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The Influence of Donor Lymph Nodes on Immune Rejection and Tolerance in Vascularized Composite Allotransplantation

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Declaration of Authorship

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Bern, January 2019

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Abstract

Vascularized composite allotransplantation (VCA) is the transplantation of multiple tissues together such as skin, bone, muscle and blood vessels. As with solid organ transplantation there is a need for immunosuppression which bears long term risks for the recipient. To avoid immunosuppression and reduce risks, tolerance to the transplant must be established. Natural tolerance is mediated by T regulatory cells (T_{reg}) and deletion of effector T cells. Recent evidence suggests that lymph nodes (LN) and lymph node stromal cells (LNSC) also play a role in tolerance induction. The contribution of LN and LNSC to chimerism and rejection of VCA has not been investigated extensively and understanding of the role of LN and lymphatic vessels is lacking. We examined whether the transfer of donor LN within the VCA induces chimerism, prolongs grafts survival and accelerates lymphatic reconstitution. Furthermore, we investigated the role of LNSC in the alloresponse. This was studied in a rat hind limb transplantation model where the leukocyte populations in the blood, LN, bone marrow (BM), spleen, thymus and skin were identified, and the graft survival was assessed daily. To further examine the role of LNSC we performed mixed lymphocyte reactions in vitro. With this study, we have shown that the transfer of donor LN within the VCA leads to higher chimerism in secondary lymphoid organs (i.e LN and spleen) as well as blood and delays the onset of graft rejection. The higher chimerism in the blood and LN of grafts containing donor LN was lost when rejection occurred, supporting the idea that donor LN transfer is transiently associated with donor cell engraftment and acceptance. Furthermore, we demonstrated that the transfer of donor LN accelerated the lymphatic reconstitution with an increase in lymphaniogenesis and established a directed lymphatic flow towards the recipients draining LN. In vitro experiments demonstrated that LNSC have immunosuppressive functions.

Taken altogether, these results indicate that the transfer of donor LN within the VCA delays rejection and improves graft survival. A possible mechanism by which this is achieved is the increased lymphangiogenesis due to the presence of donor LN and by donor specific immunosuppressive effect of LNSC within the LN environment. Further studies are needed to confirm the immunosuppressive effect of LNSC in vivo.

1 Introduction

1.1 Vascularized Composite Allotransplantation (VCA)

The field of transplantation has advanced quickly in the last years and now includes next to solid organ transplantations also vascularized composite allotransplantation (VCA). This term is used for transplantation of body structures such as limbs that consist of multiple tissues like skin, subcutaneous tissue, muscle, blood vessels and bone marrow. VCA stands in contrast to solid organ transplantation which involves only one organ and one type of tissue. The aim of VCA is to replace loss of function resulting from loss of a limb or reconstruct appearances in case of congenital abnormalities and thereby improve quality of life for the individual. The first successful hand transplant was performed in 1998 by a group in Lyon, France [1] and up to date more than 100 hand/upper extremity and 21 face transplantations have been performed worldwide[2,3]. However, in contrast to solid organ transplantation, VCA is not a life-saving procedure and therefore the consequences of a transplantation must be weighed against the possible benefits. One of the biggest challenges currently are the side effects and risk of malignancy of immunosuppressive therapy after VCA. The drugs that are widely used today are glucocorticosteroids, azathioprine, mycophenolic acid, calcineurin inhibitors, mTOR inhibitors and monoclonal antibodies and with their use the 1-year graft survival has risen to over 90%. However, some of the possible side effects include increased susceptibility to opportunistic infections and impaired wound healing which reduce life expectancy compared to the normal population[4]. Another major drawback of immunosuppressive therapy is the increased risk for malignancy which is higher in transplant recipients than in the normal population and causes 10-47% of the late mortality after transplantation[5]. A reduction of immunosuppression could therefore reduce mortality related to infections or malignancy and increase recipient survival. But with a reduction in immunosuppression the problem of graft rejection would become more prominent since the immune system of the recipient would recognize the transplant as foreign. It would be necessary to induce tolerance towards the transplant, a task that is undertaken by the immune system and its cells.

1.2 Basic Immunology

Before discussing the more specific case of rejection in the context of transplantation it is important to understand the more broader mechanism of how the immune system deals with foreign tissues and antigens. The body has established multiple lines of defence against pathogens such as innate and adaptive immunity. Innate immunity comprises cells that express germline encoded pattern recognition receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs). Some examples of such cells include dendritic cells (DC), macrophages and neutrophils. Signalling via these PRRs leads to activation of innate immunity and either to

direct effector function of innate cells or production of inflammatory mediators and induction of inflammation which helps to clear the pathogen and induce an adaptive immune response[6]. The innate immune response is the initial defence and paves the way for the adaptive immune response which is directed towards more diverse antigens and with a higher specificity. Adaptive immunity mainly comprises T cells with their T-cell receptor (TCR) and B cells with their B-cell receptor (BCR). Those cells recognize antigens in different manners, T cells recognize antigen that is presented via the membrane bound major histocompatibility complex (MHC) whereas B cells recognize antigen via the membrane bound immunoglobulin (Ig) receptor (BCR)[7]. Activation of T cells requires recognition of a specific antigen that is presented to the T cell as an MHC:peptide complex together with co-stimulatory molecules on the surface of antigen-presenting cells (APC) such as dendritic cells (DC). With subsequent proliferation and differentiation, the T cell will become an effector T cell ready for specific antigen recognition. There are two major functional classes of T cells, the CD8 T cells which recognize antigen presented on MHC class I and differentiate into cytotoxic T cells that directly kill infected or non-self cells and CD4 T cells which recognize antigen presented on MHC class II and differentiate into either effector cells such as T_H1, T_H2, T_H17 and T_{FH} or into T-regulatory cells (T_{reg}) which may constrain immunity[8] (Figure 1). Activation of B cells requires binding of the BCR to an antigen and – with some exceptions – the help of T_{FH} which recognize the MHCII:peptide complex expressed by B cells on their surface. Activated B cells differentiate into plasma cells which secrete specific antibodies for the humoral defence[9].

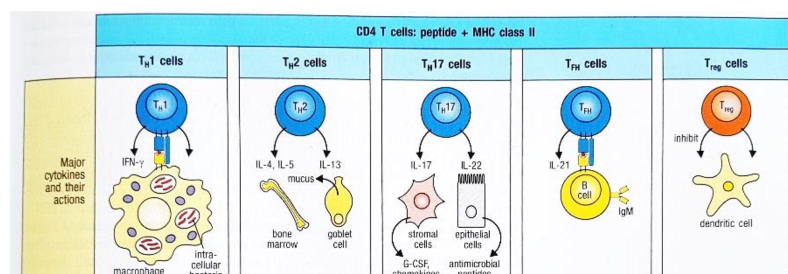


Figure 1: Subsets of CD4 effector T cells. CD4 effector cells enhance functions of other cells in order to kill foreign or infected cells. T_H1 cells produce cytokines which activate macrophages. T_H2 produce cytokines that activate neutrophils, basophils and mast cells and T_H17 cells lead to recruitment of neutrophils. T_{FH} form interactions with naïve B cells and traffic to B-cell follicles where they promote a germinal centre response. T_{reg} cells suppress T cell and innate immune cell activity thereby preventing autoimmunity during an immune response

Figure and text adapted from "Janeway's Immunobiology" 9th edition, Figure 9.30

1.3 Immunology of Organ Rejection

The reason why transplanted organs are rejected is that the immune system discriminates self from non-self tissues and mounts an immune response towards tissues that are recognized as non-self. This recognition of self is acquired during the maturation process of immature T cells, the thymocytes, in the thymus by undergoing positive selection where thymocytes recognize and bind self-peptide:self MHC complexes. Those cells that pass the

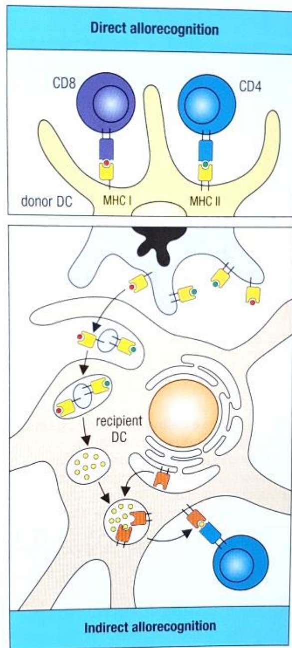


Figure 2: Direct and indirect pathway of allorecognition leading to T cell activation. Donor DC in recipient secondary lymphoid tissues stimulate direct and indirect allorecognition. Upper panel: allogeneic MHCI and II on donor DC interact directly with the TCR of alloreactive CD4 and CD8 T-cells. Lower panel: Endocytosis of alloantigen by recipient DC upon death of donor DC. Peptide sof donor origin (yellow) are presented to CD4 T cells on the recipients MHCII molecules (orange) *Figure and text adapted from "Janeway's Immunobiology" 9th edition Figure 15.49*

positive selection step further undergo negative selection where thymocytes that react strongly with self-antigens are deleted thereby removing self-reactive T cells[10]. Mature T cells emigrating from the thymus are therefore primed to strongly react against any non-self antigen and especially against alloantigen such as donor MHC molecules that are present on donor cells in the transplant.

Three different pathways lead to activation of alloreactive T cells: indirect, direct and semi-direct (Figure 2). The indirect pathway follows a similar mechanism as recognition of pathogenic antigens, namely the presentation of alloantigen (in this case from the donor) on self-MHC (from the recipient) expressed by recipient APC which is recognized by the TCR of alloreactive T cells. The direct pathway involves allo-MHC (from the donor) expressed by donor APC presenting alloantigen (from the donor) to recipient T cells, this kind of activation only appears in transplantation as it relies on the presence of donor APC and donor-MHC. The semi-direct pathway is similar to the direct pathway as it involves alloantigen bound to allo-MHC, the difference being that the allo-MHC is located on autologous APC (from the recipient) rather than APC of donor origins[11,12].

How exactly does transplantation and the presence of foreign tissue induce rejection? Both innate and adaptive immune cells as well as foreign MHC play a role in rejection. First, there is a non-specific innate immune response which involves inflammation mediated by cells of the innate immune system that, via the PRRs, recognize DAMPs which are produced by damaging of tissue during the transplantation and ischemia/reperfusion injury (I/R)[13]. Second, there is a donor-specific adaptive immune response which involves presentation of alloantigen to recipient T cells according to the three pathways described above (indirect, direct, semi-direct). The innate and adaptive response together lead to acute and chronic graft rejection, the former manifests within a week of transplantation whereas the latter can occur years after transplantation.

The main components of acute rejection are chemokines, non-specific effector cells, T cells and the acute humoral response. Chemokines attract immune cells and are important for homing of APC to the LN with subsequent antigen presentation, non-specific effector cells

comprise DC, natural killer cells (NK), macrophages/monocytes and neutrophils and contribute to the tissue damage in acute rejection. T cells differentiate into different subsets and cause direct cytotoxicity in the case of CD8+ T cells or, in the case of CD4+ T cells, help to activate B cells which secrete specific antibodies that take part in humoral rejection and cause graft rejection mainly via the complement system.

During chronic rejection there is intragraft vasculopathy which manifests as thickening of the intima of vessels and a reduction in the lumen which can lead to ischemia of the graft and progressive dysfunction. Activation of endothelial cells due to donor specific antibodies leads to fibroblast activation and deposition of extracellular matrix which causes fibrosis also seen in chronic rejection[14].

1.4 Tolerance Induction

To prevent acute and chronic rejection of a transplant the body must tolerate the foreign tissue instead of mounting an immune response against it. The natural protection of the body against excessive or misdirected T cell responses are T_{reg} cells. T_{reg} cells are a subset of CD4+ T cells that can suppress the activation of reactive lymphocytes. There are two types of T_{reg} cells: natural T_{reg} (nT_{reg}) that are programmed in the thymus and induced T_{reg} (iT_{reg}) that develop in the periphery[15]. The main regulator of T_{reg} cells is the transcription factor FoxP3 expressed by CD4+CD8-CD25+ thymocytes in the thymus as well as by CD4+CD25+ T cells in the periphery. nT_{reg} develop, similarly to T cells, through a process of positive and negative selection in the thymus. However, contrary to T cells, T_{reg} precursors show high affinity for the self-peptide:self-MHC complex and are more readily recruited into the T_{reg} lineage[16]. iT_{reg} develop in the periphery under tolerogenic conditions, they require stimulation of the TCR and presence of cytokines such as IL2 and TGFβ and can affect regulatory function over allo- as well as autoreactive T cells in the periphery[17].

T_{reg} seem to play an important role also in VCA where infiltration of T_{reg} in the skin of a VCA graft of tolerant mice, but not of mice that rejected the skin graft, was reported. This indicates a potential role of T_{reg} in tolerance induction or maintenance both in VCA and in solid organ transplantation[18]. Further evidence shows that T_{reg} cells not only migrate to LN but also to the allograft where they suppress the alloimmune response. In a model of islet transplantation it was found that intragraft and LN homing T_{reg} cells significantly extended graft survival by reducing alloreactive T cells and inhibiting migration of DC from the graft to the draining LN[19]. Furthermore, experiments on heart allotransplantation showed that the transfer of donor T_{reg} cells (passenger T_{reg} cells) within the transplant reduces the recipient autoantibody response and prolongs graft survival. The mechanism proposed by this group was that the passenger T_{reg} cells inhibit host adaptive immune responses[20].

T_{reg} cells, however, are not the only players in transplantation tolerance. One of the best-studied mechanisms of tolerance induction today is mixed allogeneic chimerism (MAC)

achieved by simultaneous transplantation of bone marrow together with the organ. One significant drawback of MAC is the occurrence of graft-versus-host disease (GVHD) where donor immune cells attack the recipients' tissue. There is therefore a need to find other ways of inducing tolerance to avoid this issue and new recent reports focus their attention on the possibility to exploit the lymphatic system and LN to constrain alloreactivity.

1.5 The Role of Lymphatics and Lymph Nodes in Immunity

The lymphatic system is a system that helps the body to maintain tissue homeostasis mainly by regulating the drainage of the protein rich lymph. Lymph is produced in the periphery by ultrafiltration of blood from capillaries into the interstitial space and is taken up into lymphatic vessels to be transported to draining LN[21]. Lymph also transports antigens, antigen-bearing cells and pathogens from the periphery to the draining LN. An important component of the lymphatic system are the LN which are secondary lymphatic organs (SLO) where antigen presentation of DC to T cells takes place [22]. LN are interconnected by lymphatic vessels and they have a specific anatomical structure that is adapted to their function of hubs for antigen presentation and T cell activation. They are divided into a medulla, T cell zone and B cell zone and are surrounded by a lymphatic sinus and a capsule of connective tissue (Figure 3). In the lymphoid follicles of the B cell zone is where the T_H-dependent B cell activation takes place and the T cell zone is where APC reside and present antigen to naive T cells [23].

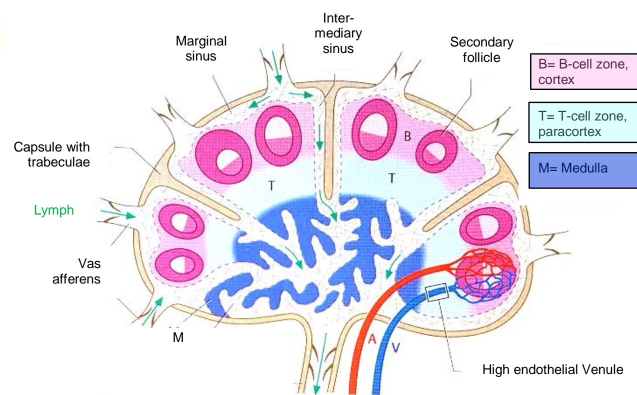


Figure 3: Schematic structure of a LN. Secondary follicles are depicted larger than proportional. *Figure adapted from "Taschenlehrbuch Histologie", Renate Lüllmann-Rauch, 4th edition 2012*

How do LN and lymphatics contribute to the immune response? One important role of the LN is that they offer a place where antigen- bearing DC and T cells can interact and mount an immune response by activation of T effector cells[24]. Another important contribution from the lymphatics is that they serve as conduits for antigens and immune cells which are transported away from the periphery so they do not accumulate and cause infections. It was observed that lymphedema increases the occurrence of infection mainly due to the stasis of lymph containing immune cells and foreign antigen which elicit an inflammatory response in the tissue where the lymph stagnates[25]. In addition, a group found that in a model of impaired cutaneous lymphatic vessels the humoral immune response – a part of the adaptive immune response – was impaired, showing the importance of the lymphatics in mounting an appropriate immune response. This group also showed that the drainage of lymph via the lymphatics plays an essential role in peripheral tolerance, in their model of defective

cutaneous lymphatics they found impaired tolerance to topically applied DNFB (a reagent used to elicit contact hypersensitivity) even with previous treatment with a tolerizing agent. They also found impaired self-tolerance with decreased T_{reg} cell trafficking to LN and development of autoimmunity[26].

