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Lymph nodes depleted grafts show better outcomes in a rat VCA model

Masterthesis

Awarding the academic title

Master of Science in Biomedical Sciences

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Abstract.

Vascularized Composite Allotransplantation is the transplantation of multiple tissues such as muscle, skin, bone, nerve and lymph nodes as a functional unit. The lymphatic system has long been considered as a passive network whose role in transplantation immunology was to promote rejection. In contrast, recent studies have shown that lymphatic endothelial cells (LECs) have tolerogenic and immunomodulatory properties and could therefore prolong graft survival.

We designed a pilot study aiming at gaining the first insight on how the inclusion of donor lymph nodes in VCA transplants influences the allograft rejection. We performed Brown Norway-to-Lewis hind-limb transplantations with lymph nodes-depleted allografts and with intact hind-limbs. At euthanasia, we collected and analyzed recipient and donor lymph nodes and skin samples.

Our study design allowed the consistent transfer of lymph nodes-depleted, or intact, allografts and the collection of recipient and donor lymph nodes at euthanasia. We were able to analyse these lymph nodes with flow cytometry and we succeeded in designing an immunofluorescence staining protocol to analyse skin samples. Moreover, our first results suggest that the inclusion of donor lymph nodes in VCA transplants promotes rejection through alloantigen delivery to the draining lymph nodes.

If these findings are verified, they would have important implications when considering VCA and immunosuppression, especially for some face transplants where lymph nodes are transplanted as part of the graft.

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1 Introduction

1.1 Vascularized Composite Allotransplantation

According to the American Society of Transplantation, Vascularized Composite Allotransplantation (VCA), also known as Composite Tissue Allotransplantation (CTA), is defined as ”the transplantation of multiple tissues such as muscle, bone nerve and skin, as a functional unit (e.g. hand, or face) from a deceased donor to a recipient with a severe injury” [AST, 2011]. From an immunological point of view, this tissue heterogeneity renders VCA transplants far more challenging as compared to solid organ transplantations (SOT), where graft composition is homologous.

In humans, the most frequent VCA allografts are hand and face. Up to date, more than 100 of these two types of transplantations have been performed worldwide [Issa, 2016, Kanitakis et al., 2016, Kaufman et al., 2016].

As any transplantation between genetically non-identical recipient and donor will lead to rejection, patients receiving a VCA must follow a life-long immunosuppressive therapy, which has side-effects such as opportunistic infections, metabolic disorders and organ damages. It is therefore of critical importance to better understand the mechanisms leading to rejection of VCA transplants in order to reduce immunosuppression, especially in a life-enhancing but not life-saving therapy such as VCA.

1.2 Allograft Rejection Mechanisms

Major Histocompatibility Complex (MHCs) are the main molecules responsible for rejection reactions. Each individual inherits two MHC haplotypes, one from each parent chromosome. MHC genes are very polymorphic and therefore the MHC molecules are different in every individual, except identical twins. Their role is to display antigens for recognition by T cells. MHC molecules can be divided in two classes: MHC class I and MHC class II.

MHC class I are present in all nucleated cells and are recognized by naive CD8+ cytotoxic T cells. Docking of the CD8 receptor to the MHC class I molecule will lead to apoptosis of the infested cell. MHC class II are normally present only on antigen-presenting cells (APCs): dendritic cells (DCs), macrophages and B cells, and are recognized by naïve CD4+ helper T cells. APCs take up, process and present antigens fractions on their MHC II molecules. Docking of the CD4 receptor to the MHC class II is the first step in CD4+ helper T cell differentiation into memory, effector or regulatory cell.

After a transplantation, graft allogeneic MHC molecules can be presented to recipient T

cells in two different ways called direct and indirect alloantigen recognition.

In the direct pathway, recipient T cells recognize intact allogeneic MHC molecules present on the donor cells surface. This could be explained by the fact that T cells, via their T cell receptors (TCRs), have an intrinsic affinity for MHC molecules, independently from their self or foreign origin. This pathway can activate both CD4+ and CD8+ cells (**Figure 1A**, adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015]).

In the indirect pathway, recipient T cells recognize pieces of allogeneic MHC molecules that have been processed by recipient APCs, like in an ordinary antigen presentation. The indirect alloantigen presentation can only activate CD4+ T lymphocytes because alloantigens are phagocytosed and presented on MHC class II molecules (**Figure 1B**, adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015]).

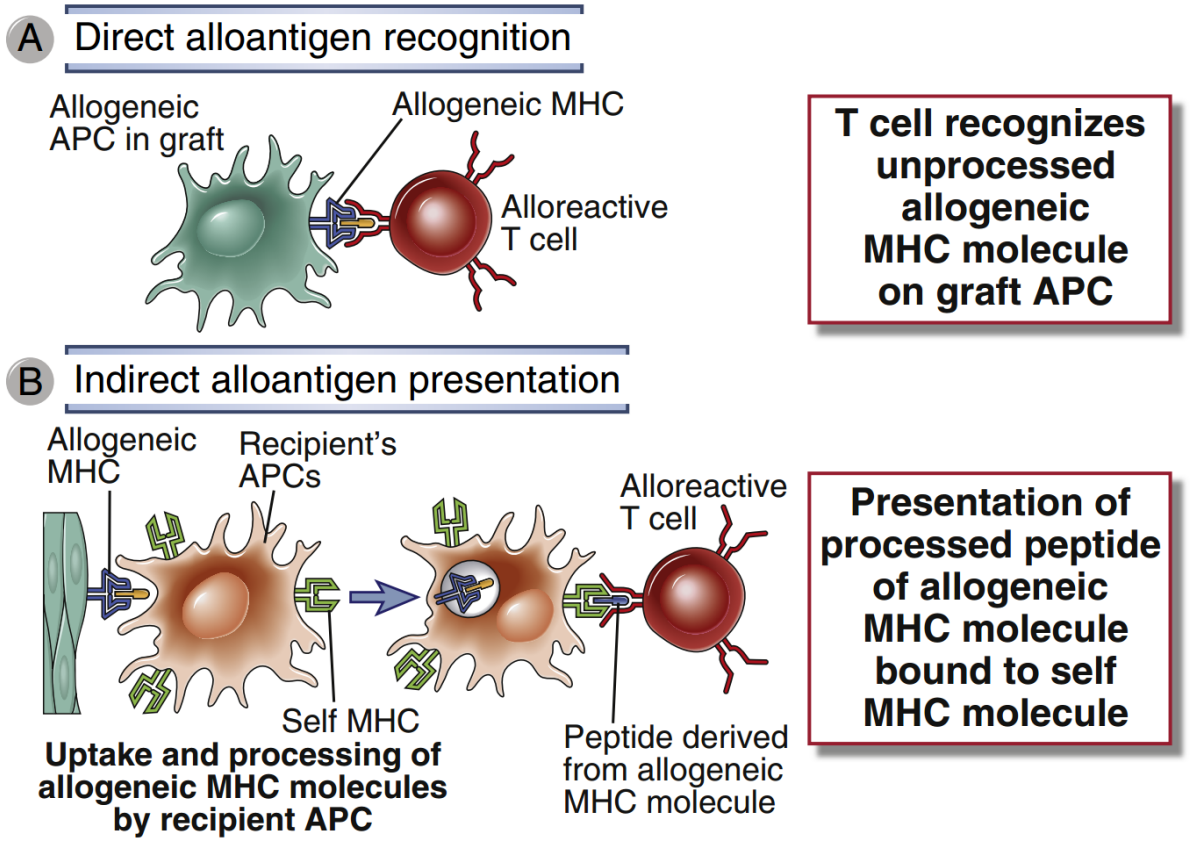

Figure 1: Schematic representation of alloantigen recognition after organ transplantation. (A) Direct alloantigen recognition occurs when a recipient T cell recognizes an intact MHC from donor origin on a donor APC. **(B)** Indirect alloantigen recognition occurs when a T lymphocyte recognizes a fraction of MHC molecule from donor origin that has been taken up and processed by a recipient APC and is presented on recipient MHC. Adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015].

Right after transplantation, donor APCs present in the graft can activate recipient T cells via the direct pathway. The number of donor APCs decreases with time and direct allorecognition is therefore an early process. On the other hand, the indirect pathway is slower because

alloantigens need to be captured and processed by recipient APCs but it lasts for life. The indirect pathway becomes then more prominent with time [Afzali et al., 2007].

More recently, a third pathway called semi-direct has been described: cell-to-cell contact between donor and recipient APCs could lead to the transfer of intact donor MHC molecules on recipient APCs surface leading to the activation of both CD4+ and CD8+ T lymphocytes. It might play a role in regulation of the allogeneic response but its exact role remains to be elucidated [Afzali et al., 2007, Sarhane et al., 2014].

1.3 Immunological Tolerance

Tolerance to self-antigens is an indispensable feature of the normal immune system. Its failure leads to autoimmune disorders including diabetes mellitus type 1, multiple sclerosis, psoriasis, inflammatory bowel disease and many others.

Central tolerance takes place in the primary lymphoid organs (thymus and bone marrow) during lymphocytes maturation. Self-reacting immature lymphocytes are deleted (apoptosis), change their specificity (B cells only), or differentiate into T regulatory cells (CD4+ T cells only). Peripheral tolerance occurs in the periphery in mature lymphocytes that escaped central tolerance. Recognition of self-antigen by a mature lymphocyte leads to its deletion (apoptosis, or to functional unresponsiveness to this antigen (anergy). T regulatory cells can also suppress self-reactive lymphocytes (**Figure 2**, adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015])

Regulatory T cells are a subset of CD4+ lymphocytes whose role is to maintain self-tolerance and suppress immune responses. They express interleukin-2 (IL-2) receptor and FoxP3, a transcription factor critical for their development. T regulatory cells mainly develop in the thymus after a CD4+ T cells recognize a self-antigen (natural regulatory T cells). Antigen recognition in the periphery can also lead, to a lesser degree, to the development of T regulatory cells from naïve CD4+ lymphocytes (adaptive regulatory T cells). T regulatory cells can suppress the activation and effector functions of other lymphocytes (**Figure 3**, adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015]). Several mechanisms of suppression such as the production of immunosuppressing cytokines IL-10 and TGF-beta, the consumption of IL-2, and the inhibition of costimulation via CTLA-4 expression have been suggested.

