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Exploring immune cell functions and ways to make use of them

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Abstract

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In addition to host defense, alternative functions of immune cells are emerging. Immune cells are crucial during healing of injured tissue, in formation of new blood vessels, angiogenesis, and also in maintaining the balance in inflammation having immune regulating functions. Over the last decade a higher degree of heterogeneity and plasticity of immune cells have been reported and immune cells develop different characteristics in different situations *in vivo*.

This thesis investigates roles for immune cells in situations of muscle hypoxia and reduced blood perfusion, wound healing in skin and at sites of transplantation of allogeneic islets of Langerhans and on top of this, ways to steer immune cell function for future therapeutic purposes. A specific neutrophil subset ($CD49d^{+}VEGFR1^{+}CXCR4^{high}$) was found to be recruited to VEGF-A released at hypoxia and these neutrophils were crucial for functional angiogenesis. In muscle with restricted blood flow macrophages were detected in perivascular positions and started to express aSMA and PDGFR1b and were found to directly assist in blood flow regulation by iNOS-dependent NO production. This essential function in muscle regain of function could be boosted by plasmid overexpression of CXCL12 where the effect of these macrophages chaperoning the vasculature was amplified improving limb blood flow regulation. The effect on macrophages accelerating tissue regeneration being amplified by CXCL12 was tested in a model of cutaneous wound healing where the administration of CXCL12 was optimized for high bioavailability. In the skin, CXCL12-treatment induced accumulation of TGFb-expressing macrophages close to the wound driving the healing process, and subsequently the wounds healed with an efficiency never reported before. In the last study means to circumvent systemic immune suppressive therapy required in allogeneic transplantation was investigated. Allogeneic islets of Langerhans transplanted to muscle were immediately destroyed by the host immune system. Co-transplanting islets and CCL22-encoding plasmids we could curb this fast rejection for 10 days by accumulating $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T lymphocytes at the site for transplantation preventing islet grafts from being attacked by the host cytotoxic T lymphocytes.

In summary this thesis outlines distinct immune cell subsets being essential for regain of tissue function in hypoxia, ischemia and post injury and ways to amplify specific immune cell functions in these situations that are feasible for clinical use.

Keywords: leukocytes, neutrophils, macrophages, regulatory T cells, chemokines, VEGF-A, hypoxia, ischemia, muscle, Islets of Langerhans, diabetes, transplantation, wound healing

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Massena S*, Christoffersson G*, **Vågesjö E***, Seignez C, Gustafsson K, Binet F, Herrera Hidalgo C, Giraud A, Lomei J, Weström S, Shibuya M, Claesson-Welsh L, Gerwins P, Welsh M, Kreuger J, Phillipson M. ***equal contribution**. Identification and characterization of VEGF-A-responsive neutrophils expressing CD49d, VEGFR1, and CXCR4 in mice and humans. *Blood*, 2015 Oct 22;126(17):2016-26.

II. **Vågesjö E**, Seignez C, Christoffersson G, Korsgren O, Eriksson U-J, Essand M, Holm L, Phillipson M. Uncovering a new role for immune cells: macrophages assist in regulation of blood flow in ischemic muscle. *Manuscript*.

III. **Vågesjö E**, Mortier A, Huss F, Proost P, Roos S, Phillipson M. Accelerated skin wound healing by CXCL12 1a delivered on site by lactic acid bacteria. *Manuscript*.

IV. **Vågesjö E**, Christoffersson G, Waldén TB, Carlsson PO, Essand M, Korsgren O, Phillipson M. Immunological shielding by induced recruitment of regulatory T-lymphocytes delays rejection of islets transplanted in muscle. *Cell Transplant,* 2015;24(2):263-76.

Contents

Abbreviations

ANOVA	One way analysis of variance
aSMA	Alpha smooth muscle actin
CD	Cluster of differentiation
ECM	Extra cellular matrix
eNOS	Endothelial nitric oxide synthase
FoxP3	Forkhead Box P3
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HIF	Hypoxia inducible factor
HMGB1	High-mobility group box 1 protein
iNOS	Inducible nitric oxide synthase
LB	*Lactobacillus reuteri*
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
MMR	Macrophage mannose receptor
MQ	Macrophages
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PFU	Perfusion units
SEM	Standard error of the mean
TCR	T cell receptor
TGF	Transforming growth factor
Treg	Regulatory T lymphocyte
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild type

Introduction

The immune system comprises together with skin and mucosa the security system of the body and is crucial for maintaining the host healthy and resistant to pathogens. It consists of different cells (leukocytes) and humoral components that communicate and collaborate through multiple signaling routes. Upon activation, the immune system can quickly amplify signals and mobilize forces but it also possesses conduits to develop specific memory to antigens (*1*). Leukocytes of the innate immune system directly kill and phagocytose pathogens or infected cells whereas leukocytes of the adaptive system also utilizes opsonizing antibodies and antigen specific receptors to detect and facilitate killing of e.g. infected cells (*2*). In addition to host defense, alternative functions of leukocytes in tissue remodeling have been described and Professor Ilya Mechnikov was rewarded the Nobel Prize in 1908 for the finding that phagocytes, depending on the setting ingested bacteria or the tail of the tadpole through the same fundamental process (*3*).

The work presented in this thesis deliniates new roles for immune cells in situations of muscle hypoxia and reduced blood perfusion, wound healing in skin and at sites of transplantation with allogeneic islets of Langerhans. In addition strategies to steer immune cell function for future therapeutic purposes are investigated.

Immune cells

Leukocytes, originate from hematopoietic stem cells residing in bone marrow before being released to the circulation. Granulocytes and monocytes are derived from the myeloid linage and belong to the innate system whereas cells derived from the lymphoid linage, lymphocytes, are further educated in the secondary lymphoid organs and have the capacity of clonal selection and memory formation (*1*). Tissue resident immune cells can be replenished by circulating monocytes or renewed through in situ proliferation (*4*).

Neutrophil granulocytes are the most abundant leukocyte in the circulation of humans (5×10^6 / ml blood) and up to 2×10^{11} neutrophils are released from bone marrow per day with a lifespan of 5.4 days (*5-7*). Except for being the major population acutely recruited to bacterial infections (*8*), neutrophils were recently demonstrated to be involved in tissue remodeling by directly influencing the microenvironment and delivering enzymes releasing

growth factors bound to the extra cellular matrix (ECM) (*9-12*). Neutrophils are a heterogeneous population with distinct phenotypes in regards to surface receptors, adhesion molecules, enzyme content, efficiency in activating other leukocytes and production of reactive oxygen species (ROS) (*6, 9, 13-16*).

In circulation, multiple monocyte subsets have been described, where monocytes are discriminated by expression of surface markers e.g CCR2, CX_3CR1 and Ly6C (*17*). Different types display distinct behaviors where a small subset slowly patrols the vasculature and responds immediately to stimuli (*18, 19*). Monocytes recruited to tissue can but does not always undergo differentiation into macrophages (*20-23*). Macrophages are residing in most organs and exhibit specific phenotypes at homeostasis (*24*). Macrophages are plastic (*25*) and phenotypes span from characteristic microbe assassins to macrophages specialized in tissue remodeling, angiogenesis and tumor progression (*26-28*). Hallmark properties of macrophages are phagocytosis of bacteria and cellular debris as well as secretion of inflammatory agents (*29*). An arsenal of surface markers are used in defining macrophage subtypes and depending on their polarization, macrophages secrete different groups of inflammatory agents and enzymes (*25, 30*). Specific roles have been assigned macrophages in different organs, however the full spectrum of effector functions remains to be uncovered.

Immune cells of the lymphoid adaptive, memory forming immune system are divided in B- and T-lymphocytes. B-lymphocytes, maturing and selected in the bone marrow have the capacity to generate antigen-recognizing antibodies when expanding in lymph nodes upon activation (*31, 32*). T-lymphocytes are selected in thymus and are functionally divided into killer T-lymphocytes ($CD8^+$) and helper T-lymphocytes ($CD4^+$). Only about 2 % of T-lymphocytes are present in the blood whereas the remaining cells reside in the lymphatic system and secondary lymphoid organs (*1*). The $CD8^+$ T-cells recognizes proteins produced by cells or intracellular pathogens and directly kills altered cells or cells infected by intracellular pathogens utilizing e.g FAS-ligand and perforin (*33*). A subset $CD8^+$ T-lymphocytes common in epithelial tissues express γδ T cell receptor (TCR) as opposed to the more common αβ TCR (*34, 35*). The $CD4^+$ T cells are further classified e.g Th1, 2, 9, 17, 22 and more based on response to- and production of inflammatory mediators and pattern of receptor expression (*36-39*). However whether there is high plasticity or distinct subsets remains to be investigated. In addition, some $CD4^+$ T cells have immune regulating functions (Tregs) and possess the ability to suppress both activation and function of effector T lymphocytes and are therefore important in immune homeostasis (*40, 41*). Tregs are recognized by high levels of CD25 and CCR4 and the expression of the nuclear protein Forkhead Box P3 (FoxP3), which can be induced both in the thymus and at the site of inflammation (*40, 42*). Resident dendritic cells and macrophages in organs in close contact with the external environment such as skin, lungs and intestine are highly active in sampling antigens

and presenting their catch to T lymphocytes mainly in local lymph nodes. Leukocyte function in different organs is context-dependent and is influenced by several physiological factors and immune regulating soluble agents produced both locally and in peripheral lymphoid tissues

Chemokines

Chemokines, cytokines with chemotactic properties, are produced and secreted by activated cells and regulates cell actions through paracrine and autocrine signaling. The chemokines, 8-14 kilo Daltons in size, are synthetized with a secretory signal sequence transporting them to the plasma membrane. Secreted chemokines bind negatively charged extra cellular matrix glycoproteins e.g heparan sulfate and are transported into postcapillary venules where they are presented to rolling leukocytes (*43-46*). Chemokines come in families, CL, CCL, CXCL and CX_3CL (*47*), and bind to one or multiple seven loop transmembrane G-protein coupled receptors (*48*). Chemokine receptors can be abundant, e.g expressed on many cell types or close to specific to one cell type and are expressed at different densities at the cell membrane (*49, 50*). Secreted chemokines are subjected to posttranslational modifications by extracellular enzymes by e.g nitration, glycosylation, citrulliniation or proteolytic cleavage which can change *in vivo* chemokine activity and also induce loss of G-protein activation upon receptor binding (*51*). Recent reports describe chemokines as monomers, or forming dimers or multimers either with itself or with other chemokines (*52*). Intriguingly, the different combinations and conformations can elicit different cellular responses upon receptor binding and which form that dominates depends to some extent on local pH (*53*). Also the processing of chemokines by extracellular enzymes in tissue dependent on the local pH (*54-56*). The notion that local pH and tissue microenvironment at sites of inflammation in different situations together set the ultimate effect elicited by different chemokines remains to be fully explored in order to clarify chemokine effects in different contexts *in vivo*.