1.5.1 The Role of Lymph Node Stromal Cells (LNSC) in Adaptive Immunity and Tolerance

LNSC are a heterogeneous population of non-hematopoietic cells that play a pivotal role in the anatomical organization of LN. Not all LNSC have been identified and characterized yet. The ones that are known are: follicular dendritic cells (FDC) in B cell areas, fibroblastic reticular cells (FRC) in T cell areas, lymphatic endothelial cells (LEC), blood endothelial cells (BEC) and marginal reticular cells (MRC). These cells produce chemokines that help direct lymphocytes within the lymphoid organs and provide structural support and guidance for the immune cells[27].

For a long time, it was believed that the LNSC were only a structural component without active participation in immune reactions. This view has changed as it has been recognized that LNSC participate in adaptive immunity by facilitating DC – T cell interactions, directing antigen distribution to T cells and promoting APC and DC entry into LN as well as by providing structure for efficient immune cell interactions[28]. This important role of LNSC in the immune response was further proven by the observation that knock-out models for LNSC subsets presented decreased lymphocyte numbers, disrupted LN structure and impaired adaptive immune function. More recently, a more direct influence on immunity by LNSC was proposed. Several groups have shown that LNSC may present antigens on MHC-I and MHC-II to promote peripheral self-tolerance. LNSC can present self-antigen in the form of peripheral tissue antigen (PTA) on MHC-I but they can also cross-present exogenous antigen on MHC-I or MHC-II which they acquire by active uptake similar to professional APC[29]. Therefore, LNSC may influence both CD4 and CD8 activation and are currently investigated as promising players in tolerance induction.

1.5.2 CD8+ T cell Tolerance

The expression of PTA on LNSC is mainly regulated by the Aire gene (autoimmune regulator gene), which also regulates expression of self-antigen on thymocytes in the thymus and is effected via MHC-I[30]. LNSC are extra-thymic Aire-expressing cells (eTAC) located in peripheral LN that serve as an additional mechanism to reduce autoreactive T cell generation[28]. Similarly to what has been described in the thymus, it was shown that eTAC also promote peripheral tolerance by deletion of self-reactive CD8+ T cells indicating that LNSC are a player in peripheral tolerance[31].

1.5.3 CD4+ T cell Tolerance

Notably, LNSC not only express MHC-I but also MHC-II via which they can influence CD4+ T cells[32]. The expression of MHC-II on LNSC is regulated by the regulatory factor CIITA (class II transactivator) but the MHC-II can also be acquired from DC by MHC-II transfer. Presentation of self-antigen on the MHC-II subsequently induces CD4+ T cell apoptosis and thereby tolerance to the corresponding self-antigen[33].

All together, these findings lead to the conclusion that LNSC are important for maintaining self-tolerance by keeping autoreactive T cells in check thereby complementing the thymus in its role of establishing central tolerance to self-antigens.

1.6 The Lymphatic System in Transplantation

Lymphatic vessels and networks serve as conduits for antigens and regulate the movement of antigens from the tissue towards LN where tolerance or rejection induction can take place. Upon transplantation the lymphatic network is interrupted, whereby flow of lymph containing immune cells and antigens from the periphery to the recipient LN is impaired. However, due to the inflammation process triggered by the surgical intervention and ischemia reperfusion injury, the process of lymphangiogenesis (i.e generation of new lymphatic vessels from pre-existing ones) is rapidly started. A crucial factor for lymphangiogenesis is the presence of inflammatory factors such as TNFα, which induce VEGF-C (vascular endothelial growth factor C), the main cytokine driving lymphatic vessel growth via the VEGFR3 (VEGF receptor 3). In addition, macrophages at sites of inflammation are equally able to produce VEGF-C and contribute to lymphangiogenesis[34]. Evidence suggests that lymphangiogenesis in solid organ transplantation promotes rejection due to infiltration of inflammatory cells and alloantigen drainage to recipient LN with an increase of the allogeneic response[35, 36, 37, 38].

However, more recently a role of LN and lymphatic vessels in transplantation tolerance has been proposed. It is known that in the case of tumours, lymphatic vessels are crucial in the establishment of an impaired anti-tumour immune response since they transport tumour-specific antigen to the LN where T cells against the tumour can be primed[39]. Moreover, there is evidence that lymphangiogenesis and the presence of lymphatics can ameliorate allograft rejection also in the case of solid organ transplantation. Recently, it has been demonstrated that in lung transplants the density of lymphatic vessels decreased during acute rejection causing accumulation of metabolites and T cells in the graft and thus inflammation and graft rejection[40]. It was also found in a liver transplant model that increase in lymphatic vessels help resolve cellular rejection and have a beneficial effect on graft tolerance[41]. Clinical observations in renal and heart allografts showed that increased lymphatic vessel density correlated to less rejection and better graft survival[42,43]. This shows the binary role of LN and lymphatic vessels in solid organ transplantation – on one hand they promote graft rejection by increasing alloantigen presentation and immune

cell migration and on the other hand they may reduce inflammation by more efficient lymph clearance and regulation of the immune system.

In contrast to solid organ transplantation, the role of lymphangiogenesis in VCA is less clear. Lymphatic reconstitution was observed in face transplants where donor and recipient lymphatic vessels reconnected but the effect on the immune response was not investigated. Importantly, since the lymphatic system not only influences antigens and APC but also T_{reg} cells, it is possible that it plays a role not only in rejection but also in tolerance. However, this possibility has so far not been investigated systematically in VCA.

1.7 Aims

In order to advance with the success of VCA it is important to find ways to decrease rejection and prevent graft loss. There are different ways to address this issue, a possible mechanism being the induction of tolerance. There is increasing evidence that lymphatics and LN play a role in rejection and tolerance, but their role has not been investigated in VCA. Therefore, in this study we will investigate the effect of LN transfer on the acute rejection process after VCA using a fully MHC mismatched rat hind limb transplantation model. More specifically, we will verify whether the transplantation of donor LN within the VCA graft can influence the alloresponse and prolong graft survival without administration of immunosuppressive drugs. Furthermore, we will investigate the development of lymphatic vessels in the graft after transplantation in order to better characterize the process of lymphatic reconstitution as well as reconnection between donor and recipient lymphatics and how this process influences graft rejection. In addition, we will examine the influence of LNSC on proliferation of lymphocytes of recipient origin in an in vitro model.

2 Materials and Methods

2.1 Overall Study Design

The Brown-Norway (BN) to Lewis (LEW) rat hind limb transplantation model was used to test whether the transfer of donor LN including the LNSC within the hind limb allograft would influence rejection of the transplanted limb and/or affect chimerism levels. 8 LEW rats received grafts with intact inguinal and popliteal LN and 8 rats received lymphadenectomized grafts. These two rat strains were used because of a complete MHC-class mismatch to provoke acute rejection in absence of immunosuppression. After transplantation, rats were observed, and rejection was graded macroscopically until full rejection (grade 3 rejection, see below) was reached at which point the rats were sacrificed. Whole blood was sampled at post-operative day 7 (POD7) from the sublingual vein and at rejection (endpoint) from the heart during the sacrifice. At the endpoint other organs were sampled as well, namely the LN, BM and skin from the transplanted and contralateral limb as well as spleen and thymus. These organs were characterized by means of flow cytometry. Special attention was payed

to leukocytes of donor origin to determine chimerism of the graft. To assess the lymphangiogenic process and reconnection of the lymphatic system to the recipients draining basin the rats underwent daily lymphography with near-infrared imaging starting from POD2. For this purpose the transplanted leg was injected with a fluorescent dye that is taken up by the lymphatic conduits and drains into the LN.

In addition, to assess the effect of LNSC on leukocytes in vitro, a mixed lymphocyte reaction (MLR) was performed in the presence of LNSC from LEW or BN rats. The goal was to measure the amount of T_{reg} cells and the proliferation of monocytes isolated from peripheral blood of naïve LEW and BN rats by means of flow cytometry and staining with a fluorescent dye.

2.2 Rat Hind Limb Allotransplantation

The hind limb transplantation was performed as previously described [44]. Briefly, 8 inbred male BN (donor) and 16 inbred male LEW (recipient) rats aged 8-12 weeks were purchased from Charles River. Before the transplantation buprenorphine (50µl/kg) was administered as an analgesic and anesthesia was induced with 5% isoflurane in pure oxygen and maintained with 1.5% isoflurane. During the operation rats were kept at body temperature by a heat pad and an ophthalmic ointment was applied to prevent the eyes from drying out. The BN rats (donors) received 300 IU of heparin intravenously to reduce blood coagulation.

The hindlimb was amputated at mid-femur level, the artery and vein were sharply divided close to the inguinal ligament and kept at 4°C in a gauze wetted with saline. The LEW rats (recipient) were prepared by amputating the corresponding hindlimb at the same level. A blunt 18 Gauge needle was used for osteosynthesis and the femoral vein was anastomosed by cuff technique as previously described[45]. The femoreal arteries were anastomosed using an end-to-en interrupted suture technique using 10.0 suture. After vascularization, muscles and skin were sutured with 4/0 resorbable sutures. Nerves were not anastomosed. In the hindlimb including the donor LN the inguinal fatpad containing the LN from the BN rat (donor) was transplanted together with the leg to the LEW rat (recipient) whereas in the hindlimb without LN from the donor the inguinal fatpad and popliteal LN from the BN rat were removed, and the leg transplanted without donor LN. The rats were closely monitored after the surgery and once awake kept in cages with their litter mates where they had access to food and water ad libitum. After the surgery all the rats were checked daily and a rejection score for the transplanted leg as well as a well-being score was determined based on the following criteria:

Table 1. Graft Rejection Evaluation in Rat Hind-Limb Transplantation

SCORE	SIGNS (SEQUENTIAL EVENTS)*	LEVEL OF REJECTION
0	☐ Normal	No rejection: Grade 0

2	☐ Epidermolysis ☐ Exudation ☐ Desquamation	Moderate rejection: Grade 2
3	☐ Eschar formation ☐ Necrosis ☐ Mumification	Severe Rejection: Grade 3

Rats with score 1 will be monitored daily until rejection is resolved or progress to the next levels
Rats with score 2 will be monitored every 12 hours until rejection is resolved or progress to the next levels
Rats with score 3 will be sacrificed by injecting 150 mg/kg (i.p.) pentobarbital

***These signs are not causing pain in rats**: Pain and sensory recovery have been evaluated in rat hind limb transplantation model using cutaneous pain stimulation test and walking track analysis. Results revealed that sensory recovery was near zero until one month, negligible at three months and 68% after one year. In line with this finding, it has been also reported that a well-healed allograft is not capable of generating a considerable degree of pain reaction after three months.

Adapted from: Min, Z., and N. F. Jones. 1995. Limb transplantation in rats: immunosuppression with FK-506. The Journal of hand surgery 20:77-87.

2.3 Groups

To assess the effect of LN, LNSC and lymphangiogenesis on rejection of VCA the recipient rats (LEW) were divided randomly into 2 groups of 8 rats each as calculated by power analysis. For both groups the recipients (LEW) LN were left intact. In the first group (D+) the donors' inguinal and popliteal LN were left intact and transferred with the transplanted leg to the recipient. In the second group (D-) the donor leg was lymphadenectomized by removing the fatpad (containing the inguinal LN) and the popliteal LN.

Table 2. Characteristics of the Graft Groups

Group	*Lymphatic System and LN*
D+	Recipient LN intact, donor LN (in fatpad and popliteal area) transplanted with leg to recipient
D-	Recipient LN intact, donor LN removed from leg of donor (transplanted without fatpad nor popliteal LN)

N= 8 rats in each group

2.4 Endpoint – Sacrifice of Rats

Once Grade 3 rejection was reached (as determined by the criteria in Table 1) the rats were anesthetized (induction with 5% isoflurane, maintenance with 2% isoflurane) and prepared for organ collection. The inguinal LN, skin, muscle tissue and tibial bone were collected for both the transplanted and contralateral side. Furthermore the spleen and thymus were

collected in 1x phosphate buffered saline (PBS) and the whole blood was obtained by puncturing the heart using an 18G syringe and EDTA coated tube.

The organs were then stored in formalin (for histology), cryosectioning medium (for cutting and Immunofluorescence) and liquid nitrogen or processed according to different protocols to isolate the leukocytes.

2.5 Digestion of Organs to Isolate the Cells

***Lymph Nodes*:** To help with digestion the LN were pierced with tweezers to break the outer membrane. Then they were placed in an 2ml Eppendorf tube with an enzyme mix containing culture medium (DME/F-12 from Sigma – Ref:D6421 with 10% HI FBS from Seraglob – Ref:S40500 with 1% L-glutamine from Gibco – Ref:25030-024 and 1% Penicillin/Streptomycin from Gibco – Ref:15140-122), collagenase P (final concentration 0.2 mg/ml), dispase I (neutral protease from Roche – Ref:04942086001, final concentration 0.67 mg/ml) and DNAse (from Sigma – Ref:DN25, final concentration 0.1mg/ml). The Eppendorf tube was put on a hot plate at 37°C and vortexed regularly to help with digestion and release of leukocytes. After 20 minutes the supernatant was transferred to a collection tube and fresh enzyme mix was added to the remaining undigested LN. This step was repeated 3 times. The enzyme mix containing the leukocytes was centrifuged and washed, the pellet was used for the flow cytometry staining. The LN from the transplanted side were digested separately from the LN from the contralateral (control) side.

Skin: A piece of skin of approximately 1cmx1.5cm was retrieved from the transplanted leg and minced into small pieces with scissors. The pieces were placed in a C-tube (Miltenyi Biotec – Ref:130-093-237) with a digestion solution containing DMEM, 10% HI FBS, Collagenase D (1mg/ml) and DNAse (200 μm/ml). The C-tube was placed in a dissociator (gentle MACS, Miltenyi Biotec – Ref:130-093-235) set to a program for skin digestion. At the end of the dissociation the tube was incubated for 1h at 37°C in agitation. The cell suspension was strained and centrifuged, the pellet re-suspended and layered on top of Lymphoprep Separation Media (Stem Cell Technology – Ref:07801). After centrifugation the lymphocyte cell layer was transferred to a 2ml Eppendorf, washed and the pellet kept for staining.

Bone: To obtain the BM the ends of the tibia were cut, and the bone was flushed repeatedly with PBS using a syringe with a blunt tip 18G needle. The cell suspension was filtered through a 70nm strainer, centrifuged and the pellet was kept for staining. ***Spleen and Thymus:*** the organs were separately crushed by smashing them through a 70nm strainer with a syringe plunger and washing with PBS. The cell suspension was centrifuged, and the pellet kept for staining.

Blood: Before processing whole blood was analysed with a blood analysing machine (Sysmex – Ref:KX-21N BO516) to determine absolute numbers of leukocytes per millilitre of blood. Plasma was then separated from whole blood by centrifugation, stored and replaced with the same amount of PBS. 100µl of blood was put into an 2ml Eppendorf tube for isolation of cells. Erythrocytes were lysed with erythrocyte lysis buffer (eBioscience – Ref:130-094-183, 10X diluted for final concentration of 1X), centrifuged and supernatant was discarded and then washed with PBS. The pellet was kept for staining.

2.6 Staining of Cells and Flow Cytometry Analysis

In general, flow cytometry measures cell characteristics by means of fluorescent labels specific to proteins on single cells. Cells are labelled with antibodies conjugated to a fluorescent dye which binds to surface proteins such as cluster of differentiation (CD) or intracellular molecules. The fluorochromes on the antibodies are excited by laser light of a certain wavelength in the flow cytometry machine and the fluorescent signal emitted is detected by the machine. The light that is emitted has a certain wavelength ("colour") and gives information about the antibody bound to the surface and thereby about the nature of the cell. By using a combination of fluorochromes that bind different proteins present on different cells multiple cells in one sample can be characterized[46].

