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(54) Title: LYMPHATIC VESSEL ENDOTHELIAL HYALURONIC ACID RECEPTOR-1 (LYVE-1+) MACROPHAGES AND USES THEREOF

(57) Abstract: The invention is directed to a method for maintaining structure, homeostasis, repairing or treating media thinning of one or more blood vessels as well as treating atherosclerosis or ischaemic heart disease. The method comprises contacting the blood vessel with LYVE-1+ macrophages either directly or by local administration of M-CSF to recruit endogenous LYVE-1 macrophages to the blood vessel, wherein the blood vessel's media comprises smooth muscle cells.



LYMPHATIC VESSEL ENDOTHELIAL HYALURONIC ACID RECEPTOR-1
(LYVE-1⁺) MACROPHAGES AND USES THEREOF

RELATED APPLICATION(S)

5 This application claims the benefit of U.S. Provisional Application
No.61/552,847, filed on October 28, 2011.

 The entire teachings of the above application(s) are incorporated herein by
reference.

BACKGROUND OF THE INVENTION

10 Major killers in developed countries such as vascular heart disease, aging
and cancer, are associated with loss of blood vessel homeostasis and function which
leads to tissue/organ damage. Thus, there is an urgent need to find means to
maintain and/or repair the function and structure of the blood vessels.

15 SUMMARY OF THE INVENTION

 The invention is based on the utility of lymphatic vessel endothelial
hyaluronic acid receptor-1 (LYVE-1⁺; LYVE-1⁺) macrophages to repair and
normalize blood vessels in an individual in need thereof.

 Accordingly, in one aspect, the invention is directed to method of
20 maintaining structure and homeostasis of a blood vessel in an individual in need
thereof, wherein the blood vessel's media comprises smooth muscle cells,
comprising contacting the blood vessel with an effective amount of lymphatic vessel
endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.

 In another aspect, the invention is directed to a method of repairing one or
25 more blood vessels in an individual in need thereof, wherein the blood vessel's
media comprises smooth muscle cells, comprising contacting the blood vessel with
an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1
(LYVE-1⁺) macrophages.

In another aspect, the invention is directed to a method of treating media thinning of one or more blood vessels in an individual in need thereof, wherein the blood vessel's media comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.

In another aspect, the invention is directed to a method of treating atherosclerosis in an individual in need thereof, comprising contacting one or more diseased blood vessels wherein the one or more blood vessels' media comprise smooth muscle cells with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.

In another aspect, the invention is directed to a method of treating ischaemic heart disease in an individual in need thereof comprising contacting one or more diseased blood vessels wherein the one or more blood vessels' media comprise smooth muscle cells with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows distribution of LYVE-1⁺ macrophages on mouse skin whole mounts associated with smooth muscle cells. Confocal z-stack images of skin whole-mount in WT mice, demonstrating the localization of LYVE-1⁺ cells in close association with SMCs. LYVE-1⁺ and α -SMA were used as markers for initial lymphatics, LYVE-1⁺ cells and SMCs, respectively. LYVE-1⁺ cells did not associate with the initial lymphatic vessel. Instead, they were observed in close association with SMCs that covered both the collecting lymphatics and blood vessels. Bars: 100 μ m.

Figure 2 shows mouse skin fluorescent images of the phenotype of LYVE-1⁺ macrophages, a type of macrophage expressing CD68, CD11b, F4/80, CD163 and CD206. Fluorescent imaging showing that LYVE-1⁺ cells were macrophages. (A) LYVE-1⁺ cells in the skin of WT mice expressed the macrophage markers CD68, CD11b and F4/80. In the skin, LYVE-1⁻ macrophages were also present. LYVE-1 also stains for lymphatic vessels. (B) LYVE-1⁺ macrophages were both CD163⁺ and CD206⁺, where both markers are highly expressed.

(C) LYVE-1⁺ macrophages expressed CD115, also known as macrophage colony stimulating factor receptor (MCSF-r). However, they did not express CX₃CR1, a marker commonly found on monocyte. Bar 10µm.

Figure 3 shows mouse skin fluorescent images evidencing that LYVE-1⁺ macrophages are not dendritic cells: MHC II⁻, CD11c⁻. Fluorescence imaging illustrating that LYVE-1⁺ cells are not dendritic cells. LYVE-1⁺CD68⁺ cells did not express CD11c and MHCII. CD11c⁺ as well as MHCII⁺ cells could be detected via IHC, hence serving as positive controls. Bar 10µm.

Figure 4 shows mouse skin fluorescence imaging evidencing that LYVE-1⁺ macrophages are not endothelial cells: CD31⁻, Podoplanin⁻, VEGFR2⁻ & R3⁻. Fluorescence imaging showing LYVE-1⁺ macrophages were not endothelial progenitor cells during steady state. (A) It was observed that LYVE-1⁺ macrophages did not express CD31 and podoplanin. Positive staining of CD31 and podoplanin were seen expressed by lymphatic vessels. CD68 was used to distinguish LYVE-1⁺ cells from lymphatic vessels. (B) VEGFR2 was positively expressed on blood vessels but not on LYVE-1⁺ macrophages. LYVE-1⁺ lymphatic vessels but not LYVE-1⁺ cells expressed VEGFR3. Bars 10µm.

Figure 5 shows mouse skin whole mount fluorescent images of LYVE-1⁺ macrophages associated with smooth muscle cells. Phenotype characterization of LYVE-1⁺ cells associated with vascular SMCs on skin whole-mounts. CD68 and CD206 were used to identify macrophages while MHCII was used to identify dermal dendritic cells. LYVE-1⁺ cells were CD68⁺CD206⁺MHCII⁻, indicative that they were macrophages. In contrast, LYVE-1⁻ macrophages do not exhibit a preferential association with vascular SMCs.

Figure 6 are fluorescent images of LYVE-1⁺ macrophages associated with smooth muscle covered blood vessels in human skin. Confocal z-stack images of skin whole-mount from humans, demonstrating the localization of LYVE-1⁺ cells in close association with SMCs. LYVE-1 and α-SMA were used as markers for the initial lymphaticism LYVE-1⁺ cells and SMCs, respectively. LYVE-1⁺ cells did not associate with the initial lymphatic vessel. Instead, they were observed in close association with SMCs that covered blood vessels. Bars: 30µm.

Figure 7 shows the construct expressed by the MAFIA mouse model.

Figure 8 show a fluorescent image evidencing that LYVE-1⁺ macrophages express CD115 and a graph evidencing that treatment of (T) MAFIA mice effectively depletes CD115⁺ macrophages in the skin causing LYVE-1⁺ macrophages to decrease significantly. LYVE-1⁺ macrophages are MCSF-
5 dependent. (A) Immunostaining of skin section from MAFIA mouse in which all cells expressing CD115 (MCSF-R) would be GFP⁺. This revealed that LYVE-1⁺ express CD115. (B) Quantification of LYVE-1⁺ and LYVE-1⁻ macrophages by immunostaining for LYVE-1 and CD68 revealed that in treated (T) MAFIA mice LYVE-1⁺ macrophages were significantly depleted in the skin; $p < 0.0001$.

10 Figure 9 shows macroscopic images depicting edema in MAFIA treated mice. Loss of LYVE-1⁺ cells in these mice was associated with signs of swelling, a characteristic of edema due to “leaky” vessels.

Figure 10 shows fluorescent images evidencing that smooth muscle cells (SMCs) are affected in MAFIA treated mice when LYVE-1⁺ macrophages are lost.
15 Confocal z stack images showing SMCs degradation in MAFIA treated mice. Distinct layers of SMCs could be clearly delineated in the artery and vein of NT mice. In treated MAFIA mice, the SMCs lost their morphology and the layers became thinner and degraded. Defective SMCs affects vascular integrity. Bar: 50µm.

20 Figure 11 shows a schematic diagram of how KI20227 prevents autophosphorylation of macrophage stimulating growth factor receptor (MCSF-R) and a graph of quantification data which shows significant depletion of LYVE-1⁺ but not LYVE-1⁻ macrophages in KI20227 treated mice. (A) Schematic diagram showing KI20227 drug prevents the auto-phosphorylation of MCSF-R. (B)
25 Quantification after immunostaining of skin section for LYVE-1 and CD68 reveal the significant depletion of LYVE-1⁺ but not LYVE-1⁻ macrophages in KI20227 treated mice. This indicates that the LYVE-1⁺ macrophages are very dependent on the activity of MCSF-R.

Figure 12 shows macroscopic images illustrating edema in KI20227 treated
30 mice. Arrows indicate a swollen tail observed in treated mice but not in untreated mice. The observations mirror what was seen in MAFIA treated mice, where the penis was enlarged in KI20227 treated mice (lower panel).

Figure 13 shows fluorescent images of degraded SMCs in KI20227 treated mice. Confocal z stack images illustrating poorly spread and degraded SMCs in KI20227 treated mice. SMCs in treated mice underwent thinning and lost their defined shape whereas SMCs in non-treated mice were well-distributed and organized.

Figure 14 shows fluorescent images illustrating depletion of cells that are heavily dependent on the activity of CSF-1R in mice treated for three weeks with KI20227. Skin section from untreated (top panels) and KI20227 treated mice for 3 weeks (bottom panels) were immunostained for LYVE-1 and CD68. LYVE-1⁺ macrophages were significantly reduced in treated mice compared to untreated mice but LYVE-1⁻ seemed to be unaffected by KI20227 treatment.

Figure 15 shows images of vascular leakage in the aorta from KI20227 treated mice for 3 weeks after intravenous injection of EVANS blue. Increased accumulation of EVANS blue was observed in KI20227 treated mice compared to untreated mice.

Figure 16 is a graph showing alterations of LYVE-1⁺ macrophages in skin of untreated apoE^{-/-} mice compared to WT and in apoE^{-/-} mice treated with the lowering-cholesterol drug, ezetimibe. Alterations of LYVE-1⁺ macrophages in the skin of mice deficient for apolipoprotein E (apoE^{-/-}). Quantification of LYVE-1⁺ and LYVE-1⁻ macrophages (CD68) on skin section from WT, apoE^{-/-} mice and apoE^{-/-} treated with ezetimibe which restores plasma cholesterol levels and reduces aortic lesions in apoE^{-/-} mice. LYVE-1⁺ macrophages were significantly decreased in apoE^{-/-} mice which developed dysfunctional lymphatics due to the loss of SMCs coverage on the collecting lymphatics (Lim *et al.*, *Am J Pathol*, 175(3):1328-1237 (2009)). Treatment with ezetimibe restored SMC coverage and lymphatic function and the LYVE-1⁺ macrophages were restored to that of WT.

Figure 17 is a schematic diagram of the aorta and a colorimetric image of aorta whole-mount staining showing adventitial layer of the normal aorta is fully covered with LYVE-1⁺ cells.

Figure 18 shows a schematic and fluorescent image of the composition of the vascular wall of the aorta.

Figure 19 shows fluorescent images evidencing that LYVE-1 macrophages are closely associated with medial SMCs. Cross section of normal aorta for smooth muscle actin, LYVE-1⁺, CD68 revealed that LYVE-1⁺ macrophages are closely associated with medial smooth muscle cells whereas LYVE-1⁺ macrophages are absent or rarely found in the adventitia.

Figure 20 shows atherosclerosis in a cross section of an artery.

Figure 21 shows a picture and schematic of the three sites where atherosclerosis develops with a focus on the aortic root, ascending aorta and carotid (innominate/brachiocephalic artery).

Figure 22 shows fluorescent images evidencing that LYVE-1 macrophages are closely associated with medial SMCs in aortic root from WT mice.

Immunostaining of aortic root section from 25 week old WT mice for SMA, CD68 (macrophage), LYVE-1 revealed that LYVE-1⁺ macrophages are closely associated with medial SMCs.

