Het kan in principe de bloedtoevoer voldoende herstellen en op deze manier het verstopte bloedvat compleet vervangen. Helaas is deze groei van nature niet snel genoeg waardoor dit proces op het moment nog weinig mensen kan helpen. Verder onderzoek is dus noodzakelijk om dit proces beter te leren begrijpen en waar mogelijk te versnellen om zo in de toekomst mensen te kunnen helpen.

Om het onderzoek in dit proefschrift te kunnen begrijpen is het van belang om te weten wat arteriogenese inhoudt. Omdat de bouw en groei van een bloedvat nu niet echt tot de algemene kennis behoort wil ik gebruik maken van een wat bekender fenomeen: de snelweg A1. Deze grote snelweg tussen Oost en West Nederland vervoert dagelijks grote hoeveelheden auto's net zoals een bloedvat dagelijks grote hoeveelheden bloed vervoert. Omdat de A1 door de Veluwe loopt zijn er een aantal ecoducten over de weg gebouwd. Als één van deze ecoducten in zou storten ontstaat er een groot verkeersinfarct en komt geen van de automobilisten nog op zijn bestemming via de A1. Natuurlijk kunnen de auto's omrijden via allerlei N-wegen, maar zij komen dan door file vorming wel veel later op hun bestemming aan of zelfs helemaal niet. Dit probleem kan ook niet snel worden opgelost. Het opruimen van een ingestort ecoduct kost tijd evenals het verbreden van een N-weg. De doorstroom van auto's is dus langere tijd verminderd. Precies hetzelfde proces vindt plaats in het lichaam bij het verstoppen van een bloedvat. Het bloed met daarin de benodigde zuurstof kan nog wel op zijn bestemming komen via de kleinere vaten, maar het is veel minder dan daarvoor en dus vaak onvoldoende. Daarnaast is ook deze verminderde doorstroom van lange duur.

Stel je nu echter eens voor dat de overheid weet dat het ecoduct gaat instorten, of ze

hebben zelfs het besluit genomen om de A1 te sluiten en al het verkeer om de Veluwe heen te leiden. In dat geval is er voldoende tijd om maatregelen te nemen. De verkeersstroom op de A1 kan dan langzaam worden afgebouwd terwijl men op een andere plek de N-wegen vergroot. Hetzelfde proces kan plaatsvinden in het lichaam als het afsluiten van het bloedvat maar langzaam genoeg gaat. Op dat moment kan een kleiner bloedvat voldoende groeien om de volledige bloedstroom op te vangen net zoals een verbouwde N-weg de volledige verkeersstroom over kan nemen. De groei van een klein bloedvat onder deze omstandigheden wordt arteriogenese genoemd en is dus het proces waar ik de afgelopen jaren onderzoek aan heb gedaan.
Arteriogenese treedt alleen op wanneer je een bloedvat in het lichaam afsluit en die omstandigheden zijn helaas nog niet in een laboratorium te creëren. Daarom had ik voor veel van mijn onderzoek muizen nodig waarin ik door middel van een kleine operatie een bloedvat in de achterpoot afsloot. Vervolgens bepaalde ik hoe snel en goed de bloedtoevoer zich herstelde en wat het effect van verschillende behandelingen hierop was. Ook heb ik in enkele hoofdstukken gekeken naar de rol van verschillende bloedcellen in het herstel. Door meer over hun rol te leren hopen we in de toekomst medicijnen te ontwikkelen die arteriogenese versnellen of verbeteren.