We use a specific antibody panel put together to label leukocytes, more specifically T cells and T_{reg} cells as well as distinguish between donor and recipient cell origin to determine the chimerism.

Table 3. Characteristics and Amounts of Antibodies used for Staining of Cells

Antibody	*Fluorochrome*	*Target Protein*	*Quantity used per sample (µl) and Reference*
CD3	PerCp	TCR associated protein	0.5 (Miltenyi Biotec – Ref:130-102-674)
CD4	APC-Cy7	CD4 receptor	0.25 (BioLegend – Ref:201518)
CD8	PE-Cy7	CD8 receptor	2 (Miltenyi Biotec – Ref:130-102-685)
CD25	FITC	IL2 receptor alpha chain (ILR2RA)	2 (BioLegend – Ref:202103)
CD31		PECAM	0.25 (eBioscience – Ref:50-0310-82)
CD45	AlexaFluor700	CD45 receptor-type protein tyrosine phosphatase	2 (BioLegend – Ref:202218)
Helios	PE-Vio615	Helios protein (hematopoietic specific transcription factor)	1 (Miltenyi Biotec – Ref:130-112-636)
FoxP3	Pacific Blue	FoxP3 transcriptional regulator	0.5 (eBioscience – Ref:48-5773-82)
RT1Ac	PE	Rat MHC class I	2 (AbD Serotec – Ref:MCA156PE)

Live cells are distinguished from dead cells with Viobility fixable dye (Miltenyi Biotec – Ref:130-109-816) which binds to intracellular unfixed proteins, strongly staining cells with a damaged cell membrane (i.e dead cells). The flow cytometry staining was used to characterise cell populations in the LN, skin, BM, spleen, thymus and whole blood. After the cells were isolated from the different organs as described above, the procedure for the staining was the same for all the organs including blood. Due to the lower number of leukocytes isolated from skin only half the amount of antibody was used in this tissue. First, the fixable viability dye was added to the cell mix and incubated 15 minutes at 4°C. Cells were washed and 8.5 µl of the antibody mix containing all the antibodies from the table 3 – except FoxP3 and Helios – were added to the Eppendorf tubes (only 4.25µl for the skin tubes). The tubes were incubated at 4°C in the dark for 15 minutes and a fixation/ permeabilization solution (eBioscience, 1X Fixation/Permeabilization concentrate – Ref:00-5123-43 diluted in Fixation/Permeabilization diluent – Ref:00-5223-56) was added to the tubes at the end of the incubation. Then the tubes were incubated for 30 minutes at room temperature (RT) in the dark. The cell suspension was washed with permeabilization buffer (eBioscience, Permeabilization Buffer 10X – Ref:0-8333-56, working dilution 1X) and the two antibodies FoxP3 Pacific Blue and Helios PE-Vio615 were added to the cell suspension and incubated at RT for another 30 minutes in the dark. The cell suspension was washed with permeabilization buffer and cells were kept at 4°C in the dark until flow cytometry analysis. The flow cytometer used for the analysis was the LSRII/SORP (BD Bioscience) and all data were analysed with the FlowJo software. Gating strategies for all the organs are in the supplementary data (Supplementary Figures 1-8)

2.7 Lymphography

Daily lymphography was performed from POD2 and under inhalation anaesthesia (1.5% isoflurane) using the near-infrared imaging system Visionsense™ VS3 Iridium (Medtronic). Before acquisition, 3µl of the fluorescent dye indocyanine green (ICG, Verdye Diagnostic Green – Ref:25DE05601, 5mg/ml in ultrapure water) were injected in the foot pad of the transplanted leg and light pressure was applied to the foot to help with lymphatic uptake. During the following 15 minutes the leg was filmed with the Visionsense™ VS3 Iridium and pictures were taken every 5 minutes for 15 minutes to capture any lymphatic drainage of the dye in the leg, the crossing of the suture line between the donor and recipient was of special interest. The pictures of all the rats were analysed to find the exact day at which crossing of the suture line could first be observed, which served as an indicator for a connection between the donor and recipient lymphatic system. The time until crossing was compared between the two groups using GraphPad Prism 7.

2.8 Immunofluorescence

Immunofluorescence staining is used to visualize specific structures or cells within a tissue using a fluorescent dye bound to an antibody. This antibody can either directly bind the structure or cell of interest in the tissue (so called direct label) or an intermediary antibody that is bound to the structure or cell of interest (so called indirect label). Excitation of this dye in a fluorescent microscope gives off a signal which is measured and can be used for quantitative analysis. In this study, the lymphatic vessels in the skin were of special interest. For this reason, a lymphatic vessel specific antibody was used to stain skin samples retrieved from the graft: Mo mAb anti-Podoplanin (Merck, IgG1k – Ref:MABT850) which binds to podoplanin expressed on lymphatic vessels. Skin samples were cut into approximately 1x1.5cm pieces and frozen in cryosectioning medium for cutting with a cryotome (5µm thick sections), slides with samples were kept at -20°C. Before staining, they were laid out to dry and fixed with acetone for 10 minutes then re-hydrated with 1x Tris buffered saline TBS (prepared from 10X TBS) and tissues were circled with a Dako pen (Dako, cat. s-2002) followed by blocking with 3% BSA-TBS for 1 hour at RT. The ideal antibody concentrations were established with a trial staining, for the final staining a dilution of 1:200 was used for the primary antibody anti-Podoplanin. The antibody was diluted in TBS-PBS 1%-BSA added on top of the tissue and incubated at 4°C over night. The next day, the slides were washed with 1xTBS and the secondary antibody for anti-Podoplanin (goat anti-mouse Alexa fluor 546 – Ref:A11030) together with DAPI (dilution of 1:1'000) was added diluted 1:500 in TBS-PBS 1%-BSA and incubated for 90 minutes at RT in the dark. After washing, the slides were mounted using glycergel (Dako, Ref:C0563). Images were acquired with the LCI LEICA DMI4000 B.

2.9 ELISA – Enzyme-linked Immunosorbent Assay

To determine the amount of VEGF-C present in the skin and plasma an ELISA was performed using the Rat VEGF-C Platinum ELISA kit (thermofisher – Ref:BMS626/2TEN). First, the protein was extracted from snap-frozen skin. For this, the skin was minced, added to an M-tube containing a lysis buffer (RIPA buffer, Sigma – Ref:R0278 with protease inhibitor cocktail, Sigma – Ref:P8340) and the M-tube was placed in a dissociator (gentle MACS, Miltenyi Biotec – Ref:130-093-235) set to a program for protein extraction. The samples were then sonicated and centrifuged, the supernatant containing the proteins was removed and protein content was quantified for the skin as well as the plasma. To quantify the protein in skin a 1:2 and 1:5 dilution of the protein extract was prepared and added to a 96 well plate. A standard dilution series was prepared in duplicate on the same plate using albumin standard (ThermoScientific – Ref:23209, concentration of 2 mg/mL). To 5µl of sample and the standards, the reagents A and B (BioRad – Ref:500-0113 and 500-0116) were added and incubated for 15 minutes at RT on a shaker. The optical density (OD) was

measured at 750nm (Tecan reader, Ref:30034301) and analysed using the Graphpad Prism software. For the ELISA approximately 1.5 mg of protein for the skin samples and 1:2 diluted plasma was loaded per well. A standard dilution was performed starting from 3000pg rat VEGF-C per ml down to 47pg per ml. Before use, the ELISA plate was washed with wash buffer and then the sample and biotin-conjugate (1:100 dilution) were added and incubated at RT for 2 hours on a microplate shaker set to 400 rpm. The plate was washed and streptavidin-HRP (1:100 dilution) was added and incubated for another 1 hour at RT on a microplate shaker set to 400rpm. The plate was washed again, TMB substrate solution was added to all the wells and colour development was monitored. Once the highest standard developed a dark blue colour the reaction was stopped by adding stop solution and the OD was measured at 450nm using a spectro-photometer (Tecan infinite M1000, Ref: 30034301)

2.10 Bioplex

Protein concentration in skin and plasma was calculated as described above (see ELISA – Enzyme Linked Immunosorbent Assay). The ProcartaPley Multiplex Immunoassay kit (Invitrogen – Ref:PPX-15-MX7DPVA) was used to measure 15 different cytokines in skin and plasma. Plasma samples were diluted 1:2, skin samples were diluted so that approximately 1.5mg of protein were loaded per well. The standard was prepared according to the user guide, the plate was prepared by adding the magnetic beads and washing with wash buffer. To each well sample type-specific buffer was added with the sample – 25µL for plasma samples, 20-40µL of sample for skin samples– and the plate was incubated at RT for 1.5 hours on a shaker set to 500 rpm. The plate was again washed, and detection antibody was added followed by an incubation of 30 minutes at RT on a shaker set to 500 rpm and another wash. Streptavidin-PE (SAPE) was added to all wells, incubated as before and washed after which the plate was prepared for analysis with the Bio-Plex 3D suspension array system (Biorad) by adding reading buffer to each well, incubating 5 minutes as before and reading the plate. Setup was according to the user guide.

2.11 Modified Mixed Lymphocyte Reaction (MLR)

To assess the effect of LNSC on the proliferation of recipient (LEW) lymphocytes a modified MLR with cells from naïve BN and LEW rats was performed in vitro. The cells of interest were: peripheral blood mononuclear cells (PBMC) from LEW rats (recipients) as responder cells, splenocytes from BN (donors) and Wistar (third party control) as stimulator cells and LNSC isolated from a mix of LN retrieved either from LEW or BN rats. To obtain LNSC from a mix of LN, the LN were digested as described above (see digestion of organs to isolate the cells – lymph nodes) but instead of leaving the pellet for staining it was resuspended in culture medium (DMEM/-12 with 10% HI FBS, 1% P/S and 1% L-glutamine), cultured in flasks at 37°C and kept until confluent. One day after seeding, the medium was changed to

wash away all cells except the LNSC which were attached to the flask by that time. For the MLR 3 different interactions were realized: LEW PBMC without splenocytes, LEW PBMC with BN splenocytes and LEW PBMC with Wistar splenocytes. This interaction was tested in presence of either LEW or BN LNSC and without LNSC as a negative control (see Figure 4). The MLR was performed in a 48 well plate in which first the LNSC from a primary culture were seeded into the wells with a density of $3x10^5$ cells/well and left to attach overnight. Then the stimulator and responder cells were prepared as follows: stimulator cells (from BN and Wistar) were thawed and washed. Both stimulator cells were then irradiated (GammaCell 40 extractor, Theratronics) at 3000cGy, washed, counted and re-suspended in MLR medium (DMEM/F-12 plain medium with 0.05mM final concentration of 2-mercaptoethanol, Gibco – Ref:31350-010) for a final concentration of $3x10^5$ cells/100µl. Responder cells (LEW PBMC) were thawed and washed. To track the proliferation of the responder cells they were stained with the proliferation tracker CellTrace CFSE (eBioscience – Ref:65-0850-85, final concentration of 5µM). After staining the cells were washed, counted and re-suspended in MLR medium for a final concentration of $3x10^5$ cells/100µl. Responder (LEW PBMC) and stimulator (either BN or Wistar splenocytes) cells were added in a 1:1 ratio into each well, including wells containing the LNSC ($3x10^5$ repsonders:$3x10^5$ stimulators: $3x10^5$ LNSC). The plate was incubated for 4 days at 37°C and 5%CO_2. On the 5th day the cells were collected for flow cytometry analysis.

The cells were stained with fixable viability dye, CD4 APC-Cy7 (Bio Legend – Ref:201518), CD8 PE-Cy7 (Miltenyi Biotec – Ref: 130-102-685), CD3 PerCP (Miltenyi Biotec – Ref: 130-102-674), FoxP3-A450 (v- eBioscience – Ref: 48-5773-82) and Helios-PE (Miltenyi Biotec – Ref: 130-104-001). The FACS was acquired with the flow cytometer LSORPII and the data were analysed with the FlowJo software.

	1	2	3	4	5	6	7	8	9
	Negative control			Test			Positive control		
Lewis LNSC test	Lewis LNSC **Responder cells (Lewis T cells)** **MLR medium**			Lewis LNSC **Responder cells (Lewis T cells)** **BN stimulator**			Lewis LNSC **Responder cells (Lewis T cells)** **Wistar stimulator**		
Control	**Responder cells (Lewis T cells)** **MLR medium**			**Responder cells (Lewis T cells)** **BN stimulator**			**Responder cells (Lewis T cells)** **Wistar stimulator**		
BN LNSC test	BN LNSC **Responder cells (Lewis T cells)** **MLR medium**			BN LNSC **Responder cells (Lewis T cells)** **BN stimulator**			BN LNSC **Responder cells (Lewis T cells)** **Wistar stimulator**		

Figure 4: Experimental Set-up for MLR. Table showing the cell origins and population going into every well in the MLR

3 Results

3.1 *In Vivo – Graft Survival and Rejection*

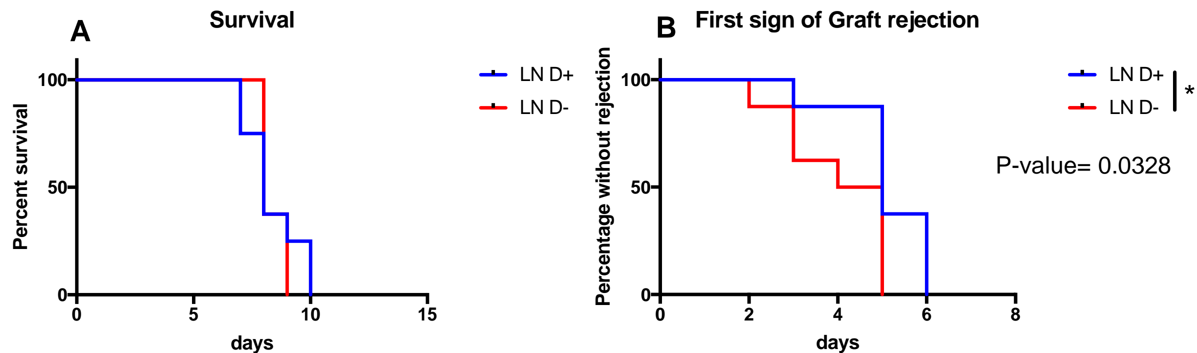


Figure 5: A. Graft survival of donor LN depleted grafts (D-) and grafts containing donor LN (D+). Grade of rejection defined as: 0= no rejection; 1= erythema, edema, hair loss; 2= epidermolysis, exudation, desquamation; 3= eschar formation, necrosis, mummification. Grade three rejection was considered as definitive loss of the graft and represented the endpoint of the experiment. Graft survival represented with Kaplan-Meier curve **B. First occurrence of graft rejection and progression for donor LN depleted grafts (D-) and grafts containing donor LN (D+).** The time until appearance of the first sign of rejection (i.e Grade 1) represented with Kaplan-Meier curve, *p-value <0.05 by Mantel-Cox test

In order to investigate the effects of LN transfer on VCA survival, graft survival of rats receiving hind limbs depleted of donor LN (D-) was compared to graft survival of hind limbs containing donor LN (D+). There was no significant difference in the graft survival between the D+ and the D- group as shown in Figure 5A. However, when looking at the rejection free period (Figure 5B) there was a significant difference (*p-value= 0.0328) between the two groups. This is measured as first sign of rejection which is defined as the day the graft presents with grade 1 rejection. Grade 1 rejection as well as grade 3 rejection was observed one day earlier in the D- compared to the D+ group (mean first day of rejection D- group= 4.5, D+ group= 5).

3.2 *In Vivo – Effect of Lymph Node Transfer on the Immune Compartment*

The effect of inguinal LN transfer within the graft on immune cells was measured in peripheral blood on post-operative day 7 (POD7) and in blood from the heart, in inguinal LN (transplant or contralateral side), BM (transplant or contralateral side), spleen, thymus and skin (transplant or contralateral side) at endpoint (i.e Grade 3 rejection).

