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Comparison between stromal vascular fraction and adipose derived stem cells in a mouse lymphedema model

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ABSTRACT

Background: Lymphedema is one of the most common complications following breast cancer. Axillary lymph node dissection and radiotherapy are two well-known risk factors resulting in either removal or damage to the lymph nodes. As stem cells are known for their regenerative capabilities, they could theoretically repair/restore the damaged lymph vessels leading to a decrease in lymphedema.

Methods: We evaluated the treatment of SVF and ASC on a mouse lymphedema model. Forty-five mice were allocated into three groups containing 15 mice each. The SVF group was injected with 100 µl containing 1×10^6 SVF, the ASC group with 100 µl ml containing 1×10^6 ASC and the NS with 100 µl ml of NS. Volumes of the mice were assessed weekly by µCT hindlimb volumetry for a total of 8 weeks. Lymph vessel morphometry was assessed by cross-sections of both hindlimbs stained for anti-LYVE1. Lymphatic function was assessed by lymphatic clearance.

Results: The volume change between the groups was non-significant throughout all 8 weeks. The immunohistochemistry showed a statistically significant difference between the hindlimbs in ASC vs. NS group $p = 0.032$, 95% CI [-2121, -103].

Conclusion: The volume of the hindlimbs showed that treatment with SVF or ASC yielded very similar results compared to the control group when assessed after 8 weeks. In week two the biggest difference between ASC and NS was seen but the difference diminished during the 8 weeks. The secondary outcomes showed that the lymph vessel lumen decreased when treated with ASC compared to the control group. Lymphoscintigraphy yielded non-significant results.

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Introduction

Roughly 20% of women worldwide diagnosed with breast cancer each year will develop lymphedema [1]. Lymphedema is a condition in which protein rich fluid accumulates in soft tissue after interruption of the lymphatic flow [2]. Risk factors include having a mastectomy, axillary lymph node dissection and radiotherapy [1]. The primary treatment today is conservative care. Compression, manual lymph drainage and skincare are the main self-care treatments [3]. In cases where conservative treatment is insufficient microsurgical treatments such as lymphovenous anastomoses or lymph node transplants could be applied [4]. The microsurgical aim is to drain the lymphatic flow or reconstruct the lymphatic pathways but the success rates varies [4]. Currently, there is no known curable treatment for lymphedema [5].

Regenerative medicine holds great promise in repairing damaged tissues and organs and restore functionality by stimulating the body's own regenerative capacity [6]. As a new source for multipotent stem cells [7], adipose tissue has been introduced and considered as a great candidate for cellular therapy due to the following criteria: (a) it is easily harvested [8]; (b) it can be harvested from the patient themselves [8]; (c) possibility of harvesting an adequate number of cells for transplantation, due to

the high cellular proliferation *in vitro* [9], (d) multipotent capacity of cell differentiation [7], (e) they have little immunogenicity [10]. It is a rich source of stem cells obtained by liposuction and subsequent enzymatic digestion. The generated heterogeneous cell population from adipose tissue is termed stromal vascular fraction (SVF) which consist of more than 10 different types of cells including adipocytes, stem cells, endothelial cells, other progenitor cells, fibroblast, T-regulatory cells and macrophages [10]. Upon *in vitro* culturing of SVF, a cell population emerges, termed adipose derived stem cells (ASC) although less heterogenous they are not homogenous [11].

Comparison of SVF and ASC cells has until this point been limited. A 2018 study on a rat flap model showed that SVF are superior in their regenerative potential compared to ASC [12]. To our knowledge, no one has compared the two cell types in a mouse lymphedema model.

Knowledge of lymphedema and its treatment options have until recently been studied in rodent models, where lymphedema was induced by different means. A single irradiation of 30 Gray (Gy) and various surgical methods involving the lymph nodes have been applied. However, such a radiation dose has shown to induce severe skin and soft tissue damage distinguishing itself

from the clinical lymphedema [13]. This study was conducted on a lymphedema model shown to mimic the clinical setting with no skin or soft tissue damage and prolonged lymphedema through 8 weeks [13].

The aim of the study was to compare SVF with ASC in the treatment of lymphedema in a mouse hindlimb model. The primary endpoint was decrease in volumes of lymphedema when treated with SVF or ASC. Secondary endpoint was decrease in the lymph vessel lumen when treated with SVF or ASC and finally, whether the lymphatic function in mice with lymphedema increased when treated with SVF or ASC.

Materials and methods

Animals

This study was approved by The National Animal Inspectorate (2018-15-0201-01445) in Denmark and all experiments were conducted according to national laws of animal research.

Forty-five 9-week old female C57BL6 mice from Janvier (Janvier Labs, Le Genest-Saint-Isle, Saint-Berthevin Cedex, France) were used in this study. The mice were inbred for more than 20 generations making them ideal for research where the same genetic material is wanted [14]. The mice were acclimatized for seven days before the study began.

Postoperatively the mice were housed individually and received oral analgesic treatment (Buprenorphine, 0.2 mg/g) daily for 3 days. On the fourth day they were transferred to cages of 3–8 mice. They were maintained at a normal 12-h day/night cycle at 21 degrees Celsius with a humidity of 45–55%. They were fed a standard diet and had access to unlimited water. The mice were euthanized by cervical dislocation under anesthesia at the end of the study.

Study design

Lymphedema was induced in right hindlimb of the mice using the same procedure. The mice were then earmarked from 1-45 and randomized into three different groups with 15 mice in each (Figure 1). We used www.randomization.com for the allocation with seed number 26493 for reproduction. The mice were divided into groups. The treatments were blinded for all investigators, except the person solely dealing with the injections. All analyses (volume and lymphoscintigraphy) were done blindly. Group A injected with 0.1 ml containing 1×10^6 SVF. Group B injected with 0.1 ml containing 1×10^6 ASC. Group C (control) injected with 0.1 ml of normal saline (NS).

The mice were injected with the allocated treatment 1 week after surgery. Treatment was given as 0.1 ml solution injected

subcutaneously slightly proximally and distally to the wound gap (Figure 2).

All mice underwent weekly micro-computed tomography (μ CT) scans for 8 weeks to assess the change in volume in the operated and unoperated hindlimbs. To assess the lymphatic function at the eighth and ninth week of the study, four mice from each group underwent technetium ^{99m}Tc human serum albumin (^{99m}Tc -HSA) lymphoscintigraphy (Figure 3).

Forty-five mice were used based on our power calculation for 80% power and a significance level of 5%. A previous study [13] using the exact same method of inducing lymphedema, was used in the power calculation along with our own pilot study (data not shown). The results showed that with 15 mice in each group, we would have an 80% chance of detecting a volume reduction of 10 mm^3 , or around 5%, with a 5% significance level.