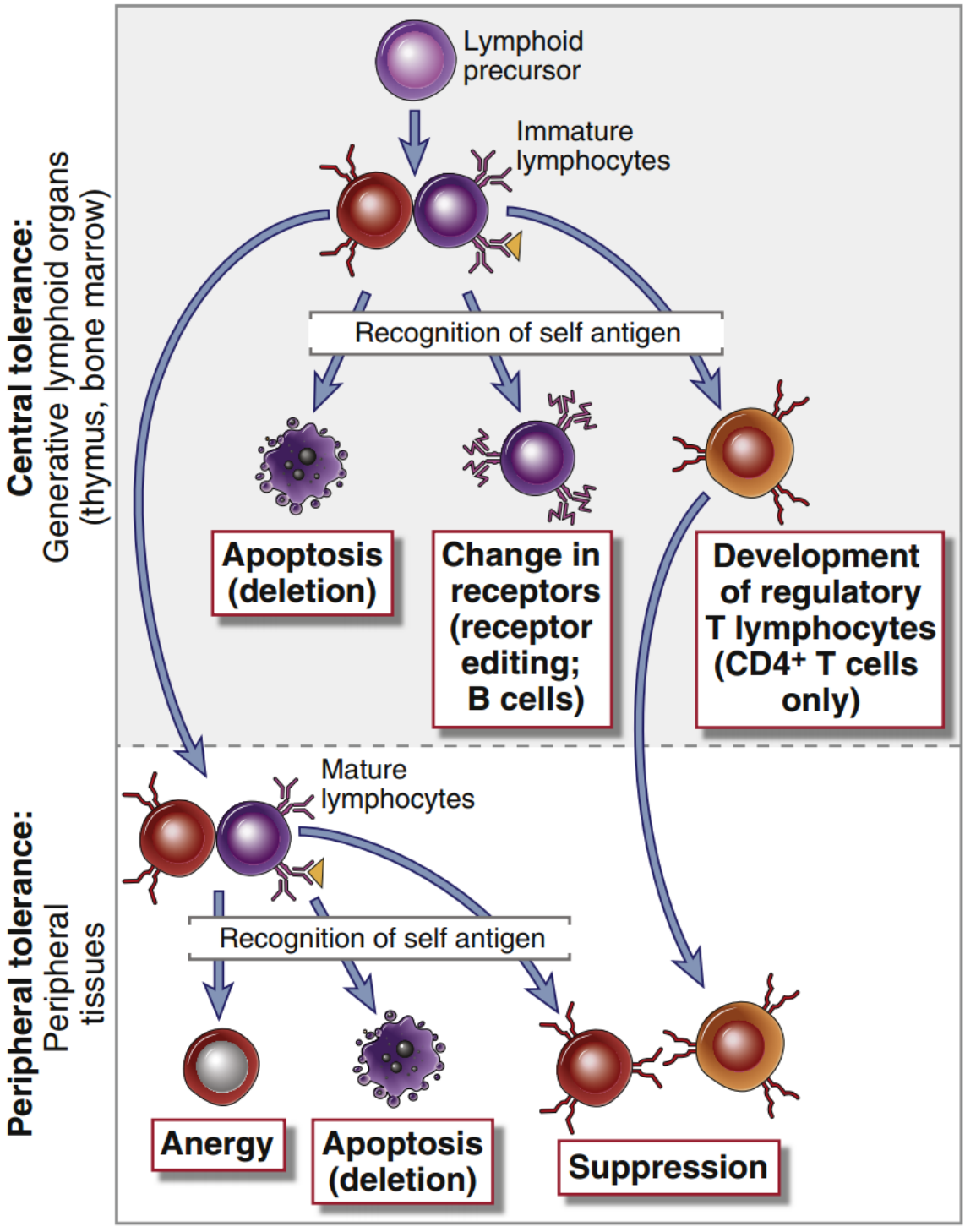

Figure 2: Tolerance to self-antigens. Central tolerance: immature self-reactive lymphocytes may undergo apoptosis (deletion), change their receptors (B cells only), or develop into T regulatory cells (CD4+ cells only). **Peripheral tolerance:** self-reactive lymphocytes that matured and reached periphery may be deleted (apoptosis), inactivated (anergy), or suppressed by regulatory T cells Adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015].

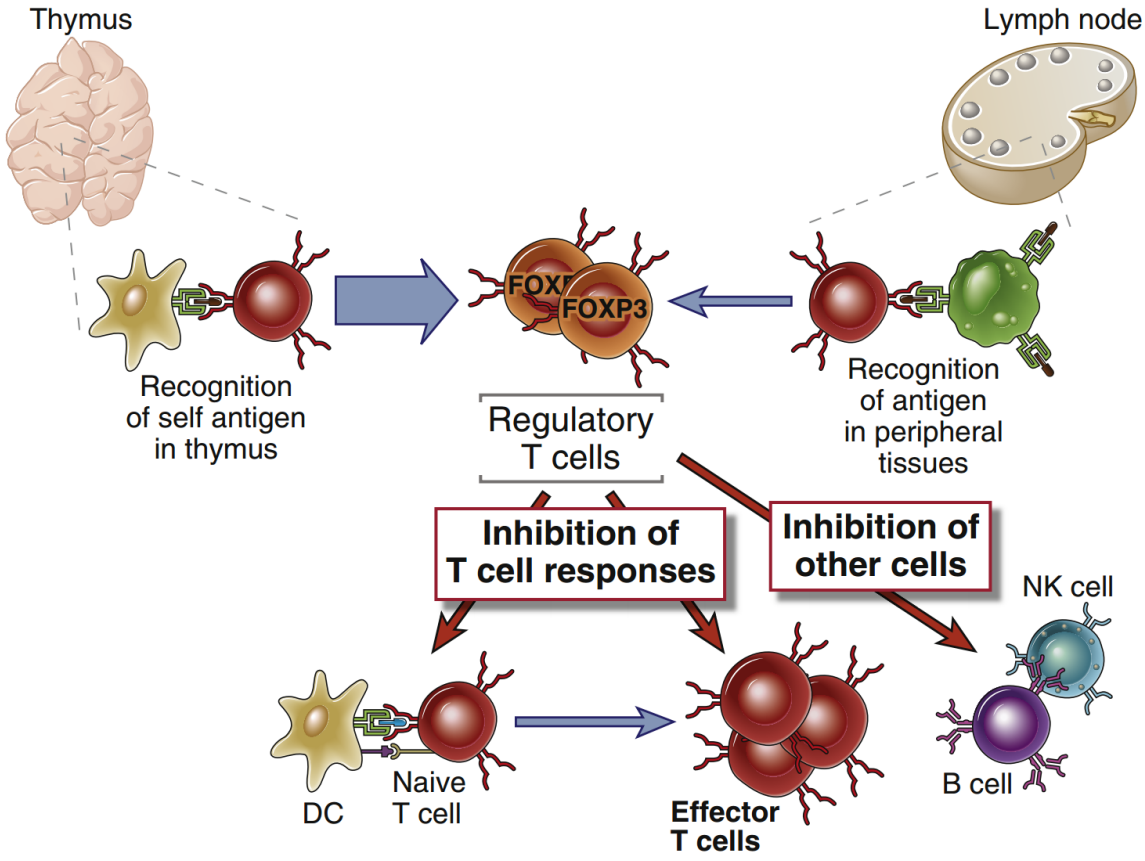

Figure 3: Regulatory T lymphocytes. Regulatory T lymphocytes can develop after self-antigen recognition in the thymus (natural T regulatory cells) or after antigen recognition in the periphery (adaptive T regulatory cells). They express IL-2 receptor and FoxP3. They can suppress the activation and the effector functions of T cells but can also directly inhibit B cells and NK cells. Adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015].

1.4 Lymphatic System Generalities

The lymphatic system is a network composed of specialized vessels and organs. It contains lymph, which is drained from tissue to lymph nodes, and from lymph nodes to the blood. It plays a crucial role in tissue fluid homeostasis, lipid metabolism and in immune response. Hence, the lymphatic system drains soluble antigens and APCs that have captured foreign antigens from the sites of infection and transports them into the draining lymph node via the afferent lymphatic vessel, where they can activate lymphocytes and start an immune response.

After their maturation in the thymus, naïve T lymphocytes enter the bloodstream and migrate to secondary lymphoid tissue such as spleen (through open arterioles), lymph nodes or mucosa-associated lymphoid tissue (MALT), through high endothelial venules (HEVs). If no foreign antigen is recognized in these organs, naïve T cells leave and eventually drain into the circulation. Once in the bloodstream again, they repeat this cycle until they recognize a foreign antigen. When T cells recognize a foreign antigen they become activated, proliferate and differentiate into memory and effector lymphocytes. These activated lymphocytes go back into the circulation in order to arrive at the sites of infection in the periphery (T cells recirculation through lymph nodes is explained in **Figure 4** adapted from [Abul K. ABBAS, 2015]).

1.5 Lymphatic system as a passive draining network potentially harmful for the allograft

In line with what is mentioned above, it has long been thought that the lymphatic system was exclusively harmful to the allograft. Indeed, the formation of new lymphatic vessels (lymphangiogenesis) following transplantation allows the trafficking of soluble alloantigens and APCs bearing alloantigens to secondary lymphoid organs, where an immune response against the allograft occurs [Hos and Cursiefen, 2014].

In human kidney transplantation, lymphangiogenesis is associated with a lymphocyte-rich inflammatory infiltrate, in which antigen presentation by dendritic cells activates B and T lymphocytes [Kerjaschki, 2004]. Increased density of the PROX-1 lymphatic endothelial marker is associated with organ rejection in human lung transplantation [Dashkevich et al., 2010].

According to this, it has been shown in the mice model of corneal transplantation that preoperative specific and selective inhibition of lymphangiogenesis prolongs graft survival, indicating that lymphatic vessels, not blood vessels, are the most important mediators of rejection after corneal transplantation [Dietrich et al., 2010]. Similar results were observed in mice pancreatic islets transplantation where the targeting of lymphangiogenesis, using diverse inhibitors, limited graft destruction and prolonged allograft survival [Yin et al., 2011].