Peripheral Blood

The absolute white blood cell counts as well as the amount of donor leukocytes in blood in the D+ and D- group showed no difference. There were significantly more donor monocytes and donor T_{reg} cells in the D+ than the D- group as well as a significantly higher percentage of T_{reg} cells (including both donor and recipient T_{reg}) among the T_{helper} cell population for the D+ group at POD7. However, this difference was only observed in the frequency but not in absolute number. Furthermore, the absolute number of T_{reg} cells of donor origin in the D+ was significantly higher than in the D-. This indicates that the presence of donor LN increases the amount of donor T_{reg} cells and influences the number and composition of leukocytes in the blood of the recipient (Figure 6).

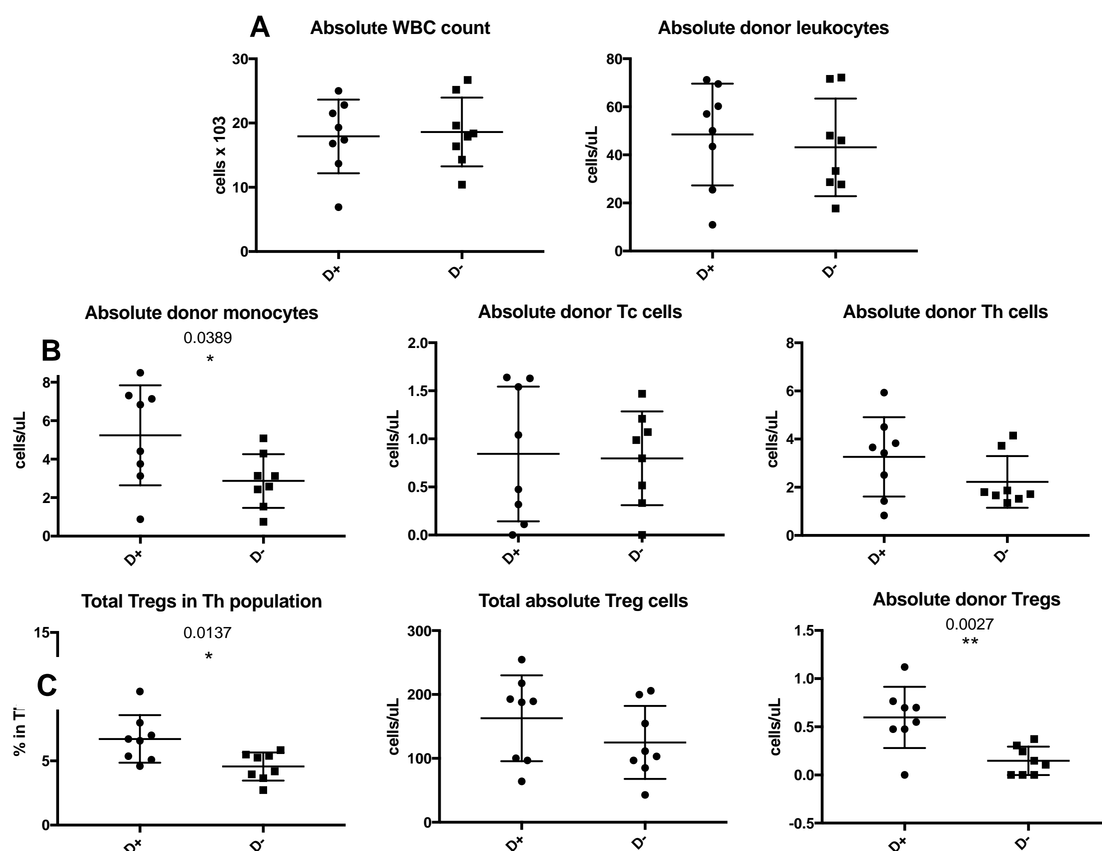


Figure 6: Effect of donor LN transfer on the immune compartment in recipient blood at POD7. Whole blood was stained with antibodies specific for CD3, CD4, CD8, CD25, CD31, CD45, FoxP3 and RT1Ac at post-operative day 7 (POD7). The samples were analysed by flow cytometry as described in the methods **A.** Total amount of white blood cells (WBC) in the whole blood (expressed as cells x 10^3) and cell count of donor leukocytes per microliter of blood. **B.** Evaluation of chimerism by absolute number of donor cells ($RT1Ac^+$, expressed as cells x 10^6/µL) comparing D+ to D- group. **C.** Percentage of T_{reg} cells ($CD45^+CD3^+CD25^+FoxP3^+$) in T_{helper} cell population ($CD45^+CD3^+CD4^+$) as well as absolute number of recipient and donor T_{reg} cells ($CD45^+CD3^+CD25^+FoxP3^+RT1Ac^+$). *p-value <0.05 by unpaired students t-test. All data as mean ±SD

Interestingly, when blood at POD7 was compared to blood at endpoint there was a significant decrease in the number of donor immune cells for the D+ group which was not observed in the D- group (Figure 7), indicating a change in cell composition of the blood during rejection. Moreover, the high percentage of T_{reg} in the T_{helper} population decreased significantly in the D+ group at the endpoint compared to POD7. This shows that chimerism is only increased transiently in the blood.

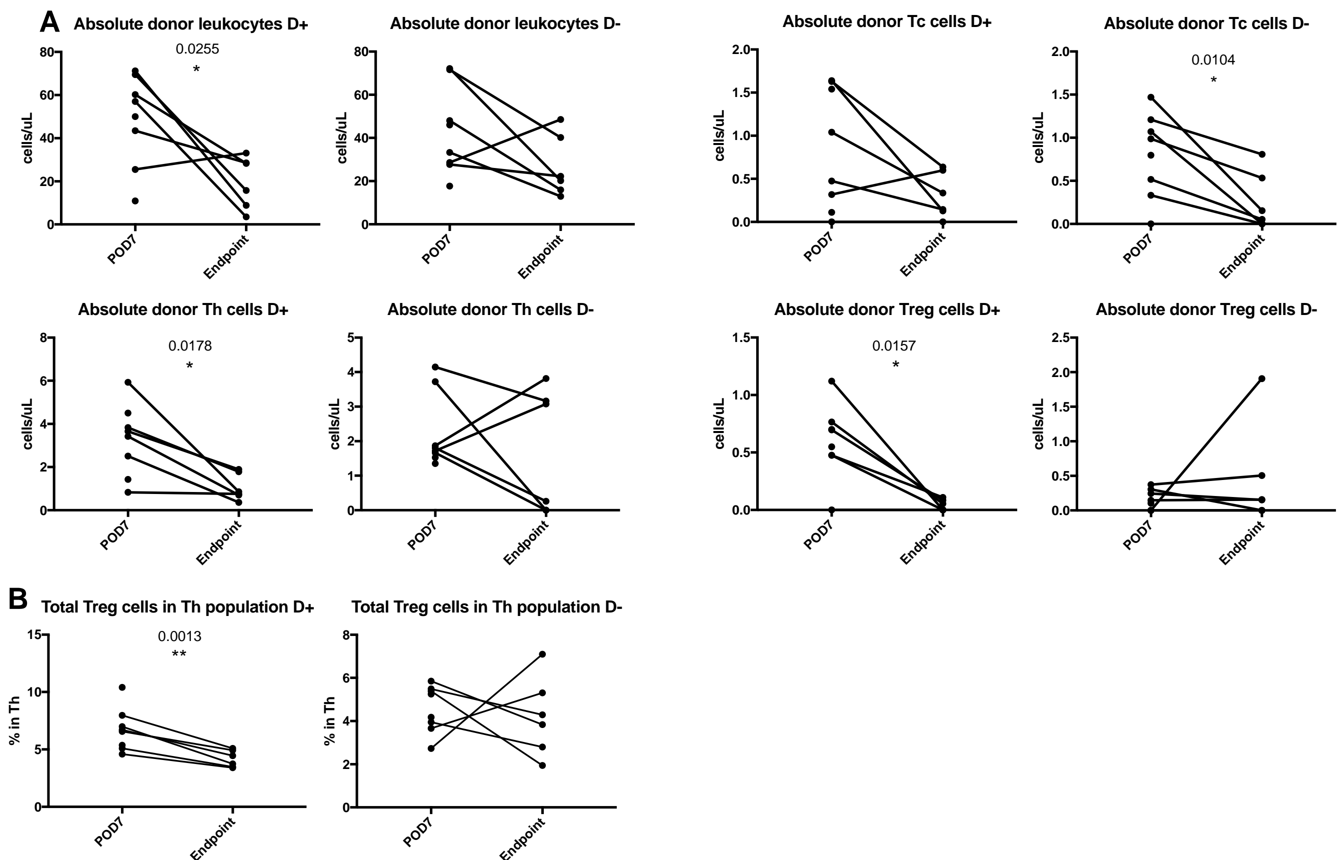


Figure 7: Change of donor cells from POD7 to rejection in peripheral blood
A. Blood on POD7 was compared to blood at endpoint to see changes in leukocyte population during rejection for the grafts containing LN (D+) and depleted of LN (D-) **B.** Change of percentage of T_{reg} cells in T_{helper} cells from POD7 to endpoint for total amount of T_{reg} cells (recipient and donor origin) *p-value <0.05 **p-value <0.01 by paired students t-test.

Lymph Nodes

Analysis of the LN showed no difference in the recipient leukocyte composition between the D+ and the D- group (supplementary Figure 9). There was a general trend but no significant difference towards more donor cells in the LN retrieved from the graft of the D+ group for all the other measured leukocyte populations including stromal cells (Figure 8).

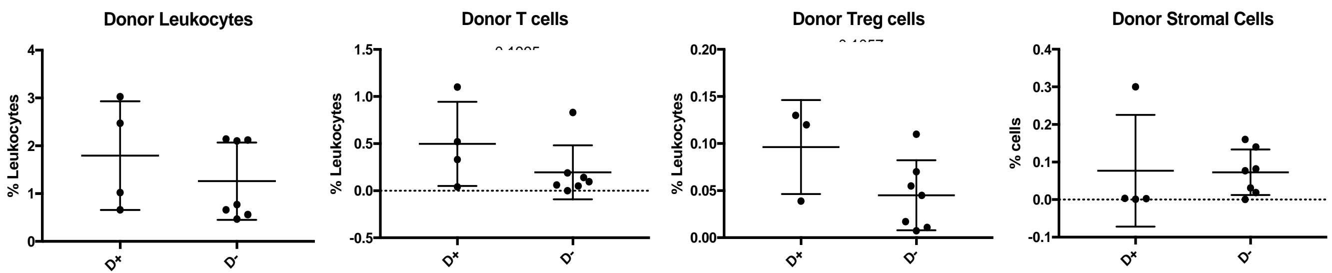


Figure 8: Effect of donor LN transfer on the immune compartment in LN from the graft (transplanted side). Donor immune cells isolated from LN retrieved from the graft with donor LN (D+) or without donor LN (D-) were analysed by flow cytometry as described in the methods

When we look at the percentage of donor leukocytes within total leukocytes for the D+ group we see that this number is low but that there are still more donor leukocytes in the LN retrieved from the graft with donor LN (Figure 9). This shows that a significant fraction of donor leukocytes migrates to the recipient LN meaning specific homing takes place, which would be expected since the donor LN contain many immune cells that can potentially migrate. In order to understand the changes in lymphocyte composition during rejection within the LN we compared the LN retrieved from the transplanted side to the ones retrieved from the contralateral side. There were no differences for the ratio of leukocytes in either the contralateral or the transplanted side for either group (Figure 9 upper panel). We observed significantly more donor leukocytes and T_{reg} cells in the transplanted side LN of the D+ group than in the D- group (Figure 9). This shows that the transfer of donor LN increases the ratio of donor leukocytes and that this increase – as with peripheral blood – is lost during rejection since there is no difference between the LN from the D+ or D- at the endpoint (Figure 8), meaning the donor leukocytes do not persist in the LN even though the percentage of donor leukocyte is still higher in the LN retrieved from the transplanted than the ones retrieved from the contralateral side.

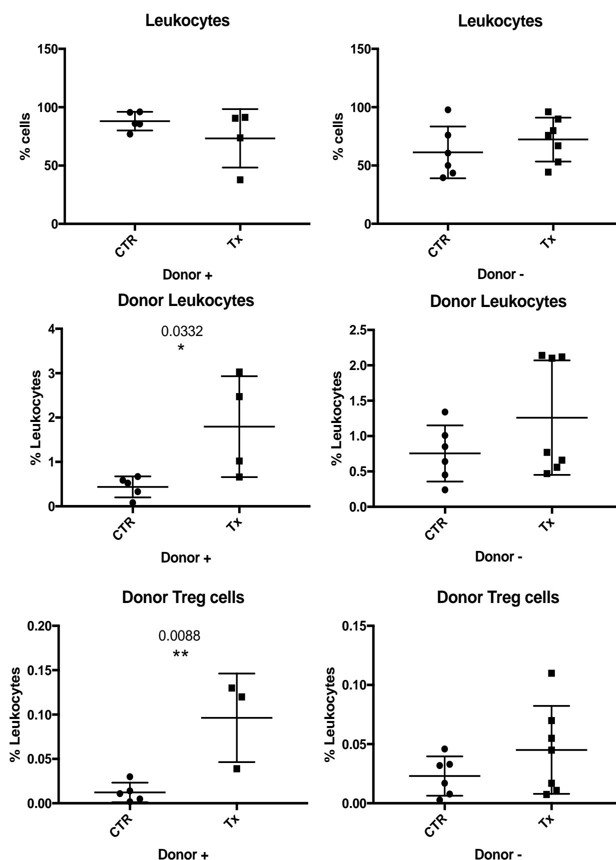


Figure 9: Comparison of leukocyte populations in the LN retrieved from the CTR and the Tx side for the D+ and the D- group. Comparing leukocyte and donor immune cell populations in the LN from D+ (Donor +) and D- (Donor-) group. *p-value<0.05 **p-value<0.01 by unpaired t-test

Bone Marrow (BM)

Analysis of the BM composition of the tibia from the graft showed no significant difference between the D+ and D- group (Figure 10)

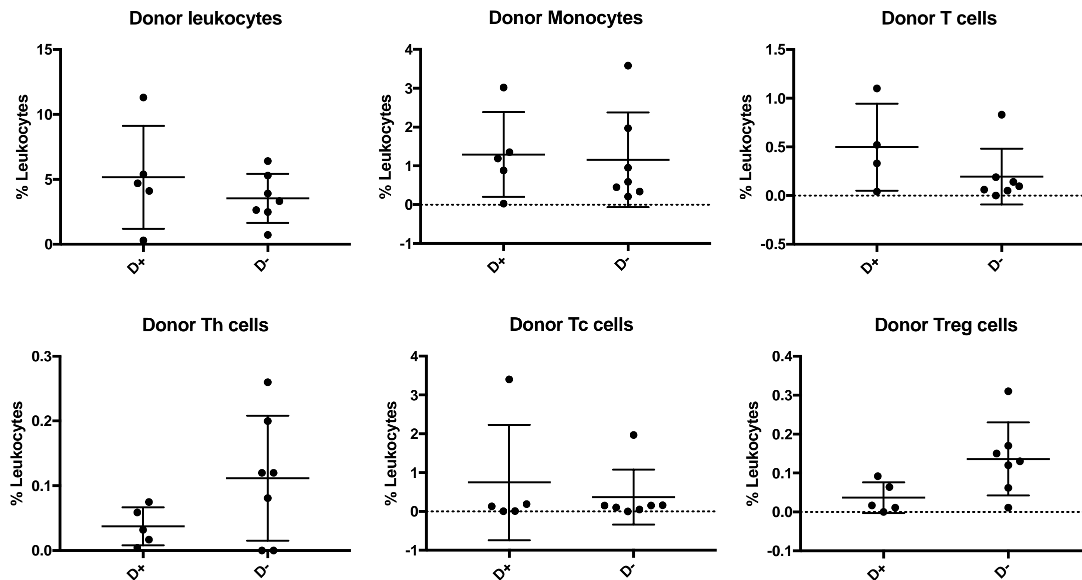


Figure 10: Effect of donor LN transfer on the immune cell compartment in graft BM.