Figure 23 shows fluorescent images evidencing that loss of LYVE-1 macrophages is accompanied by loss of medial SMCs in aortic root from apoE^{-/-} mice. Immunostaining of aortic root section from 25 week old apoE^{-/-} mice for SMA, CD68 (macrophage) LYVE-1 revealed that LYVE-1⁺ macrophages are lost in adventitia from atherosclerotic mice. The only LYVE-1⁺ structure remaining are lymphatic vessels which are CD68⁻.

Figure 24 shows fluorescent images evidencing that loss of LYVE-1 macrophages is accompanied by loss of medial SMCs in aortic root from Ldlr^{-/-} mice. Immunostaining of aortic root section from 40 week old Ldlr^{-/-} mice for SMA, CD68 (macrophage) LYVE-1 revealed that LYVE-1⁺ macrophages are lost in adventitia from atherosclerotic mice. The only LYVE-1⁺ structure remaining are lymphatic vessels which are CD68⁻.

Figure 25 shows a schematic and pictures of atherosclerosis in apoEKO mice with a focus on the ascending aorta.

Figure 26 shows fluorescent images evidencing that loss of LYVE-1 macrophages is accompanied by loss of medial SMCs in ascending aorta from apoE^{-/-} mice. Immunostaining of ascending aorta section from 25 week old apoE^{-/-} mice for SMA, CD68 (macrophage) LYVE-1 revealed that LYVE-1⁺ macrophages are

lost in adventitia from atherosclerotic mice. The only LYVE-1⁺ structure remaining are lymphatic vessels which are CD68⁻.

Figure 27 shows fluorescent images evidencing that loss of LYVE-1 macrophages is accompanied by loss of medial SMCs in ascending aorta from Ldlr^{-/-} mice. Immunostaining of ascending aorta section from 40 week old Ldlr^{-/-} mice for SMA, CD68 (macrophage) LYVE-1 revealed that LYVE-1⁺ macrophages are lost in adventitia from atherosclerotic mice. The only LYVE-1⁺ structure remaining are lymphatic vessels which are CD68⁻.

Figure 28 shows a schematic and pictures of atherosclerosis in apoE^{-/-} mice with a focus on the carotid 1.

Figure 29 shows fluorescent images evidencing that loss of LYVE-1 macrophages is accompanied by loss of medial SMCs in carotid 1 from apoE^{-/-} mice. Immunostaining of carotid 1 from 22 week old apoE^{-/-} mice for SMA, CD68 (macrophage) LYVE-1 revealed that LYVE-1⁺ macrophages are lost in adventitia from atherosclerotic mice particularly on the side of the aorta bearing the lesions. The only LYVE-1⁺ structure remaining are lymphatic vessels which are CD68⁻.

Figure 30 shows fluorescent images evidencing that loss of LYVE-1 macrophages is accompanied by loss of medial SMCs in carotid 1 from apoE^{-/-} mice and precedes the formation of the fibrous cap. Immunostaining of carotid 1 section from 17 week old apoE^{-/-} mice for SMA, CD68 (macrophage), LYVE-1 revealed that LYVE-1⁺ macrophages are lost in adventitia from atherosclerotic mice before the formation of fibrous cap which characterizes more advanced lesion.

Figure 31 shows fluorescent images evidencing that loss of LYVE-1 macrophages precedes the loss of SMCs in carotid 1 from apoE^{-/-} mice. Immunostaining of carotid 1 section from 14 week old apoE^{-/-} mice for SMA, CD68 (macrophage), LYVE-1 revealed that the loss of LYVE-1⁺ macrophages precedes the loss of SMCs in atherosclerotic mice.

Figure 32 shows FACS data evidencing that monocytes from blood and bone marrow do not express LYVE-1. Flow cytometric data illustrating absence of LYVE-1 expression on monocytes. (A) Monocytes in the bone marrow do not express LYVE-1 (B) Both Gr-1^{high} and Gr-1^{low} blood monocytes are also LYVE-1⁻. (C) A small subset of macrophages present in the peritoneal cavity are LYVE-1⁺.

Figure 33 shows FACS data evidencing that a small population of macrophage derived in vitro from mouse bone marrow express LYVE-1. Flow cytometric analysis of bone marrow derived macrophage culture with M-CSF (10ng/ml). The analysis showed that a small proportion of F480⁺ macrophages (18%) generated in vitro from mouse bone marrow expresses LYVE-1⁺.

Figure 34 shows FACS data showing that 50% of macrophages from mouse adipose tissue are LYVE-1⁺ macrophages. Flow cytometry analysis of cells isolated from mouse adipose tissue (fat pad) showed that 50% of the macrophages are LYVE-1⁺ macrophages. Moreover, LYVE-1⁺ macrophages express higher level of MCSF receptor compared to LYVE-1⁻ macrophages supporting the finding that in skin where LYVE-1⁺ cells are very dependent on MCSF.

Figure 35 show fluorescent images of rat ischaemic heart untreated or treated with a vascularized graft evidencing that LYVE-1⁺ macrophages are only accumulating in treated rat with vascularized graft which ameliorates heart function. This indicates the therapeutic utility of LYVE-1⁺ macrophages. Immunostaining of section from rat ischemic hearts for LYVE-1 revealed that the group of rats treated with vascularized graft in which heart function was ameliorated also exhibited a significant accumulation of LYVE-1⁺ cells compared to rat untreated (without vascularization graft). This indicates that LYVE-1⁺ cells are associated with therapeutical effects mediated by the graft in ischemic heart from rats.

Figure 36 shows depletion of LYVE-1⁺ cells in MAFIA treated mice induces aortic stiffness. Aortic sections from non-treated and treated MAFIA mice were immunostained for smooth muscle action (SMA). Elastic lamina in media is autofluorescent. In treated MAFIA mice where LYVE-1⁺ cells are depleted elastin fibers in the aortic media appeared more rigid compared to untreated MAFIA mice.

Figure 37 shows depletion of LYVE-1⁺ cells induces aortic stiffness. Scanning electronic microscopy of aorta from untreated and treated MAFIA mice to analyze of the structure of collagen fiber and elastin. This analysis revealed thinner collagen fibers and increased elastic laminae fenestrations in treated MAFIA mice which are depleted in LYVE-1⁺ cells compared to untreated mice. These findings further support the aortic stiffness observed in absence of LYVE-1⁺ macrophages.

Figures 38A-38B shows loss of LYVE-1⁺ cells in hypertensive mouse and normotensive mouse. Figure 38A: Aortic section from WT and Smad3 deficient mice (S3KO) at steady state (normotensive) were stained for LYVE-1, CD68 and DAPI to identify LYVE-1⁺ macrophages. As observed in WT mice, aorta from

5 S3KO also exhibit the presence of LYVE-1⁺ macrophage in adventitia under normotensive conditions. Figure 38B: Aortic section from WT and Smad3 deficient mice (S3KO) under hypertensive conditions (treated with angiotensin II) were stained for LYVE-1, CD68 and DAPI to identify LYVE-1⁺ macrophages. In contrast to normotensive conditions, hypertensive conditions in both WT mice and S3KO

10 were associated with the loss of LYVE-1⁺.

Figure 39 shows depletion of LYVE-1⁺ cells induces aortic stiffness. mRNA was extracted from aortic media of untreated and treated MAFIA mice isolated by laser capture microdissection. Expression of mRNA for tropoelastin (TropE, enzyme synthesizing elastin), collagen (coll1a1) and III (col3a1) and metalloproteinases

15 (MMPs) 2, 3, 9 and 12 which are known to degrade collagen was analyzed by real-time quantitative PCR. A significant reduction in TropE and collagen was observed whereas increased expression of several MMPs was detected. These results indicate that in absence of LYVE-1⁺ cells there is an imbalance between synthesis and degradation of elastin and collagen.

20 Figure 40 shows LYVE-1⁺ macrophages express TGFbeta. Aortic section from WT mice at steady state was stained for LYVE-1 and TGFbeta which is a factor known to maintain SMCs. LYVE-1⁺ macrophages expressing TGFbeta were identified in the adventitia of aorta.

Figure 41 shows high expression of MCSF in aortic media from WT mice at

25 steady state. mRNA was extracted from aortic adventitia and media of WT mice and analyzed for MCSF expression. mRNA for MCSF was exclusively expressed in the media which is composed of smooth muscle indicating that smooth muscle cells are the major source of MCSF which is critical to maintain LYVE-1⁺ cells.

Figure 42 shows LYVE-1⁺ can be isolated from mice and human fat. Flow

30 cytometric analysis of cells isolated from (a) mouse adipose tissue (epididymal fat) and (b) human subcutaneous fat showed the presence of LYVE-1⁺ macrophages.

Figure 43 is a schematic showing that LYVE-1⁺ macrophages maintain smooth muscle cells by producing TGFbeta, and inversely, smooth muscle cells secrete factors to maintain LYVE-1⁺ cells.

5 DETAILED DESCRIPTION OF THE INVENTION

Originally recognized as an essential part of the innate and acquired immune system, macrophages emerge as omnipresent regulators of tissue homeostasis, growth and regeneration. Although lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) is considered as a specific marker for initial lymphatic vessels,
10 it is also expressed by some tissue macrophages. It has been proposed that LYVE-1 positive macrophages (LYVE-1⁺ macrophages) are a source of endothelial progenitor cells particularly in inflammatory settings. However, this concept has been recently challenged during development. Thus, the function of LYVE-1⁺ macrophages remains unknown.

15 Demonstrated herein is that LYVE-1⁺ macrophages were required to maintain blood vessel structure and homeostasis as depletion of these cells induced loss of smooth muscle cells and edema. Depletion of LYVE-1⁺ macrophages resulted in macroscopic effects that included peripheral edema, vascular leakage and lymphatic dysfunction. These macroscopic observations were accompanied by
20 significant microscopic changes in the blood vessels. As shown herein, smooth muscle cells in arteries and veins lost their integrity, appeared disorganized and the smooth muscle cell layer of the blood vessels was thinned.

Accordingly, in one aspect, the invention is directed to method of maintaining structure and homeostasis of a blood vessel in an individual in need
25 thereof, wherein the blood vessel's media comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺; LYVE-1+) macrophages. In particular aspects, the methods provide for maintaining a blood vessel's integrity, function, structure, homeostasis or a combination thereof.

30 In another aspect, the invention is directed a method of repairing one or more blood vessels in an individual in need thereof, wherein the blood vessel's media

comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of LYVE-1⁺ macrophages.

In yet another aspect, the invention is directed to a method of treating media thinning of one or more blood vessels in an individual in need thereof, wherein the
5 blood vessel's media comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of LYVE-1⁺ macrophages.

As described herein, LYVE-1⁺ macrophages can be utilized to maintain the integrity and function of blood vessels (*e.g.*, existing blood vessels; newly formed blood vessels). These findings are relevant to therapies aimed at revascularization of
10 a tissue/organ (*e.g.*, infarcted hearts) and/or normalization of vessels (*e.g.*, in cancer and vascular diseases).

For example, shown herein is that atherosclerosis, a known world-wide vascular disease affecting the arteries, was associated with a loss of LYVE-1⁺ macrophages which preceded the loss of medial smooth muscle cells characteristic
15 of early stage of atherosclerosis. Moreover, in ischaemic heart rat models, the beneficial effects of vascularized graft on heart function were associated with the accumulation of LYVE-1⁺ macrophages. Thus, LYVE-1⁺ macrophages can be used to repair and normalize blood vessels which are altered in conditions or diseases such as arteriosclerosis, atherosclerosis, ischemic heart, venous disease, chronic
20 venous inflammatory disease, diabetes, aneurysms, cancer, aging.