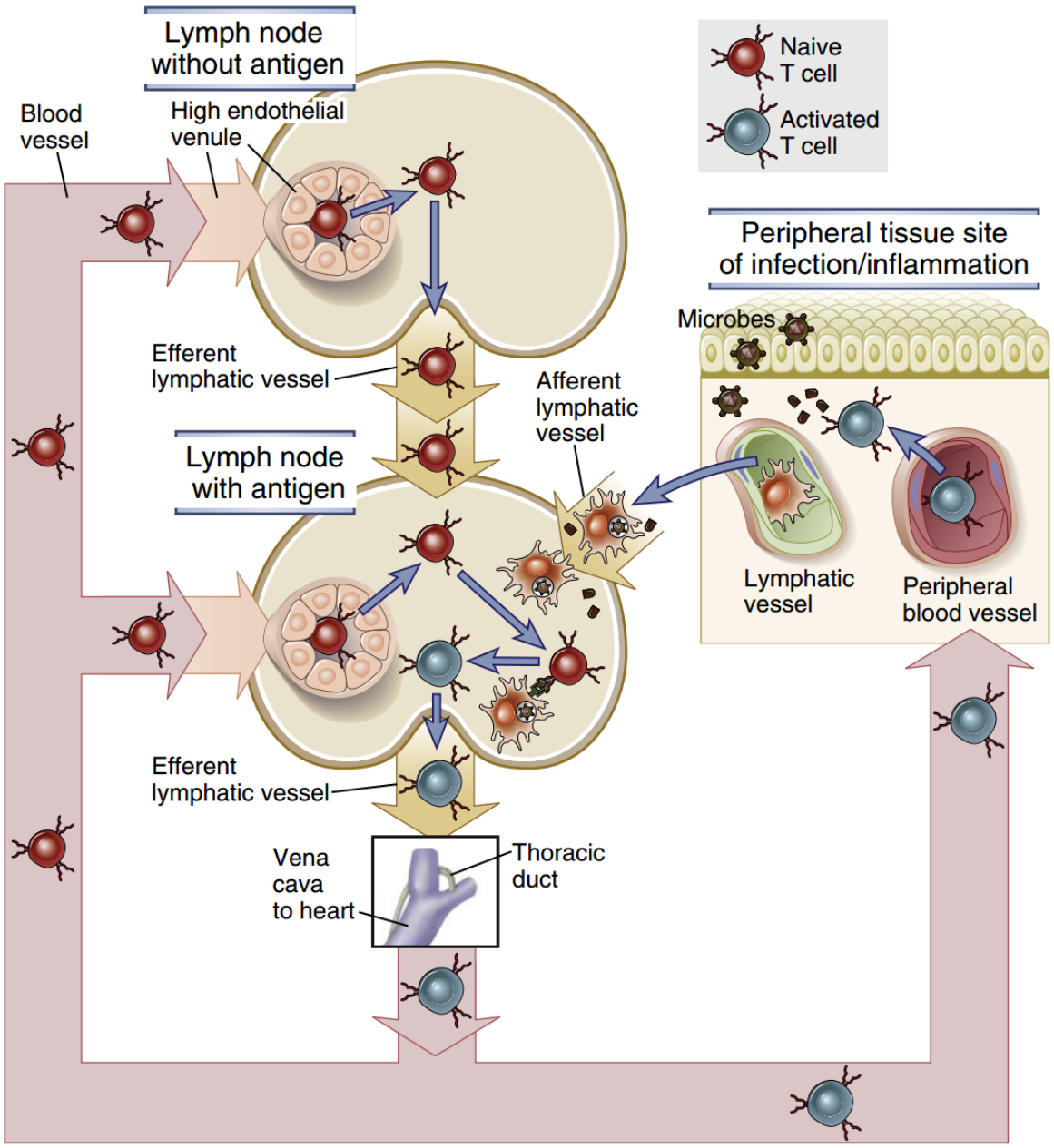

Figure 4: Schematic representation of the recirculation of T lymphocytes. After leaving the thymus, naïve T lymphocytes enter the bloodstream and migrate to lymph nodes through high endothelial venules. Dendritic cells that have taken up foreign antigens in the periphery migrate to lymph nodes through afferent lymphatic vessels. If the T lymphocytes recognize a foreign antigen, they get activated and go back into the lymphatic circulation through the efferent lymphatic vessel and the into the blood stream via the thoracic duct. If the T lymphocytes don't recognize a foreign antigen, they remain naïve and do another cycle. T cell recirculation through other secondary lymphoid organs than lymph nodes is not shown. Adapted from Cellular and Molecular IMMUNOLOGY [Abul K. ABBAS, 2015].

1.6 Lymphatic system as an active player in immunomodulation and tolerance possibly prolonging graft survival

Since the recent discovery of new specific markers for lymphatic endothelial cells (LECs), the study of the lymphatic system has gone a step further [Ezaki et al., 2009]. Consequently, the previously discussed passive role of the lymphatic system is currently being challenged by recent studies suggesting that the lymphatic system, especially lymphatic endothelial cells (LECs), play an active role in modulating immunity and tolerance [Card et al., 2014, Shields, 2011].

Indeed, it has been shown in mice that LECs express peripheral tissue antigens (PTA) and costimulatory molecules allowing them to induce CD4+ and CD8+ T cell tolerance [Hirosue et al., 2014, Rouhani et al., 2015, Tewalt et al., 2012]. In VEGF-C overexpressing mice, dendritic cell maturation and CD8+ lymphocytes activation are inhibited under inflammatory conditions, whereas Tregs are elevated [Christiansen et al., 2016]. Moreover, these properties are controlled by the lymph node environment and therefore restricted to the lymphatic system [Cohen et al., 2014]. Similar roles have been observed in human LECs [Nörder et al., 2012].

In a mice lymph node transplantation model, it has been shown that the absence of MHC-II expression on LECs was leading to CD4+ and CD8+ T cells activation, leading eventually to graft rejection. MHC-II expression on LECs is therefore primordial for self-antigen presentation, resulting in homeostatic maintenance of regulatory T cells (Tregs) and maintenance of immune quiescence [Baptista et al., 2014].

The role of LECs in VCA has not been assessed yet but with regard to what is mentioned above, we can expect that they could be a strong actor in this field. We can speculate that LECs, via presentation of PTA from donor origin, could serve as an antigen reservoir for induction of CD4+ and CD8+ T cell tolerance. Moreover, recent studies have shown that the presentation of self-specific antigen in the peripheral tissue leads to activation and proliferation of Treg cells that differentiate into more potent suppressors, mediating resolution of organ-specific autoimmunity in mice [Davis, 2015, Legoux et al., 2015, Rosenblum et al., 2011]. LECs could therefore also promote the expansion and maintenance of donor-specific Tregs.

1.7 Aim of the study

In face and hand transplantation, different tissues containing lymph nodes and lymphatic vessels are transplanted to the recipient. According to this, we could presume that the lymphatic system would play an essential role in this field. Thus, considering the previously discussed antagonists roles of the lymphatic system in organ transplantation, it is crucial to understand

whether the lymphatic system of donor origin may promote rejection of the VCA transplant via antigen delivery or induce donor tolerance via the immunomodulatory roles of the LECs. To our knowledge, there are no published reports focusing on the role played by donor lymphatic and lymph node in VCA rejection. Therefore, we designed a pilot study aiming at gaining the first insight on how the inclusion of donor lymph nodes in VCA transplants influences VCA rejection. This preparatory study will be used to test the performance characteristics and capabilities of our study designs, measures and procedures and will be the base for the development of a subsequent, larger, study.

1.8 Experimental approach

To test our hypothesis, we performed Brown Norway-to-Lewis hind-limb transplantations with lymph nodes-depleted allografts and with intact hind-limbs. This method allowed us to specifically address the role of donor lymph nodes in VCA transplantation.

Rat orthotopic hind-limb transplantations models have been used for years to study rejection mechanisms [Shapiro and Cerra, 1978]. Hence, the large diameter of rat vessels allows better success rates in microvascular anastomosis. Strong MHC mismatch and differences in skin colour make Brown Norway-to-Lewis hind-limb transplantation a widely used model in VCA research.

At euthanasia, we collected lymph nodes and skin samples from donor and recipient origin. We analysed the leukocyte composition of the lymph nodes with flow cytometry and compared both groups. We also compared the grafts macroscopically with our own scoring and microscopically with immunofluorescence staining of the skin samples.

2 Materials and Methods

2.1 Materials

2.1.1 Devices

Table 1: Devices

Device	Model	Company
Cryostat	Hyrax C60	Zeiss, Germany
Flow cytometer	SORP LSR II	Becton, Dickinson and Company (BD), USA
Light microscope	Leica DMI 4000 B	Leica Camera AG, Germany

2.1.2 Chemicals and consumptives

Table 2: Chemicals and consumptives

Chemical/Consumptive	Reference	Company
Acetone pure	G002	Dr. Grogg Chemie AG, G002
Bovine Serum Albumin	A7030	Sigma-Aldrich
DAKO Glycergel	C0563	Agilent Technologies
H2O ultra-pure	N/A	Sartorius
HCl 25%	1.00316	Merck Millipore
KCl	1.04936	Merck Millipore
KH2PO4	1.04873	Merck Millipore
Na2HPO4·2H2O	1.0658	Merck Millipore
NaCl	1.06404	Merck Millipore
NaCl 0.9% sterile	100 0 090	Laboratorium Dr. G. Bichsel AG
NaN3	13412	Riedel-de-Häen
Tissue-Tek® O.C.T compound	4141	Sakura Finetek USA Inc
Tris	T1378	Sigma-Aldrich

2.1.3 Drugs

Table 3: Drugs

Drug	Generic name	Manufacturer	Concentration	Solvent	Dose	Route
Buprenorphine	Temgesic®	Reckitt Benckiser AG	0.3mg/ml	NaCl 0.9%	50µg/kg	s.c.
Enrofloxacin	Baytril®	Bayer	25mg/ml	NaCl 0.9%	5-10mg/kg	s.c.
FK-506 (Tacrolimus)	N/A	LC laboratories	1mg/ml	Ethanol/Kollifor 1:1	1mg/kg	s.c.
Heparin	N/A	Inselspital	20'000 units E/48	NaCl 0.9%	200ul/kg	i.v.
Isoflurane	Forene®	AbbVie AG	pure	N/A	**Induction:** 5% with 1L/min O2 **Maintenance:** 1-1.5% with 0.6L/min O2	inh.
Patent blue V sodium salt	Patent Blue V®	Guerbet	25mg/ml	N/A	25-50 µl/kg	s.c.
Pentobarbital	Esconarkon ad us. vet. Injecktionslösung	Streuli Pharma AG	300mg/ml	Ethanol	150mg/kg	i.p.

2.1.4 Buffers

Table 4: Buffers

PBS 10x 1L stock solution:	TBS 10x 1L stock solution:
NaCl: 80.0 g	Tris: 30.3 g
KCl: 2.0 g	NaCl: 80.8 g
Na2HPO4·2H2O: 14.2 g	Add 900 ml ultra-pure H2O
KH2PO4: 2.0 g	Adjust pH to 7.5 with HCl
Ultra-pure H2O to 1000 ml	Ultra-pure H2O to 1000 ml
To prepare 1x working solution:dilute 10x working solution 1:10 with ultra-pure H2O	To prepare 1x working solution:dilute 10x working solution 1:10 with ultra-pure H2O
PBS1%BSA 1L:	TBS1%BSA 1L:
BSA: 10.0 g	BSA: 10.0 g
NaN3: 1.0 g	TBS 1x to 1000 ml
PBS 1x to 1000 ml	TBS3%BSA 1L:
	BSA: 30.0 g
	TBS 1x to 1000 ml

2.2 Methods

2.2.1 Overall study design

To determine whether donor lymph nodes play a role in allo-transplant rejection, we studied 7 rats during 35 days after orthotopic hind-limb allotransplantation. We distributed the recipient animals into two groups: the treatment group received a limb containing no regional lymph nodes (n = 3); the control group received a limb containing regional lymph nodes (n = 4).

We injected the rats daily with tacrolimus (FK-506) during 21 days and we sacrificed them at post-operative day 35

Without treatment, rejection starts at day 11 after surgery [Gajanayake et al., 2014]. Re-establishment of the lymphatics occurs around day 7 after surgery [Buretta et al., 2013]. This model allows therefore the study of the rejection mechanisms after lymphatics are re-established and without any influence of the surgically-induced stress response.

All experiments were performed in compliance with the Swiss Legislation for Animal Exper-

imentation and approved by the Veterinary Service of the Office for the Agriculture and Nature of the Canton Bern.

2.2.2 Animals and housing

7-to-8-weeks-old Male Brown-Norway (BN) rats (RT1Ac, donor) and 7-to-8-weeks-old Male Lewis (LEW) rats (RT1Al, recipient) weighing around 250g were purchased from Charles Rivier Laboratories (Sulzfeld, Germany). They were kept in a pathogen-free environment at the University of Bern and the experimental protocol was approved by the cantonal authority. During the experiment, all animals were allowed access to regular food and water ad libitum.

2.2.3 Anaesthesia and pain management

Anaesthesia was induced with Isoflurane (Forene®) inhalation (5% with 1L/min O2) in an induction chamber and was maintained through a nose-cone adapter (1-1.5% with 0.6L/min O). Analgesia was obtained by injecting Buprenorphine (Temgesic® 50µg/kg s.c.) 30 min before surgery and every 12h through post-operative day 2. Further doses of Buprenorphine were given if animals were showing signs of pain. Fluoroquinolone antibiotic enrofloxacin (Baytril®, 5-10mg/kg s.c.) was administered for 14 days in two animals showing signs of infection. Animals were placed on thermal pads during surgery to maintain body temperature and ophthalmic ointment was used to prevent desiccation.

2.2.4 Orthotopic hind-limb allotransplantation

The orthotopic rat hind-limb VCA transplantation model was used [Sacks et al., 2012]. All rats were anaesthetized and analgesized as described above and 50 µl of Heparin was given by penile vein injection. Both donor legs were shaved and opened through a circumferential skin incision at mid-femoral level. One donor BN served as donor for two LEW recipients. The femoral vein, artery and nerve were dissected precisely to ensure adequate length for ensuing anastomoses.