CXCL12 (stromal derived factor 1) expression is induced by VEGF-A (vascular endothelial growth factor A) which in turn is controlled by pO_2 sensitive signaling of HIF-1α (hypoxia inducible factor 1α), and there is constitutive expression of CXCL12 in some organs (*57, 58*). CXCL12 exerts effect by binding to the receptor CXCR4, and in some cases CXCR7, where CXCR4 is expressed by leukocytes, endothelial cells and epithelial cells and can induce multiple cellular actions (*59-63*). CXCL12 is involved in many processes such as circadian neutrophil trafficking from bone marrow (*64*) and can be found in high levels in macrophages specialized in tissue remodeling (*65*). CCL2 (monocyte chemotactic protein 1) is also upregulated by hypoxia (*66, 67*), and signals through CCR2 and recruits $CCR2^+$ monocytes

to sites of ischemia (*68*). CCL22 (macrophage-derived chemokine) is produced by alternatively activated macrophages and dendritic cells (*41*) binds CCR4 found in high levels on regulatory T cells (*40*) that are recruited to sites of expression (*30, 69*).

Immune cell recruitment from blood to sites of infection and inflammation

Leukocyte recruitment from circulation to sites of infection or tissue damage is a multistep process and depends of the cues elicited by the afflicted site (*10, 70*), the type of leukocyte, the threshold for adhesion, involved adhesion molecules and the mechanism of transmigration seems specific to the situation and organ (*71-73*). Evolutionary conserved cues for leukocyte recruitment are transmitted through leukocyte pattern recognition receptors, which binds pathogen-associated molecular pattern signals as well as endogenous molecules including chemokines that signal tissue damage (*74, 75*) or hypoxia (*9, 15*). Both bacteria and eukaryotic mitochondria contain formulated peptides (*76*), which are chemotactic to leukocytes expressing the formyl peptide receptor independently (*77, 78*).

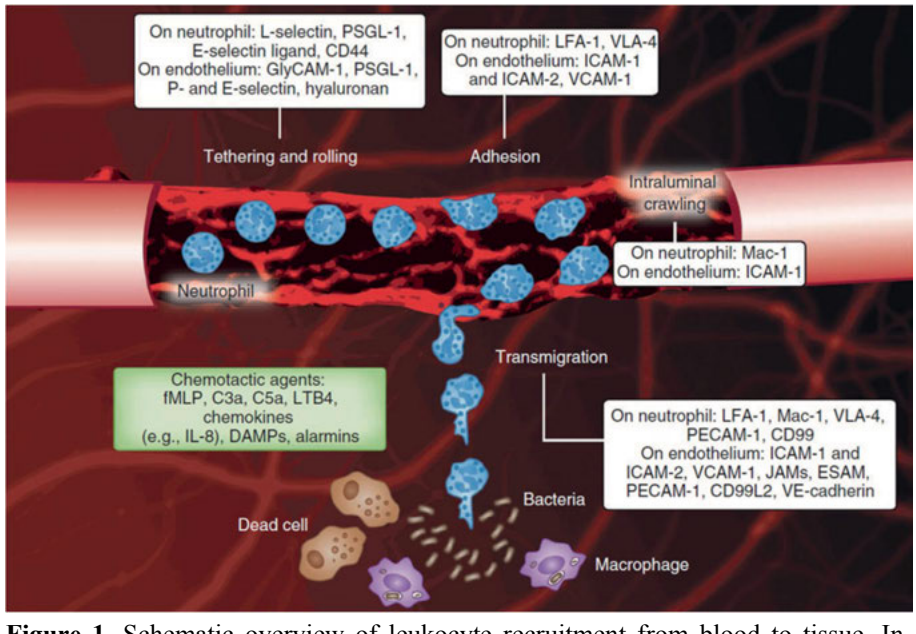

Figure 1. Schematic overview of leukocyte recruitment from blood to tissue. Intravital confocal rendering of a cremasteric venule where PECAM-1 in endothelial junctions are visualized in red and cartoon cells illustrating the steps in the recruitment cascade with chemo-attractants and adhesion molecules are described in the boxes. Image originally published in Nature Medicine by Phillipson and Kubes (*10*).

The recruitment cascade is characterized by upregulation of adhesion molecules e.g P- and E-selectins on the endothelium in postcapillary venules close to the afflicted site and this leads to that leukocytes starts to tether to and later roll on the endothelium (*79*). Leukocytes then adhere to the endothelium using β_2 integrin LFA-1 binding ICAM-1 and 2 (intercellular adhesion molecule) and VCAM-1 (vascular adhesion molecule) and start to crawl to optimal site of transmigration using β_2 integrin Mac-1 binding ICAM-1 (*10, 71, 79*). Leukocytes transmigrate from blood to tissue mainly through endothelial cell junctions (*71*) at sites with low expression of matrix proteins (*80*) even though transcellular migration also have been reported (*81*) and these steps are dependent on multiple leukocyte integrins and endothelial proteins (*10, 71, 82-85*). In addition perivascular cells have been reported to amplify inflammatory signals and thereby guide leukocytes to sites for transmigration (*86, 87*).

Neutrophils are quickly mobilized from blood circulation to afflicted sites (*8*). It has been suggested that monocytes slowly patrolling the vasculature initiate the neutrophil recruitment (*18*) however depletion of monocytes did not impact neutrophil recruitment to acute lung injury (*88*). The majority of monocytes are recruited to afflicted sites after the neutrophil influx (*18, 19*). Lymphocytes patrol the body utilizing both blood and lymph circulation, as well as secondary lymphoid tissues. If foreign proteins are detected by dendritic cells in tissue, effector T-lymphocytes specifically accumulate at the afflicted site (*89*) and recruitment is also driven by chemokine gradients and at later stages the antigen (*30, 90, 91*).

Sterile inflammation: Impaired perfusion and muscle hypoxia

Tissue damage results in hypoxia, shortage of oxygen, as blood flow is disrupted. Compensatory mechanisms include vessel neo-formation, angiogenesis, and vessel remodeling and the involvement of neutrophils and macrophages in these situations is established (*9, 12, 27, 92, 93*). Sprouting angiogenesis has been shown to dominate in areas where the blood flow is completely disrupted whilst vessel splitting/vascular intussusception occurs at sites where the flow is reduced (*94*). Insufficient blood supply and altered intraluminal shear was reported to induce growth of existing collateral arterioles (*93, 95*). Ischemia can also occur when the need for oxygen and nutrients is increased e.g by muscle work, and when blood flow regulation is compromised (*96-98*). Impaired regulation of blood flow comprise a problem for patients post myocardial infarction, with peripheral artery disease, type 2 diabetes mellitus or endothelial dysfunction (*99-101*). Muscle blood flow is regulated on the arteriolar level by sympathetic nerve activity and

tissue O_2 consumption whereas capillary perfusion is regulated in response to local vasoactive signals and metabolites (*102*). Vascular remodeling to regain functional regulation of blood flow is highly dependent on local instructions, where VEGF-A (*103*), angiopoetin-2 (*104*) and a range of chemokines, e.g. CXCL12 and CCL2 regulated downstream pO_2 sensitive HIF-1a signaling (*58*). Constitutes of the microenvironment recruit specific neutrophils (*9, 12, 15*) monocytes (*20, 105, 106*) and enhance specific functions in macrophages (*25, 28, 107*) that facilitates and drive the restitution process.

There is great clinical interest in therapeutic induction of angiogenesis and regain of functional blood flow regulation. Results from clinical trials assessing growth factors show modest results while studies using cells based therapies report more promising results (*108, 109*). In experimental models administration of $Gr1^{dim}CD11b^{+}$ cells or endothelial progenitor cells (EPCs) results in improved angiogenesis post ischemia induction in the mouse hindlimb model (*110-112*). The effect of different cells with regenerative function are being explored and delivery of mesenchymal stromal cells (MSCs) in the coronary circulation in dogs induced local micro-hemorrhagic spots (*113*).

Wound healing and impaired wound healing

The healing of skin follows the overlapping phases of coagulation, inflammation, proliferation and remodelling (*114*). Acute trauma to the skin activates the sympathetic nervous system (*115*) and in parallel, coagulation starts and platelets forms blood clots and release signals, e.g PDGF (platelet derived growth factor) and TGF (transforming growth factor), to the immediate environment (*114, 116*). Cells in the wound release alarm signals and chemokines that initiate the recruitment of circulating immune cells (*10, 74, 75, 78*). When in tissue, neutrophils and macrophages secrete additional chemokines, growth factors such as VEGF-A, FGF (fibroblast growth factor), and EGF (epidermal growth factor), matrix digesting enzymes as well as new components of the extracellular matrix (*117-120*). The wound microenvironment changes over time, and new blood vessels are formed in the granulation tissue during the proliferative phase and blood flow to the area is elevated (*121*). Remodelling of the tissue also occur after wound closure and immune cells then leave the site. The structure and composition of skin differs between species as do the rate of healing (*122*).

There are situations where the microenvironment no longer follows the sequential changes and the wound fail to heal. These wounds have high levels of proteolytic enzymes (*123*) and cell signaling is altered (*116*). This is often associated with underlying pathologies that increase susceptibility to acquire wounds, impairment in the healing process, such as arterial- or venous insufficiencies or type I and II diabetes and this is a large clinical prob-

lem. There are limited therapies to stimulate healing used today and available standard care of chronic ulcers in patients with diabetes is mechanical removal of dead tissue together with antibiotic treatment (oral and systemic) and repeated wound dressings (*124*). For treatment of chronic ulcers have trials investigating the effect of growth factors (*116, 125*) and protease activity inhibitors (*126*) not been successful enough to yet be fully implemented in wound treatment.

Type I diabetes and islet transplantation

The only curative treatment of type I diabetes is replacement of the islets of Langerhans and this is offered to patients with brittle diabetes (*127-129*). The apparent drawback is that islet grafts come from genetically different and deceased donors whereby life-long treatment with immunosuppressant therapy is needed which is associated with systemic adverse effects (*130*) and negatively influences the graft directly and hamper islet function (*131*). A protocol excluding glucocorticoids was developed, which prolong graft survival. The remaining components of the currently used immunosuppressive therapy have anti-angiogenic and pro-diabetogenic effects that impede islet engraftment and long-term function (*128, 130*).

Islets are traditionally transplanted intraportally, whereby islets immediately face the immune components of the blood of the recipient and then end up scattered throughout the liver (*132*). Striated muscle was, in contrast to the liver, recently shown to promote prompt restoration of the intraislet capillary network and improved islet functionality (*133, 134*). If graft recognition and rejection following intraportal or intramuscular transplantation are similar, remains to be described. In contrast to islets transplanted to liver, islets transplanted to muscle can be placed together and thereby can be retrieved if necessary. In addition, striated muscle as a site for transplantation allows for non-invasive bioimaging (*135*) and tissue engineering before, during and after islet implantation (*136, 137*). Recent studies aiming to reduce the need of immunosuppressant therapy include administration of suppressive autologous *in vitro* expanded Tregs (*138-140*), islet encapsulation (*141*) and modulation of the islets or the muscle implantation site with agents recruiting regulatory T-cells (*69, 142*) or induction of peripheral graft tolerance using antibodies targeting host T-lymphocytes (*143*). Regulatory T-cells, also a heterogeneous population, are sparse in circulation. Expansion of Tregs *in vitro* induces alterations during the culture period (*144, 145*) and systemic administration of these Tregs cause general immune suppression. So far, humans receiving *in vitro* expanded Tregs have not demonstrated increased susceptibility to infections (*146*). Which strategy that will be feasible in clinical islet transplantation remains to be elucidated though local immunosuppression close to the islet graft is to aim for.