Well established mouse models of atherosclerosis were used to demonstrate that loss of LYVE-1⁺ macrophages is associated with atherosclerosis. Therefore, in another aspect, the invention is directed to a method of treating hardening and/or loss of elasticity of a blood vessel in an individual in need thereof, comprising
25 contacting one or more diseased blood vessels, wherein the one or more blood vessels' media comprise smooth muscle cells, with an effective amount of LYVE-1⁺ macrophages. In yet another aspect, the invention is directed to a method of treating arteriosclerosis in an individual in need thereof, comprising contacting one or more diseased blood vessels, wherein the one or more blood vessels' media comprise
30 smooth muscle cells, with an effective amount of LYVE-1⁺ macrophages. As used herein "arteriosclerosis" refers to a condition comprising hardening and/or loss of elasticity of one or more medium and/or large arteries in an individual. In still

another aspect, the invention is directed to a method of treating arteriolosclerosis in an individual in need thereof, comprising contacting one or more diseased blood vessels, wherein the one or more blood vessels' media comprise smooth muscle cells, with an effective amount of LYVE-1⁺ macrophages. As used herein

5 "arteriolosclerosis" refers to a condition comprising hardening and/or loss of elasticity of one or more arterioles (small arteries) in an individual.

In a particular aspect, the invention is directed to a method of treating atherosclerosis in an individual in need thereof, comprising contacting one or more diseased blood vessels, wherein the one or more blood vessels' media comprise
10 smooth muscle cells, with an effective amount of LYVE-1⁺ macrophages. As used herein, "atherosclerosis" refers to a hardening and/or loss of elasticity of an artery due to a (one or more) plaque (*e.g.*, an atheromatous plaque). Thus, the diseased blood vessel can be a (one or more) blood vessel comprising one or more atherosclerotic plaques.

15 Using a rat ischemic (or ischaemic) heart model, it was also shown herein that improved the function of the ischemic heart is attributed to LYVE-1⁺ macrophages. Therefore, in yet another aspect, the invention is directed to a method of treating ischaemic heart disease in an individual in need thereof comprising contacting one or more diseased blood vessels wherein the one or more blood
20 vessels' media comprise smooth muscle cells with an effective amount of LYVE-1⁺ macrophages. Ischemic heart disease or myocardial ischemia occurs when blood flow to the heart muscle is decreased by a partial or complete blockage of an (one or more) artery that carries blood to your heart. The decrease in blood flow reduces the heart's oxygen supply. In one aspect, a (one or more) myocardial infarction (MI)
25 preceded the ischemic heart disease in the individual. In another aspect, the one or more diseased blood vessel is a blood vessel of the ischemic heart.

LYVE-1⁺ macrophages are macrophages that express CD68, Cd11b, F480, CD206 and CD163. In the methods of the invention, a blood vessel is contacted with a composition comprising, consisting essentially of, or consisting of LYVE-1⁺
30 macrophages. The LYVE-1⁺ macrophages can be used in a partially purified, substantially purified, homogeneous or pure form.

LYVE-1⁺ macrophages can be generated *in vitro* (e.g., from bone marrow cells in culture with MCSF); isolated *ex vivo* and introduced (e.g., administered; transplanted) into an individual in need thereof (e.g., introduced or contacted (e.g., directly) with a diseased blood vessel in the individual) and/or expanded (e.g., *in vitro*; *in vivo*) at, and/or recruited to (e.g., induced), the site of interest can be induced.

In particular aspects, exogenous or endogenous LYVE-1⁺ macrophages can be used in the methods of the invention. For example, the LYVE-1⁺ macrophages for use in the methods of the invention can be exogenous (obtained from a source other than the individual to whom the LYVE-1⁺ macrophages are being administered). As will be appreciated by those of skill in the art, LYVE-1⁺ macrophages can be obtained (e.g., separated, isolated) from a variety of sources for use in the methods. For example, as described herein, LYVE-1⁺ macrophages can be separated and/or isolated (e.g., partially, substantially purified) from tissue such as adipose tissue or bone marrow.

In addition, or alternatively, LYVE-1⁺ macrophages can be generated *in vitro*, for example, by obtaining and contacting bone marrow with macrophage colony stimulating factor (MCSF) and maintaining the bone marrow under conditions in which LYVE-1⁺ macrophages are produced by the bone marrow. The LYVE-1⁺ macrophages so produced are then contacted with the blood vessel. In addition, as shown herein, a tissue (e.g., graft such as a myocardial artificial graft (MAG)) comprising LYVE-1⁺ macrophages can be used in the methods of the invention.

As will be appreciated by one of skill in the art, the method of contacting a blood vessel with LYVE-1⁺ macrophages can also be accomplished by stimulating the production of LYVE-1⁺ macrophages at the site of the (one or more) blood vessel to be treated (e.g., stimulating endogenous LYVE-1⁺ macrophages). For example, macrophage stimulating colony factor (M-CSF) can be administered to the individual (e.g., at the site of the blood vessel being treated) to stimulate the production and/or recruitment of the individual's LYVE-1⁺ macrophages at that site.

LYVE-1⁺ macrophages are contacted with one or more blood vessels for example, by administering to an individual in need thereof (e.g., by introduction of

exogenous LYVE-1⁺ macrophages and/or mobilization of endogenous LYVE-1⁺ macrophages), to promote vascularization (*e.g.*, collateral vascularization) and/or arteriogenesis (*e.g.*, for individuals with limb ischemia, heart ischemia, diabetes and/or hypercholesterolemia). In addition, LYVE-1⁺ macrophages can be used in
5 combination with current angiogenic strategies to improve angiogenesis. For example, administration of LYVE-1⁺ macrophages can be used in combination with current angiogenic strategies such as introduction of vascular growth factors (*e.g.*, vascular endothelial growth factor) to stimulate angiogenesis).

In a particular aspect, the LYVE-1⁺ macrophages are mammalian LYVE-1⁺
10 macrophages. Examples of mammalian LYVE-1⁺ macrophages include primate, canine, feline, rodent, and the like LYVE-1⁺ macrophages. Specific examples of mammalian LYVE-1⁺ macrophages include human, pig, dog, cat, horse, cow, sheep, goat, rabbit, guinea pig, rats and mice LYVE-1⁺ macrophages.

Determining or confirming that LYVE-1⁺ macrophages have been obtained
15 for use in the methods can be performed using methods described herein and routine skills. For example, the phenotype of the macrophage can be determined using one or more antibodies that specifically bind to one or more surface markers of LYVE-1⁺ macrophages, such as one or more antibodies that bind CD68, Cd11b, F480, CD206, CD163 or a combination thereof and analyzed by immunohistochemistry or
20 flow cytometry.

As used herein a (one or more) blood vessel includes any of a variety of blood vessels that are comprised of smooth muscle cells and present in an individual (*e.g.*, mammal) such as a collecting lymphatic or blood vessel. As used herein a collecting lymphatic vessel is a vessel in the lymph system that collects lymph (*e.g.*,
25 from interstitial fluid). Examples of a blood vessel include an (one or more) artery, a vein and/or an arteriole. In one aspect, the blood vessel is an existing blood vessel. In other aspects, the blood vessel is a newly formed blood vessel. In another aspect, the method can be used at a site where a blood vessel is about to be formed (*e.g.*, at an injury or wound site).

30 Specific examples of an artery include a pulmonary artery, aorta, mesenteric artery, carotid artery, subclavian artery, coronary artery, renal artery, and iliac artery. Specific examples of a vein include a jugular vein, hepatic portal vein, subclavian

vein, inferior vena cava, pulmonary vein, hepatic vein, coronary vein, iliac vein, superior vena cava, renal vein and arteriole. In particular aspects, the LYVE-1⁺ macrophages are contacted with the adventitia of the aorta. In another aspect, the LYVE-1⁺ macrophages are contacted with a (one or more) blood vessel of the heart.

5 As known to those of skill in the art, a blood vessel's "media" or "medial layer", also known as the "tunica media", is the middle layer or coat of the blood vessel wall, comprised of smooth muscle cells and elastic tissue (Figure 19). It lies between the tunica intima (intimal layer) on the inside of the blood vessel, and the tunica externa or tunica adventitia (adventitial layer) on the outside of the blood
10 vessel. In some aspects, the media accounts for the bulk of the wall of a blood vessel. In some vessels, the smooth muscle cells of the media are arranged in circular layers around the vessel, and the thickness of the coat varies with the size of the vessel. In particular aspects of the invention, the LYVE-1⁺ macrophages are contacted with the outer side of the blood vessel(s).

15 In the methods of the invention, the blood vessel can be contacted with LYVE-1⁺ macrophages in a variety of ways. For example, the LYVE-1⁺ macrophages can be administered systemically or locally to the individual. In one embodiment, the LYVE-1⁺ macrophages are administered locally to the one or more blood vessels (*e.g.*, placed in direct contact with the one or more blood vessels). As
20 will be appreciated by those of skill in the art, in one aspect, the blood vessel can be contacted with exogenous LYVE-1⁺ macrophages using a variety of methods. For example, LYVE-1⁺ macrophages could be transplanted alone or incorporated within, or as part of, a support (*e.g.*, a scaffold; a gel) into the targeted tissue/organ (*e.g.*, heart). As will also be appreciated by those of skill in the art, in other aspects, the
25 blood vessel can be contacted with endogenous LYVE-1⁺ macrophages. For example, the blood vessel can be contacted with endogenous LYVE-1⁺ macrophages by administering (*e.g.*, locally, systemically) macrophage stimulating growth factor (M-CSF) to the blood vessel and maintaining the blood vessel under conditions in which endogenous LYVE-1⁺ macrophages are recruited to the blood vessel or
30 induced to grow at the blood vessel.

Any suitable route of administration can be used to administer, either systemically or locally, a composition comprising, consisting essentially of, or

consisting of, LYVE-1⁺ macrophages or an agent that recruits or induces LYVE-1⁺ macrophages (*e.g.*, MCSF). Examples of suitable routes of administration include oral, dietary, topical, transdermal, rectal, parenteral, intraarterial, intravenous, intramuscular, subcutaneous injection, intradermal injection), inhalation (*e.g.*,
5 intrabronchial, intranasal or oral inhalation, intranasal drops), ocular, pulmonary, nasal, and the like. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending on the particular agent chosen. Suitable dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, aerosols, and the like. In a particular aspect, LYVE-1⁺
10 macrophages are administered locally to the one or more blood vessels. The mode of administration will vary depending on the particular agent chosen.

The LYVE-1⁺ macrophages can be administered in a single dose (*e.g.*, in a day) or in multiple doses. In addition, the LYVE-1⁺ macrophages can be administered in one or more days (*e.g.* over several consecutive days or non-
15 consecutive days).

The LYVE-1⁺ macrophages used in the methods described herein can be administered to a subject as part of a pharmaceutical composition. Formulations will vary according to the route of administration selected (*e.g.*, solution, emulsion or capsule). A "pharmaceutical composition" comprises a (one or more)
20 composition or compound described herein as the active ingredient and inert ingredient(s), such as pharmaceutically acceptable excipients, that make up the carrier. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral
25 administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's lactate and the like. Formulations can also include small amounts of substances that enhance the effectiveness of the active ingredient (*e.g.*, emulsifying, solubilizing, pH buffering, wetting agents). Methods of
30 encapsulation compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art. For inhalation, the agent can be solubilized and loaded into a

suitable dispenser for administration (*e.g.*, an atomizer or nebulizer or pressurized aerosol dispenser).