In animals without regional lymphatics in the transplant, the epigastric vessels were ligated and the vascular inguinal lymphatic was removed. The popliteal lymph node was also removed through a medial incision over the muscles (**Figure 5**). In animals with regional lymphatics in the transplant, the epigastric vessels were kept and the vascularized inguinal lymphatic tissue was transferred as part of the graft.

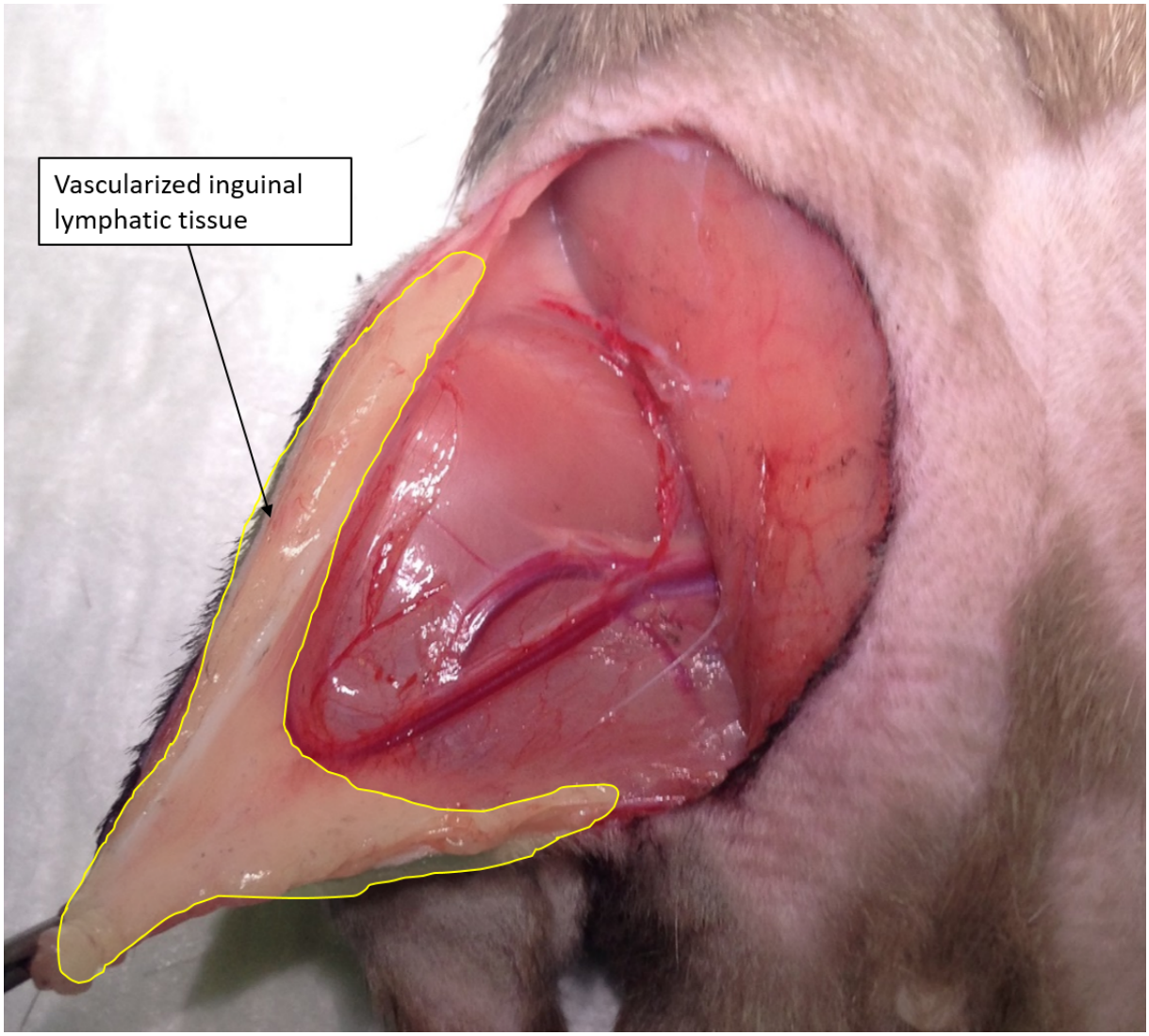

Figure 5: Picture of a dissected Brown-Norway hind-limb before transplantation. Vascularized inguinal lymphatic tissue was removed prior to transplantation in allografts of the VCA-LN group. Popliteal lymph node was also removed (not shown).

After transection of the sciatic nerve, a transverse osteotomy was performed at the mid-femoral level to conclude the allograft harvest. The donor animal was euthanized using pentobarbital (150mg/kg i.p.) and death was confirmed by bilateral thoracotomies.

The recipient's hind-limb was prepared in a similar manner and was discarded with the donor inguinal lymphatic tissue and popliteal lymph node. The femoral vessels were prepared for microvascular anastomosis and the femoral and sciatic nerves were prepared for neurosuture.

Transplantation of the allograft started with the femoral osteosynthesis achieved using an 18-gauge needle as intramedullary rod. 10-0 nylon sutures were used for the anastomosis of the femoral vessels and end-to-end neurorrhaphy.

2-5ml of sterile normal saline (Laboratorium Dr. G. Bichsel AG) was given sub-cutaneously post-operatively to replenish blood loss. After surgery, the animals received Buprenorphine

(50µg/kg s.c.) routinely. They were observed until recovery and all animals were monitored daily to detect signs of pain or rejection.

2.2.5 Immunosuppression and monitoring

Daily immunosuppression with Tacrolimus (1mg/kg s.c.) was maintained for 21 days after surgery in all recipient animals.

Animals were checked daily to detect signs of pain or infections such as weight loss and agitation. The transplanted limbs were visually examined to detect signs of surgical failure.

2.2.6 Euthanasia and samples collection

Rats were sacrificed at post-operative day 35. Anaesthesia and analgesia were performed as previously described. Both legs were shaved, pictures were taken, and 100-200 µl Patent Blue V®, was injected in the donor planta pedis to color lymph nodes in blue, allowing their identification.

In the VCA+LN group, incisions were made in both donor and recipient popliteal area. Popliteal lymph nodes from donor and recipient origin were easily visualized and isolated. Inguinal incisions were made on the contralateral and ipsilateral side to collect fat-pad lymph nodes from recipient and donor origin. In the VCA-LN group, recipient popliteal and fat-pad lymph nodes were extracted in the same way.

Skin samples were collected from both donor and recipient legs in the two groups and directly covered by O.C.T compound on dry ice. Samples were then stored at -80°C.

Table 5: Lymph nodes collected

Sample name	Location	Side	Origin	Group	Amount
Popliteal contralateral lymph node (POP CL LN)	Poplitea	Contralateral	Recipient	Both	VCA+LN: 4, VCA-LN: 3
Popliteal donor lymph node	Poplitea	Ipsilateral	Donor	VCA+LN	4
fat-pad contralateral lymph node (FP CL LN)	Vascularized inguinal lymphatic tissue	Contralateral	Recipient	Both	VCA+LN: 5, VCA-LN: 2
fat-pad ipsilateral lymph node (FP IL LN)	Vascularized inguinal lymphatic tissue	Ipsilateral	Recipient	Both	VCA+LN: 2, VCA-LN: 2
fat-pad donor lymh node	Vascularized inguinal lymphatic tissue	Ipsilateral	Donor	VCA+LN	2

2.2.7 Rejection grading scores

Dermatological evaluation of allograft rejection was performed using our own grading score:

Table 6: Rejection grading score

0:	**no signs of rejection**
1:	**erythema and oedema**
2:	**epidermolysis and exudation**
3:	**desquamation, necrosis and mummification**

2.2.8 Fluorescence-activated cell sorting (FACS)

Freshly collected lymph nodes were smashed on 50ml Falcon tubes using 70µm filters and 5ml syringes pistons. Falcon tubes were filled up to 50ml with PBS 1%BSA and centrifuged for 5 min at 1500 rpm. Supernatant was discarded, cells were counted using a Neubauer chamber and transferred into 2ml Eppendorf's tubes. Cells were then stained for CD45R, RT1Ac, CD3, CD11b/c, CD45, and DAPI was added right before acquisition. Finally, tubes were acquired

with the BD LSR II Special Order System flow cytometer and data was analysed using Flowjo software. Gating strategy is described in **Figure 6**.

Table 7: Antibodies and dyes used for flow cytometry

Name	Volume (µl) per sample	Reference number	Company
CD45R FITC	2	130-106-778	MACS
RT1AC PE	2	MCA156PE	ABd Serotec
CD3 PerCP	2	130-102-674	MACS
CD31 PE-Cy7	0.5	25-0310-82	eBioscience
CD11b/c AF647	0.5	201814	Biolegend
CD45 APC-Cy7	5	10-107-792	MACS
DAPI	1	32670-25MG-F	Sigma