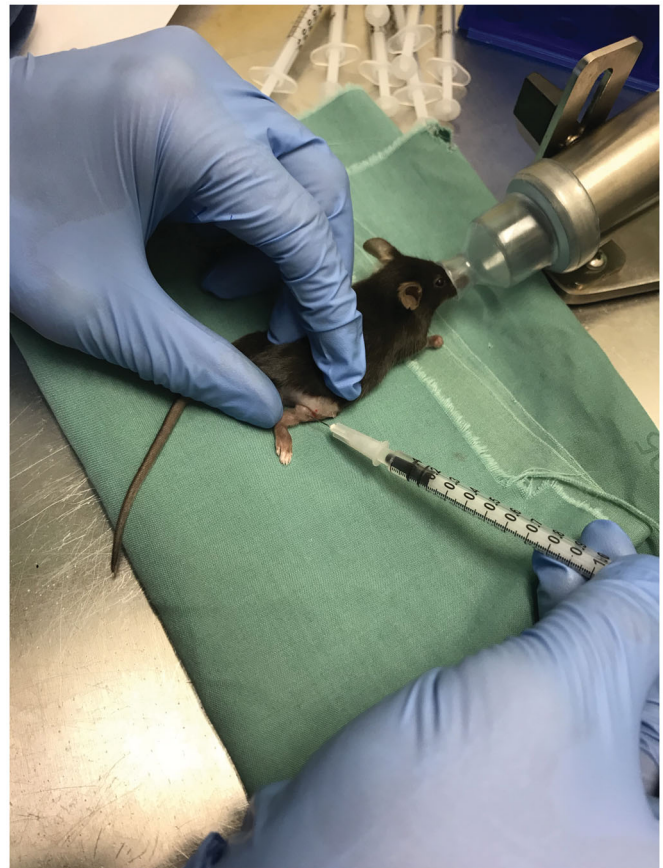


Figure 2. Injection technique of the treatment. The mice were injected one week after surgery.

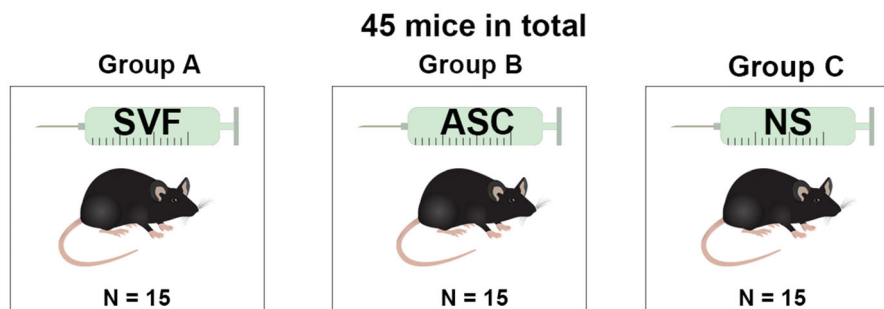


Figure 1. Overview of the groups, the total number of mice and number of mice per group. N: number; SVF: stromal vascular fraction; ASC: adipose-derived stem cells; NS: normal saline.

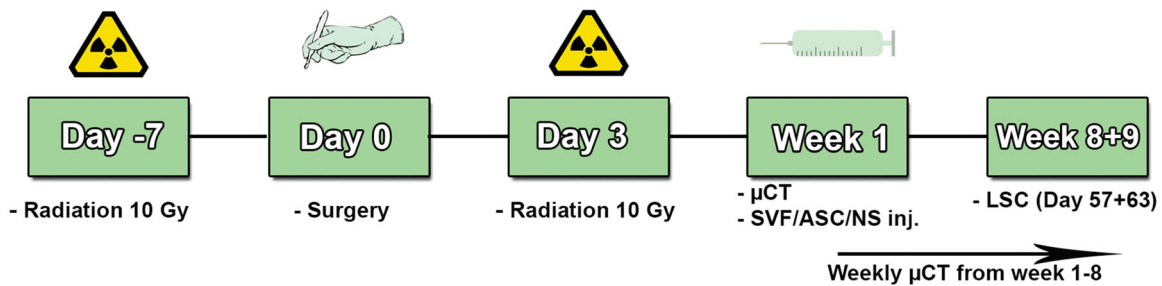


Figure 3. Study design. LSC: lymphoscintigraphy; SVF: stromal vascular fraction (Group A); ASC: adipose-derived stem cells (Group B); NS: normal saline (Group C); Gy: gray; μCT: microcomputed tomography.