In order to understand the change in BM composition following transplantation and rejection in the two groups, we compared the tibial BM retrieved from the transplanted side (Tx) to the one from the contralateral (CTR) side. We observed a lower percentage of total leukocytes in the transplanted BM compared to the CTR side, conversely, we found higher frequencies of T_H and T_{reg} cells in the BM retrieved from the transplanted side of rats receiving LN depleted grafts (D- group) as compared to their contralateral side (Figure 11).

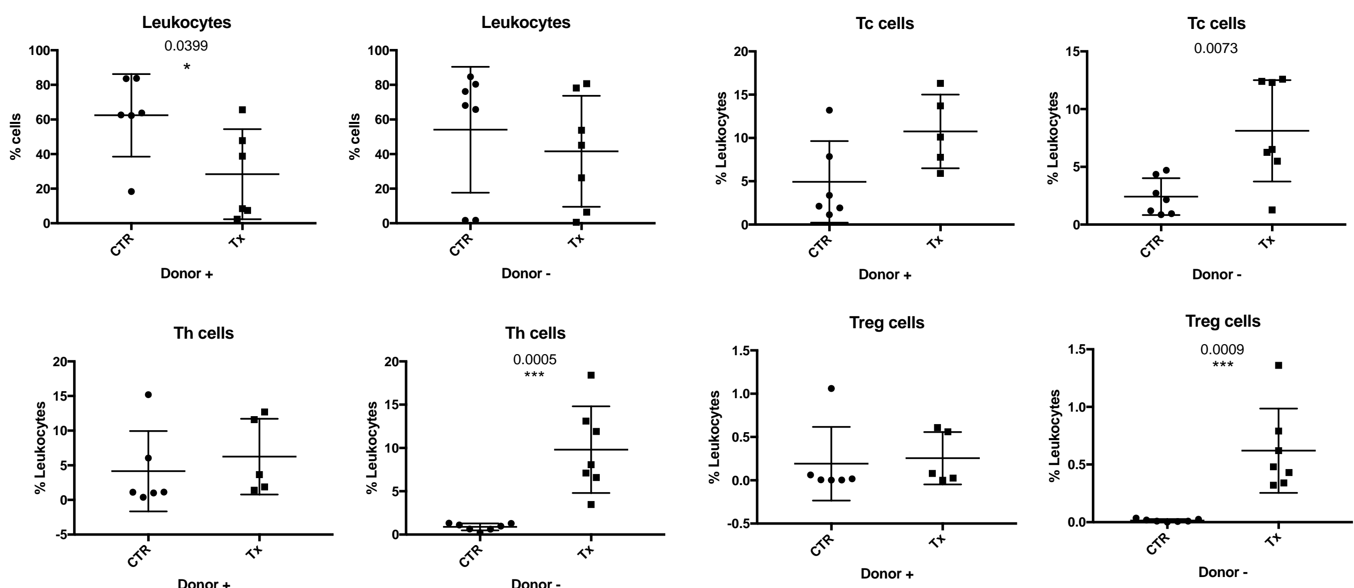


Figure 11: Comparison of contralateral (CTR, recipient origin) to transplant (Tx, donor origin) BM of the tibia for grafts containing donor LN (Donor +) and grafts depleted of donor LN (Donor -) All the cells analysed are of recipient origin.

Considering that most of these cells are of recipient origin (i.e less than 1% of leukocytes are of donor origin, as shown in Figure 10) this finding suggests that in the VCA depleted of donor LN there is a higher infiltration of recipient T_H and T_{reg} cells in the BM.

Moreover, there was a significantly higher percentage of leukocytes of donor origin and T_H and T_{reg} cells in the Tx side of the D- group but not in the D+ group (Figure 12). However, the increase in T_H and T_{reg} cells was most probably due to the higher T_H and T_{reg} cell population in the transplanted side.

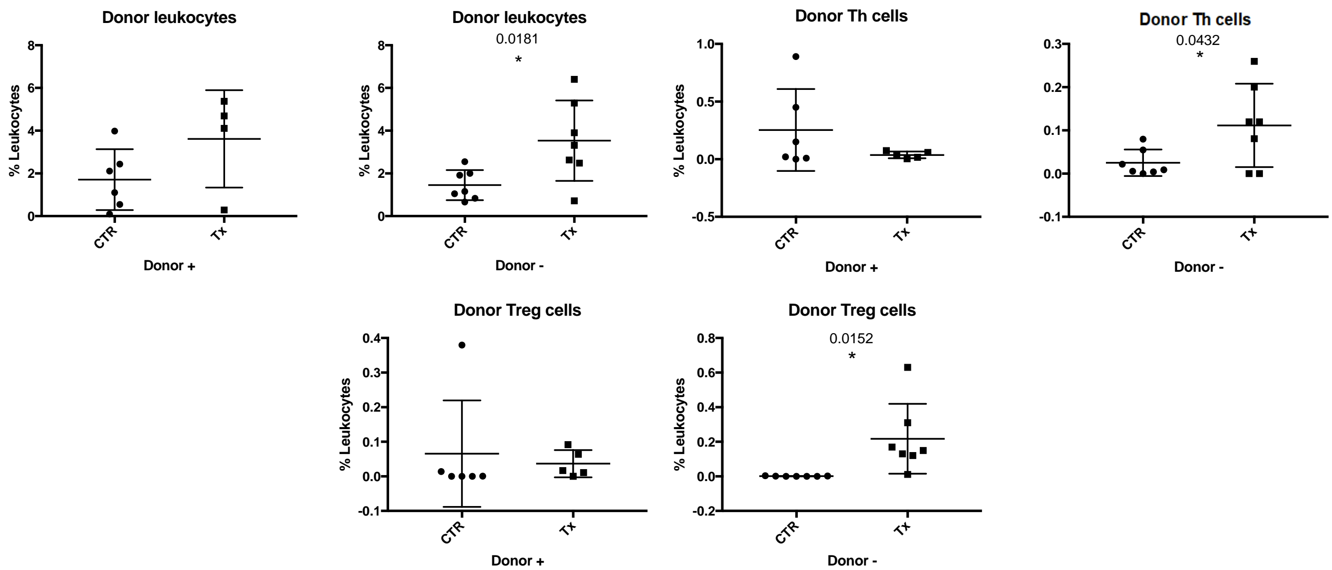


Figure 12: Comparison of BM from the contralateral side to the transplanted side for grafts containing donor LN (Donor +) and grafts depleted of donor LN (Donor -) Donor leukocyte populations. *p-value <0.05 **p-value <0.01 ***p-value<0.001 by students t-test.

This reveals that the ratio of different lymphocyte populations in the BM is negatively influenced by the transfer of donor LN.

Spleen, Thymus and Skin

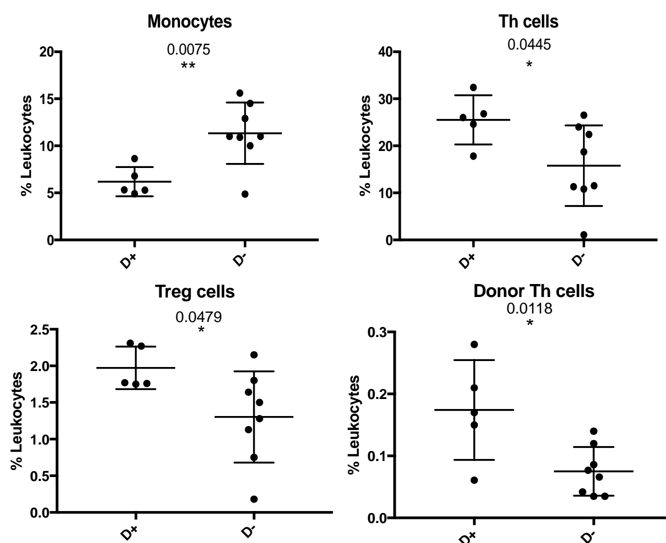


Figure 13: Comparison between D+ and D- group of monocyte, T_H and donor T_H and T_{reg} cell population in the spleen.

Analysis of the spleen thymus and skin showed differences in only some of the leukocyte populations when comparing the D+ to the D- group. For the spleen there was a lower percentage of monocytes and a higher percentage of T_H of recipient and donor origin as well as T_{reg} cells in the

group containing donor LN (D+) as compared to the group without donor LN (D-) (Figure 13). The presence of donor cells in the spleen (Supplementary Figure 10) shows that they not only migrate to draining LN but also to other secondary lymphoid tissue such as the spleen and the transfer of donor LN increased the percentage of donor T_H cells significantly (Figure 13). There was no difference for other recipient and donor cell populations in the spleen. (supplementary Figure 10). Thymus and skin showed no difference in any cell population when comparing the D+ to the D- group meaning the presence or absence of donor LN did not change any leukocyte populations in these organs (see supplementary Figure 11 and 12).

3.3 In Vivo – Lymphography

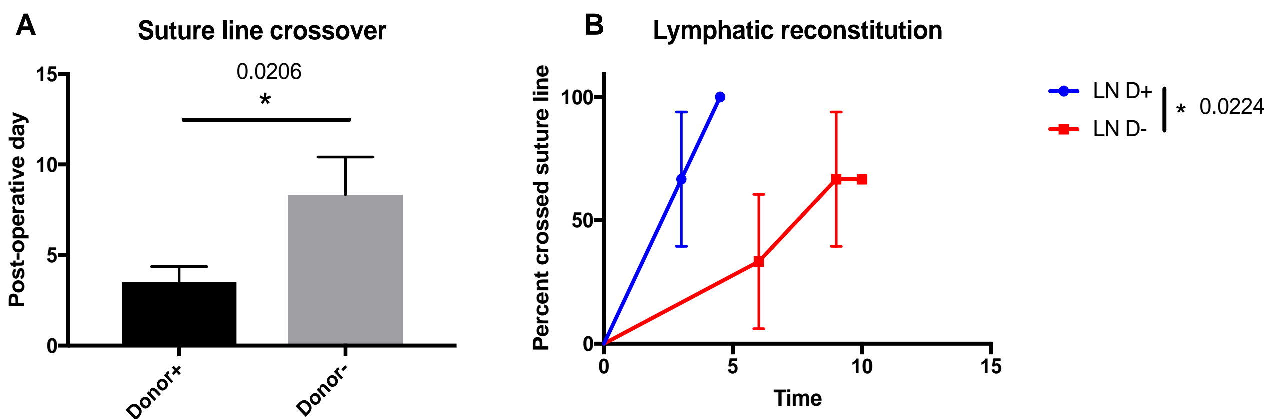


Figure 14: Time until crossing of the suture line and lymphatic reconstitution comparing the groups with and without donor LN. A. Mean time needed for crossover of lymphatics comparing the D+ and D- group (mean ± SEM for D+ group is 3.5±0.5 days and for D- group 8.3 ± 1.2 days) **B.** Percentage of animals showing crossover measured per post-operative day for the D+ and the D- group (mean for D+ group is 3 days and for D- group 9 days). *p-value< 0.05

Crossing of the suture line was measured as an indicator for lymphatic reconstitution, meaning a connection between the donor and recipient lymphatic system with flow of antigen-bearing lymph. In the group with donor LN the crossover occurred around POD3 which was 5 days earlier than in the group without donor LN where crossover first occurred around POD8 showing that the presence of donor LN leads to a significantly quicker crossing of the suture line by the lymphatic vessels (Figure 14). Crossover in the D+ group was not only earlier but also directed towards LN of the recipient whereas a specific direction was not observed in the D- group (Figure 15 and 16). Furthermore, in the D+ group all animals showed a crossover before POD5 whereas in the D- group even at the endpoint (around POD9-11) not all the animals showed crossover (Figure 16).

In addition, at Grade 3 rejection a change in the appearance of lymphatics and movement of fluorescent dye could be observed. The directionality of lymphatic vessels was lost and subcutaneous spreading of the dye (“splashing”) could be seen which occurred due to a disruption of the lymphatic vessels which were unable to take up the dye and transport it in a

directed flow towards draining LN. This showed that at rejection a disruption of the lymphatic vessels and hence lymphostasis occurred. Disruption of lymphatic vessels ("splashing") was observed for both groups at the rejection timepoint showing that during rejection the difference due to presence or absence of LN was lost

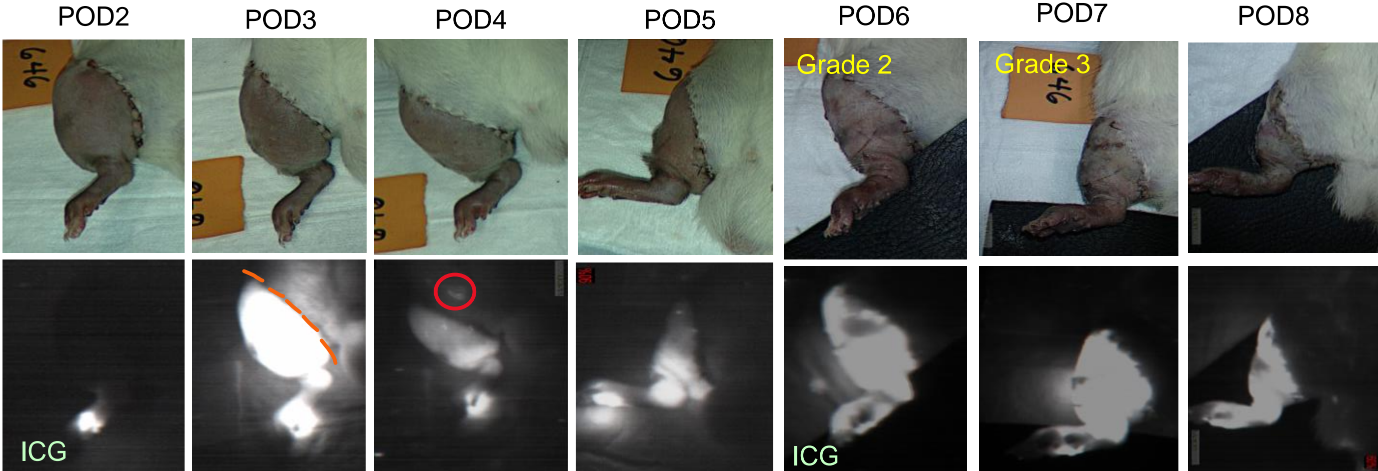


Figure 15: Near-infrared imaging of transplanted leg containing donor LN. ICG dye was injected at post-operative days 2 and 5 according to the clearance from the lymphatic system. Suture line is depicted as an orange intermittent line, diffuse crossing can be observed on POD3, and on POD4 accumulation of dye in a LN (red circle) is visible due to directional transport of dye. The picture of POD6 shows "splashing" of ICG dye which occurred when the dye stayed subcutaneously instead of being taken up into lymphatic vessels.

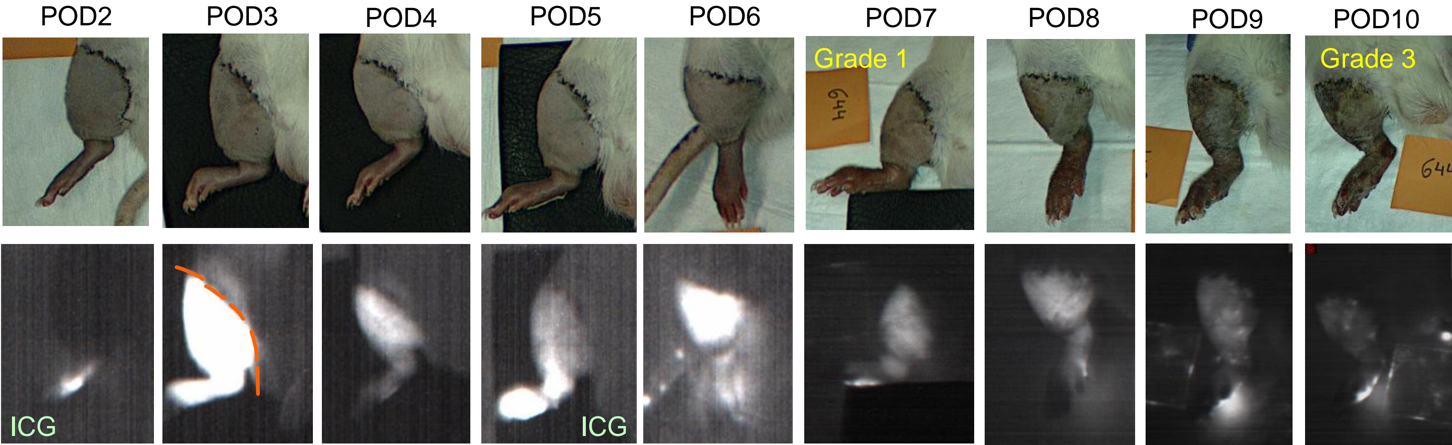


Figure 16: Near-infrared imaging of transplanted leg without donor LN. ICG dye was injected at post-operative days 2 ad 5 according to the clearance from the lymphatic system. Suture line depicted as orange intermittent line. No crossing or clear lymphatic tracks can be observed until the endpoint at POD10

3.4 Immunofluorescence

Immunofluorescence staining was performed to analyse the lymphatic system in the skin by measuring the expression of podoplanin, a marker specific for lymphatic vessels. Skin from the graft was retrieved at the endpoint (i.e. Grade 3 rejection). At this timepoint there was no difference in the lymphatic vessel density between the D+ and the D- group in skin samples taken from the graft.