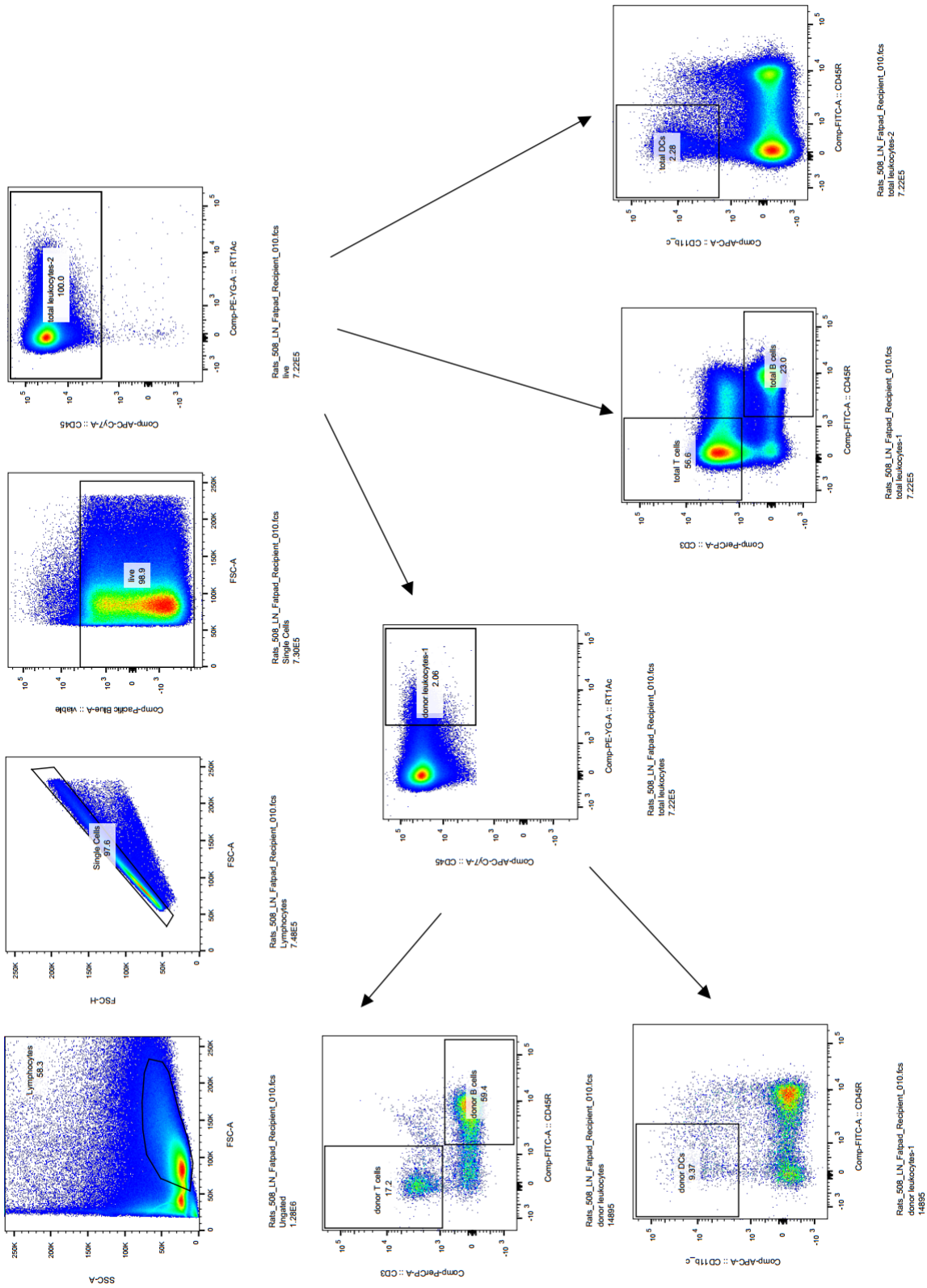

Figure 6: Flow cytometry gating strategy

2.2.9 Immunofluorescence

OCT embedded skin samples were cut in 5µm slices using the Zeiss cryostat hyrax c60. Sections were placed on slides and stored at -20°C. Samples were then fixed in -20°C cold Acetone for 10 minutes, rehydrated in TBS, and blocked with TBS-3%BSA during 1 hour at room temperature. Slides were then rinsed with TBS and incubated overnight at 4°C with primary antibody diluted in TBS-1%BSA (1:100). Slides were rinsed and incubated 1 hour at RT protected from the light with secondary antibody diluted in TBS-1%BSA (1:500) and DAPI (1:1000). After incubation, slides were washed 3 times, dried and mounted. Pictures were taken with the Leica DMI 4000 microscope using the Leica AF software and quantified with ImageJ software.

Table 8: Antibodies and dyes used for immunofluorescence

Name	Dilution	Reference number	Company
CD3 mouse anti rat	1:100	14-0030	eBioscience
CD68 mouse anti rat	1:100	MCA341GA	AbD Serotec
CD11 b/c AF647	1:100	201814	Biolegend
Goat anti-Mouse IgG AF 546	1:500	A-11030	Invitrogen
DAPI	1:5000	32670-25MG-F	Sigma-Aldrich

2.2.10 Statistical analysis

Data were analysed with the GraphPad Prism 7.02 software and results are expressed as mean ± standard deviation. Differences were assessed using the unpaired parametric Student's t test. A p-value <0.05 was considered statistically significant.

3 Results

3.1 Macroscopic evaluation of allograft-rejection

Transplanted limbs were evaluated macroscopically at euthanasia to detect dermatological signs of rejection such as erythema, oedema, epidermolysis, exudation, desquamation, necrosis and mummification. Allografts were then graded with our rejection criteria. The group who received allograft containing lymph nodes (VCA+LN) was compared to the group who received lymph nodes depleted allografts (VCA-LN). Out of the 4 animals in VCA+LN, 3 showed signs of epidermolysis (rejection grade 2) and one showed signs of necrosis (rejection grade 3). Out of the 3 animals in VCA-LN, none showed signs of epidermolysis. The mean rejection score of VCA+LN group was higher than in VCA-LN group (1.75 ± 1.258 vs 0.33 ± 0.5774), but this difference did not reach statistical significance (**Figure 7**).

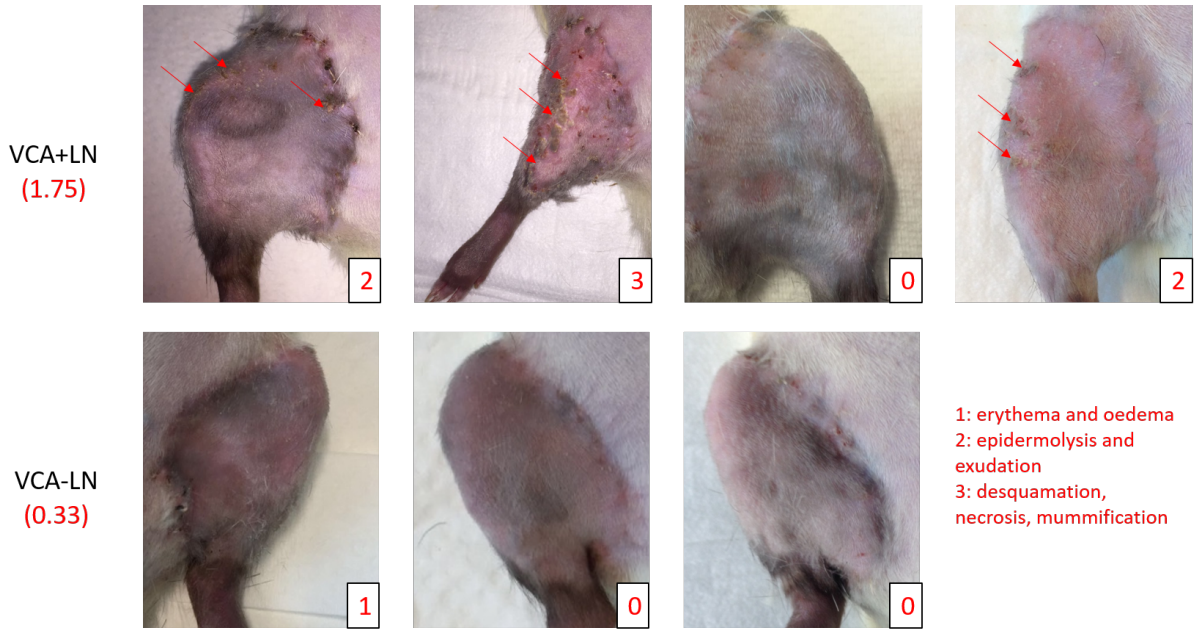

Figure 7: Macroscopic evaluation of transplanted limbs in VCA+LN group (top panels) and VCA-LN group (bottom panels) at post-operative day 35. Signs of epidermolysis (red arrows) were observed only in the VCA+LN group. The mean rejection score of VCA+LN group was higher than in VCA-LN group (1.75 vs 0.33), but this difference did not reach statistical significance.)

3.2 Microscopic evaluation of leukocytes infiltration in the allograft skin

3.2.1 T cells infiltration

T cells infiltration in the allograft skin was compared in VCA+LN (n = 4) and VCA-LN (n = 3) groups. Fluorescence intensity was higher in the VCA+LN group as compared to the VCA-LN, but this difference did not reach statistical significance (integrated density: 454.5 ± 329.6 vs 207.1 ± 103.5) (**Figure 8**).

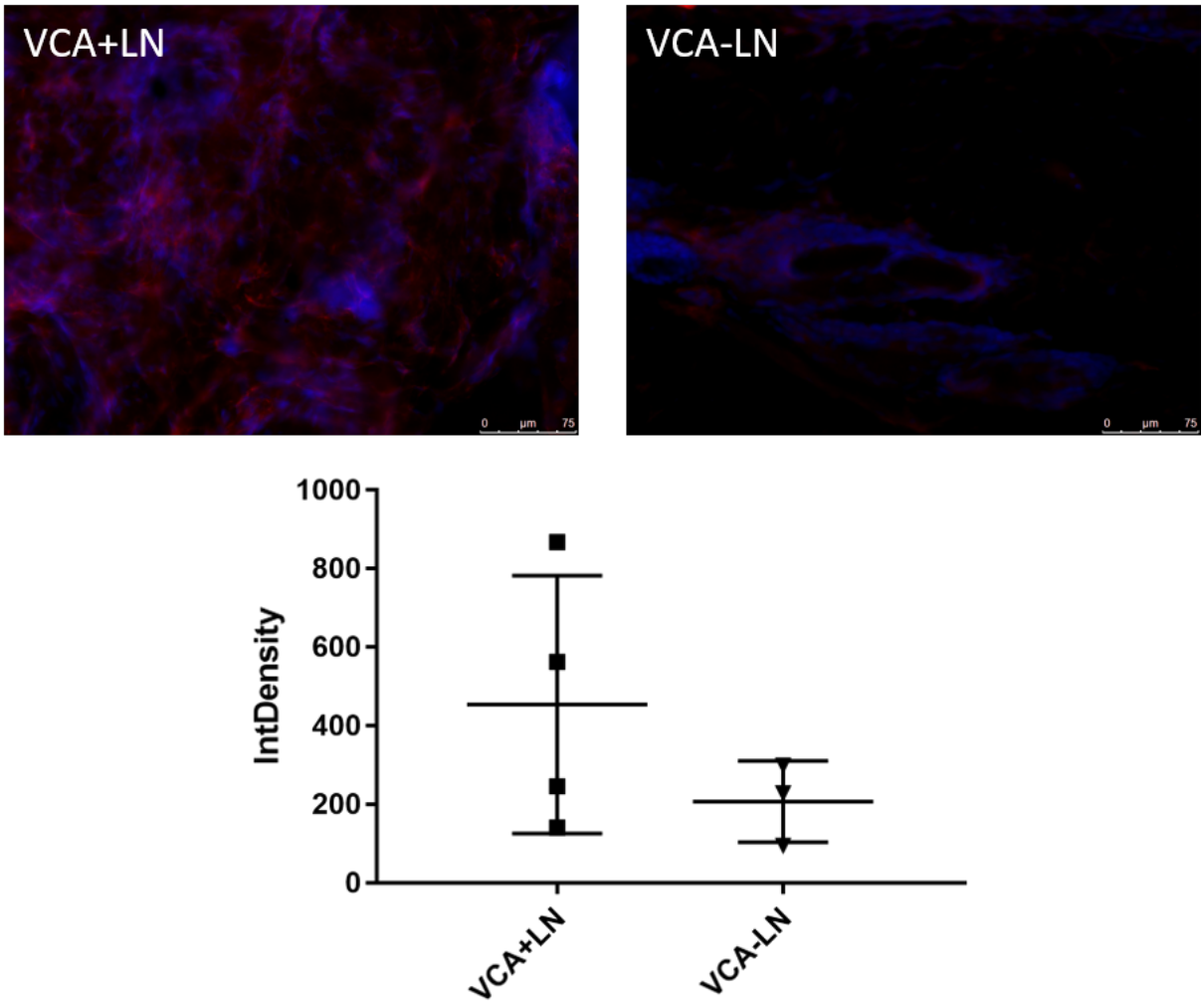

Figure 8: Immunofluorescence micrographs of T cells infiltration in allograft skin tissue. Skin sections were stained for CD3 (red) and DAPI (blue). ImageJ software was used to quantify the fluorescence intensity. Integrated density was higher in the VCA+LN group compared to VCA-LN but this difference did not reach statistical significance. Values are mean±SD.

3.2.2 Dendritic cells infiltration

Dendritic cells infiltration in the allograft skin was compared in VCA+LN (n = 4) and VCA-LN (n = 3) groups. No significant difference in fluorescence intensity was observed (integrated density: 522.9 ± 314.4 vs 519.9 ± 209.8) (**Figure 9**).