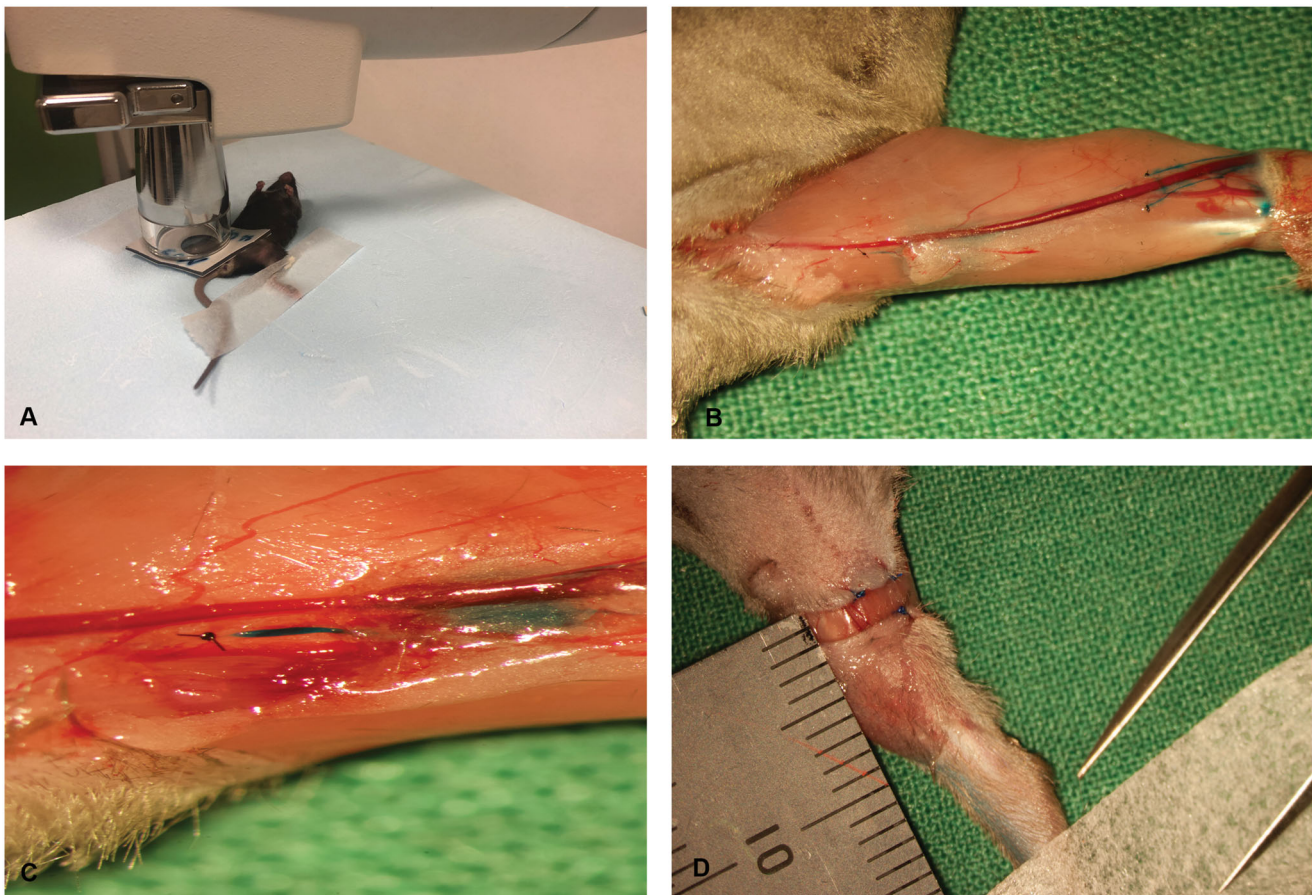


Figure 4. Brief overview of the lymphedema model. A: Radiation of the mice. B: 3 lymph vessels sutured, and blockage confirmed. C: Up close of blocked postnodal lymph vessel. D: 2 mm. circumferential wound left at the end of the surgery.

Establishment of lymphedema

All animals were anesthetized with a subcutaneous injection of Hypnorm (fentanyl 788 μg/kg; fluanisone 25 mg/kg) and Dormicum (midazolam 12.5 mg/kg). The establishment of lymphedema was conducted in three separate procedures. Radiation before and after surgery, and surgery itself [13]. Three surgeons (AB, FD, AW) operated all animals. Prior training preceding the experiment ensured that all surgeons had a similar experience. Two of the three surgeons conducted a pilot study and found similar results as our previous study [13] (data not shown). During treatment, the mice were anaesthetized with a mixture of 1.5–2% isoflurane and 100% oxygen.

Radiation

The hindlimbs of the mice were irradiated with a dose of 20 Gy in two fractions (Gulmay D3100 X-ray instrument (Xstrahl, Camberley, UK) with a dose rate of 5.11 Gy/min (100 kVp, 10 mA, HVL 2.53 Al) (Figure 4). The diameter of the irradiated field was 25 mm. The radiation was performed 7 days before and 2 days after surgery.

Surgery

Surgery was performed according to a previously described model [15], but with minor modifications [13]. Briefly, a circumferential

incision was made approximately at the mid-thigh area. The skin was then carefully dissected from the underlying muscle both proximally and distally. Approximately 0.01 ml patent blue V was injected between the second and third toe almost instantly revealing a postnodal and two prenodal lymph vessels along with the popliteal lymph node. Lymph vessels were sutured with 10-0 suture and the popliteal lymph node was resected. The mouse was then fixated on the supine position and the subiliac lymph node along with the fat pad was resected.

Lastly, to block the superficial lymph flow, the skin was sutured leaving a 2 mm gap using 6-0 nylon suture [16,17].

Isolation, culture and characterization of cells

SVF

To isolate SVF, subcutaneous fat pads from individual eight-week old C57BL6 female mice were dissected and rinsed in 3 ml Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, BE12-604/U1). Tissue was cut into small pieces of 2–4 mm and enzymatically digested using an adipose tissue dissociation kit (Milteny Biotec, MACS, 130-105-808) according to the manufacturer's protocol on a gentle MACS Octo Dissociator for 40 min. Digestion was stopped by adding 5 ml complete DMEM containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma, Cat. no. 217135) with 1% penicillin/streptomycin (Lonza) and filtered through a 100- μ m nylon mesh. SVF cells were pelleted (400 g, 4 °C, 10 min) and washed in 10 ml complete DMEM/F12, then used immediately for the desired application. For injections of freshly isolated SVF, the cells were resuspended immediately at 1×10^6 cells in 100 μ l sterile phosphate-buffered saline (PBS) and kept cold until injected.

ASC

To culture ASCs, SVF cells were plated in Petri dishes with 20,000 cells/cm² in complete DMEM medium and incubated at 37 °C in humidified CO₂. These primary cells were defined as passage 0 (P0) and cultured for 3 days to reach up to 90%

confluency. Thereafter, every third day cells were passaged by trypsinization (Sigma, Cat no.200590) and reseeded at 10,000 cells/cm² in petri dishes. These cultured primary cells showed uniform spindle shaped morphology and were referred to as ASC. ASC at passage 2 (P2) were used to inject into mice at density of 1×10^6 cells in 100 μ l sterile PBS. The animals used for extraction of adipose tissue were euthanized after the procedure by cervical dislocation under anesthesia.

Characterization of cells

To characterize mouse SVF and ASC, mouse mesenchymal stem cell marker antibody panel was used (R&D system, Catalog number SC018). Protocol was performed as per manufactures instruction. Briefly, cells were fixed with 4% NBF (neutral buffered formalin) and 1×10^6 cells were resuspended in 1 ml staining buffer (HBSS (Hank's Balanced Salt Solution) with 5% serum). For each marker 90 μ l of cell suspension was mixed with 10 μ l of antibody and incubated at room temperature for 30 min. Following incubation with the primary antibody, cells were washed with staining buffer and incubated with corresponding secondary antibody tagged with Alexa Fluor-647 in dark for 30 min according to manufacturer's instruction. Cells were washed and resuspended in 300 μ l staining buffer for flow cytometric analysis. Data were analyzed on FlowJo (FlowJo Software, Ashland, OR, US). Flowcytometry is graphed in Figure 5.