Aims

The overall aim of the work presented in this thesis was to challenge the view of the different roles of immune cells in clinically relevant experimental models and to investigate ways to utilize specific immune cell functions therapeutically.

I. To uncover the identity of neutrophils accumulating at sites of hypoxia, and to delineate their means of recruitment and functional importance of this population in an *in vivo* model of angiogenesis.

II. To investigate the functional role of macrophages chaperoning blood vessels in ischemic muscle and find means to promote angiogenesis and accelerate restoration of functional blood flow regulation utilizing macrophage functions.

III. To accelerate skin wound healing by developing techniques for sustained onsite delivery and bioavailability of chemokine CXCL12, and to delineate the downstream mechanisms and the involvement of macrophages.

IV. To induce local immune privilege for allogeneic islet grafts during engraftment by accumulation of immune suppressive regulatory T lymphocytes at the site of transplantation.

Methods

This thesis investigates immune cell functions in different clinically relevant experimental models, as well as ways to utilize specific immune cell functions by changing the tissue microenvironment *in vivo*. All experiments were approved by Uppsala University Laboratory Animal Ethical Committee and Uppsala Ethical Review Committee.

Study II and IV used the same experimental approach to achieve local accumulation of specific immune-competent leukocytes in muscle, namely local injection of DNA plasmids encoding specific chemokines. The plasmids were constructed for high *in vivo* expression in muscle (*136*) from commercial backbone pVAX1 (Invitrogen), and inserts encode reporter luciferase and one of the chemokines CXCL12, CCL2 or CCL22 where the endogenous secretory sequence were substituted for the IgG secretory sequence (*69*).

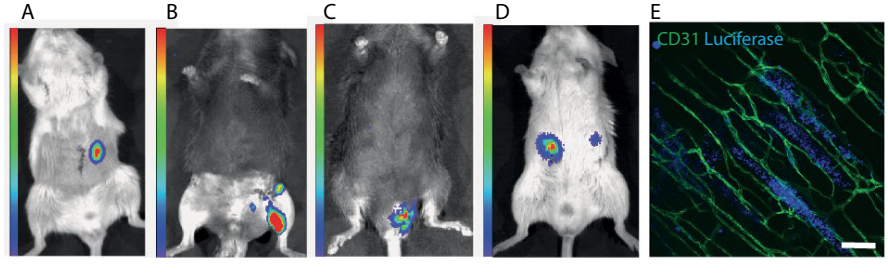

Figure 2. Intramuscular plasmid expression and plasmid encoded luciferase enzyme. Luminescent signal corresponding to plasmid expression in the left oblique external abdominal wall muscle (**A**), left quadriceps and gastrocnemius muscle (**B**) and left cremaster muscle (**C**) in an anesthetized mice. Dose dependent expression of oblique external abdominal wall muscles (left: 100 µg; right, 10 µg) (**D**). Images acquired 5 minutes following i.p. injection D-Luciferin by non-invasive bioimaging (IVIS Spectrum). Plasmid encoded luciferase (Firefly-Luc2) is seen in blue within the myocytes of transfected striated muscle tissue, with muscle vasculature seen in green (**E**). Image acquired by laser scanning confocal microscopy, bar: 100 µm, z=60 µm.

In study III, CXCL12 was delivered topically to the wounds as recombinant protein or by applying genetically modified lactic acid bacteria transformed with a plasmid encoding CXCL12 to the wound surface. Control bacteria

were transformed with a luciferase-encoding plasmid. All plasmids were modified from the pSIP400-series optimized for expression in lactic acid bacteria (*147*). Plasmid expression was quantified using non-invasive bio imaging of the luminescent reporter signal (IVIS Spectrum, Living Image 3.1 software, PerkinElmer) (Fig. 2A-E).

The recruitment of leukocytes to the sites of expression (study II, III and IV), hypoxia (study I and II), or the wound (study III) was assessed in tissue homogenates by single cell suspensions by flow cytometry (FACSCalibur or LRS II, FACS Aria II, all BD Bioscience), in tissue sections using immunohistochemistry (Nikon C1 on a TE2000-U base with Plan APO VC 20X/0.75 respectively or Zeiss 780 with 20x0.8 objective) or *in vivo* using intravital video- or confocal microscopy (Leica DM5000B with a 20/0.5 or 40x/0.5W HCS Apo objective connected to a Hammamtsu Orca R2 video camera; Zeiss LSM 5 Live, with a piezo motor-controlled WPlanApo 40x/1.0 with 0.5 optical zoom) in the exteriorized cremaster (*71*) or in exposed hind limb muscle immobilized by light vacuum suction. Leukocytes were labelled using antibodies targeting markers: Gr1, Ly6G, Ly6C, CD49d, CXCR4, VEGFR1, VEGFR2, CX_3CR1, CD10, CD16, Siglec-8, CCR3, CD115, F4/80, MMR, aSMA, PDGFR1, iNOS, RAGE, CCR2, CD34, CD45, CD68, TGFb, CD4, CD8, CD25 or FoxP3. Leukocytes were depleted using either repeated injections of anti CD49d antibody or clodronate liposomes (Encapsula Nanosciences). Circulatory levels of CXCL12, CCL2 and CCL22 chemokines were measured in serum using ELISA (Quantikine, RnD Systems).

The functional properties of the accumulated leukocytes were challenged in four different experimental models *in vivo;* Study I) transplantation of hypoxic syngeneic islets of Langerhans to muscle, Study II) restoration of blood perfusion in ischemic hindlimb muscle, Study III) cutaneous wound healing and Study IV) transplantation of allogeneic islets of Langerhans to muscle.

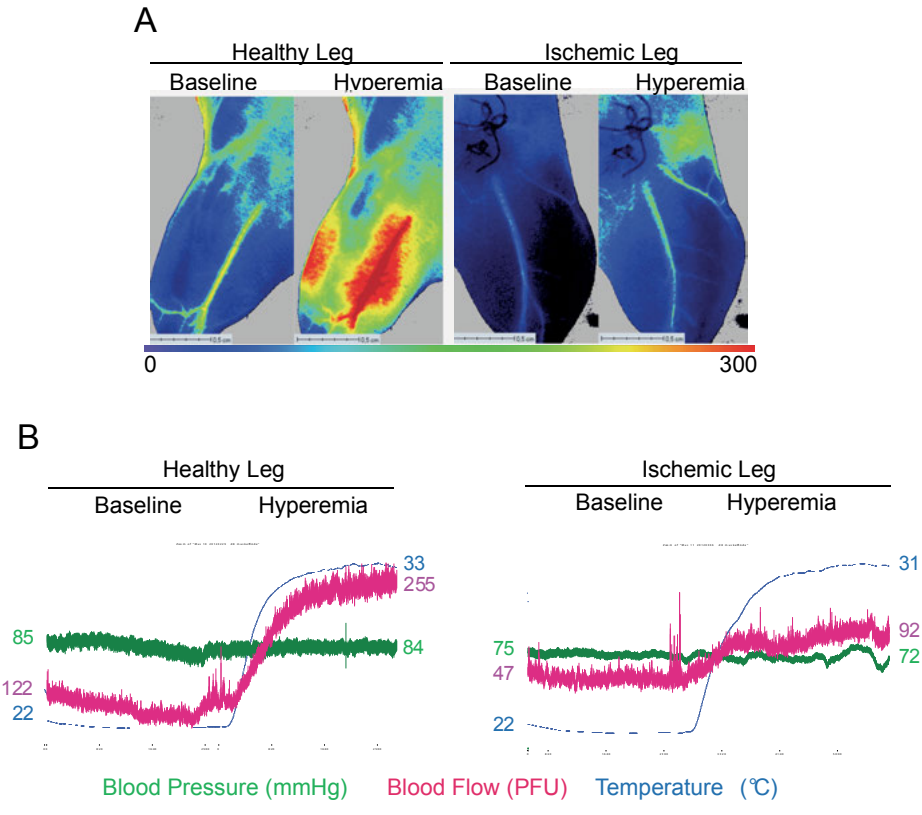

Figure 3. Blood flow regulation in response to heat-challenge in hindlimbs and foot pads. Protocol assessing heat-induced (Δ9.9±0.3°C, n=87) blood flow increase in healthy and ischemic limbs using Laser Speckle Contrast Analysis (**A**) and Laser Doppler Flowmetry (**B**). After baseline recording, the entire leg was heated through adjacent tubing of circulating pre-warmed water. Skin temperature was continuously recorded at all time points and blood pressure was registered on the last day of experiment as seen in representative plots assessing healthy- and ischemic mice 7 days post-ischemia induction (**A**). Red indicates higher perfusion in A. Protocol developed in Study II.

Islets were isolated from murine C57Bl/6 donors and transplanted in the abdominal wall muscle of C57Bl/6 recipients or diabetic Balbc recipients (*69, 133*). In study I, graft revascularization was assessed 4 days post transplantation where specific $CD49d^{+}$ neutrophils were depleted. Mice were rendered hyperglycemic using alloxan-monohydrate in Study IV and different strategies of transplantation settings and timelines of CCL22-plasmid administration were investigated. Blood glucose levels were measured post transplantation to follow graft function.

In study II, hindlimb ischemia was induced by ligation of the femoral artery (*95*) and tissue restoration was measured as blood flow regulation to

heat stimuli over time using Laser Speckle Contrast Analysis and Laser Doppler Flowmetry (Perimed) (Fig. 3A-B) in the limb- and footpad.

In study III, full thickness wounds were induced in the skin of the hindlimb using a punch biopsy needle and wound size and skin blood flow were followed over time (Fig. 4A-B).

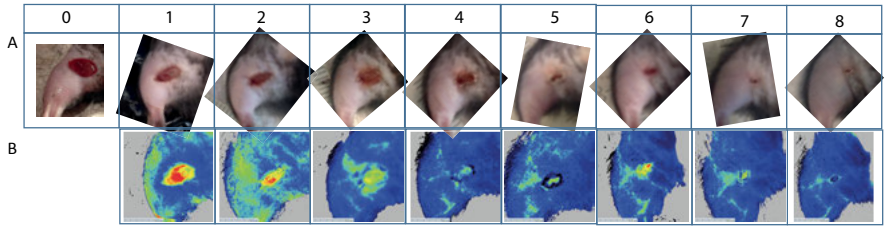

Figure 4. Healing of cutaneous wounds in mice. Daily measurement of the wound size in a healthy control mice (**A**). Corresponding images from assessment of cutaneous blood flow using Laser Speckle Contrast Analysis in the same individual (**B**). Red indicates higher perfusion in B. Method developed in Study III.