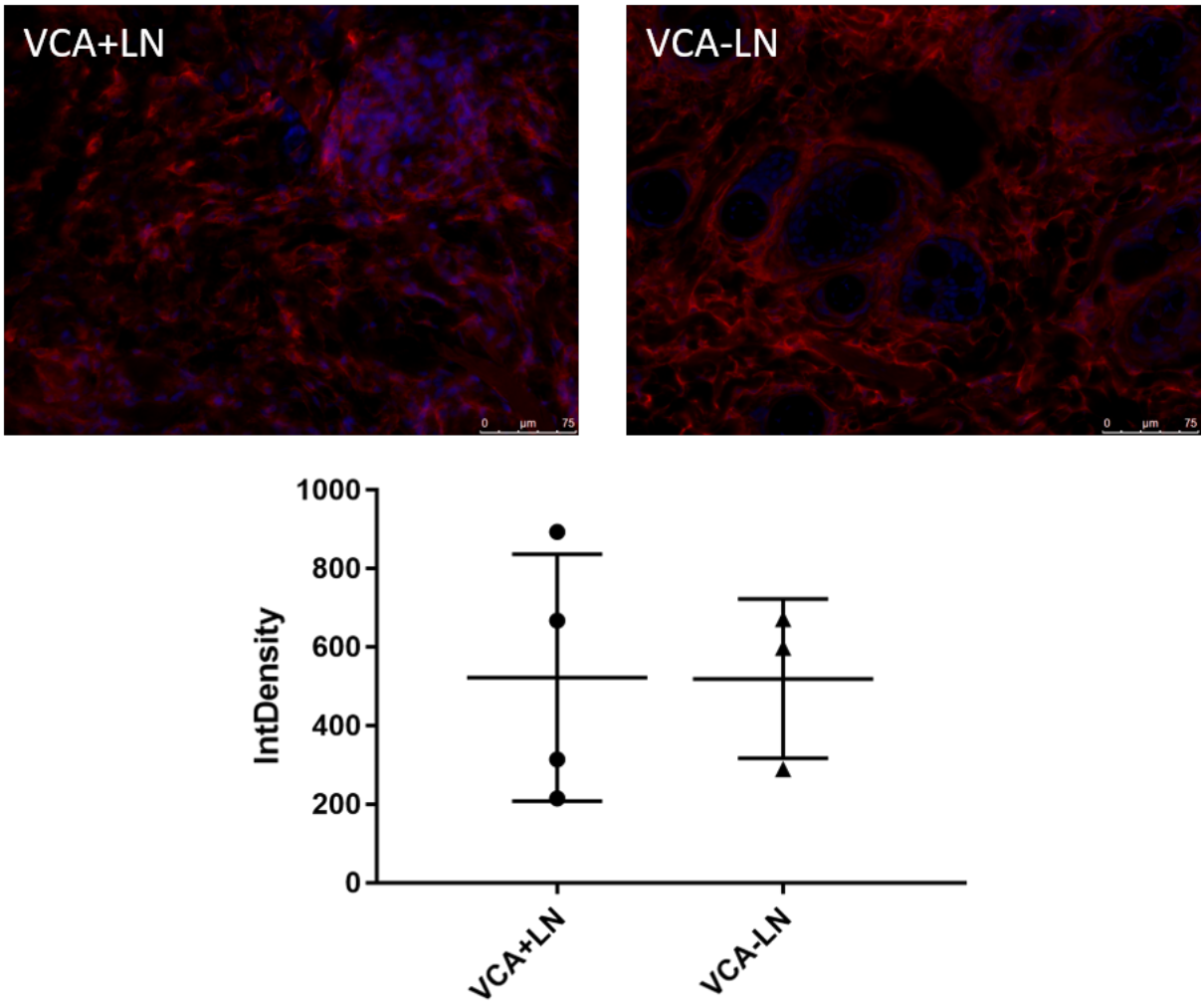

Figure 9: Immunofluorescence micrographs of Dendritic cells infiltration in allograft skin tissue. Skin sections were stained for CD11b/c (red) and DAPI (blue). ImageJ software was used to quantify the fluorescence intensity. No significant difference was observed between VCA+LN (n = 4) and VCA-LN (n = 3) groups. Values are mean±SD.

3.2.3 Macrophages infiltration

Macrophages infiltration in the allograft skin was compared in VCA+LN (n = 4) and VCA-LN (n = 3) groups. No significant difference in fluorescence intensity was observed (integrated density: 1208 ± 786.7 vs 1142 ± 501.9) (**Figure 10**).

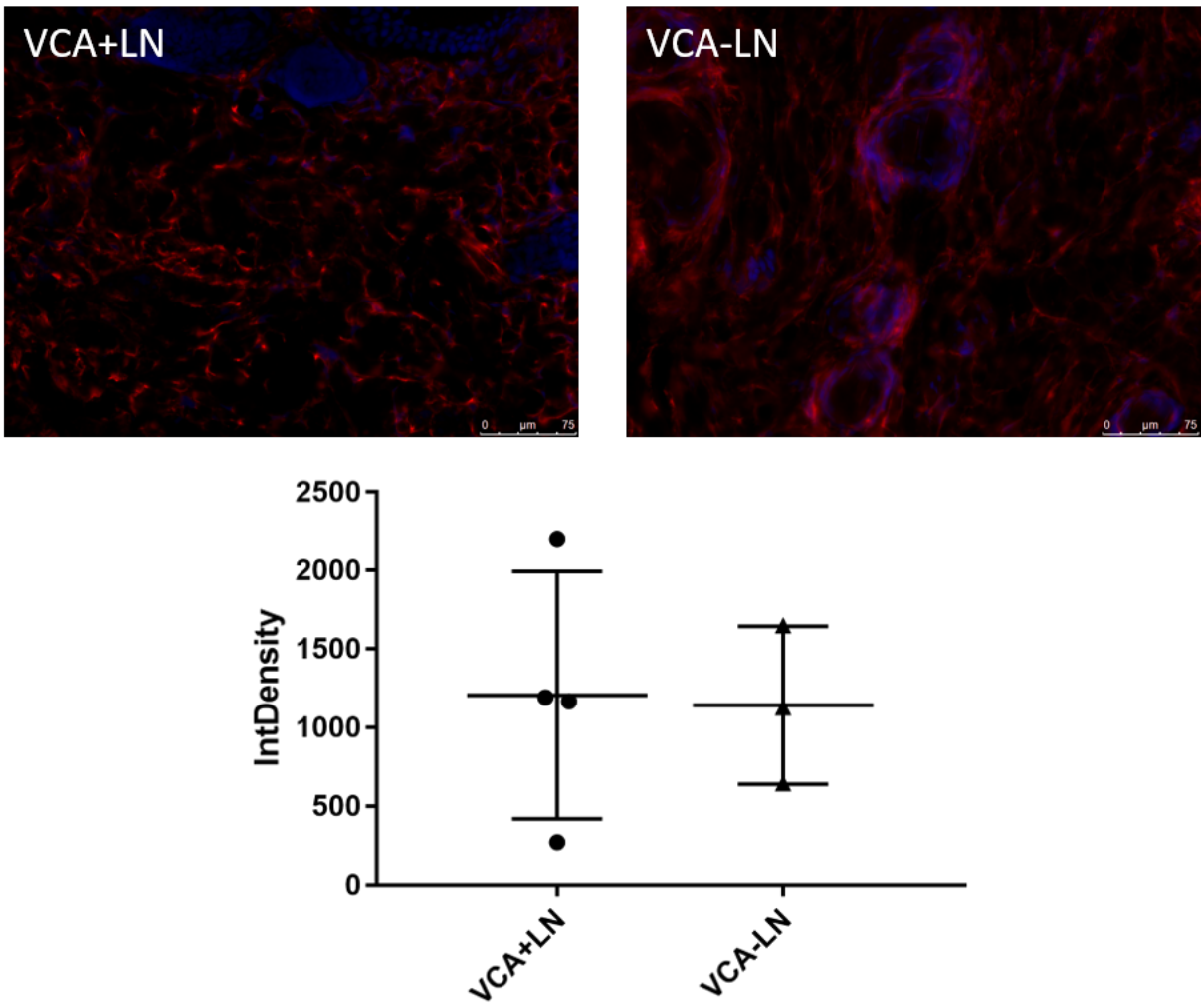

Figure 10: Immunofluorescence micrographs of Macrophages infiltration in allograft skin tissue. Skin sections were stained for CD68 (red) and DAPI (blue). ImageJ software was used to quantify the fluorescence intensity. No significant difference was observed between VCA+LN (n = 4) and VCA-LN (n = 3) groups. Values are mean±SD.

3.3 Transferred lymph nodes as site of activation for recipient T cells

In the VCA+LN group, lymph nodes from donor origin were larger than recipient lymph nodes and therefore easily identifiable. In order to understand if the lymph node presented hypercellularization, we counted the number of cell obtained by two of each type of donor lymph nodes (e.i. popliteal and fat-pad lymph node) and compared them to recipient lymph nodes. The numbers of cells in popliteal donor lymph nodes were $2.05*10^8$ and $6.43*10^8$. The numbers of cells in fat-pad donor lymph nodes were $3.40*10^8$ and $1.75*10^9$. In the same animals the numbers of cells in popliteal lymph nodes from recipient origin were $8.75*10^5$ and $1.73*10^6$ and the numbers of cells in fat-pad lymph nodes from recipient origin were $2.68*10^6$ and $3.65*10^7$.

In order to characterize the cell composition of the retrieved LN, we analyzed the percentage of B, T and dendritic cells by flow-cytomtery. In popliteal ipsilateral lymph nodes from donor

origin (n = 4), mean percentage of B cells was 40.35 ± 5.338 % of total leukocytes. 36.468 ± 5.115 % were from recipient origin and 3.883 ± 1.712 % were from donor origin. Mean percentage of T cells was 37.48 ± 7.323 % of total leukocytes. 36.870 ± 6.967 % were from recipient origin and 0.605 ± 0.406 % were from donor origin. Mean percentage of DCs was 4.23 ± 1.604 % of total leukocytes. 3.455 ± 1.391 % were from recipient origin and 0.775 ± 0.367 % were from donor origin (**Figure 11A**).

In fat-pad ipsilateral lymph nodes from donor origin (n = 2), mean percentage of B cells was 33.9 ± 3.536 % of total leukocytes. 29.9 ± 2.814 % were from recipient origin and 4 ± 0.721 % were from donor origin. Mean percentage of T cells was 35.75 ± 5.586 % of total leukocytes. 35.065 ± 5.438 % were from recipient origin and 0.685 ± 0.148 % were from donor origin. Mean percentage of DCs was 1.755 ± 1.252 % of total leukocytes. 1.290 ± 1.075 were from recipient origin and 0.465 ± 0.177 % were from donor origin (**Figure 11B**).

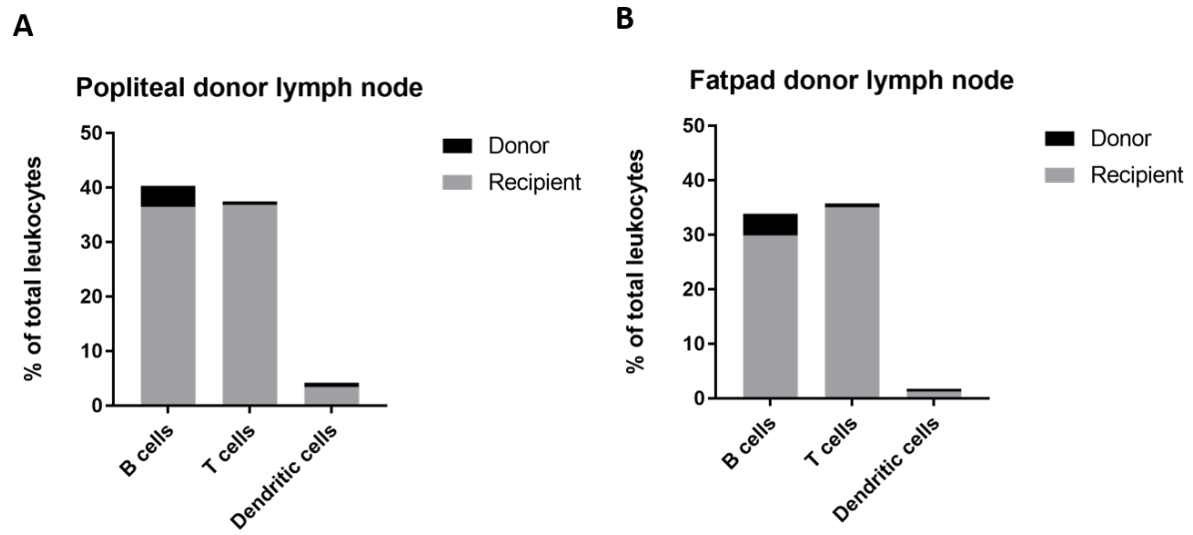

Figure 11: Flow cytometry analysis of leukocytes frequencies in lymph nodes from donor origin. B, T and Dendritic cells from donor and recipient origin were analyzed in fat-pad donor lymph nodes (A) and in popliteal donor lymph nodes (B). Values are mean (n = 4 for popliteal donor lymph node and n = 2 for fat-pad donor lymph node).