CT and SPECT lymphoscintigraphy

CT and single-photon emission computed tomography (SPECT)-lymphoscintigraphy was performed following a previously described model [13] with minor modifications. Briefly, the scans were performed on a Siemens INVEON multimodality pre-clinical scanner (Siemens pre-clinical solutions, Knoxville, TN, US). The minor modifications included the projection which was set to 1200 ms (950 ms previously) and the transaxial field of view which was 44 mm (38 mm previously). Through each imaging session,

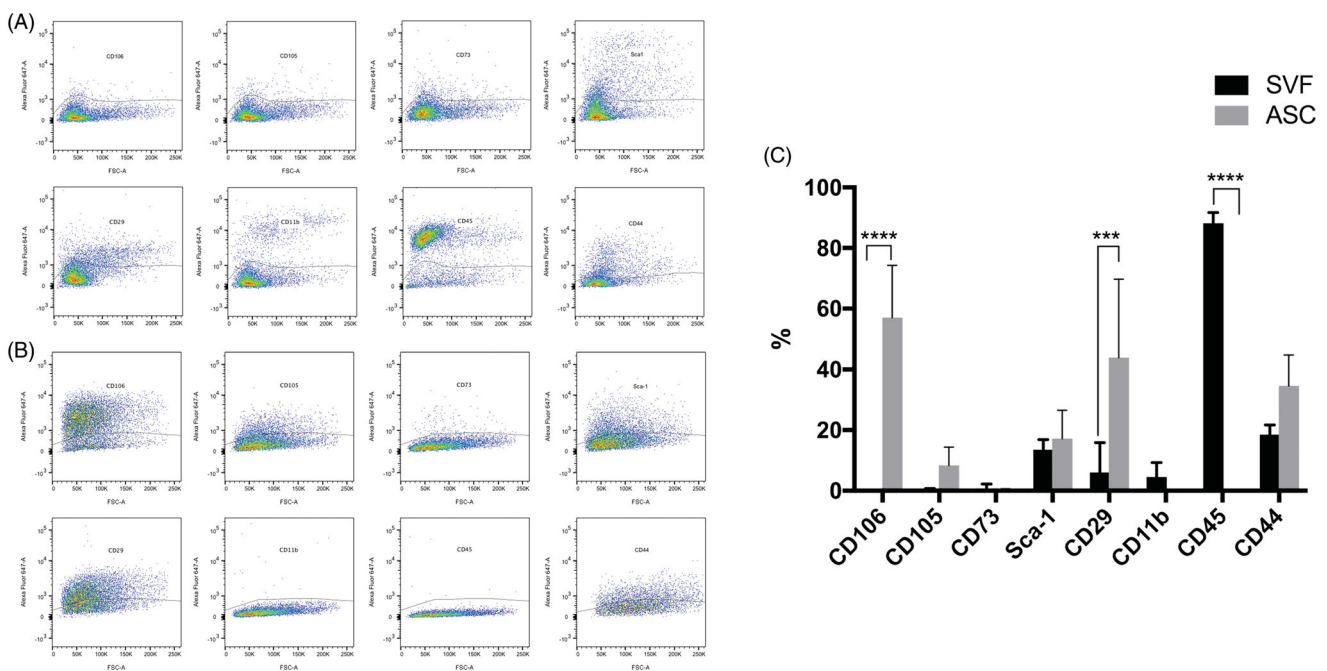


Figure 5. Characterization of mouse SVF and ASC: representative images of flow cytometric analysis of (A) SVF and (B) ASC with indicated mesenchymal stem cell markers are shown. (C) The level of mesenchymal stem cell markers of SVF and ASC cells were assessed and found to be significantly different for CD106 ($p^{***} < 0.0001$), CD45 ($p^{***} < 0.0001$) and CD29 ($p^{***} < 0.001$), $n = 3$. SVF: stromal vascular fraction; ASC: adipose-derived stem cells.

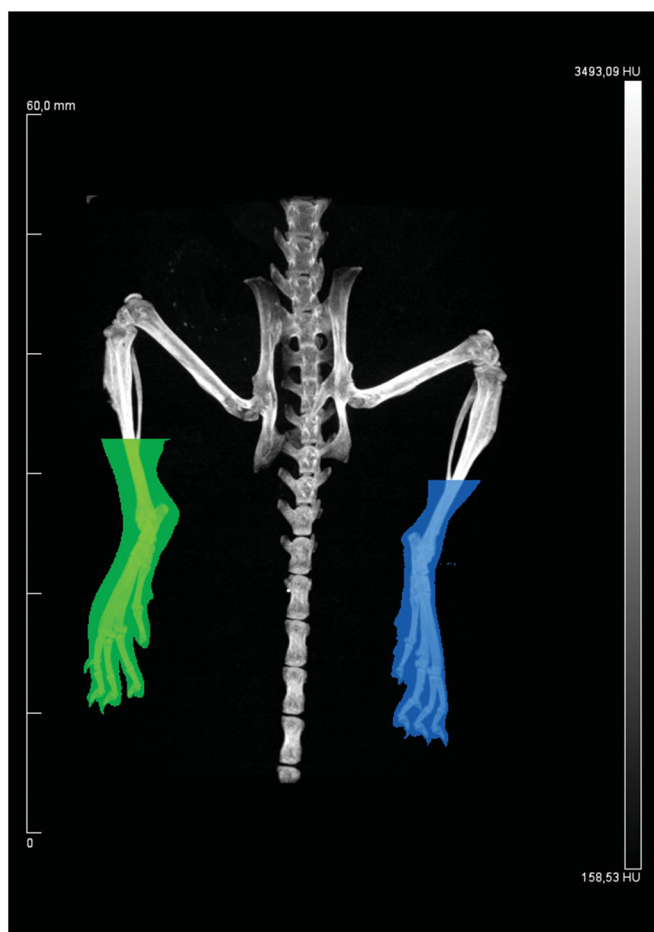


Figure 6. μ CT scan showing the volumetric measures of both hindlimbs from mouse number 13 (SVF-group) from week 2. The distal tibiofibular joint was chosen as the upper volumetric boundary limit and can be seen just superior to the highlighted areas. The volume of the hindlimb distally to this joint was then calculated using simple thresholding techniques. All voxels within the Hounsfield interval of -500 Hounsfield Units and 4000 Hounsfield Units were included in the hindlimb volume. μ CT: micro-computed tomography; SVF: stromal vascular fraction.

the animals were anesthetized with a mixture of 1.5–2% isoflurane and 100% oxygen and placed front feet first in a prone position on a heated SPECT/CT animal bed (38 mm wide).

The animals received an injection of 0.02 ml ^{99m}Tc -HSA subcutaneously between the second and third toe to measure the lymphatic removal rate. The mean radioactive activity was approximately 14.9 ± 1.9 MBq for the left hindlimb and 15.1 ± 1.5 for the right MBq (Vasculocis, CIS Bio International) and administered using a 0.5 ml 30 G insulin syringe (Covidien) under general anesthesia with a mixture of 1.5–2% isoflurane and 100% oxygen.