Results and discussion

Neutrophils express VEGFR1 and are recruited to VEGF-A through a distinct mechanism

At sites of hypoxia, levels of VEGF-A are increased and the importance of VEGF-A in angiogenesis is well established (*148, 149*). Neutrophils have previously been reported to be recruited to sites of hypoxia to initiate angiogenesis (*9, 12*) but the mechanism was not known. Using the exteriorized cremaster muscle model to follow leukocyte recruitment to VEGF-A administered through the superfusion buffer showed that $Ly6G^{+}$ neutrophils were recruited already at 30 minutes (*10, 71*). Intrigued by the rapid recruitment indicating direct signaling, the presence of receptors for VEGF-A on neutrophils was investigated. Functional signaling of VEGFR1 but not VEGFR2 was detected in neutrophils isolated from the circulation. To investigate this signaling on neutrophils *in vivo,* chimeric mice were created where neutrophils from mice with impaired signaling of VEGFR1 (Flt-1 $tk^{-/-}$) or VEGFR2 ($tsad^{-/-}$) were transplanted into wild type (WT) irradiated mice. The recruitment to VEGF-A was then studied following the same protocol, and we could conclude that neutrophils are recruited to VEGF-A through VEGFR1 expressed on neutrophils and that VEGFR2 signaling on endothelial cells is required for neutrophil transmigration (Fig. 5A-D). During the 60 minutes of cremaster superfusion with VEGF-A, no change in the number of emigrated monocytes was detected. Recruitment to VEGF-A have been suggested for monocytes in a model of VEGF-A long-term overexpression in liver and heart (*20, 105*). The role of the established neutrophil integrins, CD11a, CD11b and CD49d were investigated by blocking the respective integrin with neutralizing antibodies prior to VEGF-A superfusion of the cremaster. After blocking CD49d, similar numbers of neutrophils adhered in the postcapillary venules but adhered neutrophils were found to detach when crawling. Also anti-CD49d treatment completely inhibited neutrophil transmigration in response to VEGF-A, suggesting that neutrophils use CD49d to firmly adhere to the vessel wall and that it is required to prevent detachment during intraluminal crawling.

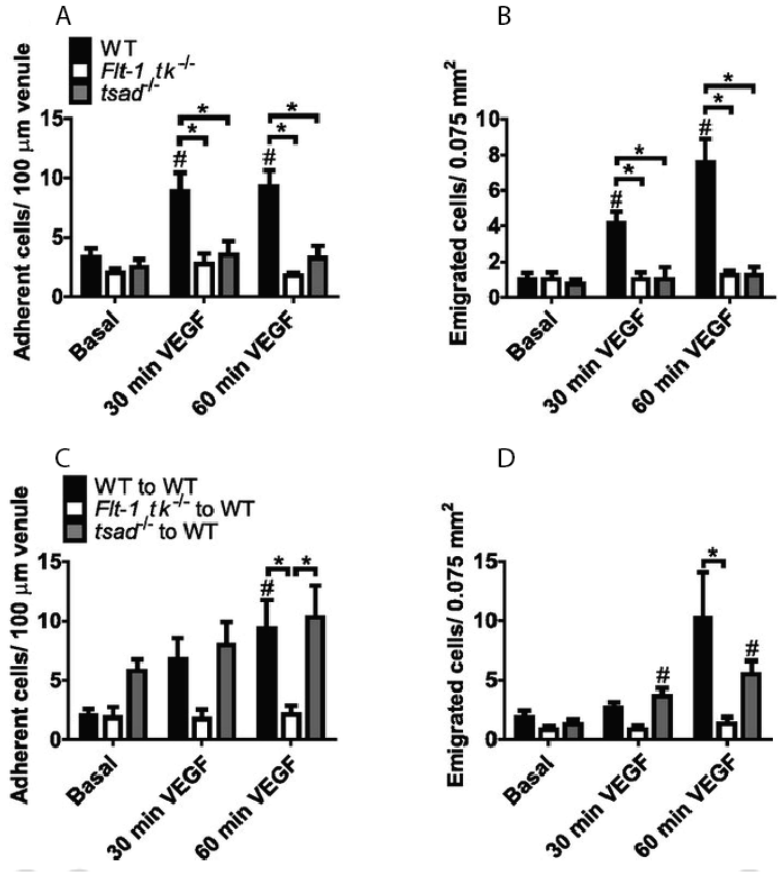

Figure 5. Activation of VEGFR1 on neutrophils and VEGFR2 on endothelial cells is necessary for VEGF-A-induced neutrophil recruitment *in vivo*. Specific inhibition of either VEGFR1 (*Flt-1 tk*$^{-/-}$) or VEGFR2 signaling (*tsad*$^{-/-}$) inhibited VEGF-A-dependent neutrophil recruitment to the cremaster muscle (**A-B**, n=4-7 per group). VEGF-A-dependent neutrophil recruitment was inhibited in WT mice transplanted with *Flt-1 tk*$^{-/-}$ bone marrow whereas neutrophil recruitment to VEGF-A was unaffected in WT mice transplanted with *tsad*$^{-/-}$ bone marrow (**C-D**, n=5-6 per group). #Indicates difference ($p<0.05$) to basal values in the same group, *Indicates difference ($p<0.05$) to other groups. Graphs originally published in Blood (*15*).

A specific subtype of neutrophils ($CD49d^+VEGFR1^+CXCR4^{high}$) essential for functional angiogenesis are found in circulation of mice and humans

The rapid recruitment of neutrophils to VEGF-A and the dramatic effect of blocking CD49d on preventing emigration to tissue lead us to investigate if there was a pool of CD49d-expressing neutrophils in the circulation. CD49d, also known as very late antigen 4, was previously thought to be expressed mainly by immature granulocytes in bone marrow (*150*). We found a small population of mature circulating neutrophils to express CD49d in circulating neutrophils in human and mice (human: 3.2±0.5%, n=12; mice: 2.8±0.6%, n=16). These $CD49d^+$ neutrophils expressed higher levels of both VEGFR1 and CXCR4 compared to the $CD49d^-$ neutrophils and displayed increased chemokineses *in vitro* when stimulated with VEGF-A. Neutrophils expressing high levels of CXCR4 have previously been demonstrated to be recruited to VEGF-A secreted by hypoxic islets of Langerhans transplanted to muscle where they were essential for revascularization of the transplanted islets (*9*). Isolation of islets from pancreas disrupts the intra islet vasculature which have to be reformed post transplantation (*133*). The importance of the $CD49d^+VEGFR1^+CXCR4^{high}$ neutrophils was challenged in the same model of syngeneic islet transplantation to muscle where $CD49d^+VEGFR1^+CXCR4^{high}$ neutrophils appeared at the hypoxic islet graft. Blocking CD49d during islet engraftment prevented this population from being recruited to the hypoxic site, and resulted in fewer new vessels surrounding the transplanted islets as well as altered vascular morphology of the few new vessels formed 3 days post transplantation (Fig. 6A-D), demonstrating the importance of this population for functional angiogenesis.

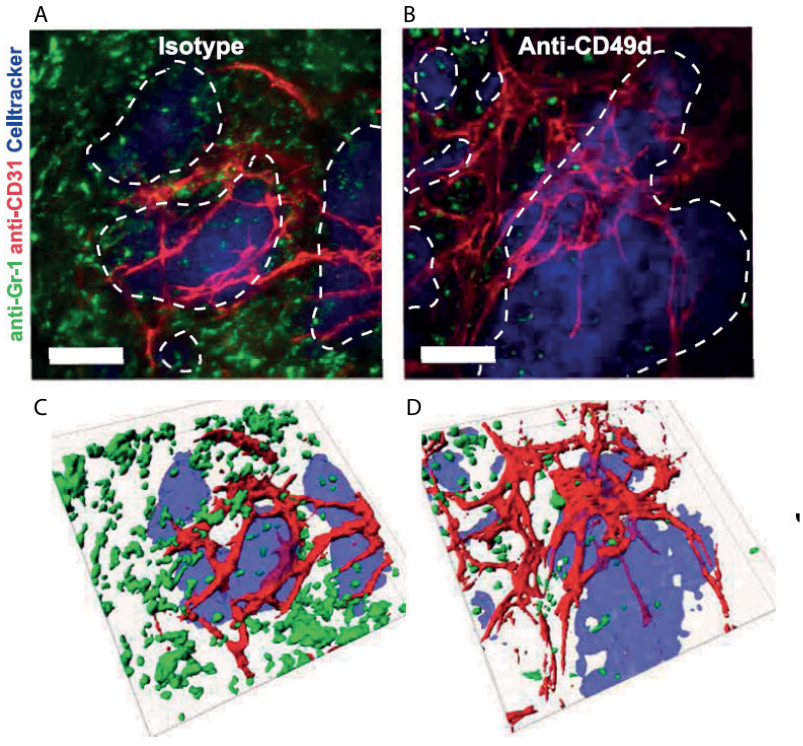

Figure 6. Recruitment of CD49d^{+}VEGFR1^{+}CXCR4^{high} neutrophils is essential for functional angiogenesis. Representative confocal Z-projections of transplanted pancreatic islets (blue, dashed lines), recruited neutrophils (green) and newly formed vasculature (red) in the cremaster muscle of mice treated with isotype- or anti-CD49d antibodies during islet engraftment (**A-B**) with corresponding surface renderings (**C-D**). Images were acquired *in vivo* 4 days post transplantation. Bar: 50µm. Figure originally published in Blood (*15*).