3.4 Analysis of the donor and recipient lymphocytes frequencies in recipient lymph nodes

3.4.1 Total donor cells frequencies

Cells from donor origin were found in both groups in every recipient lymph node. In the popliteal contralateral lymph node, mean percentage of donor cells was 4.285 ± 3.113 % of total leukocytes in the VCA+LN group and 3.213 ± 1.038 % in the VCA-LN group. In the fat-pad ipsilateral lymph node, mean percentage of donor cells was 6.575 ± 1.676 % of total leukocytes in the

VCA+LN group and 3.68 ± 0.3677 % in the VCA-LN group. In the fat-pad contralateral lymph node, mean percentage of donor cells was 3.784 ± 2.432 % of total leukocytes in the VCA+LN group and 2.92 ± 0.3818 % in the VCA-LN group (**Figure 12**).

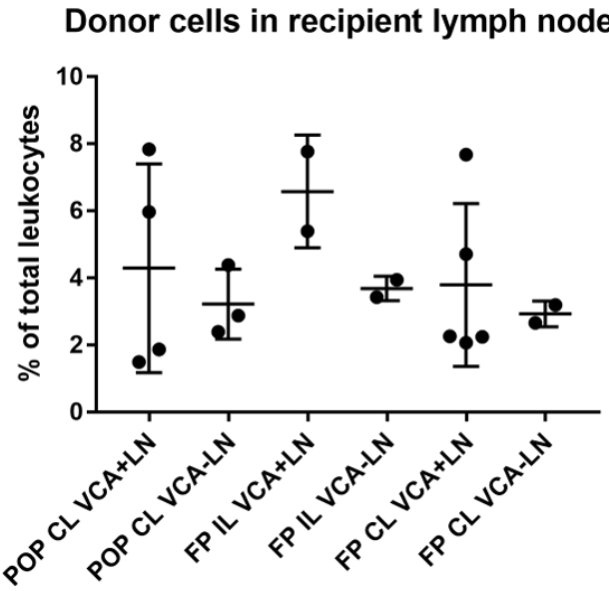

Figure 12: Flow cytometry analysis of donor cells frequencies in lymph nodes from recipient origin. Donor cells were found in both VCA+LN and VCA-LN groups in popliteal contralateral (POP CL), fat-pad ipsilateral (FP IL) and fat-pad contralateral (FP CL) lymph nodes. Values are mean ± SD.

3.4.2 Dendritic cells frequencies

Mean total dendritic cells frequency in popliteal contralateral lymph nodes was significantly higher in the VCA+LN group compared to the VCA-LN group (5.5 ± 1.324 % of total leukocytes vs 0.7967 ± 0.1901 %, p value = 0.0019). No significant difference was observed between both groups in fat-pad ipsilateral lymph nodes (0.945 ±0.2475 % of total leukocytes vs 1.03 ± 0.07071 %) and in fat-pad contralateral lymph nodes (1.672 ± 0.4169 % of total leukocytes vs 1.22 ± 1.245 %) (**Figure 13A**).

Mean recipient dendritic cells frequency was significantly higher in popliteal contralateral lymph nodes in the VCA+LN group compared to the VCA-LN group (5.333 ± 1.303 % of total leukocytes vs 0.719 ± 0.142 %, p value = 0.0019). No significant difference was observed between both groups in fat-pad ipsilateral lymph nodes (0.770 ± 0.198 % of total leukocytes vs 0.850 ± 0.057 %) and in fat-pad contralateral lymph nodes (1.506 ± 0.462 % of total leukocytes vs 1.212 ± 1.256 %) (**Figure 13B**).

No significant difference was observed in any of the recipient lymph nodes when comparing donor dendritic cells frequencies. In popliteal contralateral lymph nodes, mean donor dendritic cells frequency was 0.168 ± 0.054 % of total leukocytes in the VCA+LN group and 0.077 ±

0.054 % in the VCA-LN group. In fat-pad ipsilateral lymph nodes, mean donor dendritic cells frequency was 0.175 ± 0.049 % of total leukocytes in the VCA+LN group and 0.180 ± 0.014 % in the VCA-LN group. In fat-pad contralateral lymph nodes, mean donor dendritic cells frequency was 0.166 ± 0.117 % of total leukocytes in the VCA+LN group and 0.008 ± 0.011 % in the VCA-LN group (**Figure 13B**).

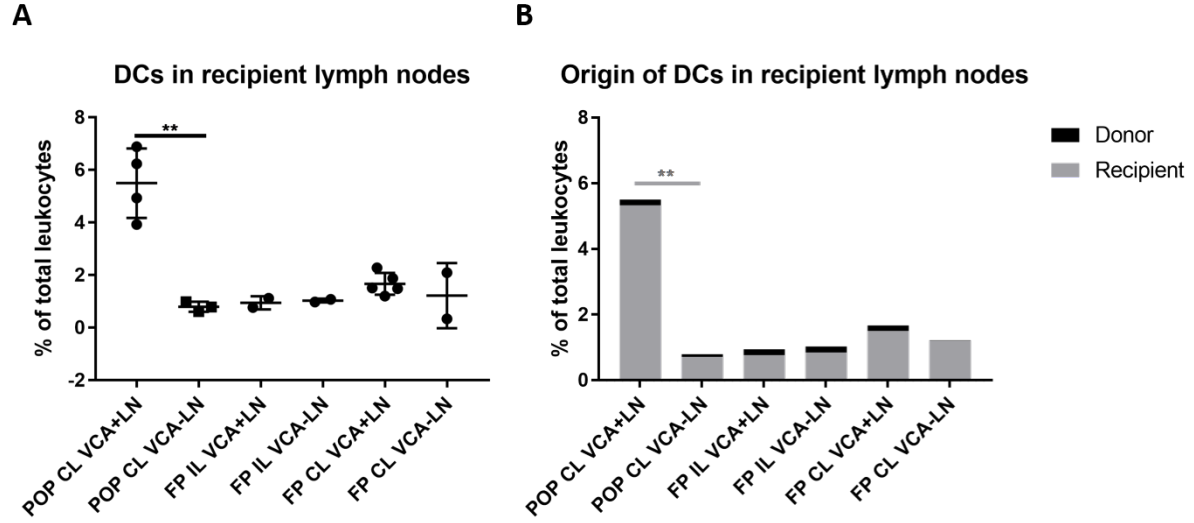

Figure 13: Flow cytometry analysis of Dendritic cells frequencies in lymph nodes from recipient origin. (A) Dendritic cells frequencies were compared in both VCA+LN and VCA-LN groups in popliteal contralateral (POP CL), fat-pad ipsilateral (FP IL) and fat-pad contralateral (FP CL) lymph nodes. Total number of Dendritic cells was significantly higher in the VCA+LN group compared to the VCA-LN group (p value = 0.0019). Values are mean±SD.**(B)** Their origin was also analysed in these same lymph nodes and compared in both groups. Number of Dendritic cells from recipient origin was significantly higher in the VCA+LN group compared to the VCA-LN group (p value = 0.0019). Values are mean.

3.4.3 B cells frequencies

Mean total B cells frequency in popliteal contralateral lymph nodes was significantly higher in the VCA+LN group compared to the VCA-LN group (43.28 ± 8.076 % of total leukocytes vs 30.1 ± 2.254 %, p value = 0.0434). No significant difference was observed between both groups in fat-pad ipsilateral lymph nodes (35.8 ± 2.263 % of total leukocytes vs 32.55 ± 4.879 %) and in fat-pad contralateral lymph nodes (33.94 ± 8.921 % of total leukocytes vs 39.3 ± 6.364) (**Figure 14A**).

Mean recipient B cells frequency in popliteal contralateral lymph nodes was significantly higher in the VCA+LN group compared to the VCA-LN group (40.000 ± 7.449 % of total leukocytes vs 27.333 ± 1.914 %, p value = 0.0374). No significant difference was observed between both groups in fat-pad ipsilateral lymph nodes (31.250 ± 3.182 % of total leukocytes vs 29.800 ± 4.667 %) and in fat-pad contralateral lymph nodes (31.440 ± 8.756 % of total leukocytes vs 36.600 ± 5.798 %) (**Figure 14B**).

No significant difference was observed in any of the recipient lymph nodes when comparing donor B cells frequencies. In popliteal contralateral lymph nodes, mean donor B cells frequency was 3.305 ± 2.498 % of total leukocytes in the VCA+LN group and 2.793 ± 1.002 % in the VCA-LN group. In fat-pad ipsilateral lymph nodes, mean donor B cells frequency was 4.555 ± 0.912 % of total leukocytes in the VCA+LN group and 2.765 ± 0.247 % in the VCA-LN group. In fat-pad contralateral lymph nodes, mean donor B cells frequency was 2.512 ± 1.691 % of total leukocytes in the VCA+LN group and 2.720 ± 0.552 % in the VCA-LN group (**Figure 14B**).

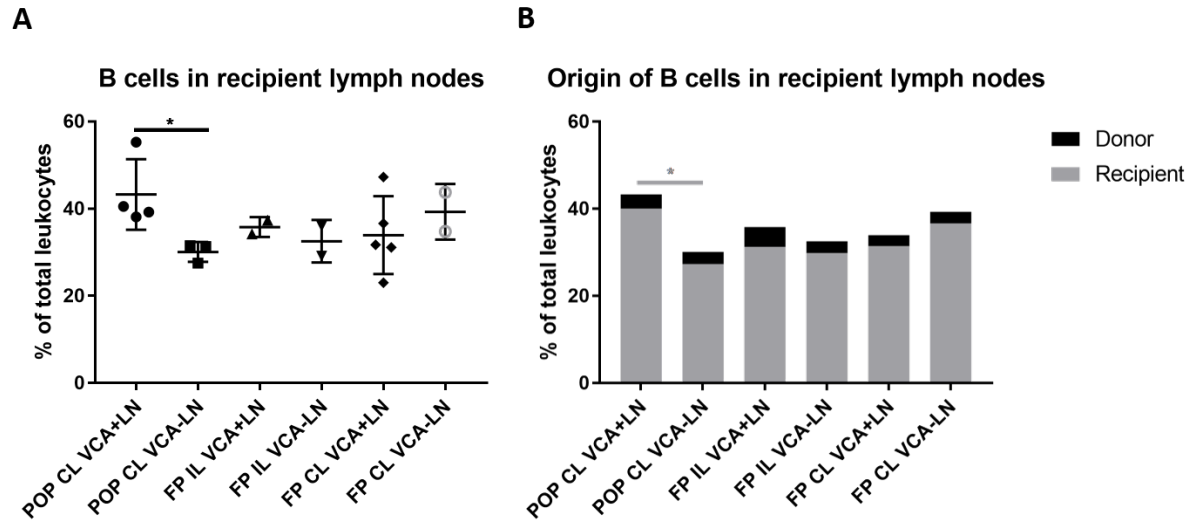

Figure 14: Flow cytometry analysis of B cells frequencies in lymph nodes from recipient origin. (A) B cells frequencies were compared in both VCA+LN and VCA-LN groups in popliteal contralateral (POP CL), fat-pad ipsilateral (FP IL) and fat-pad contralateral (FP CL) lymph nodes. Total number of B cells was significantly higher in the VCA+LN group compared to the VCA-LN group (p value = 0.0434). Values are mean±SD. **(B)** Their origin was also analysed in these same lymph nodes and compared in both groups. No significant difference was observed. Values are mean.