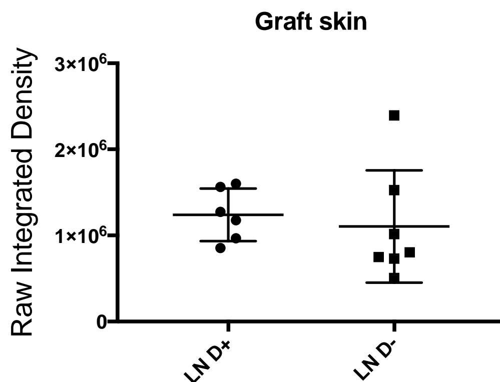


Figure 18. Evaluation of the fluorescent lymphatic marker podoplanin in skin at the endpoint. Immunofluorescence was performed to quantify the lymphatic vessel marker podoplanin in skin retrieved from the graft. Raw integrated density was measured with an image analysis software (Image J) from pictures taken

3.5 Measurement of Cytokines and Lymphangiogenic Factors

In order to investigate the difference between the D+ and the D- group in respect to lymphatic vessel growth, the amount of VEGF-C in the skin of the graft was measured. The amount of VEGF-C gives an approximation of how strongly the lymphangiogenesis is stimulated by the presence of donor LN. The amount of VEGF-C in the skin from the graft was higher for the group containing donor LN – this indicates that donor LN increase lymphangiogenesis in the skin by an increase in the growth factor VEGF-C.

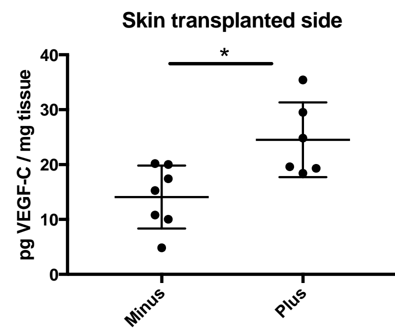


Figure 19. Amount of VEGF-C in skin from the graft. VEGF-C was measured by ELISA and is calculated as pg VEGF-C per milligram of skin

3.6 Bioplex

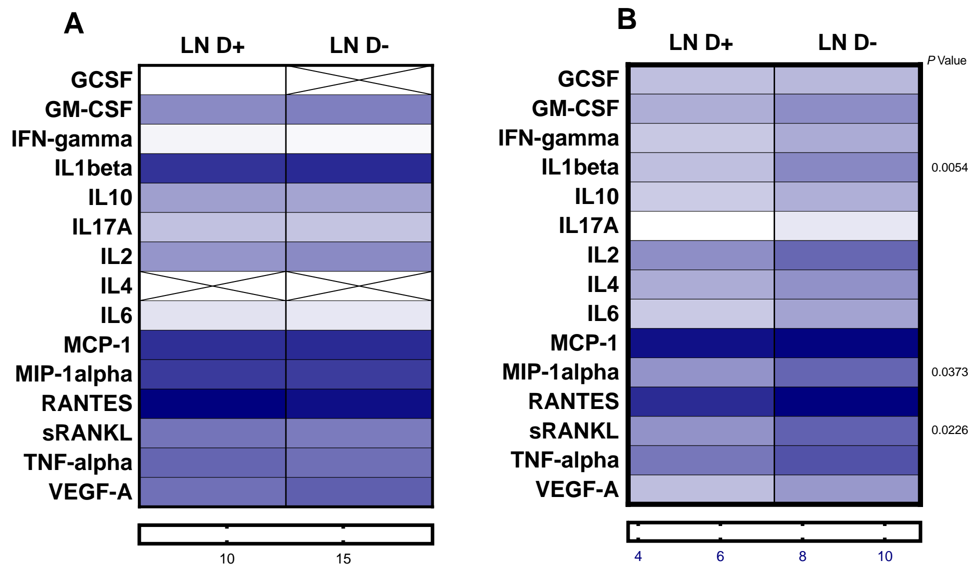


Figure 20. Inflammatory cytokines measured in the skin (A) and plasma (B) of grafts containing donor LN and grafts depleted of donor LN. Cytokines were measured by a 15-multiplex assay. LN D+ is the group with donor LN and LN D- the group without donor LN. **A. Cytokine measurement in skin retrieved from the graft.** Skin samples were taken at endpoint (i.e Grade 3 rejection) and cytokines measured as pg/mg of tissue before being log2 transformed. **B. Cytokine measurement in plasma.** Plasma samples were taken at POD7 and cytokine fluorescent intensity was log2 transformed.

To determine the amount of inflammatory cytokines in the skin and plasma a 15-Plex Luminex assay was performed. There was no difference in the amount of cytokines in the skin when comparing the group with donor LN (LN D+) to the group depleted of donor LN (LN D-) (Figure 20A). This finding is not surprising as the skin was taken at the endpoint (Grade 3 rejection) when inflammation was probably comparable between the two groups and the effect of donor LN was lost. However, the plasma which was taken before rejection occurred showed lower cytokine concentrations for the group with donor LN as compared to the group without (Figure 20B). This indicates that the transfer of donor LN within the VCA caused a less pronounced inflammatory reaction.

3.7 In Vitro – Mixed Lymphocyte Reaction (MLR)

In addition to the in vivo experiments we performed a mixed lymphocyte reaction in vitro to further assess the interaction of donor or recipient lymph node stromal cells (LNSC) with recipient T cells and their effects on T cell alloresponse. We found that in absence of LNSC T cells proliferated more than in presence of stromal cells from either origin. This showed that stromal cells have an immunosuppressive effect and reduce the proliferation of T cells regardless of the origin of the stromal cells. Furthermore, T cells proliferated less in the presence of autologous LNSC and alloantigen than in the presence of allogeneic LNSC with alloantigen (Figure 17). More specifically, LEW LNSC (LWSC) are immunosuppressive on recipient T cells (LEW origin) not only in the presence of BN (donor) stimulator but also of Wistar (third party) stimulator cells. Conversely, BN LNSC (BNSC) are only

immunosuppressive in the presence of BN stimulator but not Wistar (third party) stimulator cells. This finding could be explained by the specific inhibition of proliferation in a setting where the immune cells have the same origin as the stromal cells, meaning inhibition of recipient T cell proliferation is effectuated by recipient LNSC and that donor LNSC also inhibit recipient T cell proliferation but don't inhibit third party (Wistar) stimulation induced proliferation.

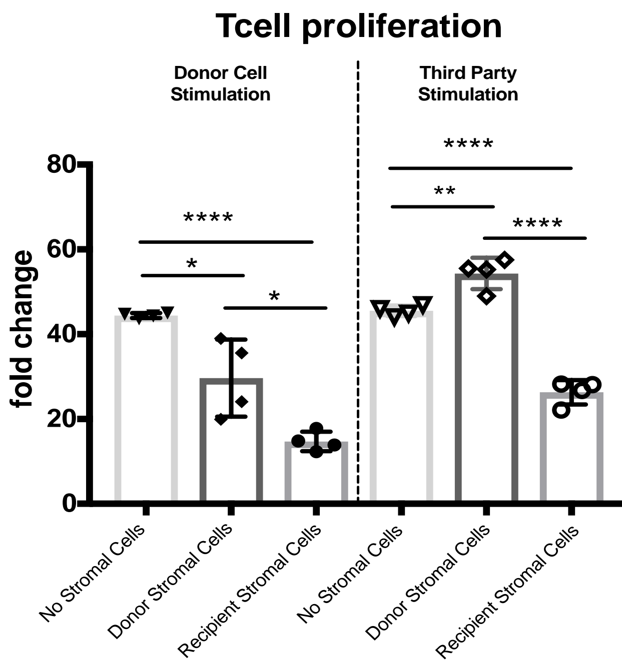


Figure 21: Analysis of MLR in presence of LEW LNSC, BN LNSC and either BN (donor) or Wistar (third party) stimulator cells. All values are normalized against the T cell proliferation in a well with LNSC but without stimulator cells (negative control). *p-value<0.05 **p-value<0.01 ****p-value<0.0001 by one-way ANOVA multiple comparisons. The Figure is a representative of n=3 experiments.

4 Discussion

Our results show that transplantation of lymph nodes within a VCA leads to a delayed onset of the graft rejection process. This is accompanied by higher levels of chimerism as shown by the increased percentage of T_{reg} cells and monocytes of donor origin in the blood (Figure 6), higher numbers of donor cells in the graft for the LN (Figure 9) and an increased number of immune cells of recipient as well as donor origin in the spleen of the recipient rats (Figure 13). This demonstrates that transplantation of donor LN within the VCA changes the immune compartments in the LN as well as in other secondary lymphoid organs (i.e the spleen) that play a role in antigen presentation and tolerance induction. Previous studies show that an important factor in tolerance induction are T_{reg} cells[18] and that intragraft T_{reg} cells reduce alloreactive T cells and prolong graft survival[19]. In agreement with this evidence we observed increased donor T_{reg} cells in the peripheral blood of rats receiving donor LN before rejection occurred (i.e at POD7) as compared to rats receiving donor LN-depleted grafts (Figure 7). Then, once rejection progressed, the frequency of those cells decreased. The question remains whether T_{reg} chimerism in the blood may help to control graft rejection and is lost during rejection or if it is only a sign of the delayed rejection, with delayed rejection of the donor circulating cells. The decrease of donor cells observed at rejection supports the second hypothesis. It is, however, likely that improved chimeric levels may play a role in the delay of rejection itself, as previously suggested[47]. However, one limitation of our experiment was that we were not able to determine the specificity of the T_{reg} cells that we identified. This information could have possibly helped to understand how the chimerism we observed contributed to tolerance or rejection.

When we quantified the amount of inflammatory cytokines in the plasma before rejection occurred we found less inflammation in the group containing donor LN, which suggests that transfer of donor LN within the VCA leads to less of an inflammatory response in the blood before full rejection occurs (Figure 20B). The same cytokines were measured in the skin at endpoint (Grade 3 rejection) but we saw no difference, most likely due to the inflammatory response caused by rejection that was similar for grafts containing or depleted of donor LN (Figure 20A). Interestingly, not only donor cells but also recipient immune cells decrease in the blood during rejection. This may be due to relocation of immune cells by traveling in the blood to sites of rejection like the skin which is the first place where rejection becomes apparent. Furthermore, we did not observe any difference in the frequency of donor and/or recipient cell populations at the endpoint in the LN of rats receiving grafts containing or depleted of donor LN (Figure 8). We speculate that at the rejection point, the chimerism that existed in the blood on POD7 was lost in the secondary lymphoid organs and could no longer contribute to tolerance. The question that remains – and warrants further investigation – is whether the decrease in T_{reg} cells triggers or influences the rejection process or only

coincides with it but is caused by a different mechanism. To investigate this question, it would have been interesting to have LN on POD7 as a comparison to LN at the endpoint.

An unexpected finding was that in the VCA BM, we found less donor cells in rats receiving donor LN containing grafts than in grafts depleted of donor LN. Notably, this difference was secondary to a decrease of T_H and T_{reg} cells in the transplanted BM in recipient rats receiving donor LN depleted grafts rather than an absolute decrease of donor cells in D+ BM. This may be explained by a decreased relocation of cells from the contralateral to the transplanted BM in the presence of donor LN within the VCA, or with an increase of the number of cells in the contralateral BM of rats receiving LN-containing grafts. Further analysis including naïve BM may help to investigate this hypothesis.

For donor cells to travel from the periphery to draining LN where antigen presentation and tolerance induction takes place, lymphatic vessels are important. It has been demonstrated that lymphatic insufficiency leads to impaired peripheral tolerance[26] and that a higher density of lymphatic vessels in allografts reduces rejection[42,43]. In agreement with these findings we showed that transplantation of donor LN within the graft delayed rejection and lead to a quicker and LN-directed reconstitution of lymphatic vessels and thus establishment of lymphatic and immune cell flow towards recipients draining LN (Figure 14). We were able to quantify the growth factor responsible for lymphangiogenesis (i.e VEGF-C) in the skin and showed that this factor is increased at the endpoint in the skin of grafts containing donor LN, therefore showing that transplantation of donor LN increases the lymphangiogenesis (Figure 19). The importance of VEGF-C in prolonging the graft survival is further supported by our finding that a block of VEGF-C lead to an earlier onset of rejection, similar to what we observed in the graft without donor LN. This shows that VEGF-C is crucial for the lymphangiogenesis leading to a delay of rejection observed in the group containing donor LN (see supplementary Figure 13).

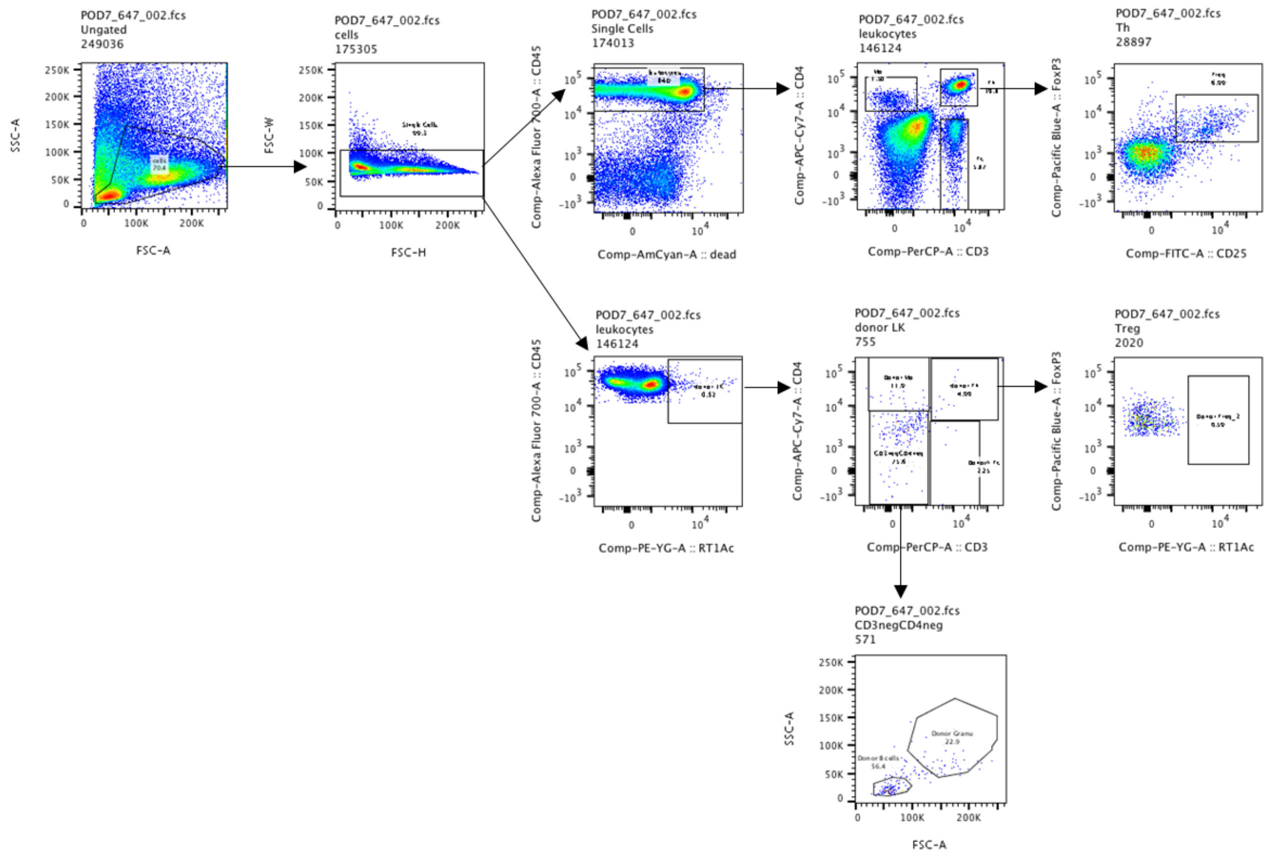
Our findings stand in contrast to earlier studies which showed that lymphangiogenesis in solid organ grafts lead to quicker rejection[35,36,37,38]. Therefore, we postulate that the role of lymphangiogenesis in VCA might be different from its role in solid organ transplantation and that in this context the reconnection of the lymphatic system of the donor to the recipient may help to delay graft rejection favouring lymphatic and inflammation clearance and improved cell migration after transplantation. Indeed, as with the changes in the immune compartment, the appearance of lymphatic vessels changed during rejection. We observed disruption of vessels and lymphatic flow at rejection in both groups we analysed, indicating that the improved drainage secondary to transfer of donor LN is lost once the immune-mediated inflammation leads to full rejection. Further studies focused on the role of lymphatic vessels in antigen trafficking and inflammation clearance are needed to fully investigate the role of lymphatic reconstitution in VCA rejection. A potential explanation for the observed delayed

rejection is provided by recent evidence suggesting that LNSC can present antigen and thereby modulate peripheral tolerance induction[33]. Our in vitro experiments show that LNSC are immunosuppressive and decrease the proliferation of recipient lymphocytes (Figure 21). Therefore, we suggest that the transfer of donor LN within the graft may be used to transfer LNSC that might delay rejection and – by a direct immunosuppressive effect exerted on lymphocytes in the donor LN environment – increase T_{reg} cells.