Macrophages accumulate in perivascular positions in the ischemic muscle, and start to express PDGFR1b and aSMA

Macrophages comprise a versatile and plastic cell type that resides in most tissues at homeostasis and is a key player in tissue remodeling (*25, 120*). In ischemic hindlimb muscle, increased numbers of macrophages were detected 3 and 7 days following ischemia induction. What directly caught our eye was that macrophages were found in perivascular positions (<1µm from the vascular lumen) to arterioles and arteriolar branches (Fig. 7A-D) where blood flow is mainly regulated (*102*). Also venules and capillaries were found to be chaperoned by macrophages and at 3 days post ischemia induction, 20.7±10.1 % (n=5) of the CX_3CR1^{+} macrophages were found in

perivascular positions. The functional importance of the increased number of macrophages in the ischemic muscle was challenged by clodrosome-depletion and severe visual signs of limb ischemia were detected 3 days following ischemia induction in mice depleted of macrophages

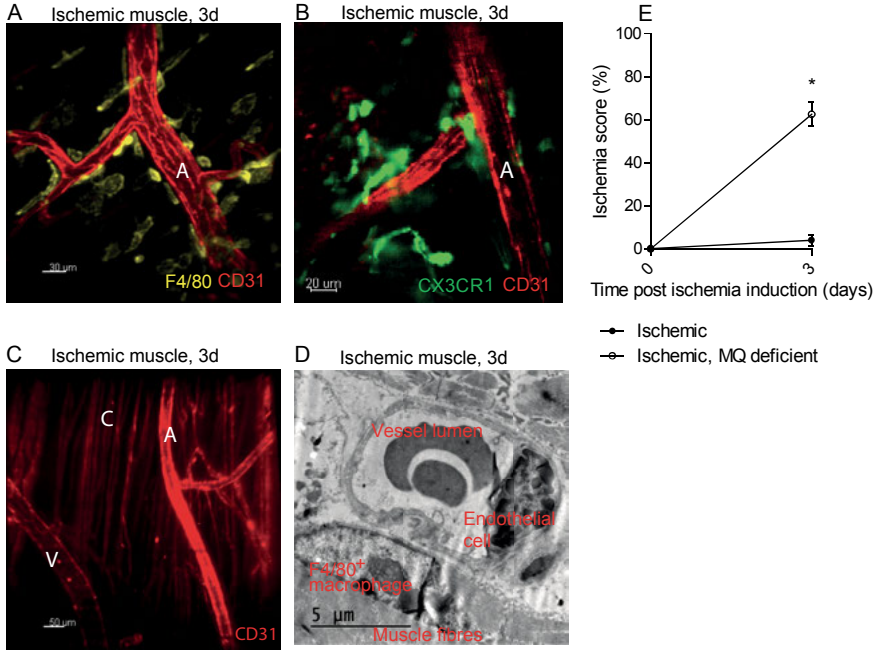

Figure 7. Macrophage numbers are increased in ischemic muscle, they are essential for limb function and are found in perivascular positions. Macrophages ($F4/80^+$ and CX_3CR1^+) were identified in ischemic muscle *in vivo* (A, B) shown in representative z-stack renderings from intravital confocal images. Panel C shows the vasculature of ischemic muscle using intravital confocal imaging, where the arteriole, capillaries and venule are marked (A, C, V). Panel D shows a close up on a $F4/80^+$ macrophage next to a capillary in ischemic muscle 3 days post ischemia induction visualized by transmission electron microscopy. Visual signs of ischemia at the time of ischemia induction and at 3 days later in WT mice (control liposomes n=7) and in mice depleted of macrophages using clodronate liposomes (n=5) (E). "A" indicates arteriole in A and B. Bar: 30 µm in A, 20 µm in B, 50 µm in C and 5 µm in D. *Indicates difference ($p<0.05$), Students two-tailed t-test.

as compared to control mice (Fig. 7E) following an ischemia scoring system (*151*). Analyzing the phenotype of these ischemia-amplified macrophages in muscle using flow cytometry revealed that all macrophages ($F4/80^+$) expressed CXCR4 where expression was higher in the population co-expressing MMR (macrophage mannose receptor). Macrophages ($CD45^+CD68^+F4/80^+$) were also found to express CCR2 and iNOS (inducible nitric oxide synthase) as well as the non-classical macrophage markers

PDGFR1b (platelet derived factor receptor 1 beta) and αSMA (alpha smooth muscle actin), normally associated with pericytes (*152*).

Perivascular macrophages assist in blood flow regulation in ischemic muscle by iNOS production of NO

Blood flow regulation upon heat-challenge was assessed in healthy and ischemic limbs (Fig. 3A-B). The basal perfusion was reduced to 1/5 of that of healthy tissue by femoral artery ligation and ischemic limbs were almost unable to respond to the heat-challenge 3 and 7 days post ischemia induction, as detected by both total tissue perfusion and arteriolar inner diameter change at branch points. The hyperemic response to prolonged warming is mediated by nitric oxide (NO) (*153, 154*). Using inhibitors to the different NOS-enzymes we detected that the heat-induced hyperemia was shifted from eNOS-dependence to iNOS-dependence in ischemic muscles (Fig. 8A-B). That macrophages can express iNOS is known, however this has been associated with inflammatory macrophages and not with macrophages specialized in tissue remodeling expressing MMR, as identified in the ischemic muscle (*155-157*). In sections of ischemic muscle, increased iNOS levels was measured at 3 days following ischemia induction in areas close to vasculature, and the perivascular F4/80^{+} macrophages expressed iNOS (Fig. 8C).

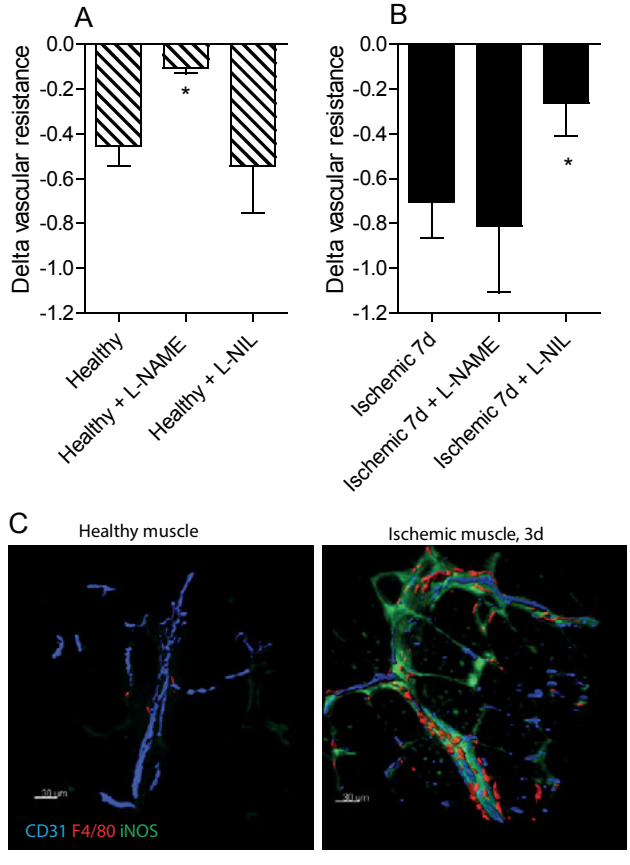

Figure 8. Assessment of the involvement of different nitric oxide synthases in blood flow regulation upon heat-stimuli in healthy (**A**) and ischemic muscles (**B**) (Healthy, n=9; Healthy + L-NAME, n=6; Healthy + L-NIL, n=3), (Ischemic 7d, n=7; Ischemic 7d + L-NAME, n=4; Ischemic 7d + L-NIL, n=7). Delta values correspond to resistance at peak hyperemia deducted by resistance at baseline. Visualization of iNOS in healthy- and ischemic muscle 3 days post ischemia induction in tissue sections (**C**). Bar: 30 µm in C. *Indicates difference ($p<0.05$) from "Healthy" or "Ischemic group", One Way ANOVA, Bonferroni post hoc test.

CXCL12 further improves the macrophage-assisted blood flow regulation in ischemic muscle and accelerate regain of function

Means to reinforce the functions of the macrophages identified in ischemic muscle was investigated by overexpressing the chemokines CCL2 and CXCL12, which are both upregulated by hypoxia, in muscle from plasmids encoding these chemokines. Intriguingly, pCXCL12 improved blood flow

regulation upon heat-challenge in ischemic muscle at all time points studied measured by the non-invasive Laser Doppler setup, while local overexpression of pCCL2 in the ischemic muscle had no effect (Fig. 9A-B). At baseline in ischemic muscle, the vascular resistance was lower in animals expressing pCXCL12 compared to mice receiving control plasmid pCTR (pCTR=1.58±0.25, n=11; pCXCL12=0.96±0.13, n=9, p=0.05) and the vasodilatory effect at the level of the small arterioles was improved by pCXCL12 overexpression in ischemic muscles 7 days post ischemia induction (Fig. 9C-D). The heat-induced hyperemia response was attenuated by specific inhibition of iNOS also in pCXCL12-expressing muscles (Fig. 9E), again demonstrating a role of macrophages in blood flow regulation. The functionality of the ischemic leg was examined by scoring ischemia-associated symptoms (*151*), and in agreement with the improved regulation of blood flow less ischemia-associated visual symptoms were observed 14 and 21 days post-ischemia induction in mice treated with pCXCL12. When looking into these muscles, we found that the ischemia-induced increase of CX_3CR1^+ macrophages was even further expanded in ischemic gastrocnemius muscle expressing pCXCL12 but not pCCL2 (Fig 9F) and pCXCL12 also induced numbers of perivascular CX_3CR1^+ macrophages as well as specifically directing macrophages to the blood vessels (Fig. 9G-H). The pCXCL12-induced improvement of blood flow regulation to heat-stimuli was absent when macrophages were depleted during ischemia induction, as compared to limbs expressing control plasmids. The levels of iNOS in ischemic muscle was further upregulated by pCXCL12, as compared to the control ischemic muscle (Fig. 9I), and significantly more $F4/80^+$ macrophages that co-expressed both MMR and iNOS were found and especially so in perivascular positions (Fig. 9J, L). pCCL2 induced non-perivascular $F4/80^+$ macrophages expressing iNOS which were negative for MMR (Fig. 9K, M). Benefits of CXCL12 in tissue remodeling have been reported before (*158-160*), however the effect of CXCL12 on blood flow regulation has not been assessed in these studies. The results of increased vascular chaperoning of macrophages producing NO through iNOS by pCXCL12 could explain the increased functional blood flow regulation and the accelerated restoration of muscle function post ischemia. The ultimate experiment to prove this remains to be performed using mice where macrophages are deficient of iNOS.