3.4.4 T cells frequencies

No significant difference was observed in any of the recipient lymph nodes when comparing total T cells frequencies. In popliteal contralateral lymph nodes, mean total T cells frequency was 45.43 ± 7.874 % of total leukocytes in the VCA+LN group and 55.27 ± 2.73 % in the VCA-LN group. In fat-pad ipsilateral lymph nodes, mean total T cells frequency was 48.6 ± 2.97 % of total leukocytes in the VCA+LN group and 53.25 ± 5.162 % in the VCA-LN group. In fat-pad contralateral lymph nodes, mean total T cells frequency was 51.6 ± 6.382 % of total leukocytes in the VCA+LN group and 45.8 ± 8.91 in the VCA-LN group (**Figure 15A**).

No significant difference was observed in any of the recipient lymph nodes when comparing recipient T cells frequencies. In popliteal contralateral lymph nodes, mean recipient T cells frequency was 44.775 ± 7.992 % of total leukocytes in the VCA+LN group and 55.167 ± 2.743

% in the VCA-LN group. In fat-pad ipsilateral lymph nodes, mean recipient T cells frequency was 47.750 ± 2.758 % of total leukocytes in the VCA+LN group and 52.900 ± 5.233 % in the VCA-LN group. In fat-pad contralateral lymph nodes, mean recipient T cells frequency was 51.040 ± 6.376 % of total leukocytes in the VCA+LN group and 45.750 ± 8.839 % in the VCA-LN group (**Figure 15B**).

No significant difference was observed in any of the recipient lymph nodes when comparing donor T cells frequencies. In popliteal contralateral lymph nodes, mean donor T cells frequency was 0.650 ± 0.532 % of total leukocytes in the VCA+LN group and 0.120 ± 0.063 % in the VCA-LN group. In fat-pad ipsilateral lymph nodes, mean donor T cells frequency was 0.815 ± 0.205 % of total leukocytes in the VCA+LN group and 0.315 ± 0.064 % in the VCA-LN group. In fat-pad contralateral lymph nodes, mean donor T cells frequency was 0.572 ± 0.294 % of total leukocytes in the VCA+LN group and 0.033 ± 0.046 in the VCA-LN group (**Figure 15B**).

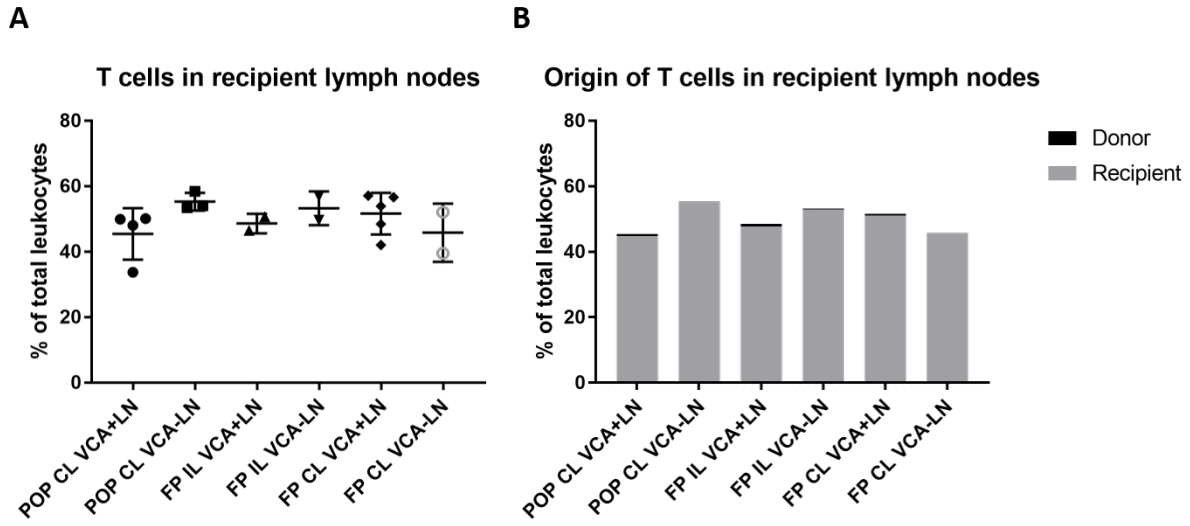

Figure 15: Flow cytometry analysis of T cells frequencies in lymph nodes from recipient origin. (A) T cells frequencies were compared in both VCA+LN and VCA-LN groups in popliteal contralateral (POP CL), fat-pad ipsilateral (FP IL) and fat-pad contralateral (FP CL) lymph nodes. No significant difference was observed. Values are mean±SD. **(B)** Their origin was also analysed in these same lymph nodes and compared in both groups. No significant difference was observed. Values are mean.

4 Discussion

4.1 Evaluation of the study design and explanation of the results

We designed a pilot study aiming at gaining the first insight on how the inclusion of donor lymph nodes in VCA transplants influences VCA rejection. We showed that we were able to consistently transfer lymph nodes-depleted, or intact, allografts and that we were capable of collecting recipient and donor lymph nodes at euthanasia. We were able to analyse these lymph nodes with flow cytometry and we succeeded in designing an immunofluorescence staining protocol to analyse skin samples.

Moreover, our pilot study gave us the first indications about how the transfer of donor lymph nodes influences VCA rejection. Indeed, we found that the inclusion of donor lymph nodes in VCA transplants promotes rejection through alloantigen delivery to the draining lymph nodes, as observed in solid organ transplantation [Dashkevich et al., 2010, Dietrich et al., 2010, Hos and Cursiefen, 2014, Kerjaschki, 2004].

First, we observed lower rejection scores and reduced T cell skin infiltration in VCA-LN allografts compared to VCA+LN allografts. Immune cell infiltration (especially T cell infiltration), epidermal and/or adnexal involvement (spongiosis, apoptosis, dyskeratosis and necrosis) are the basic features to diagnose and classify rejection in VCA [Cendales et al., 2008]. These results imply therefore that rejection was stronger in the VCA+LN group, as compared to VCA-LN. Skin changes are not limited to VCA rejection and care has been taken to consider all the conditions known in the differential diagnosis when monitoring the animals.

Second, we showed that donor lymph nodes were containing recipient B and T lymphocytes, together with APCs (DCs and B) from recipient and donor origin. We observed that donor lymph nodes were hyper-cellularized and they were therefore containing an important pool of recipient and donor cells taking part in the adaptive immune response. These results suggest that transferred lymph nodes were sites of activation for recipient lymphocytes.

Third, we observed that donor and recipient APCs frequencies in popliteal contralateral lymph nodes were higher in VCA+LN group compared to VCA-LN. The difference in donor dendritic cells did not reach statistical significance. This suggests a higher activation of the immune system in the lymph nodes of this group. If these results are confirmed, they would imply that there is an enhanced alloantigen presentation in popliteal lymph nodes of the VCA+LN group.

Finally, we observed an increased number of donor B, T and dendritic cells in recipient lymph nodes of the VCA+LN as compared to VCA-LN group, however this difference did not reach

statistical significance. APCs from donor origin can directly activate recipient lymphocytes and lead to graft rejection.

An unexpected finding was that, contrarily to what we observed in popliteal recipient lymph nodes, no difference in APCs frequencies was observed in fat-pad contralateral and ipsilateral recipient lymph nodes when comparing both group. A first explanation could come from the anatomy of the rat lymphatic system: popliteal lymph nodes are surrounded by skin and muscle, whereas fat-pad lymph nodes are surrounded by adipose tissue. Skin immunogenicity is greater than adipose tissue and this could explain why we observed differences in immune activation only in popliteal lymph nodes. A second explanation could be that these lymph nodes were not completely connected to the graft lymphatic system. We found donor dendritic cells in every recipient lymph node (except in the contralateral fat-pad of the VCA-LN group), meaning that the two lymphatic systems were connected, but we did not quantify the quality of this reconnection. In addition, during surgery, lymph nodes located in fat-pad were not coloured and therefore more difficult to extract than popliteal lymph nodes.

In the future we would need to make sure to have a way to assess which lymph nodes are connected and which are not. Lymphatic system imaging has been successfully performed in rat [Suami et al., 2011]. It has been shown that lymphatic reconstitution in a rat orthotopic hind limb transplantation model could be imaged using near-infrared lymphography and microinjections [Buretta et al., 2013]. Therefore, the replacement of our patent blue macro-injections by the use of microinjections could facilitate the extraction of lymph nodes located in the inguinal vascularized tissue. Moreover, near-infrared lymphography with microinjections of imaging dyes such as indocyanine green (ICG) or orange lead-oxide could indicate us which lymph node are connected and which are not.

Altogether, these indications suggest that an increased alloantigen presentation is going on in the VCA+LN group, leading to allograft rejection. An important role is played by donor lymph nodes, which are major sites of recipient lymphocytes activation. Interestingly, recipient lymph nodes, especially popliteal lymph nodes, appear to participate in the immune response as well. However, considering the pilot nature of the study and therefore the small numbers of animals, groups have to be expanded in order to reach higher statistical significance and confirm these findings.

4.2 Conclusion

In summary, we succeeded in designing a pilot study whose aim was to gain the first insight on how the inclusion of donor lymph nodes in VCA transplants influences VCA rejection. Our first

results showed that the inclusion of donor lymph nodes in VCA transplants promotes rejection through alloantigen delivery to the draining lymph nodes. These results could have important implications in VCA and immunosuppression, especially in some face transplants were lymph nodes are transplanted as part of the graph. Considering the pilot nature of the study, our findings have to be confirmed.

4.3 Further Research

The best outcome of this pilot study would have been to show that lympatic allotransplantations could be beneficial in inducing peripheral tolerance and therefore could prolong graft survival. In our study, the potential beneficial role of lymphatic endothelial cells was countered by the detrimental passive role of lymph nodes, as sites of activation for lymphocytes. Therefore, an interesting future research project is to specifically assess the role of lymphatic endothelial cells (LECs) in VCA transplantation. This could be achieved by the use of several methods such as the transfer of lymphocytes-depleted donor lymph nodes or the specific blocking of lymphocytes activation in donor lymph nodes. Translated to clinical practice, it would allow hand and face transplanted patients a recuded immunosuppression therapy.

5 Declaration of authorship

I herewith confirm that I wrote this thesis without external help and that I did not use any resources other than those indicated. I have clearly acknowledged all parts of the text where material from other sources has been used, either verbatim or paraphrased. I am aware that non-compliance with the above statement may lead to withdrawal of the academic title granted on the basis of this master's thesis by the Senate, according to the law governing the University of Bern.

February 9, 2017

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