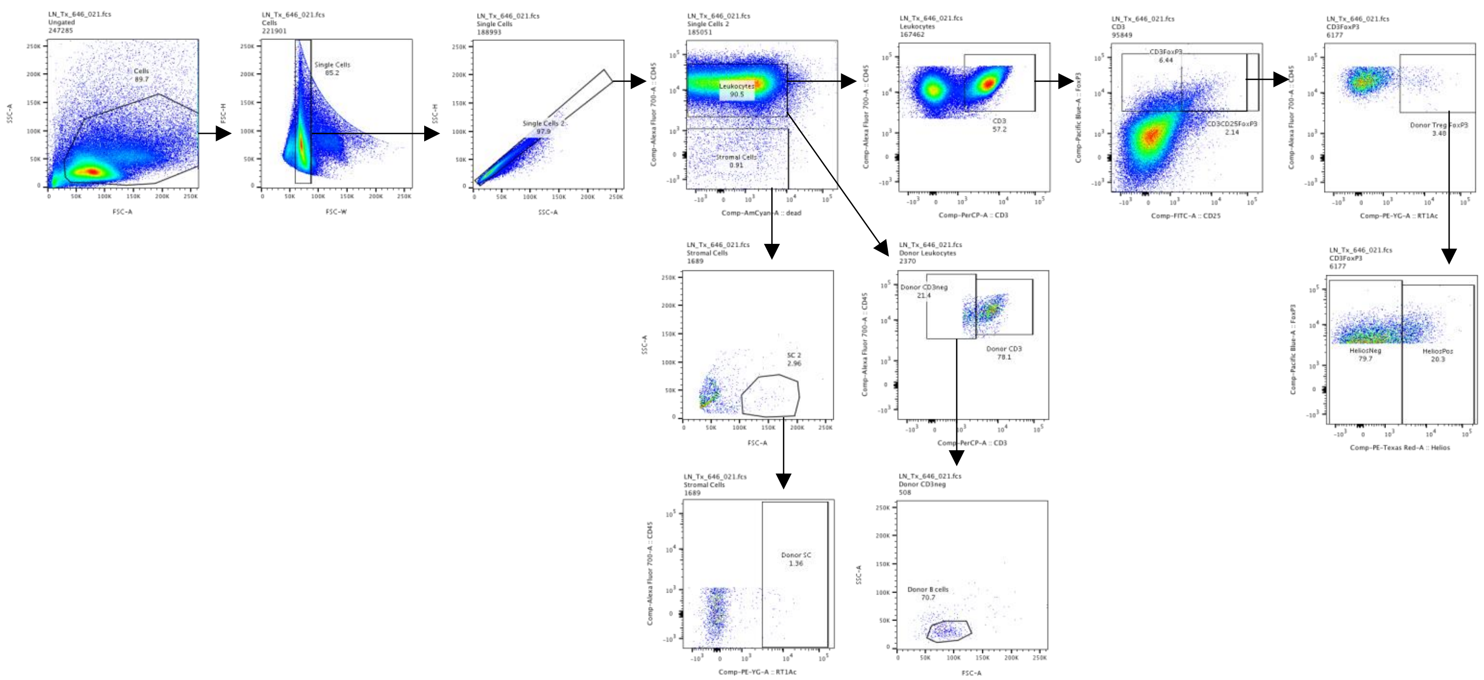
In summary, this study shows that the transplantation of donor LN within a VCA leads to delayed rejection of the graft, increased chimerism in blood and secondary lymphoid organs of the recipient and better reconstitution of lymphatic vessels with LN-directed flow. Therefore, we propose a model in which donor LN transplantation could increase acceptance of the graft thanks to improved inflammation clearance due to increased lymphangiogenesis and a direct effect of LNSC within the LN which were shown to be immunosuppressive. The knowledge gained from these experiments can be used to further investigate the role of VEGF-C, lymphangiogenesis and LNSC in tolerance induction with the goal to prolong graft survival of VCA. One possible approach would be to only transplant LNSC as an artificial LN with the goal to prolong graft survival. Our findings are also of clinical interest since we show that transplanting donor LN within the VCA leads to delayed rejection and could therefore decrease adverse events encountered in the clinics early after transplantation.

5 Supplementary Figures

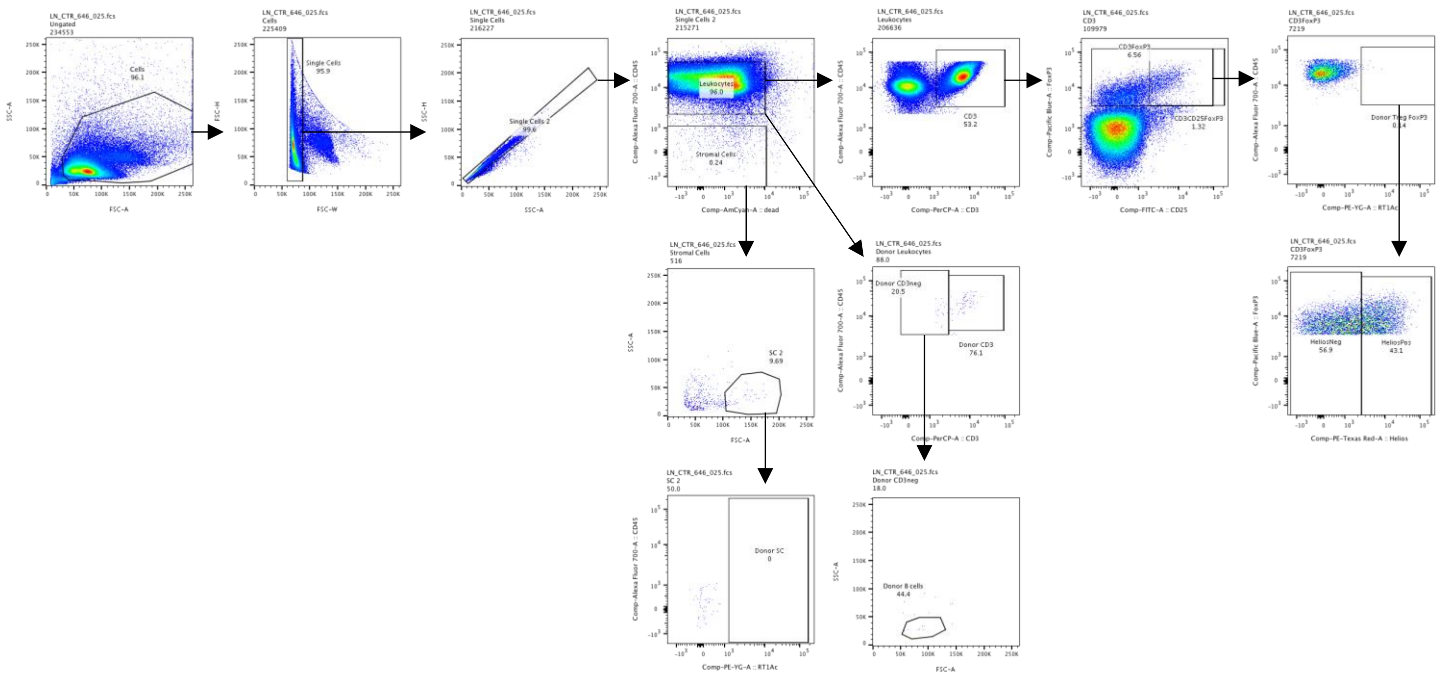
5.1 FACS Gating Strategy



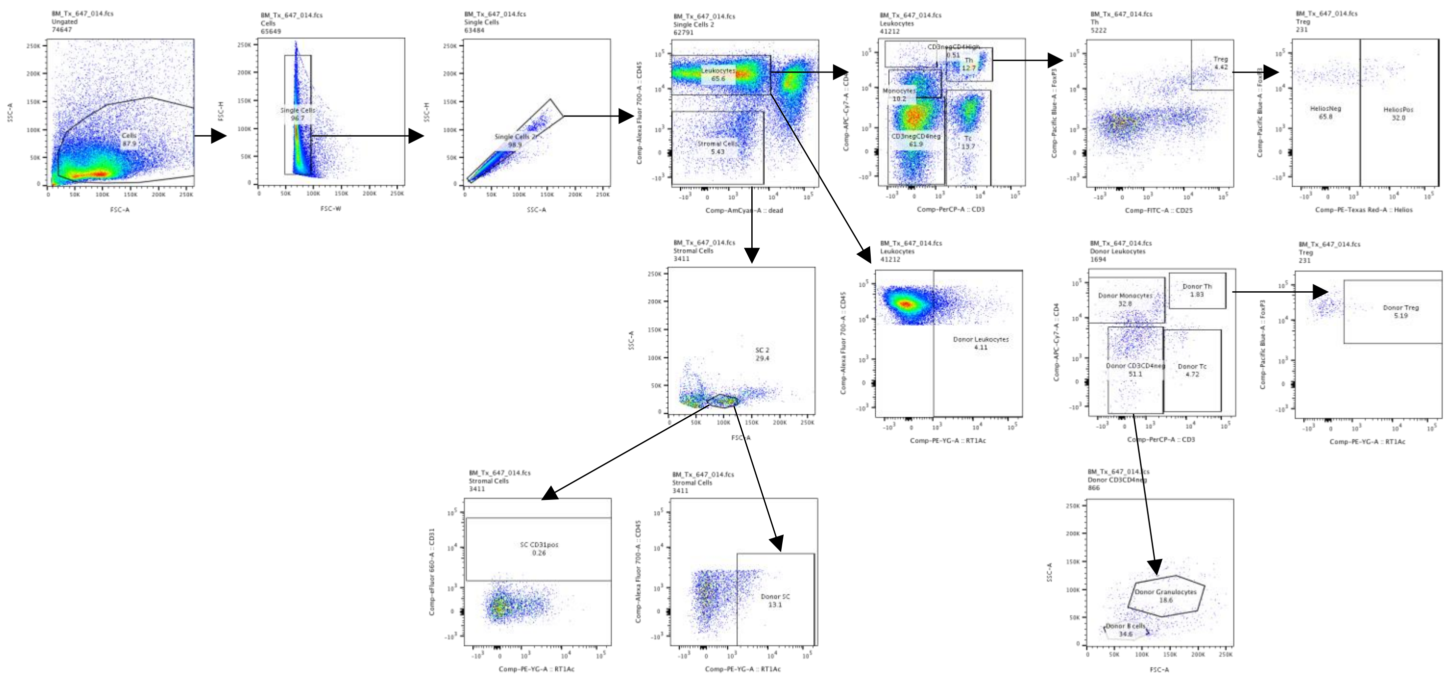
Supplementary Figure 1. Gating strategy for the blood at POD7



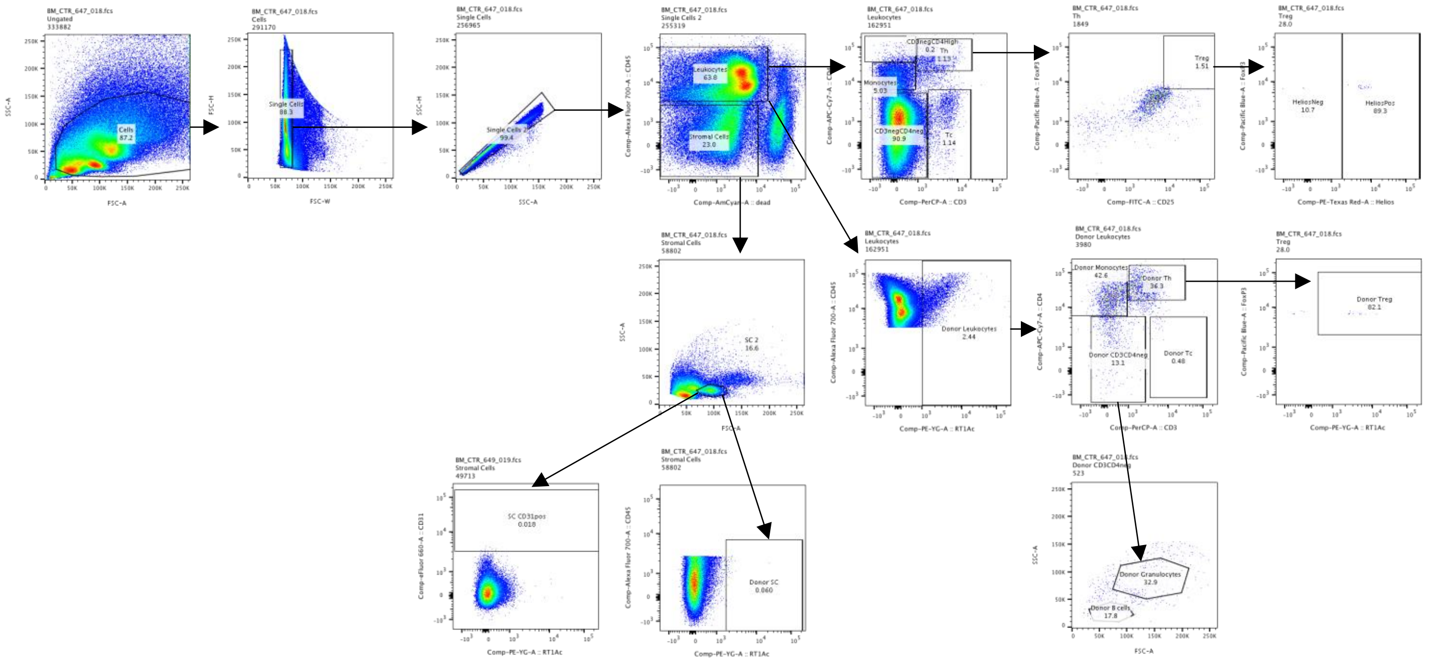
Supplementary Figure 2. Gating strategy for LN from transplanted side