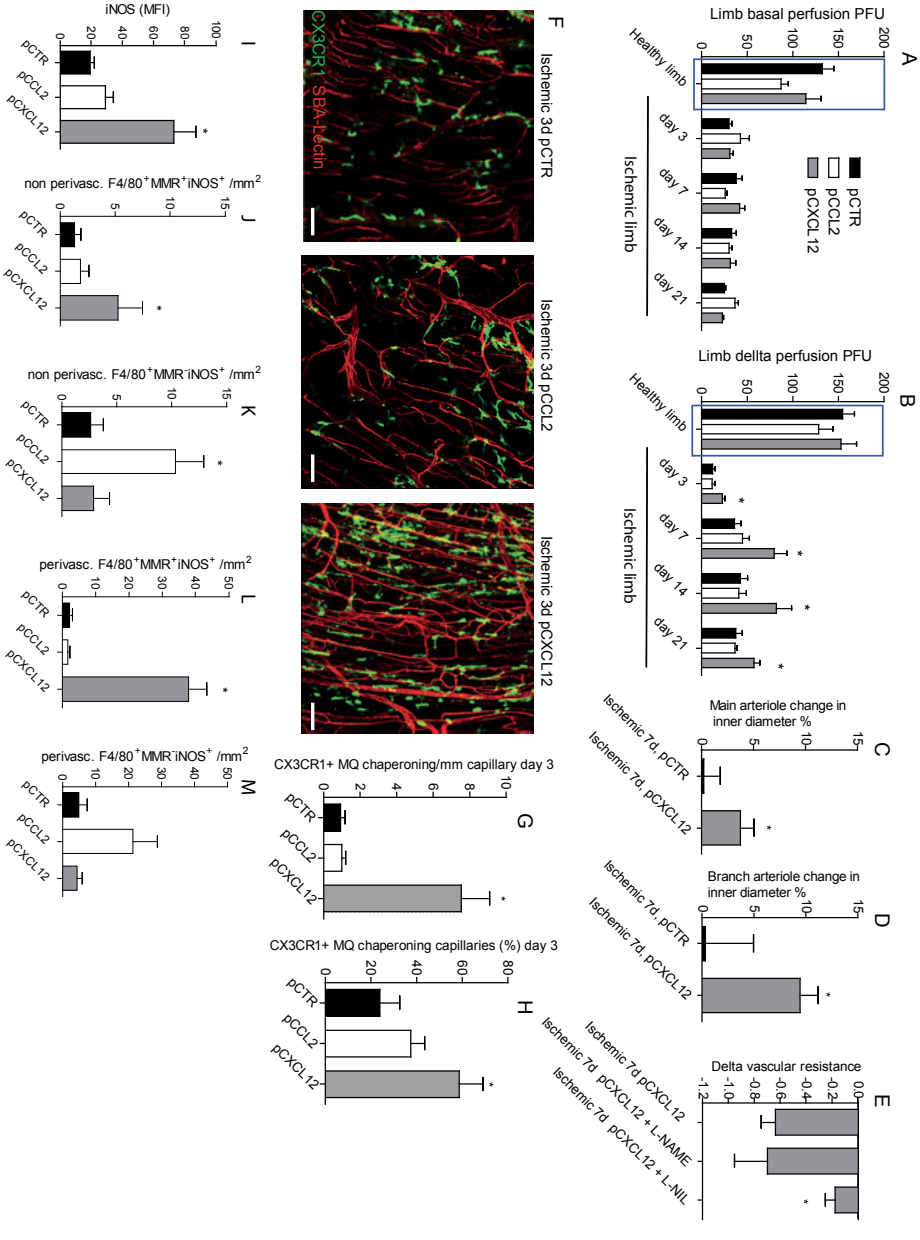
A
Limb basal perfusion PFU
Healthy limb
Ischemic limb
day 3
day 7
day 14
day 21
pCTR
pCCL2
pCXCL12
B
Limb dellta perfusion PFU
C
Main arteriole change in inner diameter %
Ischemic 7d, pCTR
Ischemic 7d, pCXCL12
D
Branch arteriole change in inner diameter %
E
Delta vascular resistance
Ischemic 7d pCXCL12
Ischemic 7d pCXCL12 + L-NAME
Ischemic 7d pCXCL12 + L-NIL
F
CX3CR1+ SBA-Lectin
Ischemic 3d pCTR
Ischemic 3d pCCL2
Ischemic 3d pCXCL12
G
CX3CR1+ MQ chaperoning/mm capillary day 3
H
CX3CR1+ MQ chaperoning capillaries (%) day 3
I
iNOS (MFI)
J
non perivasc. F4/80+MMR+iNOS+ /mm2
K
non perivasc. F4/80+MMR-iNOS+ /mm2
L
perivasc. F4/80+MMR+iNOS+ /mm2
M
perivasc. F4/80+MMR-iNOS+ /mm2

Figure 9. Improved capacity to regulate blood flow by pCXCL12 overexpression through iNOS-generated NO delivered by perivascular macrophages. Blood flow regulation in response to heat-challenge in hindlimbs following ischemia induction and transfection (pCTR, pCCL2 and pCXCL12) measured by Laser Doppler Flowmetry (**A-B**) 3 days (pCTR; n=8, pCCL2; n=6, pCXCL12; n=8), 7 days (pCTR; n=9, pCCL2; n=9, pCXCL12; n=10), 14 days (pCTR; n=7, pCCL2; n=5, pCXCL12; n=6) and 21 days (pCTR; n=6, pCCL2; n=5, pCXCL12; n=5) post ischemia induction and transfection. Panels **C** and **D** show the change in arteriolar inner diameter in ischemic muscle (pCTR; n=3, N=9, pCXCL12; n=3, N=9) in response to heat as measured from intravital confocal recordings. Muscle blood flow and blood pressure was measured in mice expressing pCXCL12 in limb muscle and delta vascular resistance (**E**) was assessed (Ischemic pCXCL12 7d, n=8; Ischemic pCXCL12 7d + L-NAME, n=3; Ischemic pCXCL12 7d + L-NIL, n=7). Macrophage number and phenotype were assessed in ischemic gastrocnemius muscle. Representative z-stack renderings of CX_3CR1 and vasculature in ischemic plasmid-expressing gastrocnemius muscle 3 days post ischemia (**F**). Bar: 100 μm. CX_3CR1^+ leukocytes quantified in regard to number and alignment with vasculature (**G-H**) (pCTR; n=9, pCCL2; n=7, pCXCL12; n=11). Expression of iNOS in ischemic muscle (**I,** pCTR, n=3, N=13, pCCL2, n=3, N=13, pCXCL12, n=3, N=13), and non-perivascular (**J-K**) and perivascular macrophages (**L-M**) analyzed based on co-expression with MMR 3 days following ischemia induction and muscle transfection (pCTR, n=3, N=9, pCCL2, n=3, N=14, pCXCL12, n=3, N=9). Delta values are hyperemia deducted with baseline perfusion or resistance. *Indicates difference ($p<0.05$) from "pCTR" group, One Way ANOVA, Bonferroni post hoc test or Students t-test (C-D).

Treatment of cutaneous wounds with lactic acid bacteria expressing CXCL12 accelerates wound healing by accumulation of macrophages secreting TGFb

Intrigued by the effect of CXCL12 on accelerating tissue restitution in ischemic muscle, we designed and produced genetically modified lactic acid bacteria expressing CXCL12 from a plasmid with the intention to investigate the effect during healing of cutaneous wounds. Plasmids encoding luciferase and CXCL12 behind an inducible promoter were transformed into *Lactobacillus reuteri* referred to as LB/LB_Luc and LB_CXCL12 respectively. CXCL12 could be detected in supernatants of activated LB_CXCL12 and administration of LB_Luc to cutaneous wounds in mice revealed high plasmid expression during one hour *in vivo*. Full thickness cutaneous wounds were induced in mice and wound appearance and skin blood flow were monitored daily. Topical administration of LB_CXCL12 once a day resulted in highly accelerated wound closure compared to untreated wounds or wounds treated with control LB, and the effect of LB_CXCL12 was most prominent during the first 24 hours (Fig. 10A-B). As a result, the wound area over time assessed as area under curve was significantly reduced in mice treated with LB_CXCL12 (Fig. 10C). This strong efficiency of wound closure were more

than 66% of the wound surface was closed during the first 24 hours has never before been reported, and wounds were examined with immunohistology at this time point.

Comparing the skin closest to the wound (area of analysis defined in Fig. 10D) to healthy unaffected skin, increased numbers of proliferating cells was detected in dermis and epidermis (dermis 20-fold increase, epidermis >1000-fold increase in $Ki67^+$ cells). The density of $F4/80^+$ macrophages was increased 20-fold in the dermis immediate to the wound 24 hours post wound induction as compared to unaffected skin. Further was the fraction of the $F4/80^+$ macrophages expressing TGFb ($F4/80^+TGFb^+$ over $F4/80^+TGFb^-$) increased six times from 8.0±1.1% (n=3) in unaffected skin to 55±10% (n=5, p=0.0002) in this area 24 hours post wound induction.

In wounds treated with CXCL12 producing *L. reuteri* the number of cells proliferating in the dermis and epidermis immediate to the wound was even further increased (Fig. 10E-F). Also the number of $F4/80^+$ macrophages present was increased compared to wounds receiving no treatment or treatment with control *L. reuteri* (Fig. 10G). The levels of TGFb in the dermis and number $F4/80^+TGFb^+$ macrophages were amplified in wounds treated with *L. reuteri* delivering CXCL12 at this time point (Fig. 10H-I). The accelerated wound closure by CXCL12 delivered by *L. reuteri* is probably due to increased tissue contraction as TGFb mediates fibroblast switch into aSMA-expressing myofibroblasts being contractile (*117, 161*) and this remains to be investigated.

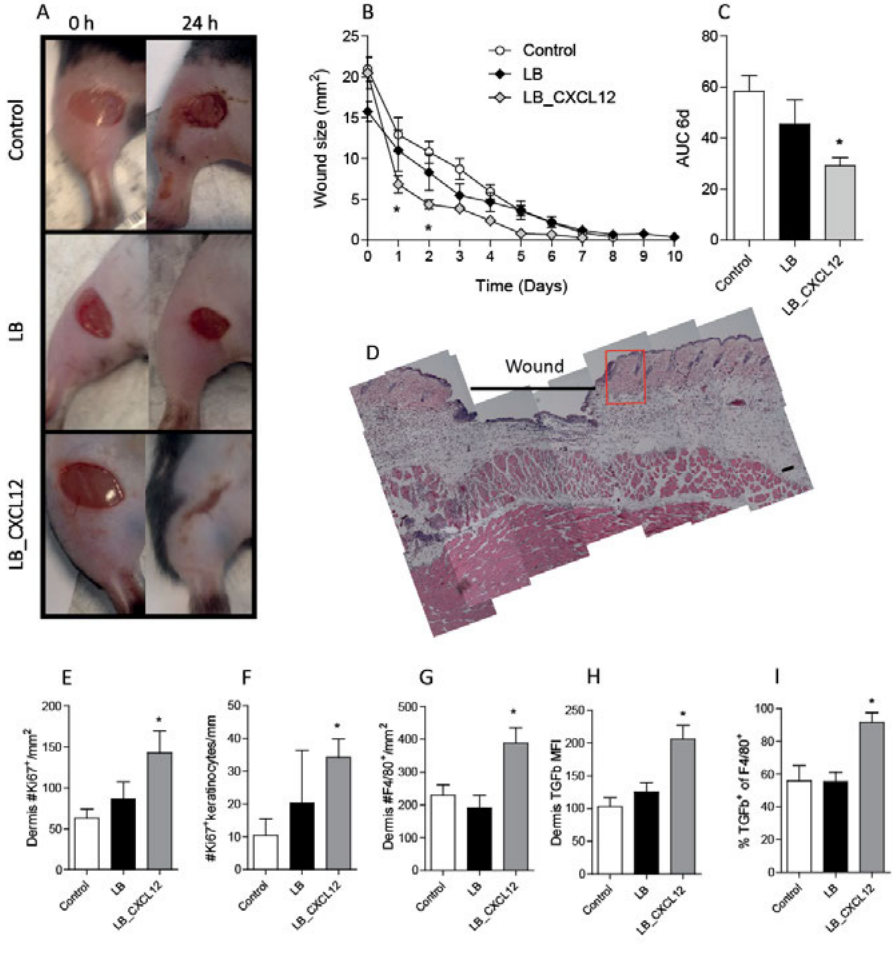

Figure 10. Accelerated wound healing with CXCL12-producing *L. reuteri* through increased numbers of macrophages expressing TGFb. Panel **A** shows representative images of wounds receiving no treatment (Control), treatment with control LB or LB_CXCL12 at time of wound induction and after 24 hours. Wound size (**B**) and accumulated wound surface (**C**) during healing in these mice (Control n=5, LB n=5, LB_CXCL12 n=5). Overview of wound area in the skin 24 hours post wound induction where area of analysis is indicated with the red rectangle (**D**). Density of proliferating cells positive for Ki67 in dermis (**E**) and epidermis close to the wound edge (0-250µm) (**F**). Density of F4/80$^+$ macrophages in dermis close to the wound (**G**). Total levels of TGFb (**H**) and fraction of F4/80$^+$ macrophages expressing TGFb in the dermis (**I**) (n=3 all groups). *Indicates difference to Control group ($p<0.05$), Two-way ANOVA, Bonferroni post hoc test (B), One Way ANOVA Bonferroni post hoc test (C, E-I).