After 45 min the tracer was assumed to have reached a steady flow with a constant removal rate [18]. Mice underwent SPECT-lymphoscintigraphy at 45 min and 4 h after injection of ^{99m}Tc -HSA and moved around freely between image sessions.

Immunohistochemistry

After euthanasia, the hindlimbs of the mice were cut off and fixed in 4% buffered formaldehyde for 48 h. The feet were then cut 1 cm distal to the heel and decalcified in 4.0 M formic acid/ 0.5 M sodium formate for 24 h. The limbs were then embedded in paraffin. We stained $3 \mu\text{m}$ thick sections with anti-LYVE1 (ab33682; Abcam, Cambridge, UK). Tris-EGTA buffer (pH 9.0) at 60°C overnight was used to achieve antigen retrieval. Specimens were

incubated with primary antibody (1:10,000) for 60 min at room temperature. Envision + HRP labelled polymer (K4003; Dako; Agilent, Glostrup, Denmark)/DAB + was used as detection system.

Analysis

SPECT lymphoscintigraphy

Data analysis of the SPECT/CT fused images were performed with the INVEON Research Workplace software, version 4.2 (IRW; Siemens Healthcare, Ballerup, Denmark). Procedure is described in a previous model [13].

μ CT

Volumetric measures of hindlimbs were calculated from acquired CT images. To standardize the analysis the distal tibiofibular joint was chosen as the upper volumetric boundary limit [19]. The volume of the hindlimb distally to this joint was then calculated using simple thresholding techniques. All voxels within the Hounsfield interval of -500 Hounsfield units and 4000 Hounsfield units were included in the hindlimb volume; see Figure 6.

Lymph vessel morphometry

The slides were scanned with Nanozoomer Digital Pathology (Hamamatsu Photonics, Boston, MA, USA) and analyzed with NDP viewer. For every mouse a cross-section of the hindlimbs was analyzed. The region of interest (ROI) was defined as the dorsal foot-pad area bounded laterally by the three middle metatarsals. Lymph vessels were identified by anti-LYVE1 staining and the lumen of the individual vessels was then obtained using the “freehand region” function; see Figure 7. Lastly, the total lumen of the lymph vessels was calculated by adding all individual lymph vessel lumens within one leg.

Statistical analysis

To assess baseline differences in week 1 along with different weights of the mice, we calculated the percentage change from baseline (week 1) and every week (X) based on:

$$\frac{(\text{Lymphedema week 1} - \text{Lymphedema week X})}{\text{Lymphedema week 1}} \times 100$$

where lymphedema is the excessive volume meaning the difference between right hindlimb and left hindlimb. In this case, we got a percentage improvement of the lymphedema; therefore, taking into consideration different baseline values along with different weight of the mice hence different ratio values between right and left leg. The Kruskal-Wallis test was used to detect significant differences between groups.

Lymph vessel volume

For each mouse, the lymph vessel lumens within the operated leg were added together. The same was done for the non-operated leg. To adjust for variability within each mouse, these two numbers were subtracted. A sum of zero indicated no difference between the two hindlimbs implying that the lymph vessels had regenerated fully. The results were listed in the three different groups and a one-way ANOVA test was used to compare differences between groups. Tukey’s multiple comparisons test was used to identify which groups differed significantly. All values were reported as a mean \pm standard deviation and graphed with a 95% confidence interval. A two-tailed p value less than 0.05 was considered significant.