Eerder onderzoek heeft aangetoond dat witte bloedcellen belangrijk zijn voor arteriogenese en dat deze cellen zich ophopen rondom de groeiende vaten. Er bestaan verschillende typen witte bloedcellen, waaronder monocyten, lymphocyten en granulocyten. Het aantal monocyten in het bloed heeft een directe relatie met het herstel van de bloedtoevoer: hoe meer cellen, hoe beter het herstel. In hoofdstuk 2 hebben we de kennis over deze cellen verder uitgebreid door te onderzoeken wat er met de aantallen van deze cellen in het bloed gebeurd na de start van arteriogenese. Daarnaast hebben we gekeken of arteriogenese ook effect heeft op de bloedstamcellen welke zorgen voor de aanmaak van witte bloedcellen. Uit onze resultaten bleek dat het aantal cellen van de verschillende typen witte bloedcellen sterk wisselde tijdens arteriogenese en dat deze wisselingen sterk verschilden per celtype. Daarnaast toonden we aan dat een toename van het aantal cellen vooraf werd gegaan door een toename van het aantal stamcellen. Deze resultaten geven aan dat er tijdens arteriogenese communicatie plaatsvindt met de bloedstamcellen. Wellicht dat er in de toekomst ingegrepen kan worden in deze communicatie om het arteriogenese proces te versnellen of te verbeteren.
In hoofdstuk 3 onderzochten we een andere methode om de positieve rol van witte bloedcellen te stimuleren. Witte bloedcellen worden naar het groeiende vat aangetrokken door specifieke signalen. Echter, deze signalen worden ook door het lichaam weer afgebroken om te zorgen dat de groei stopt als het vat klaar is. Eén van de enzymen die daar een belangrijke rol in speelt is CD26. In hoofdstuk 3 hebben we de werking van CD26 geremd met behulp van medicatie om te testen of dit het herstel zou bevorderen

of versnellen. Onze resultaten lieten zien dat de medicatie zorgde dat er meer witte bloedcellen ter plaatse kwamen en dat het herstel verbeterde. Helaas heeft dergelijke medicatie vaak een negatief effect op de onderliggende ziekte atherosclerose, met meer verstopte bloedvaten tot gevolg, waardoor je weer terug bij af bent. Daarom hebben we deze medicatie ook getest in muizen welke van nature atherosclerose ontwikkelen. Daaruit bleek dat er geen negatieve effecten op atherosclerose vorming zichtbaar waren. Vervolgonderzoek bij mensen die deze medicatie gebruiken zal moeten uitwijzen of er ook in mensen een positief effect zichtbaar is op het herstel van de bloedstroom.
In hoofdstuk 4 onderzochten we de rol van hetzelfde enzym (CD26) in atherosclerose. Tijdens atherosclerose worden er plaques gevormd, dit zijn verdikkingen van de vaatwand welke verantwoordelijk zijn voor de eigenlijke afsluiting van het bloedvat. Dit kan simpelweg door groei van de plaque, maar meestal is een plaque instabiel waardoor hij kan scheuren, het bloed stolt en de bloedprop het vat afsluit. Om meer te weten te komen over de rol van CD26 bepaalden we het gehalte CD26 in plaques afkomstig van mensen met atherosclerose. Deze resultaten legden we naast andere metingen welke aangeven hoe 'stabiel' een plaque is en dus hoe gemakkelijk deze scheurt. Hieruit bleek dat een instabielere plaque een hoger gehalte CD26 bevat. Dit zou erop kunnen wijzen dat CD26 betrokken is bij het proces dat een plaque instabieler maakt. Vervolgonderzoek zal uit moeten wijzen of dit inderdaad het geval is en of de remming van CD26 bijdraagt aan een meer stabiele plaque.
In hoofdstuk 5 onderzochten we de mogelijke rol van bloedplaatjes in arteriogenese, zij zijn onder andere betrokken bij de bloedstolling. Minder bekend is hun rol in het uittreden van witte bloedcellen uit het bloedvat. Dit uittreden van witte bloedcellen is het proces waarmee deze cellen hun uiteindelijke bestemming, de vaatwand, bereiken. Omdat eerder onderzoek heeft uitgewezen dat het aantal witte bloedcellen in de vaatwand bepalend is voor het perfusieherstel vroegen wij ons af of bloedplaatjes wellicht ook een rol spelen in arteriogenese. Om dit te onderzoeken dienden we muizen een antilichaam toe dat er voor zorgde dat alle bloedplaatjes worden afgebroken. Wanneer we vervolgens een bloedvat afsloten bleek dat muizen zonder bloedplaatjes hier minder goed van herstelden dan de controle muizen. In lijn met onze verwachtingen was daarnaast het aantal witte bloedcellen in de vaatwand lager in muizen zonder plaatjes vergeleken met controle dieren. Verder werd duidelijk uit de perfusieherstel meting en andere bepalingen aan de vaatwand dat de vaten van de dieren zonder plaatjes zich minder goed aanpasten aan de nieuwe omstandigheden. De resultaten uit dit hoofdstuk geven aan dat bloedplaatjes een tot nu toe onbekende rol in arteriogenese spelen. Deze resultaten zijn van belang voor de behandeling van patiënten met atherosclerose. Bloedplaatjes worden namelijk juist geremd bij deze patiënten omdat zij vaak een rol spelen in de uiteindelijke verstopping van het bloedvat. Verder onderzoek naar de rol van bloedplaatjes in beide processen is noodzakelijk om