As described herein, the invention is directed to therapies aimed at conditions and/or diseases which require repair and/or normalization of blood vessels in an individual in need thereof (*e.g.*, therapies involving revascularization of a tissue/organ (*e.g.*, infarcted hearts) and/or normalization of vessels (*e.g.*, in cancer and vascular diseases)). In one aspect, the therapy ameliorates the symptoms associated with the condition and/or disease in an individual. In other aspect, the therapy arrests the condition and/or disease in the individual. In yet other aspects, the therapy eradicates the condition and/or disease in an individual.

As used herein an “individual” refers to an animal, and in a particular aspect, a mammal. Examples of mammals include primates, a canine, a feline, a rodent, and the like. Specific examples include humans, dogs, cats, horses, cows, sheep, goats, rabbits, guinea pigs, rats and mice.

The term “individual in need thereof” refers to an individual who is in need of treatment or prophylaxis as determined by a researcher, veterinarian, medical doctor or other clinician. In one embodiment, an individual in need thereof is a mammal, such as a human.

The need or desire for administration according to the methods of the present invention is determined via the use of well known risk factors. The effective amount of a (one or more) particular compound is determined, in the final analysis, by the physician in charge of the case, but depends on factors such as the exact condition and/or disease to be treated, the severity of the condition and/or disease from which the patient suffers, the chosen route of administration, other drugs and treatments which the patient may concomitantly require, and other factors in the physician’s judgment.

An effective amount of LYVE-1⁺ macrophages is delivered to an individual in need thereof. As used herein, “effective amount” or “therapeutically effective amount” means an amount of the active compound that will elicit the desired biological or medical response in a tissue, system, subject, or human, which includes alleviation of the symptoms, in whole or in part, of the condition and/or disease being treated.

The composition can be administered in a single dose (*e.g.*, in a day) or in multiple doses. In addition, the composition can be administered in one or more days (*e.g.* over several consecutive days or non-consecutive days).

5 Exemplification

Example 1

Tissue distribution of LYVE-1⁺ cells

Analysis of skin whole-mounts at steady state in normal mouse revealed for the first time that LYVE-1⁺ cells are closely associated with collecting lymphatic
10 vessels and blood vessels but not initial lymphatic vessels and capillaries which are not covered with smooth muscle cells. LYVE-1⁺ cells localized on the outside of the vessels and not in the lumen of the vessels (Figure 1). These observations were confirmed in other organs including trachea, heart and intestine.

Whole-mount staining: Ears or other organs including trachea, heart and
15 intestine were isolated from intact C57BL/6 mice and fixed in 2% PFA for 2 days. Two washes of 1XPBS were carried out thereafter. Ears were split into dorsal and ventral sides and subcutaneous fats were removed using a scraper. The ears or other organs were blocked with antibody diluents containing 0.3% Triton and 0.5% BSA at 4°C overnight. Tissues were incubated with 1ml of antibodies diluted in antibody
20 diluents in the subsequent days for 2 days at 4°C. This was followed by one wash (2 hours) in 0.1% Triton and 0.2% BSA. Incubation and washing steps were repeated for subsequent antibody staining steps. After final wash, tissues were stored in eppendorf tubes containing PBS and a few drops of mounting medium and stored at 4°C. Viewing and image capture were performed using the same method as
25 mentioned for immunohistochemistry.

These data showed that LYVE-1⁺ cells are associated with vessels covered with smooth muscle cells and indicate a particular crosstalk between these two cell types. Because of this particular distribution, it was hypothesized that these cells are important to maintain vessel homeostasis.

30

Phenotype of LYVE-1⁺ cells

The phenotype of LYVE-1⁺ cells in skin was characterized by immunohistochemistry. Consistent with previous reports showing that tissue macrophages can express LYVE-1 marker, it was found that LYVE-1⁺ cells expressed known macrophage markers such as CD68, CD11b and F480 (Figure 2 and 5). They also expressed CD206 and CD163 which are markers expressed by a subset of macrophage called alternative macrophage or M2 macrophage which have anti-inflammatory properties and are involved in wound healing and tissue repair (Figure 2). However, these LYVE-1⁺ cells were not dendritic cells as they were found to be negative for CD11c and MHC class II (Figures 3 and 5). They were not endothelial cells or endothelial progenitor cells as they were found to be negative for CD31, podoplanin (lymphatic vessel marker) and VEGFR-2 and VEGFR-3 expressed by blood and lymphatic vessels (Figure 4).

Whole mount staining for macrophage markers showed that LYVE-1⁺ cells closely associated with blood and lymphatic vessels were macrophages (Figure 5).

Relevance to humans

Whether these LYVE-1⁺ macrophages existed in human tissues was investigated using human skin. As described for mouse skin, skin section from human skin exhibited LYVE-1⁺ cells, which also expressed macrophage markers such as Factor XIII but not dendritic cell or endothelial cell markers. Importantly, LYVE-1⁺ macrophages specifically lined-up blood vessels that were covered with smooth muscle cells but not initial lymphatic vessels lacking smooth muscle cells (Figure 6).

Thus, LYVE-1⁺ macrophages are also relevant to the human system, which is in agreement with a previous study (Bourlier, V., et al, 2008).

Immunohistochemistry: Mouse or human skin were embedded fresh in Tissue-Tek Optimum Cutting Temperature (Sakura Finetek) and frozen on dry ice. Embedded skins were then cut into 10 µm sections at -20°C using a cryostat (Leica CM3050S). Sections were adhered onto PolysineTM slides (Thermo Fisher Scientific). Each specimen was fixed in acetone for 5 minutes followed by two washes (5 minutes each) in 1x Phosphate Buffer Saline (1x PBS). Slides were blocked for 10 minutes in 0.2% Bovine Serum Albumin (Hyclone) and washed

twice in 1x PBS. Specimen were incubated with 50µl of antibodies diluted in 1% normal mouse serum (Jackson ImmunoResearch) for 1 hour and washed three times in 1x PBS. Incubation and washing steps were repeated for subsequent antibody staining steps. Incubation with DAPI for 5 minutes was followed by one wash in 1x PBS before mounting with fluorescent mounting media (Dako). Some tails were fixed in 4% (vol/vol) paraformaldehyde (PFA) with 30% (wt/vol) sucrose overnight. Two washes of 30% (wt/vol) sucrose (30 minutes each) were carried out the subsequent day and specimens were embedded as described above for fresh tails. Cryostat sections of 10µm were obtained. Specimens were processed and stained similarly except overnight primary and secondary antibodies were incubated at 4°C. Upon drying, slides were sealed with nail varnish and stored at 4°C. Viewing and image capture was performed using the fluorescence microscope (AxioImager Z1, AxioCam HRM camera; Carl Zeiss Micro Imaging, Inc., Jena, Germany) with Axiovision software (version 4.7; Carl Zeiss Micro Imaging, Inc.) or the confocal microscope (Leica TCS SP5; Leica Microsystems, Inc., Deerfield, IL) with LAS AF confocal software (version 1.8.2; Leica Microsystems, Inc.). The exposure time was held constant for each magnification and wavelength.

Consequences of LYVE-1⁺ macrophage ablation on vasculature at steady state

To demonstrate that LYVE-1⁺ macrophage played a role in controlling vessel homeostasis, strategies to manipulate these cells were established. It is known that macrophage differentiation and homeostasis are generally dependent on macrophage stimulating growth factor (M-CSF also known as CSF-1) as shown in mice deficient for M-CSF (op/op mice) or CSF-1 receptor deficient mice. Moreover, shown herein is that M-CSFR receptor was expressed on LYVE-1⁺ macrophages but not smooth muscle cells. MAFIA mouse (Chinnery, *et al.*, *J Immunol*, 182:2738-2744 (2009)) express green fluorescent protein (GFP) under MCSF receptor promoter, thus all cells expressing MCSF-receptor were GFP⁺ (Figure 8). Thus, the M-CSF/M-CSFR pathway was interfered with in order to alter LYVE-1⁺ macrophages. A well documented strategy known to deplete MCSF-R⁺ macrophages is use of FK506 dimerizer in MAFIA mouse model. As previously mentioned, these are transgenic mice which expressed eGFP and a suicide gene under the control of

MCSF-R promoter, and upon systemic treatment with the FK506 dimerizer, AP20187, resulted in FAS-mediated apoptosis of monocytic cells (Figure 7). In these transgenic mice, LYVE-1⁺ macrophage population was depleted by 80% whereas the LYVE-1⁻ macrophage was only depleted by 30% (Figure 8).

5 Treatment with AP20187 (Fas Ligand): Male and female C57BL/6 MAFIA mice were fed a chow diet post wean and were split into two groups each. One group of mice was treated by daily intraperitoneal injection of 10mg AP20187 dissolved in 0.05% methyl cellulose per mouse for 4 consecutive days. Another group (control) was treated with vehicle (10mg of 0.05% methyl cellulose) alone. Thereafter,
10 treatment was discontinued for 15 days before sacrifice.

Depletion of LYVE-1⁺ macrophages resulted in peripheral edema as shown by swelling of tail, paws and footpads and penis, a highly vascularized organ (Figure 9). Evans blue assays revealed vascular leakage particularly in the liver of these treated MAFIA mice and lymphatic dysfunction in skin of these mice (data not
15 shown). More importantly, these macroscopic observations were accompanied by significant microscopic changes in the blood vessels. Indeed, smooth muscle cells in arteries and veins lost their integrity, appeared disorganized and the smooth muscle cell layer of the blood vessels was thinned as shown by immunohistochemistry (Figure 10).

20 Although MAFIA treated mice resulted in a significant reduction of LYVE-1⁺ macrophages and this ablation resulted in alterations in smooth muscle cells, these mice also exhibited some reduction in LYVE-1⁻ macrophages which likely contributed to the effects observed on the vasculature.

In a second strategy, a drug known as Ki20227 was employed to treat WT
25 mice at steady state.

Ki20227 also targets M-CSF receptor but it works differently from the FK506 dimerizer in MAFIA mouse model. Ki20227 is a small molecule M-CSF receptor tyrosine kinase inhibitor, which acts to prevent the auto-phosphorylation of the M-CSF receptor tyrosine kinase and thus inhibits the cascade of downstream
30 signaling pathways which are crucial for the survival and proliferation of M-CSF-dependent cells (Figure 11).

Treatment with Ki20227, a MCSF Tyrosine Kinase Inhibitor: Male and female C57BL/6 WT mice were fed a chow diet post wean and were split into two groups each. One group of mice was daily treated by oral gavage with 30mg per kg per mouse of Ki20227. Another group (control) was treated with vehicle alone (methyl cellulose). Treatment was continued for 3 to 9 weeks prior to sacrifice.

After treatment with Ki20227 for 3 weeks, there was a significant reduction of LYVE-1⁺ macrophages as compared to the untreated mice. Interestingly, significant changes in the LYVE-1⁻ macrophage were not observed in treated mice compared to untreated mice. After 9 weeks of treatment, LYVE-1⁺ macrophages were still significantly reduced indicating that there was no repopulation of LYVE-1⁺ macrophages during the course of drug treatment and this reduction was sustained throughout the treatment. Importantly, this phenomenon was not observed in LYVE-1⁻ macrophages as no significant differences in macrophage numbers for LYVE-1⁻ population was observed between untreated and treated WT mice (Figure 11 and Figure 14). Similarly to MAFIA mice, these mice developed peripheral edema and lymphatic dysfunction (Figure 12). Interestingly, vascular leakage was observed in aorta from KI 20227 treated WT mice (Figure 15). These macroscopic changes in the periphery were accompanied by alterations in smooth muscle cells of veins and arteries from skin which appeared disorganized and degraded as shown by immunohistochemistry (Figure 13). These alterations resulted in thinning of the smooth muscle layer in blood vessels.