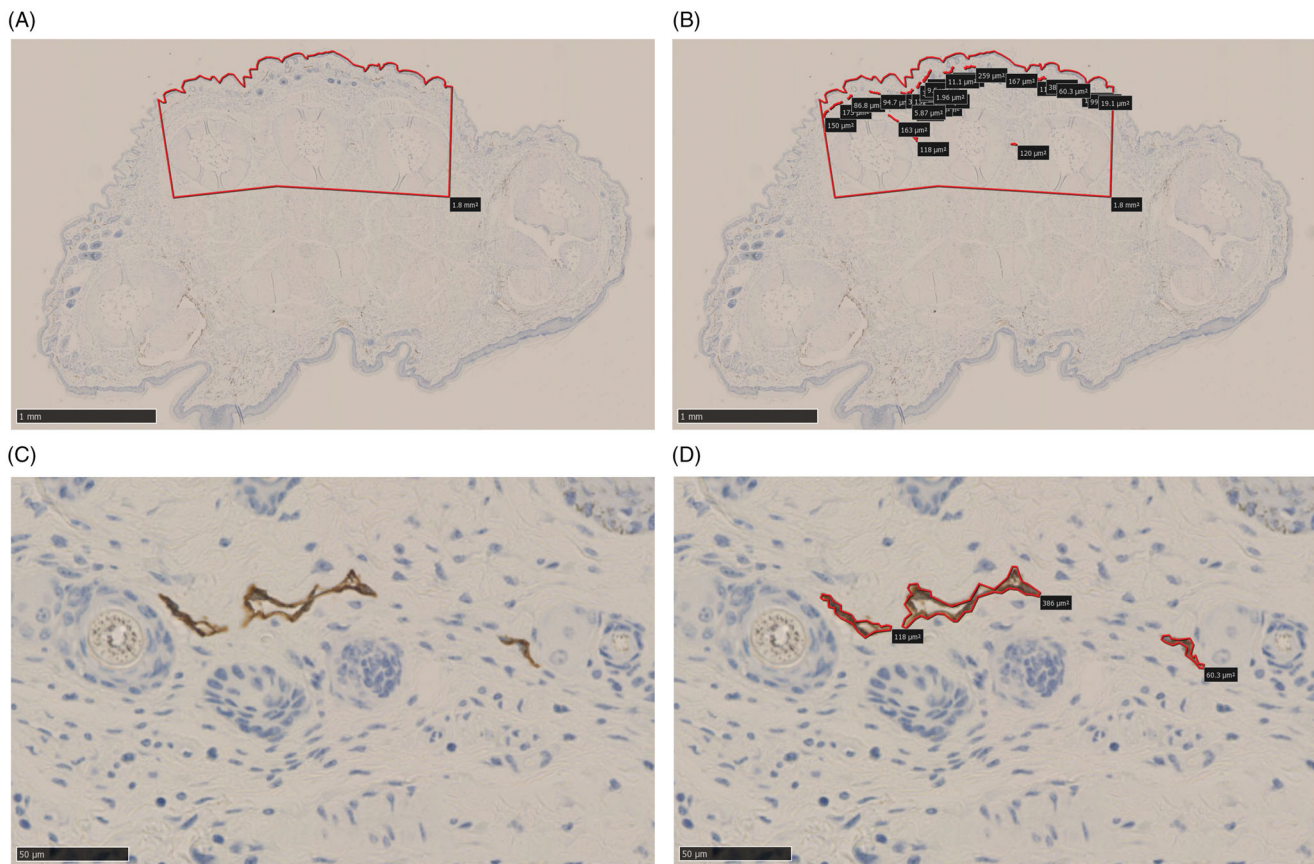


Figure 7. Anti-LYVE1-stained lymphatic vessels in a paraffin-embedded cross-section from the dorsal footpad. (A) The region of interest defined as the dorsal footpad area bounded laterally the three middle metatarsals. (B) The anti-LYVE1 stained lymphatic vessels marked within the region of interest. (C) A close up of an anti-LYVE1 stained lymphatic vessel. (D) A close up of an anti-LYVE1 stained lymphatic vessel marked with the “freehand region”-function.

Our data were analyzed using GraphPad Prism 6.0 software (Graphpad Software, Inc., San Diego, CA, USA) and STATA (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC).

Results

Two mice died during the study, both mice were from the ASC group. One of the mice died in week one during anesthesia, and the other mouse was euthanized for ethical reasons in week three due to poor healing of the circular wound. Immunohistochemistry could not be performed on one mouse from the SVF group as the tissue was lost.

Characterization of ASC and SVF cells

Flow cytometric analysis of the freshly isolated SVF showed a phenotype of Sca-1+/CD106-/CD105-/CD29+/CD11b+/CD45+/CD44+ whereas the cultured ASC showed phenotype of Sca-1+/CD106+/CD105+/CD29+/CD11b-/CD45-/CD44+. Both SVF and ASC differed significantly in the expression levels of all examined markers except Sca-1 and CD44 (Figure 5). CD106 ($p < 0.0001$) and CD29 ($p < 0.001$) were significantly higher in ASCs whereas levels of CD45 ($p < 0.0001$) were higher in the freshly isolated SVF. Our flow cytometric results are in consensus with international studies [20–23].

Limb volumes percentage change

The volume change between the groups was non-significant throughout all 8 weeks. In week 2 the biggest difference between ASC ($11.9\% \pm 45.4\%$) and NS ($-2.66\% \pm 32\%$) ($p = 0.37$) 95% CI [-11, 40], and SVF ($10.2\% \pm 49.8$) NS ($-2.66\% \pm 32\%$) ($p = 0.45$) 95% CI [-12, 38] was seen. The results are summarized in Table 1 and graphed in Figure 8.

Morphometric volume estimates

The immunohistochemistry showed a statistical significant difference between the ASC group and the NS group $p = 0.032$, 95% CI [-2121, -103]. There was no statistical difference between the NS and SVF groups or SVF and ASC groups. The lymph vessel lumen results are summarized in Table 2 and graphed in Figure 9.

Lymphoscintigraphy

The lymphatic clearance was calculated twice: from steady state (45 min) and after 4 h.

Results from the lymphatic clearance showed that unoperated limbs had a better function than the operated hindlimbs. No significant difference was able to be detected between the operated hindlimbs between groups. Results are summarized in Table 3. p values and confidence intervals were $p = 0.30$ 95%, CI [-0.31, 0.08], $p = 0.91$, 95% CI [-0.17, 0.23] and $p = 0.16$, 95% CI [-0.05, 0.34] for SVF vs. ASC, NS vs. SVF and NS vs. ASC, respectively.

Table 1. Percentage change in hindlimb volume.

	SVF (%)	SD	ASC (%)	SD	NS (%)	SD
Week 2	10.2	49.8	11.9	45.4	-2.7	32
Week 3	24.4	45.2	40.4	29.9	24.5	33
Week 4	53.1	37.5	55.8	28.8	51.4	25.3
Week 5	72.4	32.1	73.2	19.5	68.6	23.3
Week 6	79.2	20.2	77.6	17.1	74.3	21.4
Week 7	87.6	21.2	86.4	25.6	81.8	16.8
Week 8	86.7	21.2	88.0	11.6	86.6	12.7

Summarized values of percentage baseline change for every week. Baseline is week one volume difference between right and left hindlimb as the mice had not received treatment at this point. Thereby calculating a baseline excessive volume (lymphedema). Lymphedema was then calculated for the rest of the weeks. The difference between individual weeks and baseline week was then calculated (ex. lymphedema week 1–lymphedema week 2). Lastly, a percentage was calculated based on the baseline. SVF: stromal vascular fraction ($n = 15$); ASC: adipose-derived stem cells ($n = 15$); NS: normal saline ($n = 15$); SD: standard deviation.

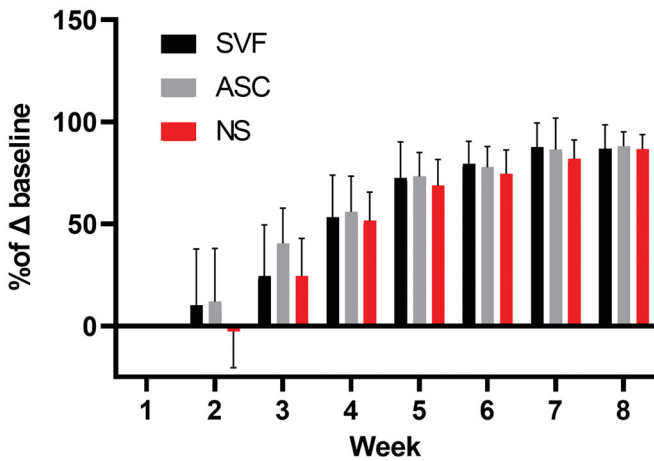


Figure 8. Percentage of baseline change. A baseline change of 100% means that the mouse has recovered completely. Baseline is week 1 volume difference between right and left hindlimb as the mice had not received treatment at this point. Thereby calculating a baseline excessive volume (lymphedema). Lymphedema was then calculated for the rest of the weeks. The difference between individual weeks and baseline week was then calculated (ex. lymphedema week 1–lymphedema week 2). Lastly, a percentage was calculated based on the baseline. No significant difference was detected. “SVF: stromal vascular fraction ($n = 15$); ASC: adipose-derived stem cells ($n = 15$); NS: normal saline ($n = 15$).

Table 2. Lymph vessel lumens.