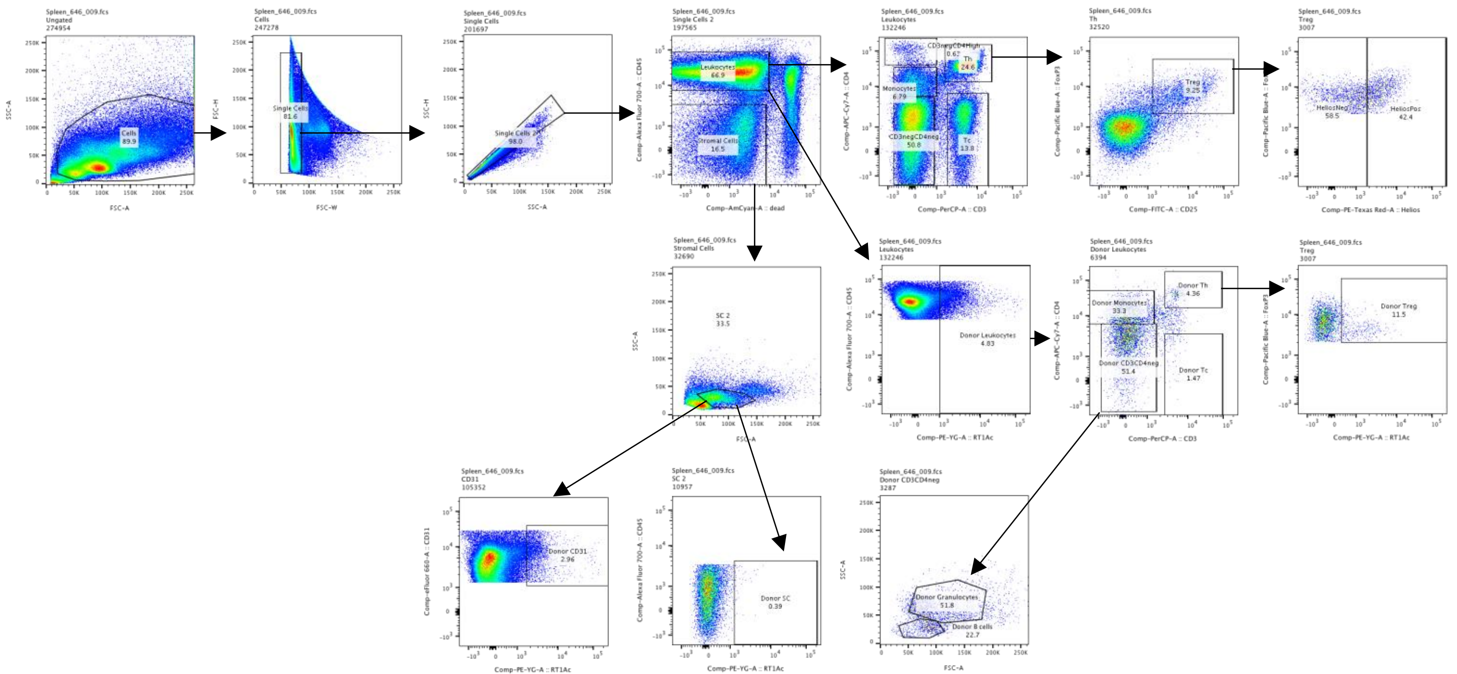
Supplementary Figure 3. Gating strategy for LN from contralateral side



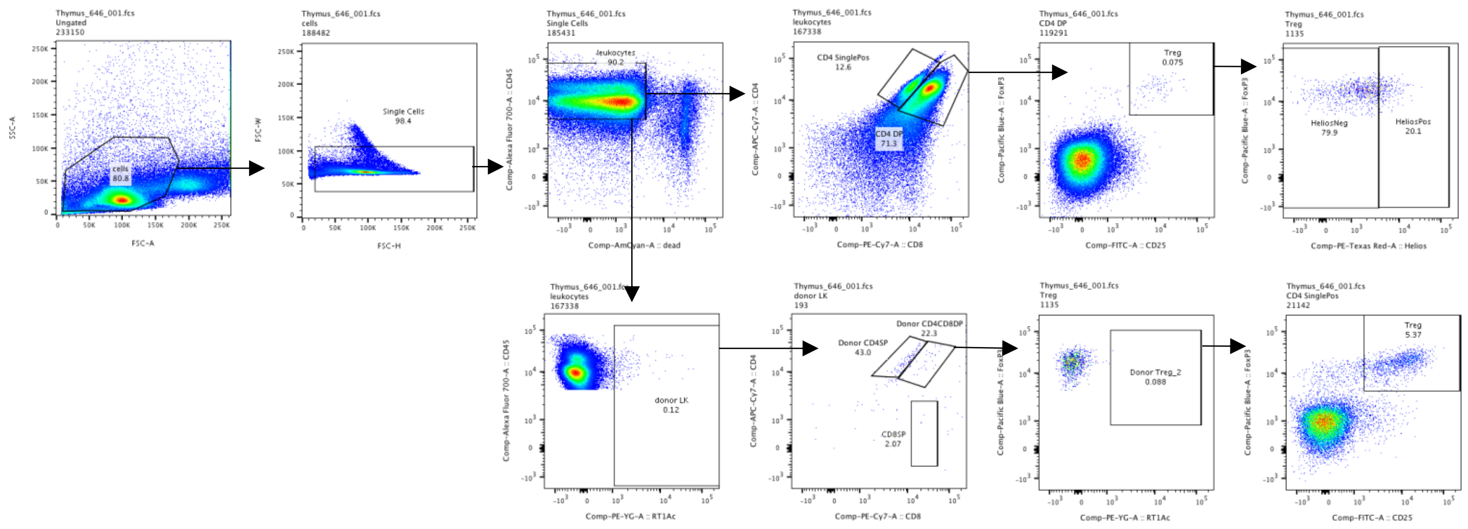
Supplementary Figure 4. Gating strategy for BM from transplanted side



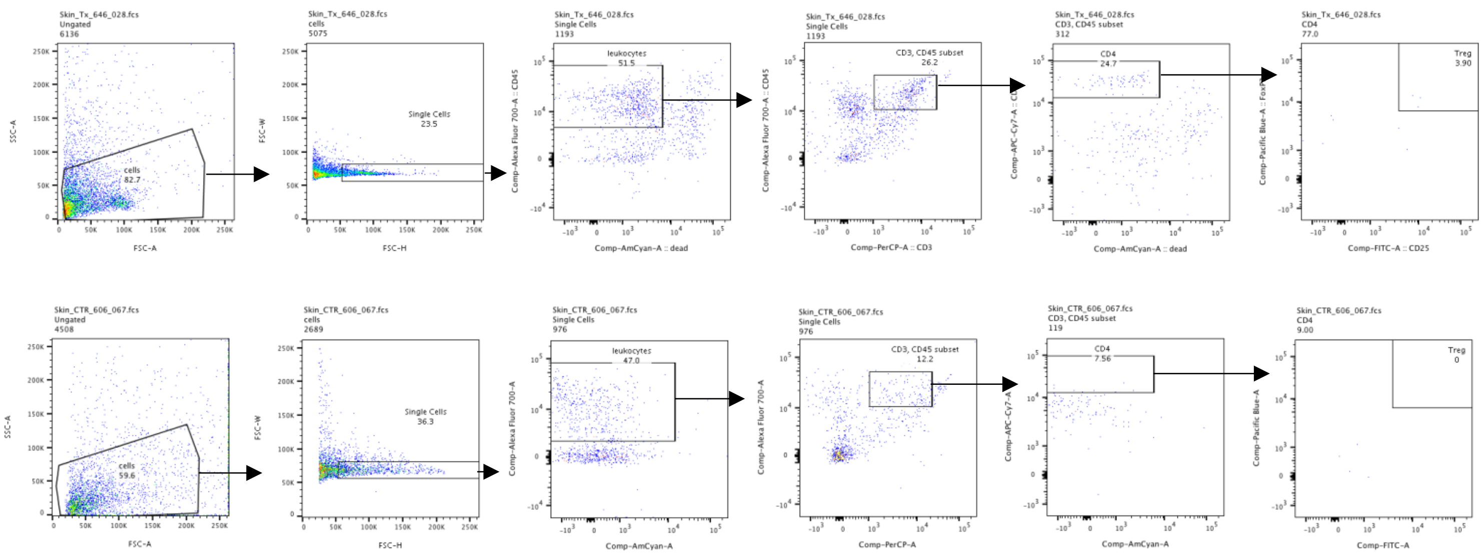
Supplementary Figure 5. Gating strategy for BM from contralateral side



Supplementary Figure 6. Gating strategy for the recipients spleen

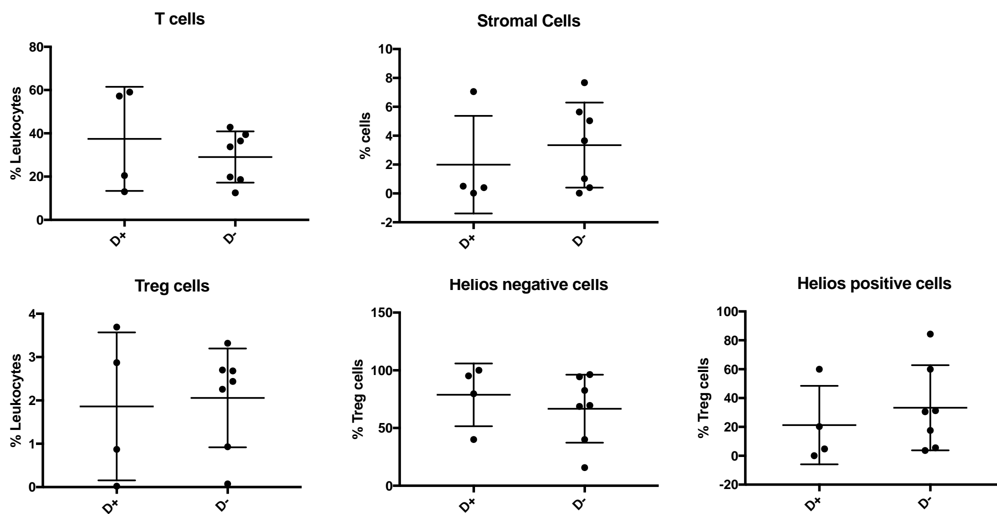


Supplementary Figure 7. Gating strategy for the recipients thymus



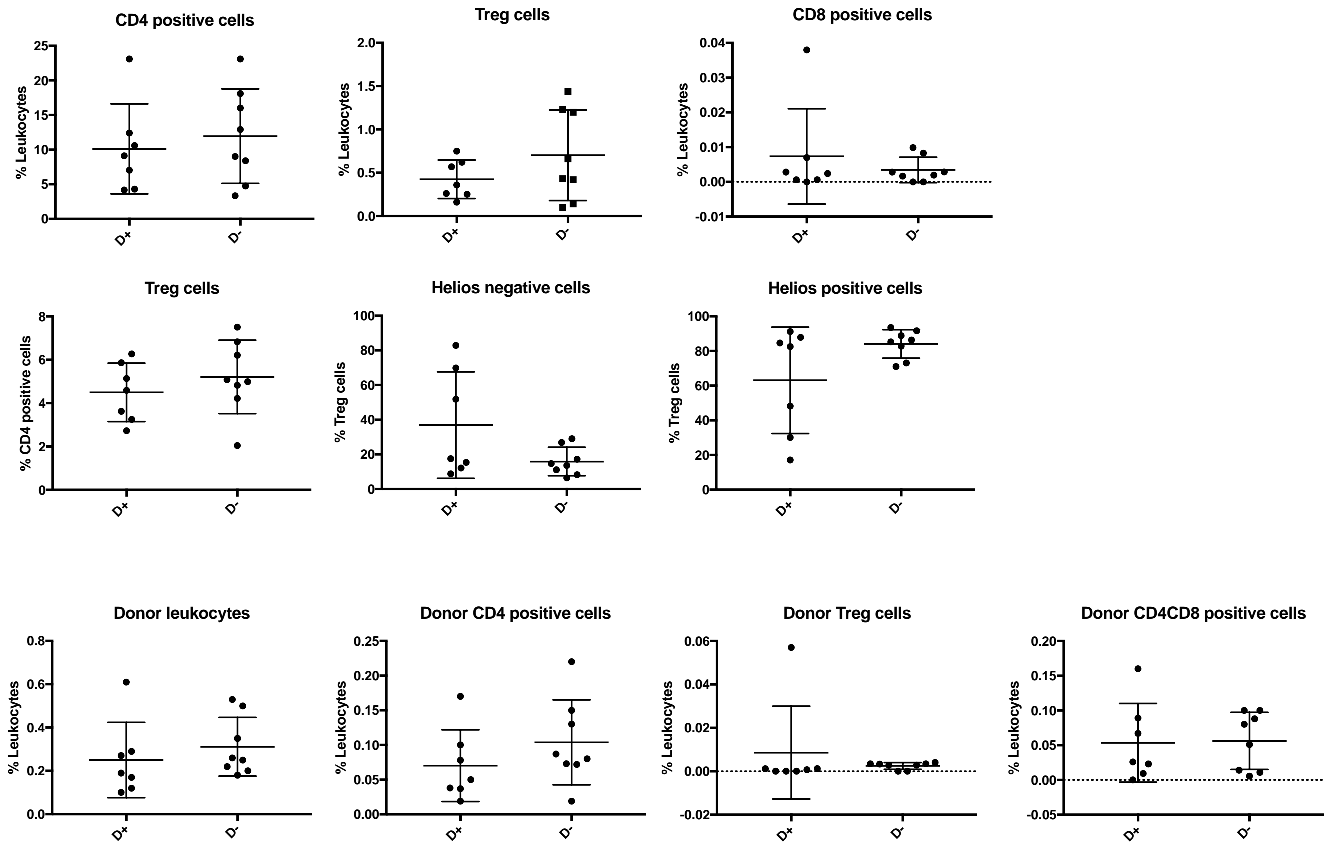
Supplementary Figure 8. Gating strategy for skin from transplanted side (Tx, upper panel) and contralateral side (CTR, lower panel)

5.2 *FACS Results*

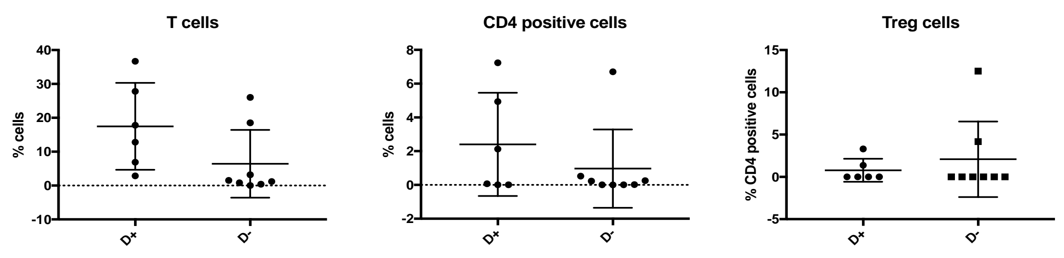


Supplementary Figure 9. Recipient cell populations in the LN from the transplanted side, comparison between the group with (D+) and without (D-) donor LN.

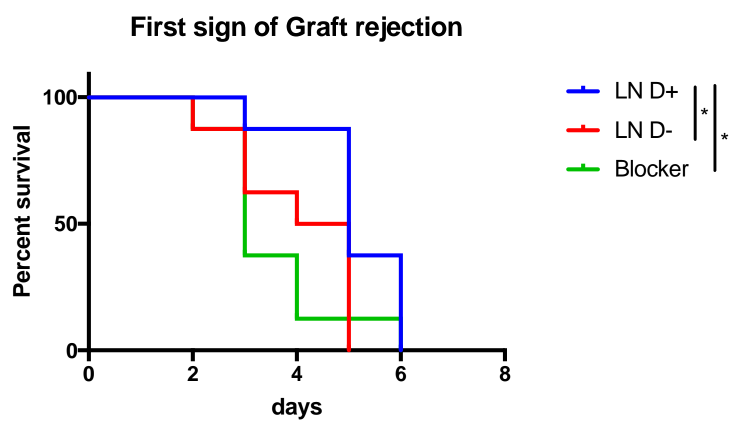
Supplementary Figure 10 Recipient and donor cell populations in the spleen, comparison between the group with (D+) and without (D-) donor LN



Supplementary Figure 11 Recipient and donor cell populations in the thymus, comparison between the group with (D+) and without (D-) donor LN.



Supplementary Figure 12. Recipient cell populations in the skin from the graft, comparison between the group with (D+) and without (D-) donor LN.



Supplementary Figure 13. Onset of graft rejection for the group with blocker compared to the D+ and the D- group. Block of VEGF-C leads to quicker onset of graft rejection.

6 Acknowledgement

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