Altogether, these data show the high dependence of these LYVE-1⁺ macrophages on M-CSF receptor activity as blocking M-CSF receptor activity totally ablated LYVE-1⁺ macrophages in tissues at steady state. Loss of LYVE-1⁺ macrophages altered smooth muscle cell integrity of blood vessels and these alterations contributed, at least in part, to vascular dysfunction at steady state (Figures 13-15).

A recent paper reported that daily subcutaneous treatment with KI20227 for 56 days reduces the abundance of LYVE-1⁺ as well as LYVE-1⁻ macrophages in mouse osteosarcoma (Kubota Y *et al.*, *J Exp Med*, 206(5):1089-1102 (2009)). Reduction of these tumor-associated macrophages decreased pathological angiogenesis and lymphangiogenesis in the tumor but did not affect healthy blood or

lymphatic vessels outside the tumors. The differences in KI20027 effect on the vasculature at steady state may be explained as follows: (1) in the experiments described herein, although the dose of KI20227 was similar (50mg/kg) and daily administrated, KI20227 was delivered by oral gavage and not by subcutaneous injection into the tail. Oral gavage likely delivered the drug more systemically than subcutaneous injection and the bioavailability and activity of the drug was likely different; and (2) to assess whether KI20227 treatment affected the vasculature outside the tumor, Kubota *et al.* analyzed the expression of CD31, a marker for blood vessels and LYVE-1 a marker of initial lymphatic vessels. However, they did not assess smooth muscle cell phenotype and the structure of collecting lymphatic vessels. Moreover, they did not mention any macroscopic effects (edema) or perform assays to assess the function of the vessels. Thus, they likely missed the changes described herein as a consequence of LYVE-1⁺ depletion. Kubota *et al.* did not comment about LYVE-1⁺ localization in relationship with blood and lymphatic vessels.

Changes in LYVE-1⁺ macrophage in atherosclerosis, a known arterial disease

Whether changes in the LYVE-1⁺ macrophages could be associated with a known vascular disease was also investigated. Atherosclerosis was chosen as a chronic inflammatory disease of the large arteries (aorta) and well established mouse models of atherosclerosis, mice lacking apolipoprotein E (apoE^{-/-}) or low density lipoprotein receptor (Ldlr^{-/-}), were used. Both models develop hypercholesterolemia and atherosclerotic plaques in the aorta that resemble those described in humans (Figure 20)

Recently, it was reported that hypercholesterolemia in apoE^{-/-} mice is also associated with poor lymphatic drainage in skin and this may contribute to peripheral edema, accumulation of lipids in skin and compromised immunity observed in these mice. Importantly, shown herein was that structural alterations in collecting vessels account in part for the reduced lymphatic transport, namely: smooth muscle cell coverage of collecting vessels was markedly reduced. Lymphatic function and structure of collecting vessels was restored in apoE^{-/-} mice by treating the mice with a FDA-approved cholesterol-lowering drug, ezetimibe. Therefore,

whether these defects in lymphatic vessels in skin of apoE^{-/-} mice could result from changes in LYVE-1⁺ macrophages as they seem to be important in maintaining smooth muscle cell integrity was assessed. Analysis of the density of LYVE-1⁺ and LYVE-1⁻ macrophages in skin from WT, non-treated apoE^{-/-} and treated apoE^{-/-} with ezetimibe by immunohistochemistry (as described above) revealed that defects in lymphatic vessels were associated with a significant reduction in LYVE-1⁺ macrophages and this was reversed by ezetimibe (Figure 16). Conversely, LYVE-1⁻ macrophages increased in non-treated apoE^{-/-} mice compared to WT mice indicating that these cells were not able to compensate for the function of LYVE-1⁺ macrophages (Figure 16).

Next, the aorta was focused upon. First, the distribution of LYVE-1⁺ macrophages in immunostained whole-mount aorta (as described above) from WT mice was analyzed. LYVE-1⁺ macrophages were only detected in adventitia of the aorta which is the outer layer of arteries lining on the media composed of the smooth muscle cells (Figure 17 and 18). LYVE-1⁺ macrophages made direct contact with smooth muscle cells (Figure 19). In contrast, no or very little LYVE-1⁻ macrophages were observed in the adventitia in normal aortas. In apoE^{-/-} mice that developed atherosclerotic plaques in aortic sinus, ascending aorta or right common carotid artery, a loss of LYVE-1⁺ macrophages was observed (Figures 20-29). Importantly, this loss coincided with loss of smooth muscle cells which is also known as “media thinning”. A more extensive study of the events occurring in the right common carotid artery during progression of atherosclerosis in apoE^{-/-} mice revealed that loss of LYVE-1⁺ macrophages preceded the changes in smooth muscle cells (Figures 30 and 31). It is important to note here that loss of LYVE-1⁺ macrophages in the adventitia of the aorta was only observed in the segment of the aorta containing a plaque. In contrast to LYVE-1⁺ macrophages, LYVE-1⁻ macrophages accumulated in the adventitia and in the intima of the vessels (Figures 30 and 31).

These data provide evidence that the loss of LYVE-1⁺ macrophages was associated with vascular disease supporting their critical role to maintain vessel function and structure. In atherosclerosis, loss of LYVE-1⁺ macrophages in the adventitia precedes medial thinning indicating that LYVE-1⁺ macrophages are

required for smooth muscle cell integrity which is consistent with the data in MAFIA mice and KI20227 treated WT mice described above (Figures 9-31).

Mechanism of action of LYVE-1⁺ macrophages

5 Because LYVE-1⁺ cells closely interact with smooth muscle cells and their depletion, at steady state and during atherosclerosis, results in the loss of smooth muscle cell integrity, it was hypothesized that LYVE-1⁺ cells produce a factor important for the maintenance of smooth muscle cells. One factor is transforming growth factor beta (TGFbeta or TGF-β). Immunohistochemistry as described above
10 for TGFbeta in wild type (WT) aorta revealed that TGF beta was expressed in the adventitia of the aorta and by LYVE-1⁺ macrophages. As mentioned earlier, LYVE-1⁺ macrophages are highly dependent on M-CSF. Thus, the spatial distribution of this growth factor in aorta from WT and apoE^{-/-} mice was analyzed. Interestingly, it was found that M-CSF was mainly expressed by smooth muscle cells in the media
15 of normal aorta whereas it was localized within the intima layer of aorta exhibiting atherosclerotic plaques.

 Thus, it is proposed that during atherogenesis, early stimuli (*e.g.*, mechanical stress, lipids) induce changes in medial smooth muscle cells including down-regulation of M-CSF. Reduced production of M-CSF in the media results in the loss
20 of LYVE-1⁺ macrophages producing TGFbeta in adventitia of the artery. Decreased TGFbeta expression in the adventitia likely in turn leads to disorganization and degradation of smooth muscle cell and ultimately their loss.

 These data uncover a mechanism controlling vessel homeostasis that involves the crosstalk between adventitial LYVE-1⁺ macrophages and medial
25 smooth muscle cells, a process dependent on TGFbeta and M-CSF.

Generation of LYVE-1⁺ macrophages

 The prerequisite for therapeutic utilization of LYVE-1⁺ macrophages is to be able to either isolate these cells from tissues or to generate them *ex vivo*. The flow
30 cytometric analysis of blood and bone marrow derived monocytes revealed that monocytes do not express LYVE-1 (Figure 32). However, a small fraction of macrophages generated *in vitro* from mouse bone marrow cells in culture with

MCSF (10 ng/ml) for 6 days expressed LYVE-1 (Figure 33). Finally, LYVE-1⁺ macrophages were isolated *ex vivo* from mouse adipose tissue and flow cytometric analysis of macrophages from adipose tissue showed that 50% of these macrophages were LYVE-1⁺ macrophages (Figure 34).

- 5 Analysis of Blood Monocytes by Flow Cytometry: Blood was drawn via cheek bleed from mice immediately following sacrifice by CO₂ asphyxiation and the volume of blood collected was noted to the nearest 10µl before being mixed well into 2ml of pre-warmed 1x Pharm Lyse™ RBC Lysing Solution (BD Biosciences). Specimens were incubated at room temperature for 5 minutes and centrifuged at
- 10 1,200 rounds per minute (rpm), 4°C for 5 minutes. The supernatant was aspirated while the cell pellet was washed with Hanks' Balanced Salt Solution without Mg²⁺ and Ca²⁺ (modified HBSS) from Sigma Aldrich. Cells were resuspended in 1.0mL modified HBSS with 5mM EDTA and 0.1% BSA. Enumeration of the total number of viable cells was performed using a hemacytometer after Trypan Blue staining.
- 15 The total number of cells was divided by the volume of blood collected to obtain the concentration. Approximately 1 x 10⁶ cells from each sample were stained according to the CD45 genotype (CD45.1 or CD45.2) as well as for the cell surface markers CD11b CD115 and F4/80.

- Analysis of Monocytes in the Bone Marrow by Flow Cytometry: After
- 20 sacrifice, the femurs and tibias of mice were separated and all extraneous muscle tissues were removed with a gauze. This was followed by a transfer of the bones into 70% Ethanol for 1 minute and thereafter two washes with HBSS were carried out. The ends of the bones were cut off using a sterile forceps and scissors. The BMs were flushed with sterile HBSS with a 27 gauge needle. Cells were then passed
- 25 through cell strainer (BD Falcon) before being centrifuged at 1200rpm, 4°C for 5 minutes. The pellet was resuspended in 2mL of HBSS and 2ml of 1.66% ammonium chloride was added for red blood cell lysis. This was followed by centrifugation at 1200 rpm, 4°C for 5 minutes and the pellet was resuspended in 2mL of HBSS. Live cells were enumerated via the use of the haemocytometer following Trypan Blue
- 30 Staining. Flow cytometric analysis was performed as described for blood monocytes.

Analysis and Quantification of Cells by Flow Cytometry: Each sample was incubated for 20 minutes at 4°C with 200µl of antibodies diluted in HBSS + 0.2% BSA. Three washes with HBSS were carried out between primary and secondary antibody incubations. Data acquisition was accomplished on the FACSCalibur™ flow cytometer (Beckton Dickinson) with online compensation and processed on FlowJo software version 7.5.4.

Isolation of cells from adipose tissue cell: fat pads from mouse were dissected out and minced in PBD containing 1%BSA and 1x liberase (Roche) and incubated for digestion at 37C for 1h. After digestion, cells were passed through a cell strainer and pelleted down. Cells were resuspended in FACS buffer and stained for LYVE-1 and macrophage markers including CD115, CD11b and F480 as described above.

Therapeutic utility of LYVE-1⁺ in ischaemic heart

A rat model for infarcted heart was used. One group of rats was left untreated and another group of rats received myocardial artificial graft (MAG) after myocardial infarction (MI). Echocardiographic and hemodynamic assessment four weeks after MI revealed that MAG treatment attenuated left ventricular (LV) remodeling (LV end-systolic volume 0.31 ± 0.13 vs. 0.81 ± 0.01 ml, $P < 0.05$; LV end-diastolic volume 0.79 ± 0.33 vs. 1.83 ± 0.26 ml, $P < 0.076$) and preserved LV wall thickness (0.21 ± 0.03 vs. 0.09 ± 0.005 cm, $P < 0.05$) compared to MI group. Cardiac output was higher in MAG than MI (51.59 ± 6.5 vs. 25.06 ± 4.24 ml·min⁻¹, $P < 0.01$), and comparable to healthy rats (47.08 ± 1.9 ml·min⁻¹). Histology showed decreased fibrosis, and a 7-fold increase in blood vessel density in the scar area of MAG compared to MI group (15.3 ± 1.1 vs. 2.1 ± 0.3 blood vessels/hpf, $P < 0.0001$). Using this model, whether the improvement of heart function observed in rat model receiving MAGs is associated with changes in LYVE-1⁺ cells was investigated. Immunostaining for LYVE-1 revealed the presence of numerous LYVE-1⁺ cells in the scar area of MAG whereas they were absent in the MI group without graft (Figure 35). Thus, these data showed that implantation of AA-enriched-prevascularized grafts enhanced vascularity in ischemic rat hearts, attenuated LV remodeling and preserved LV function. Importantly, these beneficial effects

mediated by the grafts in ischemic rat hearts were associated with the presence of LYVE-1⁺ cells indicating the therapeutic utility of these cells in ischemic hearts.