de positieve rol van bloedplaatjes in arteriogenese selectief te kunnen stimuleren.
In hoofdstuk 6 staan de eerste resultaten van ons onderzoek naar de rol van CXCL10 in arteriogenese. CXCL10 is een zogenaamde cytokine en kan verschillende typen cellen aantrekken en hun gedrag (gedeeltelijk) sturen. Om de rol van CXCL10 te onderzoeken maakten we gebruik van zogenaamde 'knock-out' muizen. In deze muizen is een stukje van de genetische code, het DNA, verwijderd zodat deze muizen geen CXCL10 meer kunnen aanmaken. Vervolgens testten we weer het herstel van de bloedstroom nadat we een bloedvat hadden afgesloten. De resultaten lieten zien dat muizen zonder CXCL10 minder goed herstelden in vergelijking met controle muizen. Om te bepalen of CXCL10 een rol speelt in de lokale cellen van de vaatwand of de circulerende cellen in het bloed vervingen we de bloedstamcellen van normale controle dieren met die van een muis zonder CXCL10. Na enkele weken wachten is dan het resultaat een muis met gewone lokale cellen, maar met circulerende cellen die geen CXCL10 kunnen maken. Ook in deze muizen bepaalden we het herstel van de bloedstroom nadat we een bloedvat hadden afgesloten. Het resultaat was vergelijkbaar met dat van een muis die CXCL10 in al zijn cellen mist. Hieruit blijkt dat CXCL10 in circulerende cellen een belangrijke rol speelt in arteriogenese. Op het moment wordt verder onderzoek gedaan aan CXCL10 om meer te weten te komen over de verdere mechanismen die een rol spelen in dit proces.

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Curriculum Vitae

René Thomas Haverslag werd op 6 juli 1983 geboren te Deventer. Hij behaalde zijn VWO diploma in 2001 aan de Christelijke Scholengemeenschap 'Revius' in Deventer. In september 2001 begon hij aan de doctoraal opleiding Biomedische wetenschappen aan de Universiteit Utrecht en behaalde zijn propedeuse het jaar daarop. Begin 2003 besloot hij een jaar te stoppen met zijn opleiding om zitting te nemen in het bestuur van de studievereniging M.B.V. Mebiose waarin hij de functie van vice ab-Actis vervulde. Na een stage in 2006 bij het onderzoekslaboratorium Orthopedie en een tweede stage in 2007 bij de groep van prof.dr. Mercola aan het Burnham Institute in San Diego behaalde hij in december 2007 zijn master diploma Biomedical Sciences. Hij vervolgde zijn wetenschappelijke carrière in april 2008 bij het laboratorium voor experimentele cardiologie aan het UMC Utrecht waar hij onder begeleiding van dr. Höfer aan zijn promotieonderzoek begon. Het praktisch gedeelte van zijn promotieonderzoek werd afgerond in april 2011 waarna hij als plaatsvervangend beleidsmedewerker bij de opleidingen Biomedische wetenschappen en Biomedical sciences aan de Universiteit Utrecht werkte. Sinds februari 2012 is René werkzaam als beleidsadviseur bij de Vereniging van Universiteiten (VSNU) in Den Haag.

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