	SVF (μm^2)	ASC (μm^2)	NS (μm^2)
	-541	-171	1974
	115	331	883
	2948	49.6	860
	577	1152	-148
	134	-452	2420
	1784	-70	-22
	822	-345	472
	-202	497	1446
	-256	-65	587
	254	615	-457
	-191	27	1584
	1162	-759	5823
	577	788	309
	89		1623
			1167
Sum	7272	1597.6	18521
Mean	519.4	122.9	1234.7
SD	933.4	534.4	1507.2

Lymph vessel lumens. The numbers are the difference between the lymphedema hindlimb and control hindlimb in terms of lymph vessel lumens after 8 weeks in the three groups. A mean and sum of zero indicates no difference between the two hindlimbs implying that the lymph vessels have regenerated fully. Values are in μm^2 . SVF: stromal vascular fraction ($n = 14$); ASC: adipose-derived stem cells ($n = 13$); NS: normal saline ($n = 15$); SD: standard deviation.

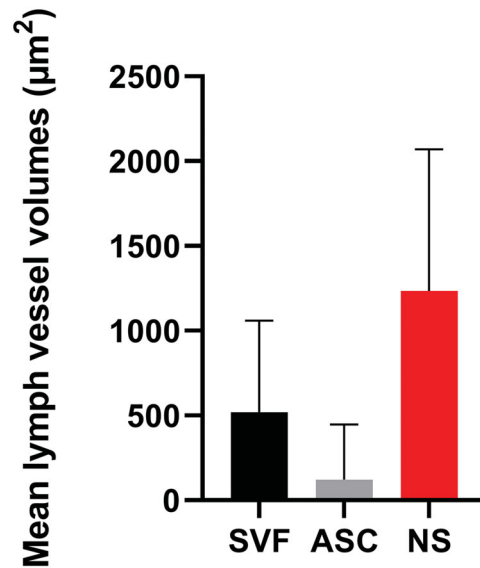


Figure 9. Lymph vessel morphometry. Mean values of the difference between lymph vessel lumens between the lymphedema hindlimb and the control hindlimb. SVF: stromal vascular fraction ($n = 15$); ASC: adipose-derived stem cells ($n = 13$); NS: normal saline ($n = 14$).

Table 3. Lymphatic clearance.

	Mouse #	Right	Left	Ratio
SVF	12	0.554	0.773	1.395307
SVF	24	0.529	0.697	1.31758
SVF	37	0.568	0.735	1.294014
SVF	4	0.525	0.713	1.358095
Average		0.544	0.7295	1.341249
SD		0.02051	0.032919093	0.044712
ASC	27	0.513	0.764	1.489279
ASC	44	0.534	0.778	1.456929
ASC	25	0.456	0.689	1.510965
ASC	43	0.561	0.764	1.361854
Average		0.516	0.74875	1.454757
SD		0.044565	0.040376354	0.065795
NS	17	0.589	0.807	1.370119
NS	42	0.579	0.637	1.100173
NS	11	0.553	0.722	1.305606
NS	7	0.521	0.765	1.46833
Average		0.5605	0.73275	1.311057
SD		0.030392	0.072656154	0.155697

Lymphatic clearance (%/min) shown for 4 mice in each group for each leg and ratio between the legs. Average and SD on a group level is marked in bold. Performed at the end of the study (week 8 + 9).

SVF: stromal vascular fraction ($n = 15$); ASC: adipose-derived stem cells ($n = 15$); NS: normal saline ($n = 15$).

Discussion

In this study, we compared the effect of SVF and ASC in a lymphedema mouse hindlimb model [13]. Forty-five mice underwent the same surgical procedure and irradiation, and were allocated to three groups with 15 mice in each group receiving SVF, ASC or NS. All mice had their hindlimbs scanned weekly for a total of 8 weeks by a μCT -scanner to assess the volume changes. At the end of the study, immunohistochemistry was performed to assess the extent of lymphedema and lymphoscintigraphy was performed to analyze lymphatic function.

The volume difference between the groups was not statistically significant when calculating the baseline lymphedema percentage change. Other studies have shown statistically significant results regarding lymphedema decrease and/or vessel density in week 2 [24,25]. However, these studies did not run for more than 2 weeks

Table 4. Hindlimb volumes.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
SVF-R	229.9 ± 30.8	210.6 ± 45.1	201.6 ± 54.7	180.9 ± 40.2	168.6 ± 12.9	167.5 ± 12.1	169.3 ± 9.47	163.9 ± 16.2
ASC-R	253.9 ± 47	233.8 ± 37.4	205.5 ± 38.9	194.6 ± 36.3	180.1 ± 20.5	177.8 ± 16.9	167.7 ± 13.7	166.4 ± 8.6
NS-R	241.8 ± 35.7	242.4 ± 45.1	216.8 ± 41.5	193.1 ± 31.3	180.0 ± 26	179.9 ± 25	176.4 ± 19.8	164.9 ± 10
SVF-RA	1.59 ± 0.32	1.55 ± 0.36	1.45 ± 0.42	1.28 ± 0.32	1.16 ± 0.09	1.11 ± 0.08	1.07 ± 0.06	1.07 ± 0.06
ASC-RA	1.71 ± 0.3	1.66 ± 0.3	1.44 ± 0.26	1.31 ± 0.23	1.18 ± 0.12	1.15 ± 0.1	1.09 ± 0.1	1.07 ± 0.07
NS-RA	1.77 ± 0.23	1.79 ± 0.33	1.57 ± 0.28	1.35 ± 0.21	1.22 ± 0.17	1.17 ± 0.14	0.12 ± 0.11	1.09 ± 0.07
SVF-L	147.8 ± 25	136.7 ± 4.8	139.4 ± 5.1	142.3 ± 5.6	145.9 ± 5.8	150.4 ± 6.1	159.2 ± 9.9	152.9 ± 10.7
ASC-L	149.4 ± 19.1	141.8 ± 7.9	143.2 ± 7.6	148.4 ± 7.6	152.2 ± 6.9	154.4 ± 8.6	153.4 ± 7.8	156.1 ± 11
NS-L	137.5 ± 21.5	135.3 ± 5.8	138.1 ± 5.3	142.4 ± 5.3	147.3 ± 6.4	153.0 ± 6.6	157.4 ± 7.5	150.9 ± 7.9

Numbers are volumes ± SD (mm³). Summarized weekly outcomes in mean ± SD and ratios ± SD.

R: right hindlimb (lymphedema); RA: ratio; L: left hindlimb (control); ratio is between right and lefthind limbs. SVF: stromal vascular fraction ($n = 15$); ASC: adipose-derived stem cells ($n = 15$); NS: normal saline ($n = 15$).

[24,25]. Interestingly, the biggest difference in our study is seen in week two, although not statistically significant.