Methods

5 Myocardial artificial grafts (MAG) were prepared as described elsewhere (Martinez et al., 2010). Briefly, 4 x10⁶ H9C2-GFP-Fluc cells were resuspended in 250 µl PBS containing 5 µmol/L L-Ascorbic acid (Sigma, St. Louis, MO, USA). The 250 µl cell-AA suspension was added under sterile conditions to Gelfoam™ squares (1.0 x 1.0 x 5 mm) placed in 8-well chamber slides. Cell solution absorption
10 into the sponge, and cell attachment were allowed for 3 hr under 5% CO₂ at 37°C prior to MAG in vivo implantation.

Subsequently, MAGs were prevascularized using a renal pouch for graft prevascularization in rats (Martinez, E. C., et al. 2010, Tissue Eng Part A 16:1349-1361). In brief, following laparotomy, a pouch was dissected in the right retrorenal
15 fat. Next, the MAG was implanted into the pouch for three days. The MAG patch was then implanted into the same rat's ischemic heart following myocardial infarction (MI). The MAG patch was then implanted into the same rat's ischemic heart following myocardial infarction (MI). MAG-treated animals (MAG group, n=6) were compared to untreated infarcted animals as injury controls (MI group,
20 n=6) and sham operated rats as healthy controls (healthy group, n=7). In the MAG (Graft) group, a mid laparotomy was performed concomitantly and the prevascularized MAG was explanted from the renal pouch and implanted into the area of myocardial ischemia. The patch was attached to the recipient heart using fibrin glue (Tisseel, Baxter Healthcare Corporation, Deerfield, IL), 30 minutes after
25 MI. The MI (injury) group, did not receive any treatment following LAD ligation, whereas the healthy (sham operated) group only underwent mid-thoracotomy and pericardectomy (Martinez, E. C., et al. 2010, Tissue Eng Part A 16:1349-1361).

Example 2

30 Mechanisms of action of LYVE-1⁺ macrophages

As shown in Example 1, MAFIA mice treated with FAS ligand exhibited a marked depletion of LYVE-1⁺ macrophages that was accompanied by vascular

leakage in the aorta. Described herein is the analysis of the structure of aorta by immunostaining from untreated and treated MAFIA mice. It was found that the elastin fiber of the media from treated MAFIA mice was more rigid and straightened compared to untreated MAFIA mice (Fig 36). This observation was followed up by a scanning electron-microscopy analysis of medial elastin and collagen fibers. Notably, in aorta from treated MAFIA mice, collagen fibers were thinner and elastin exhibited more fenestrations compared to untreated MAFIA mice (Fig 37). Such changes in elastin and collagen generally characterize aortic stiffness which is observed in hypertension, aneurysm and aging. Interestingly, mice lacking smad 3, a molecule involved in TGFbeta signaling, developed hypertension and aortic stiffness when treated with angiotensin II and they exhibited a severe depletion of LYVE-1⁺ macrophages in their adventitia layer which was not observed in normotensive smad 3 KO mice (Figs. 38A-38B). This data shows that loss of LYVE-1⁺ macrophages is associated with hypertension and aortic stiffness.

Aortic stiffness is known to result from changes in collagen and elastin. Thus, the expression of genes involved in the synthesis of collagen (procollagen 1a1 and 3a1) and elastin (tropoelastin) or conversely, in their degradation (metalloproteases, MMPs), were analyzed. This analysis revealed in the aortic media from treated MAFIA mice an increased expression of MMP-3 and MMP-9 but a decreased expression of procollagen and tropoelastin compared to untreated MAFIA mice (Fig 39). These data show that LYVE-1⁺ macrophages likely maintain smooth muscle cells by controlling the synthesis and/or degradation of collagen and elastin which are critical for smooth muscle cell survival and function.

Provided herein is direct evidence that LYVE-1⁺ macrophages maintain smooth muscle cells by producing TGFbeta and, inversely, smooth muscle cells secrete factors to maintain LYVE-1⁺ cells (see Fig. 43). Analysis of mRNA expression for M-CSF, a critical factor for LYVE-1⁺ cell survival (blocking M-CSF receptor activity with KI 20227 depletes them) in the media or adventitia from normal aorta (WT mice) revealed that mRNA for MCSF was exclusively expressed in the aortic media and not adventitia (Fig. 40). Furthermore, immunostaining of normal aorta for LYVE-1 and TGFbeta showed that LYVE-1⁺ cells can express TGFbeta (Fig. 41). These data showed that smooth muscle cell were critical for

LYVE-1⁺ by providing them MCSF and LYVE-1⁺ macrophages support smooth muscle cell by at least producing TGFbeta which in turn control the balance of collagen and elastin produced by smooth muscle cells.

5 Adipose tissue as a source of LYVE-1⁺ cells for therapeutic use

Because the presence of LYVE-1⁺ in adipose tissue can be detected by immunostaining, whether LYVE-1⁺ macrophages could be isolated from this tissue was tested. Indeed, LYVE-1⁺ macrophages were isolated and identified in adipose tissue from mouse and human after tissue disruption (Fig. 42). Being able to use
10 adipose tissue as a source of LYVE-1⁺ cells for therapeutic utility represents a serious advantage compared to in vitro generation. Indeed, liposuction is less invasive than bone marrow puncture, adipose tissue from lipoaspirate is often discarded, and since the cells are directly isolated from adipose tissue, no further expansion is required in vitro which will limit in vitro manipulation and thus
15 contamination. Thus, adipose tissue derived LYVE-1⁺ cells can be used for implantation in vivo.

Methods

Immunostaining

20 Immunostaining was preformed as described in Example 1.

Isolation of LYVE-1⁺ macrophages from adipose tissue

From Mice

Mice were sacrificed by CO₂ asphyxiation and perfused with 40ml of 1X
25 PBS through the heart. The epididymal adipose tissue was removed using forceps and scissors and the specimens were removed from bound testicles, the epididymis and associated ductus deferens. The adipose tissue was separately fully minced in pointed eppendorfs using dissecting scissors. Minced samples were then incubated at 37°C on a shaker for an hour of digestion. The epididymal tissues were incubated
30 with 9ml PBS, 1ml BSA and 100ul of 10X Liberase enzyme while the smaller Periadventitial fat samples were each incubated in 1.8ml PBS, 200ul BSA and 20ul 10X Liberase enzyme.

Upon an hour of enzymatic digestion, the suspensions were filtered through a nylon mesh followed by spinning down at 1000g in a centrifuge. At this stage, the stromal vascular fraction was collected in the form of a pellet at the bottom of the tube, while a fatty layer of adipocytes floated on top. The adipocyte layer and the digestion mix were removed by suction pump to leave behind the stromal vascular cells (SVCs). In the scenario where the pellet appeared to contain a significant amount of blood, erythrocyte lysis buffer (0.9% ammonium chloride) was used to lyse the red blood cells. Cells were then resuspended in 1.0mL modified HBSS containing 0.2% BSA. Enumeration of the total number of viable cells was performed using a hemacytometer after Trypan Blue staining. Cells were then stained for CD45, CD11b (macrophage marker) and LYVE-1 for flow cytometric analysis.

From human tissue

Subcutaneous fat from human skin was mechanically disrupted and passed through a 70µM cell strainer to prepare a cell suspension. Cell suspension was stained for CD45, HLA-DR and LYVE-1 for flow cytometric analysis.

Isolation of Aorta Vessel Compartments for Analysis by Real Time PCR

Mice were sacrificed by asphyxiation with CO₂ and perfusion was done with 40ml of 1X PBS through the heart. The entire descending aorta between the end of the aortic arch and the renal bifurcation was isolated, stored in RNeasy lysis buffer at 4°C for 2 hours. Subsequently, the adventitia and media of the aorta was separated using micro-dissecting forceps and individually stored in different tubes containing RNeasy lysis buffer. The adventitia and media were then transferred to the -80°C freezer for storage.

After isolation of the aortic vascular components, total mRNA was purified following RNeasy Micro/Mini kit guidelines (QIAGEN). RNA was quantified by Nanodrop Spectrophotometer and retrotranscribed with ABI Taqman Reverse Transcription kit (Applied Biosystems). Quantitative PCR analyses were performed with TaqMan assays from Applied Biosystems. A total of 500ng cDNA was loaded in each well of the 96-well reaction plate and each sample was run in triplicates.

Quantitative PCR was run for 40 cycles with SYBR green on standard mode using an ABI7500 apparatus (Applied Biosystems). The 7500 software v2.0.5 was used to extract raw data. The difference between the threshold cycle (Ct) of each gene and that of the endogenous control Gapdh (ΔC_t) was used to determine gene expression.

- 5 The lower the ΔC_t the higher gene expression level was indicated.

The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

- 10 While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of maintaining structure and homeostasis of a blood vessel in an individual in need thereof, wherein the blood vessel's media comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.
5
2. The method of Claim 1 wherein the blood vessel is an artery, a vein, an arteriole or a combination thereof.
10
3. The method of Claim 2 wherein the artery is an aorta.
4. The method of Claim 1 wherein the blood vessel is contacted with exogenous LYVE-1⁺ macrophages.
15
5. The method of Claim 4 wherein the blood vessel is contacted with a scaffold comprising exogenous LYVE-1⁺ macrophages.
- 20 6. The method of Claim 1 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages.
7. The method of Claim 6 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages by locally administering macrophage stimulating growth factor (M-CSF) to the blood vessel and maintaining the blood vessel under conditions in which endogenous LYVE-1⁺ macrophages are recruited to the blood vessel or induced to grow at the blood vessel.
25
8. The method of Claim 1 wherein the individual is a mammal.
30
9. The method of Claim 8 wherein the man

10. A method of repairing one or more blood vessels in an individual in need thereof, wherein the blood vessel's media comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.
5
11. The method of Claim 10 wherein the blood vessel is contacted with exogenous LYVE-1⁺ macrophages.
10
12. The method of Claim 11 wherein the blood vessel is contacted with a scaffold comprising exogenous LYVE-1⁺ macrophages.
13. The method of Claim 10 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages.
15
14. The method of Claim 13 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages by locally administering macrophage stimulating growth factor (M-CSF) to the blood vessel and maintaining the blood vessel under conditions in which endogenous LYVE⁺ macrophages are recruited to the blood vessel or induced to grow at the blood vessel.
20
15. The method of Claim 10 wherein the individual is a mammal.
- 25 16. The method of Claim 15 wherein the mammal is a human.
17. A method of treating media thinning of one or more blood vessels in an individual in need thereof, wherein the blood vessel's media comprises smooth muscle cells, comprising contacting the blood vessel with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.
30

18. The method of Claim 17 wherein the blood vessel is contacted with exogenous LYVE-1⁺ macrophages.
- 5 19. The method of Claim 18 wherein the blood vessel is contacted with a scaffold comprising exogenous LYVE-1⁺ macrophages.
20. The method of Claim 17 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages.
- 10 21. The method of Claim 20 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages by locally administering macrophage stimulating growth factor (M-CSF) to the blood vessel and maintaining the blood vessel under conditions in which endogenous LYVE-1⁺ macrophages are recruited to the blood vessel or induced to grow at the blood vessel.
- 15 22. The method of Claim 17 wherein the individual is a mammal.
23. The method of Claim 22 wherein the mammal is a human.
- 20 24. A method of treating atherosclerosis in an individual in need thereof, comprising contacting one or more diseased blood vessels wherein the one or more blood vessels' media comprise smooth muscle cells with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.
- 25 25. The method of Claim 24 wherein the blood vessel is contacted with exogenous LYVE-1⁺ macrophages.
- 30 26. The method of Claim 25 wherein the blood vessel is contacted with a scaffold comprising exogenous LYVE-1⁺ macrophages.