Prolonged bioavailability of CXCL12 in low pH by inhibition of CD26

Using different protocols to administer CXCL12 to cutaneous wound where dose, timeliness, formulation and buffer pH was altered, we could only detect similar effect on immediate wound closure as by CXCL12 delivered by *L. reuteri* when recombinant CXCL12 (rCXCL12) were given in buffer of pH 6.35 (Fig. 11), similar to what would be expected in the wound by the bacteria-produced lactic acid. Inactivation of chemokines by proteases is a large problem in chronic wounds (*123, 162*) and enzymatic activity is dependent on the local pH.

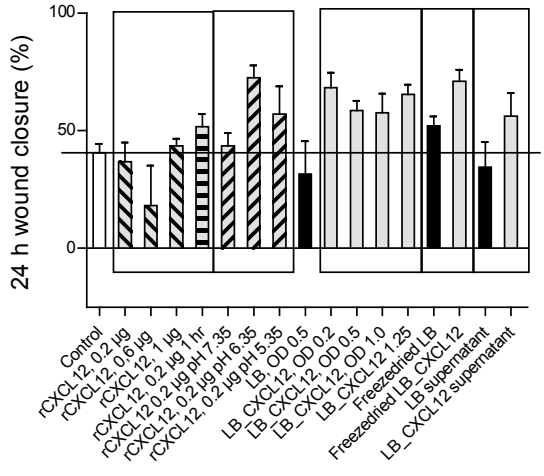

Figure 11. Wound closure during the 24 first hours in healthy mice with no or different treatments. The following groups are presented: Control, n=15; 0.2 µg rCXCL12, n=4; 0.6 µg rCXCL12, n=5; 1.0 µg rCXCL1 , n=4; 0.2 µg rCXCL12 1hr, n=6; LB OD 0.5, n=4; LB_CXCL12 OD 0.2, n=4; LB_CXCL12 OD 0.5, n=10, LB_CXCL12 OD 1.0, n=4; LB_CXCL12 OD 1.25; n=5, Freezedried LB, n=4, Freezedried LB_CXCL12, n=5, LB supernatant; n=4, LB_CXCL12 supernatant.

CD26 (dipeptidyl-peptidase 4) is an extracellular enzyme inactivating CXCL12 by cleaving the N-terminus and is most efficient at pH 8.3 (*54, 163*). A slightly basic pH is associated with the milieu in chronic wounds (*162*). When CD62 activity was measured at different pH, high enzymatic activity was demonstrated at pH 8.3 compared to at pH 7.3, as described before (*54, 163*), whereas there was no enzymatic activity at pH 6.3 or 5.3. As a result, proteolytic inactivation of CXCL12 by CD26 was demonstrated at pH 8.3, and at pH 7.3 but with lower efficiency, while no cleavage occurred at pH 6.3 and 5.3. The mechanisms underlying the observed rapid wound closure include inactivation of extracellular CD26 by the pH reduction from the lactic acid and thereby increased chemokine bioavailability

(*54, 56*), as well as CXCL12-induced cellular functions of macrophages (*156*). This can be verified *in vivo* in our model using an inhibitor of CD26 e.g sitagliptin, together with the CXCL12-producing lactic acid bacteria during wound healing. Intriguingly, different conformations of CXCL12 have been reported to elicit different cellular responses upon receptor binding (*53*), and which form that dominates depends at least partly on local pH and the specific cellular responses to CXCL12 delivered at different pH to wounds in our model remains to be investigated.

Allogeneic islets transplanted to muscle are immediately recognized and rejected

In a model of MHC-mismatched (allogeneic) islet transplantation to muscle of hyperglycemic mice using no immunosuppressive treatment, transplanted islets were not able to lower blood glucose levels at any time point (Fig. 12A). This indicates that islets are immediately recognized and destroyed by the host immune system, as detected by low insulin-positive area and islet central necrosis (Fig. 12B-E). At 7 days post transplantation, $Ly6G^+$ neutrophils, $F4/80^+$ macrophages, $CD4^+$ T lymphocytes and effector cytotoxic $CD8^+$ T lymphocytes (Fig. 12F-H) were detected in high levels surrounding the grafts. Intriguingly, intra-islet vasculature was observed at this time point in some of the transplanted islets (Fig. 12B, E). Muscle has been reported to be a site where syngeneic islets quickly attain a functional vascular network (*133*). The results indicate that the immediate graft rejection seen in muscle follows a different patterns of alloantigen recognition as compared to allogeneic murine islets transplanted intrahepatic and to the kidney subcapsular space where an initial normalization of blood glucose or even hypoclycemia is detected prior to islet rejection (*164*).

CCL22 recruits regulatory T cells shielding allogenic islets from initial rejection

The chemokine CCL22 was reported to recruit regulatory T lymphocytes inducing an immune privilege for a specific tumor (*30*). Plasmids were constructed to express CCL22 and were co-transplanted with the allogeneic islets to muscle in order to create a region of myocytes continuously secreting CCL22 around the islets. As a result, the islets were shielded from the host immune system and were able to engraft (Fig. 12E) and function, and as a result blood glucose levels were normalized (<11.1 mmol/l) for 10 consecutive days (Fig. 12A). At 7 days following transplantation, significantly higher number of insulin-positive beta cells remained (Fig. 12C) and signifi-

cantly fewer cytotoxic $CD8^+$ cells were found around and in the islets (Fig. 12G-H). However, after 10 days the host effector lymphocytes overwhelmed the islet immune privilege, resulting in graft destruction and the mice returned to hyperglycemia (Fig. 12A). Different protocols were tested and we found that key to induce the immune-privileged site was to cover of all alloantigens by the pCCL22 shield, which was best induced by co-transplanting islets and plasmids.

Finding means to reduce systemic immunosuppressive treatment by local alternatives is key in optimizing the protocol for islet transplantation. Another study presents islets that are virally transfected to express CCL22 prior to transplantation to recruit Tregs in a similar manner (*142*). This strategy does cover the islets allogens however the transfection efficiency is not optimal and the regulatory and logistic obstacles to translate this into clinical settings are significantly more complicated as compared to co-transplantation of islets and pCCL22. Endogenous $CD4^+CD25^+FoxP3^+$ Tregs have also been used to curb rejection of allogeneic islets by *in vitro* expansion and adoptive transfer at the time of islet transplantation (*165*). Unfortunately this strategy is hampered by the very few Tregs found in circulation, the lack of stable Treg surface markers impeding discrimination from other T lymphocytes, and the possible implications of *in vitro* expansion on *in vivo* suppressor effects (*144, 145*).

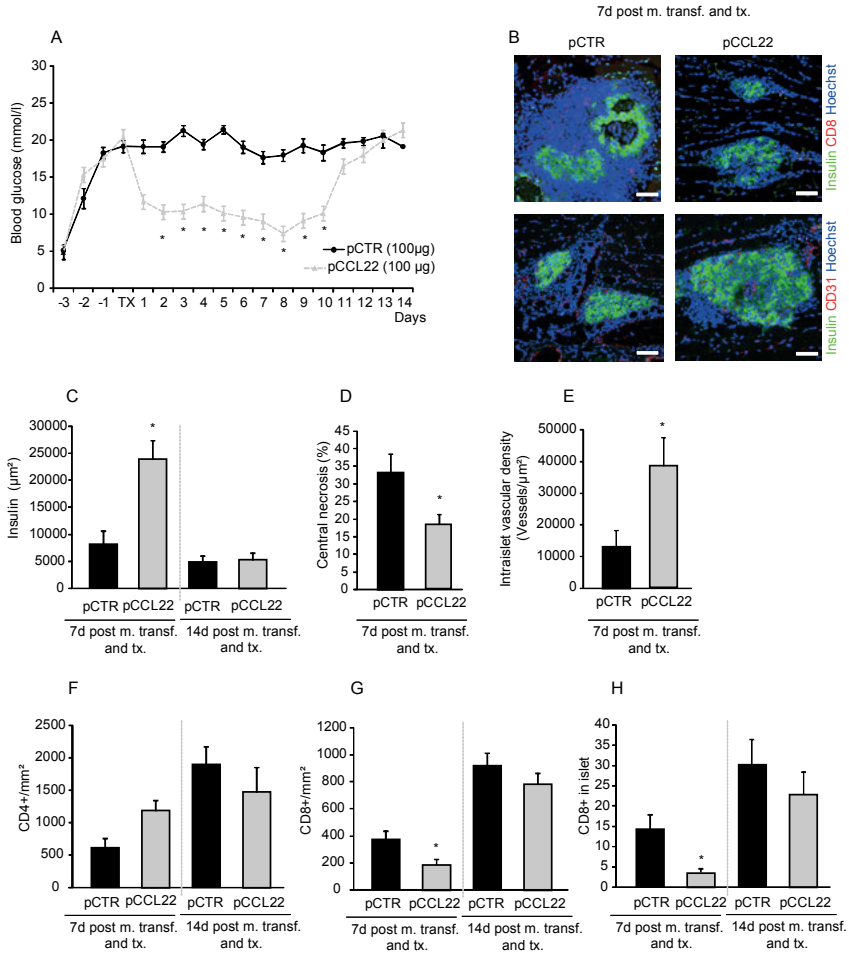

Figure 12. pCCL22 delays pancreatic islet graft rejection. Islets (C57Bl/6) were transplanted to muscle of diabetic MHC-mismatched recipients (Balb/c) together with 100 µg pCTR or pCCL22 plasmids. Panel **A** demonstrates blood glucose levels following intramuscular transplantation of 500 pre-cultured islets together with either 100 µg pCTR (n=8) or pCCL22 (n=11). Panel **B** shows representative images from sections (7 µm) of islet grafts [Insulin-A, Alexa Fluor 488 (green)] in muscle 7 days post transplantation showing $CD8^+$ T lymphocytes [Northern Light 557 (red)] (**B**, top) and $CD31^+$ vasculature [Alexa Fluor 555 (red)] (**B,** bottom), bar corresponds to 100µm. Quantifications of insulin positive area in grafts of pre-cultured islets co-transplanted with 100 µg plasmid 7 days and 14 days post transplantation (**C**) and examination of islet central necrosis 7 days post transplantation (**D**). Engraftment assessed by intraislet vascular density 7 days post transplantation (**E**). Quantification of $CD4^+$ (**F**) and $CD8^+$ (**G**) T lymphocyte density, present at the graft and the number of $CD8^+$ T lymphocytes in direct contact with the transplanted islet (**H**) 7 days and 14 days post transplantation. *Indicates statistically significant difference from the pCTR group ($p<0.05$, Student's t test). (m. transf; muscle transfection). Figure originally published in Cell Transplantation (*69*).