Our follow up of 8 weeks was based on our previous study [13], where we found lymphedema after 8 weeks. Thus, it indicated that lymphedema would be present after 8 weeks, but beyond 8 weeks was uncertain. To our knowledge, 8 weeks is the longest amount of time that an intervention on a mouse lymphedema model of this sample size has been evaluated. In a clinical setting, the evaluation should be of a prolonged period as lymphedema is a chronic disease. Further studies are needed on the optimal time period for evaluation in a mouse lymphedema model.

In terms of immunohistochemistry, we showed that lymph vessel lumen decreased when treated with ASC compared to the control group ($p = 0.032$) 95% CI [-2121, -103]. The SVF group also decreased their lumen when compared to the control group, however this was not statistically significant. ASC have previously been studied in the treatment of lymphedema in mice with mixed results. In terms of volume, Hayashida et al. [24] showed non-significant results when assessing volume 14 days after surgery/treatment between the ASC and control group. On the other hand, Yoshida et al. [25] showed significant results 14 days after surgery/treatment between the ASC groups receiving 1×10^5 and 1×10^6 cells and the control group. In term of lymphangiogenesis, an *in vitro* study by Takeda et al. [9] showed that ASC increased the formation of endothelial cells by secreting lymphangiogenic factors [9]. The number of lymphatic vessels was significantly increased in Hayashida et al. [24] in the ASC-group. Our results point in the same direction although we did not count the number of lymphatic vessels, as it was bound to bias. It was unclear whether there was one, two or even three lymph vessels at certain cross-sections. Therefore, we chose solely to include the overall lymph vessel lumen.

Lymph vessel will dilate when lymph stasis occurs [13,26]. Accordingly, a greater lymph vessel lumen will be present with increasing lymphedema [13,26]. In our study, we found no coherence between volumes in the hindlimbs and the lymph vessel lumen. We found no significant difference in the hindlimb volumes, while lymph vessel lumen was statistically significant in the ASC-group vs. NS-group.

Therefore, it should be further investigated whether the size or number of the lymph vessels is a good predictor for evaluating the *intervention* of lymphedema. Clinically, the overall size of a patient's lymph vessel lumen may be irrelevant if the volume of their lymphedema stays the same.

One advantage of our study was that all assessments were blinded. When comparing it to the two studies mentioned above, Yoshida et al. [25] found significant results in regard to hindlimb volume, but it is not mentioned whether the measurements were blinded. Hayashida et al. [24] blinded the analysis of the volume

and found non-significant results for the ASC-group when compared to a control group. The former study used a suture to measure the hindlimb circumference whereas the latter used a plethysmometer, and we used μ -CT-scans. We assume that plethysmometer and μ CT-scans are more precise than suture circumference measurement as it is prone to inaccuracy due to the small limb and risk of compression [19]. Combined with the fact that analysis was blinded and therefore less inclined to bias, it is interesting that we found the same results regarding ASC treatment in all 8 weeks as Hayashida et al. [24] as we both used blinded analysis and used a plethysmometer or μ CT-scans, indicating that our ASC treatment perhaps do not perform as well as previously seen in Yoshida et al. [25].

The mean volume of the operated hindlimbs after 8 weeks in the NS (control) group was less than our previous study [13]. This difference could be due to a number of factors such as differences in operating technique, experience, animals, radiation delivery and the scar releasing treatment. It is possible that by using three different surgeons our operating technique would slightly differ resulting in various lymphedema volumes. However, as the mice were randomized, any difference in surgery should, therefore, be diminished. It is further speculated that the mice were more inclined to use their unoperated hindlimb post-surgery, and that it might have induced hypertrophy leading to a higher volume in the unoperated hindlimb, hence decreasing the difference between the lymphedema hindlimb and the control hindlimb. In fact, there was a statistically significant difference between the volumes of the unoperated hindlimbs of the mice in week eight vs. week one (Table 4) ($p = 0.0115$) 95% CI [2.3605, 18.1595]. Whether the difference is due to hypertrophy is unclear.

In our study, the control group had an 8% volume difference in the operated vs. non-operated leg after 8 weeks. This corresponds to mild clinical lymphedema, which is the most common form [27]. Thereby, our study still has clinical relevance, even though the results varied.

All treatments were delivered through a scar-releasing procedure. In humans, it is known that a sole penetration of a needle into the tissue yields a regenerative response [28] and it is possible that such a response has had a positive effect on the hindlimb of our control group, and perhaps in the ASC and SVF groups as well. Especially since the injection was performed with 5–6 penetrations. Further studies are necessary to investigate the possible connection between the penetration of a mouse lymphedema hindlimb and the reduction of volume.

To our knowledge, no one has studied the effect of SVF in a mouse hindlimb lymphedema model. In our study, the effect of SVF and ASC were similar in all 8 weeks when observing the volume. A previous 2018 study on a rat flap model showed that SVF are superior compared to ASC [12]. A beneficial aspect of SVF cells is that they can be injected as a same-day procedure

compared to ASC, which can take seven days or more to culture. This time-scale reduction allows for liposuction and transplantation on the same day, as shown in a recent clinical study [8], making future studies and therapeutic procedures more practically realistic and manageable. ASC seems superior on a histological level compared to SVF but yields similar results when looking at volume differences. ASC might, therefore, be superior on a histological level but in the clinical setting, where the excess volume is the main problem, the two seem alike.

Our decision to treat the mice with 1×10^6 cells was based upon Yoshida et al. [25]. They compared four different volumes of cells showing that the highest concentration yielded the best results. Further studies are necessary to investigate if a higher number of cells are preferable.

The mice were injected one week after surgery slightly distally and proximally to the circular wound. Our first volume assessment following the injections yielded the biggest difference between groups. Therefore, it would be interesting to investigate if repeated injections are preferable to a single injection. Furthermore, complete wound healing was not present one week after surgery and therefore our injection could have led to minor leakage of the cell solution in the wound gap. One could speculate that a later injection would lead to less leakage due to the more healed wound, and therefore a better regenerative response. However, a later injection might have led the tissue to undergo changes from a liquid state to fibrotic tissue thereby being more difficult to treat [29]. The ideal time of injection has to be investigated in further studies.

Conclusion

The primary endpoint was the volume of the hindlimbs. It showed that treatment with SVF or ASC on a mouse hindlimb lymphedema model yielded very similar results compared to the control group when limb volume was assessed weekly for 8 weeks. The secondary endpoints showed that the lymph vessel lumen decreased when treated with ASC compared to the control group. Lymphoscintigraphy was performed, although only once at the end of the study and only on four mice in each group, and here we were not able to see a difference between groups.

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