Conclusions

In the first study of this thesis we have for the first time identified a distinct proangiogenic neutrophil subset in the circulation of humans and mice ($CD49d^{+}VEGFR1^{+}CXCR4^{high}$), which is recruited to hypoxic sites by VEGF-A. The recruitment was dependent on VEGFR1 on neutrophils and VEGFR2 on the endothelial cells and these proangiogenic neutrophils specifically used CD49d for firm adhesion and crawling. This population was demonstrated to be crucial for functional angiogenesis at hypoxic sites *in vivo*.

In the second study macrophages in perivascular positions were detected in ischemic muscle, where they started to express aSMA and PDGFR1, markers previously associated with the mural cells, pericytes. These macrophages were essential for survival of the ischemic muscle and directly assisted in blood flow regulation by iNOS-dependent NO production. Using CXCL12 overexpression in the ischemic muscles, the blood flow regulating effect of these perivascular macrophages could be amplified and the functional restoration of the muscle was accelerated.

In the third study, a system for direct delivery of CXCL12 to cutaneous wounds through genetically modified lactic acid bacteria was constructed and proven therapeutically feasible. Treatment of wounds with CXCL12-delivering bacteria induced rapid wound closure during the first 24 hours to an extent that has never before been reported. Synergistic effects of the produced lactic acid prolonged bioavailability of CXCL12 inhibiting enzymatic degradation.

In the fourth and last study it was found that allogeneic islets of Langerhans transplanted to muscle were immediately rejected. By co-transplanting islets with CCL22-producing plasmids, we could curb this fast rejection for 10 days by accumulating $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T lymphocytes at the site for transplantation. The accumulated Tregs shielded the islet grafts from being attacked by the host cytotoxic T lymphocytes and allowed the islets to engraft.

Future perspectives

A distinct and proangiogenic neutrophil subset ($CD49d^{+}VEGFR1^{+}CXCR4^{high}$) was identified. Further studies will focus on heterogeneity of the identified neutrophil subsets regarding gene expression profile, ROS-production, phagocytosis efficiency and response to different cues as well as to investigate the presence of this population in humans during conditions where high levels of angiogenesis occur. e.g post exercise, wound healing or menstrual cycle.

Intriguing findings regarding the role of macrophages assisting in blood flow regulation in ischemic muscle was presented. The mechanism will be further investigated by knocking down, iNOS signaling pathways in macrophages and macrophage adhesion molecules and to follow up on possible transitions into vascular smooth muscle cells. The effect of macrophage-assisted blood flow regulation will be studied by optical coherence microscopy in single arterioles *in vivo*.

The finding of increased bioavailability of CXCL12 when delivered by lactic acid bacteria to the wound surface will be followed up by measuring activity of CD26 and other enzymes in the wounds and delineate the role of macrophage phenotype shift in the wounds and its impact on other cell types for accelerated wound closure.

A local immune privilege was created by accumulation of anti-inflammatory immune cells shielding allogenic islets of Langerhans transplanted to muscle. The dynamics of the local rejection process of allogeneic islet grafts in muscle will be studied using state of the art *in vivo* visualization. Also means to prolong the immune privilege using repeated plasmid injections, multiple chemokine encoding plasmids and exploring the effect of other immune regulating cell types will be investigated.

Sammanfattning på svenska

Immunförsvaret är tillsammans med hud och slemhinnor vårt skydd mot patogener och håller oss friska. Det består av celler och proteiner och kommunikation sker med en mängd signalmolekyler. Vid aktivering av immunförsvaret kan många celler snabbt mobiliseras och ett immunologisk minne bildas. Immunceller använder många olika verktyg för att oskadliggöra bakterier och infekterade kroppsegna celler tillexempel fagocytos, sekretion av olika enzymer eller reaktiva syreradikaler men även antigenspecifika molekyler som antikroppar. Immunceller har även tillskrivits funktioner under fosterutveckling, vid vävnadsregenerering efter skada och vid syrebrist/hypoxi.

I detta arbete studeras nya roller för immunceller vid syrebrist och försämrat blodflöde, vid läkning av sår i huden samt vid transplantation av vävnad från en annan individ. Strategier för att använda immuncellers olika funktioner i terapeutiska syften i dessa situationer har undersökts.

Majoriteten av försöken har utförts på Institutionen för Medicinsk Cellbiologi vid Uppsala Universitet och alla försök är godkända av Etiska Nämnden i Uppsala och Uppsala Djurförsöksetiska Nämnd. Rekrytering av immunceller från blod till till vävnad samt ackumulering av immunceller i vävnaden har undersökts vid olika stimuli, tillexempel, VEGF-A, CXCL12, CCL2 och CCL22. Funktionen av de ackumulerade immuncellerna har undersökts i fyra olika modeller *in vivo*: Studie I), syngen tranplantation av hypoxiska Langerhanska öar till muskel, Studie II), återställande av funktionell blodflödesreglering i muskel med inducerat försämrat blodflöde, Studie III) läkning av sår i huden och Studie IV), allogen transplantation av Langerhanska öar till muskel.

De resultat som framkommit som är av störst vetenskaplig betydelse är följande: Neutrofiler i blodet rekryteras till vävnaden av VEGF-A redan efter 30 minuter *in vivo* och för att detta ska ske behövs VEGFR1 på neutrofilens cellyta och VEGFR2 på endotelet som klär blodkärlens insida samt att neutrofilerna kan använda sig av adhesionsmolekylen CD49d. Vi hittade en liten pool cirkulerande neutrofiler i både människa och mus som uttrycker CD49d på cellytan och dessa neutrofiler visade sig också ha höga nivåer av receptorerna VEGFR1 och CXCR4 jämfört med $CD49d^-$ neutrofiler. När CD49d inaktiverades förhindrades de $CD49d^+VEGFR1^+CXCR4^{high}$ neutrofilerna att rekryteras till de hypoxiska Langerhanska cellöarna transplanterade till mus-

kel och därmed kunde inte nya blodkärl bildas på ett effektivt sätt. Detta visar på dessa cellers proangiogena funktioner.

I muskel med försämrat blodflöde samlades en annan immuncell, makrofager, som tar bort död vävnad och medierar återställandet av normalt blodflöde och funktionell reglering av denna. Makrofagerna lade sig tätt kring blodkärlen i muskeln och började uttrycka två protein, aSMA och PDGFR1b som traditionellt ansetts höra ihop med en annan celtyp, pericyter. Utan dessa makrofager kunde muskeln inte återfå sin funktion och blev snabbt helt förstörd. En modell för att mäta maximal blodflödesreglering utvecklades och blödflödesökning vid värmestimulering mättes över tid i bakbenet på möss där femoralisartären ligerats. Denna blodflödesökning är medierad av kväveoxid, NO, och i frisk muskel producerade endotelet NO genom eNOS medan vid ischemi var det främst de perivaskulära makrofagerna som producerade NO genom iNOS. Om kemokinen CXCL12 men inte CCL2 överuttrycktes i muskeln från plasmider i muskelcellerna ökade antalet perivaskulär makrofager och blodflödesregleringen i de ischemiska musklerna blev betydligt bättre snabbare. Dessa resultat är intressanta i ljuset av att det finns flera tillstånd där blodflödesregleringen är störd så som vid diabetes och arteriell sjukdom.

Då vi sett så positiva effekter på blodflödesreglering med CXCL12-behandlingen i ischemisk muskel testade vi om vi kunde påskynda läkning av sår i huden på liknande vis genom att dra nytta av makrofagernas funktioner. För att metoden skulle vara kliniskt relevant designade och producerade vi probiotiska mjölksyrabakterier som själva kunde tillverka CXCL12. Bakterierna producerad CXCL12 under en timme när de de lades i sår inducerade i huden på möss och såren läkte betydligt snabbare jämfört med vanliga mjölksytabakterier eller ingen behandling. Denna sårläkningshastigheten har aldrig tidigare rapporterats. Det visade sig att mjölksyran från bakterierna hade synergieffekter som ökade biotillgängligheten på CXCL12 i såret genom att hämma det enzym, CD26, som annars bryter ner CXCL12. När sår behandlades med CXCL12-producerande mjölksyrabakterier ökande antalet makrofager i huden närmast sårkanten och de uttryckte TGFb, en substans som driver läkningsprocessen. Accelererad sårläkning med CXCL12 producerat i såret av mjölksyrabakterier uppmättes också i möss med försämrat blodflöde i huden och med högt blodsocker. En frystorkad formulering av bakterierna togs fram med bibehållen effekt på sårläkning och konceptet ska testas för första gången på människor under 2017. Kroniska sår som inte läker är ett stort problem i vården idag och målet är att testa den sårläkande effekten av de CXCL12-producerande mjölksyrebakterierna även i kroniska sår.

I studie IV undersöktes om vi kunde dra nytta av antiinflammatoriska egenskaper hos en viss typ av T-cell. För transplantation av organ mellan individer behövs livslång immunosuppremerande behandling, med tillhörande biverkningar, för att undvika avstötning av organet. Ett exempel är

transplantation av insulinproducerande Langerhanska öar till människor med svårkontrollerad diabetes typ I. När öar från en musstam transplanterades till muskel i hyperglycemiska möss av en annan stam utan immunosuppremerande behandling förstördes de transplanterade öarna direkt och ingen effekt på blodsockernivåer kunde mätas. Vi testade ett koncept där antiinflammatoriska T lymphocyter ($CD4^+CD25^+FoxP3^+$) ackumulerades i muskeln dit de allogena Langerhanska öarna transplanterades. De ackumulareade $CD4^+CD25^+FoxP3^+$ T-celler omringade och skyddade öarna under 10 dagar från att bli attackerade och förstörda av mottagarens egna cytotoxiska T-mördarceller. Muskel är ett lovande organ för ötransplantation då öarnas funktion är betydligt bättre om de transplanteras där jämfört med till levern, som görs kliniskt idag. Den snabba avstötningen i muskel har inte rapporterats tidigare. De flersta andra experimentella studier transplanterar öar undet njurkapseln, vilket inte är möjligt kliniskt. Resultaten från Studie IV bidrar till debatten om att finna ett optimalt protokoll för ötransplantation till diabetiker.

Nya och alternativa funktioner hos olika typer av immunceller är ett forskningsområde som växer snabbt tillsammans med antalet nya biologiska immunoterapier, främst riktade mot cancerbehandling men även inom vaccination och autoimmuna sjukdomar. Att kunna amplifiera rätt fuktion hos rätt immuncell vid rätt tidpunkt på rätt ställe i kroppen är fortfarande en utmaning men vi är många som jobbar på att förstå immuncellers fulla kapacitet och testa strategier för att terapeutiskt dra nytta av dem.

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