27. The method of Claim 24 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages.
28. The method of Claim 27 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages by locally administering macrophage stimulating growth factor (M-CSF) to the blood vessel and maintaining the blood vessel under conditions in which endogenous LYVE-1⁺ macrophages are recruited to the blood vessel or induced to grow at the blood vessel.
29. The method of Claim 24 wherein the individual is a mammal.
30. The method of Claim 29 wherein the mammal is a human.
31. A method of treating ischaemic heart disease in an individual in need thereof comprising contacting one or more diseased blood vessels wherein the one or more blood vessels' media comprise smooth muscle cells with an effective amount of lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1⁺) macrophages.
32. The method of Claim 31 wherein the blood vessel is contacted with exogenous LYVE-1⁺ macrophages.
33. The method of Claim 32 wherein the blood vessel is contacted with a scaffold comprising exogenous LYVE-1⁺ macrophages.
34. The method of Claim 31 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages.
35. The method of Claim 34 wherein the blood vessel is contacted with endogenous LYVE-1⁺ macrophages by locally administering macrophage stimulating growth factor (M-CSF) to the blood vessel and maintaining the

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blood vessel under conditions in which endogenous LYVE-1⁺ macrophages are recruited to the blood vessel or induced to grow at the blood vessel.

36. The method of Claim 31 wherein the individual is a mammal.

5

37. The method of Claim 36 wherein the mammal is a human.

DISTRIBUTION OF LYVE-1+ CELLS

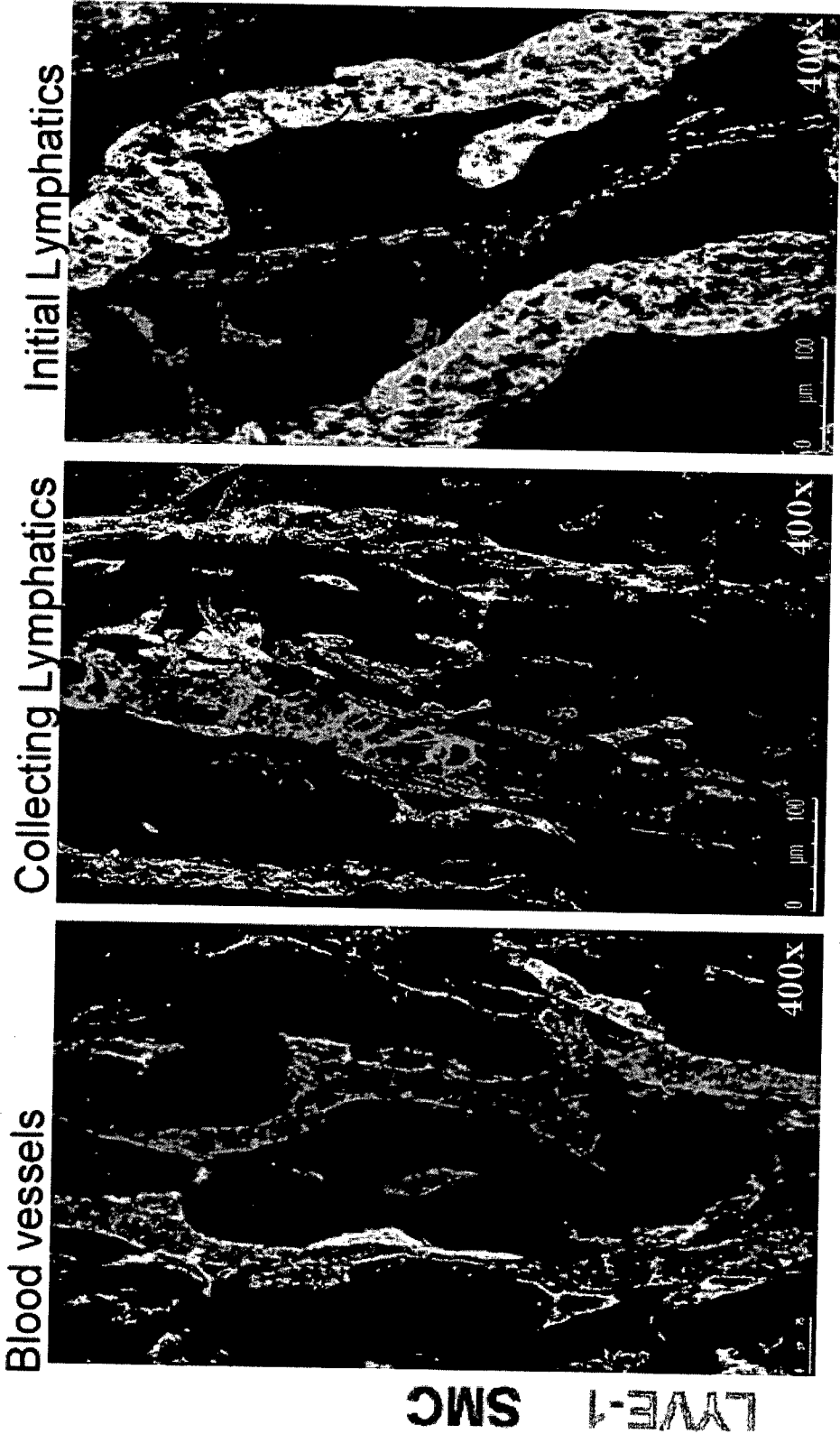


Figure 1

Figure 2

Phenotype: A type of macrophage : CD68, CD11b, F4/80, CD163, CD206

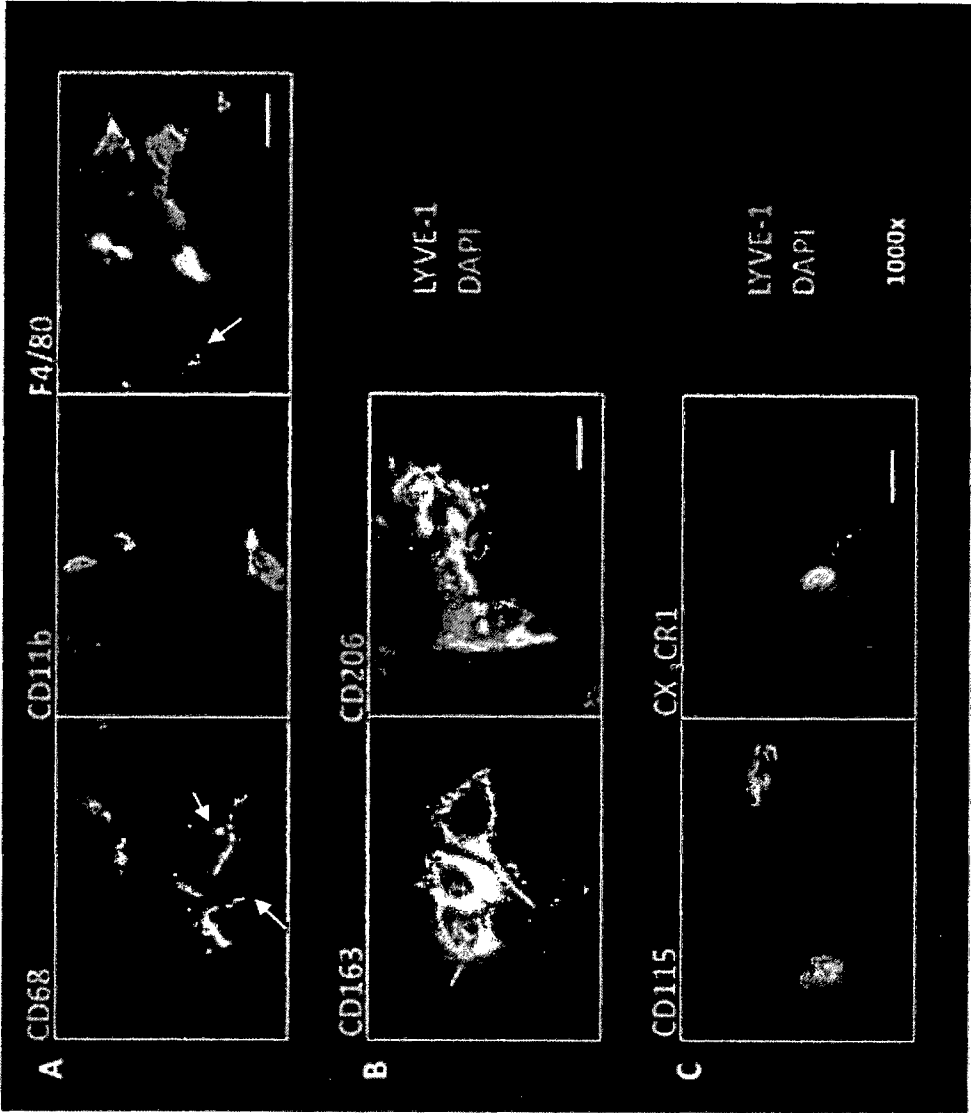


Figure 3

Phenotype: LYVE-1 expressing cell are NOT dendritic cells :MHC II⁻, CD11c⁻

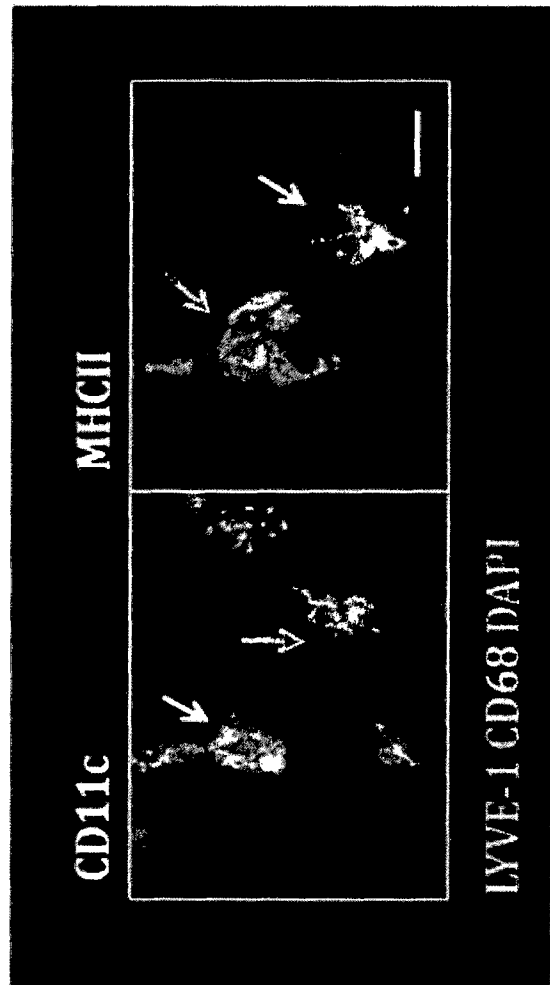


Figure 4

Phenotype: NOT endothelial cells : CD31⁻, Podoplanin⁻, VEGFR2⁻ & R3⁻

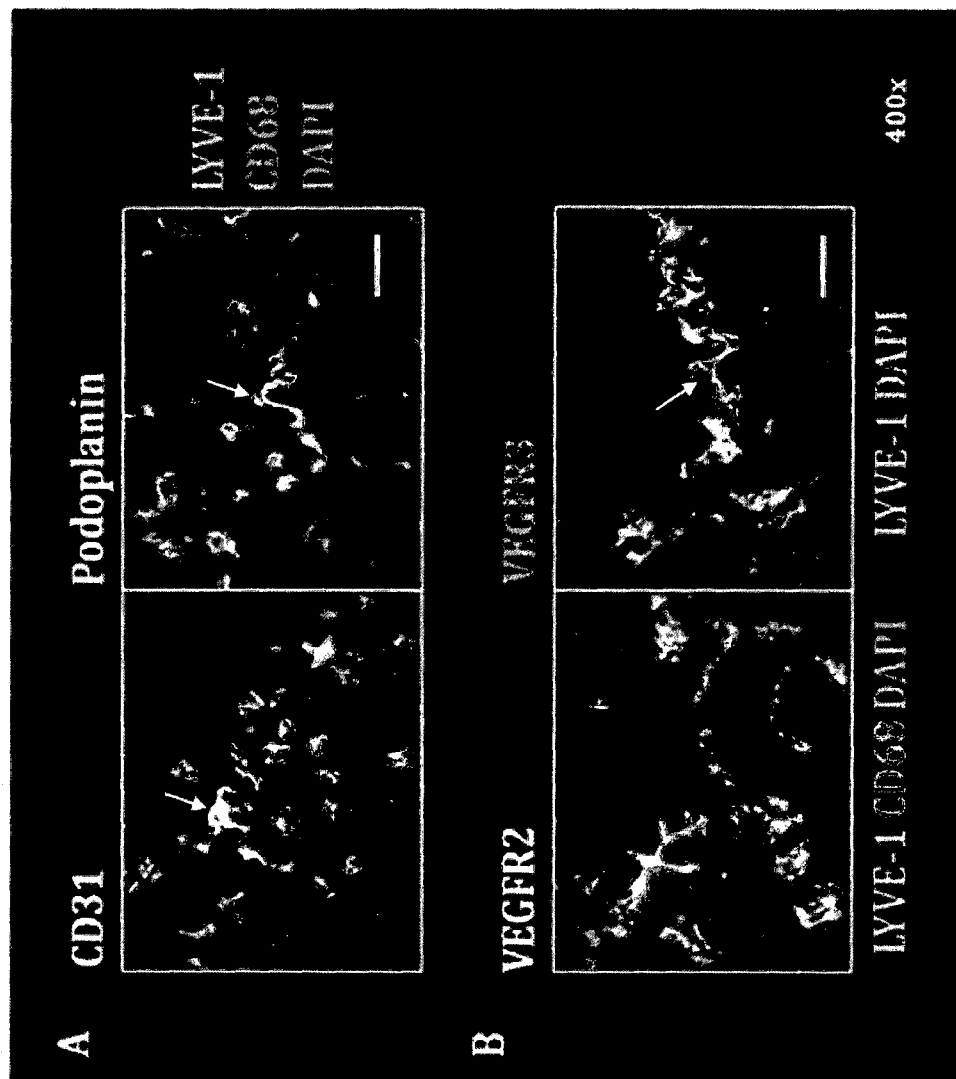
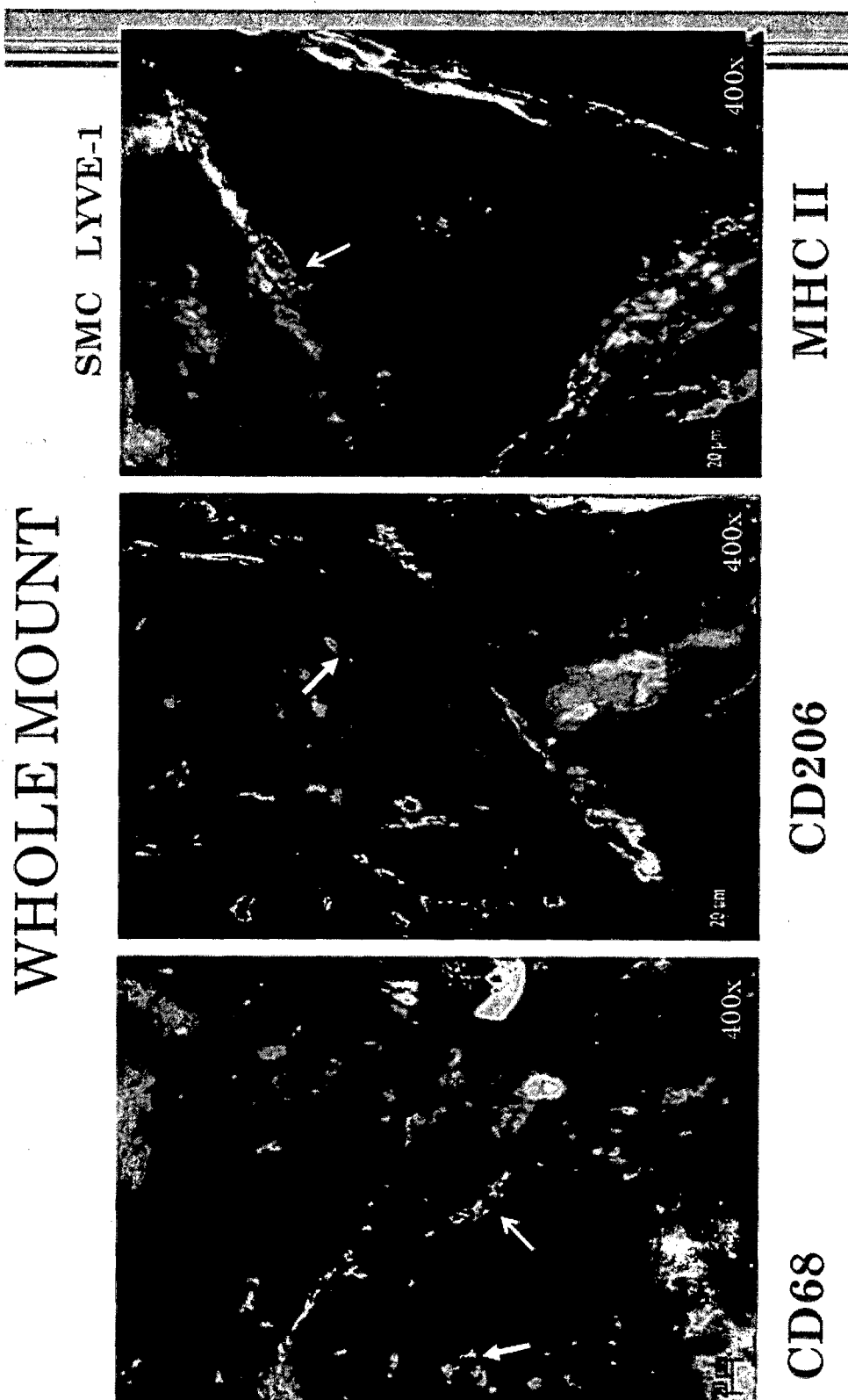


Figure 5



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Figure 6

Relevance to Humans

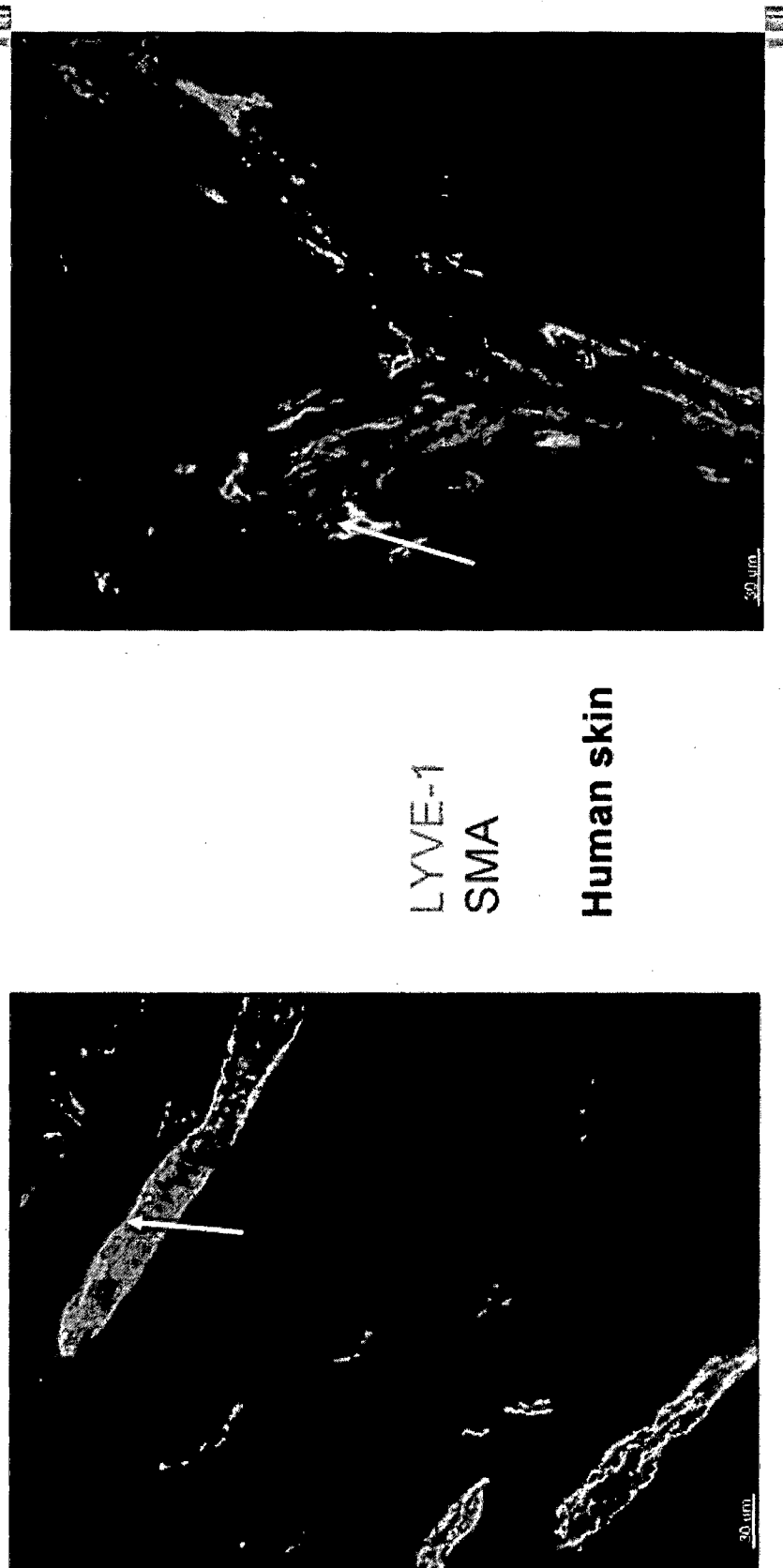
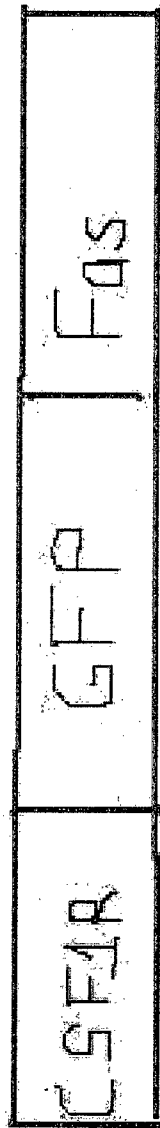


Figure 7

MAFIA MOUSE MODEL

Macrophage FAS induced apoptosis (MAFIA)

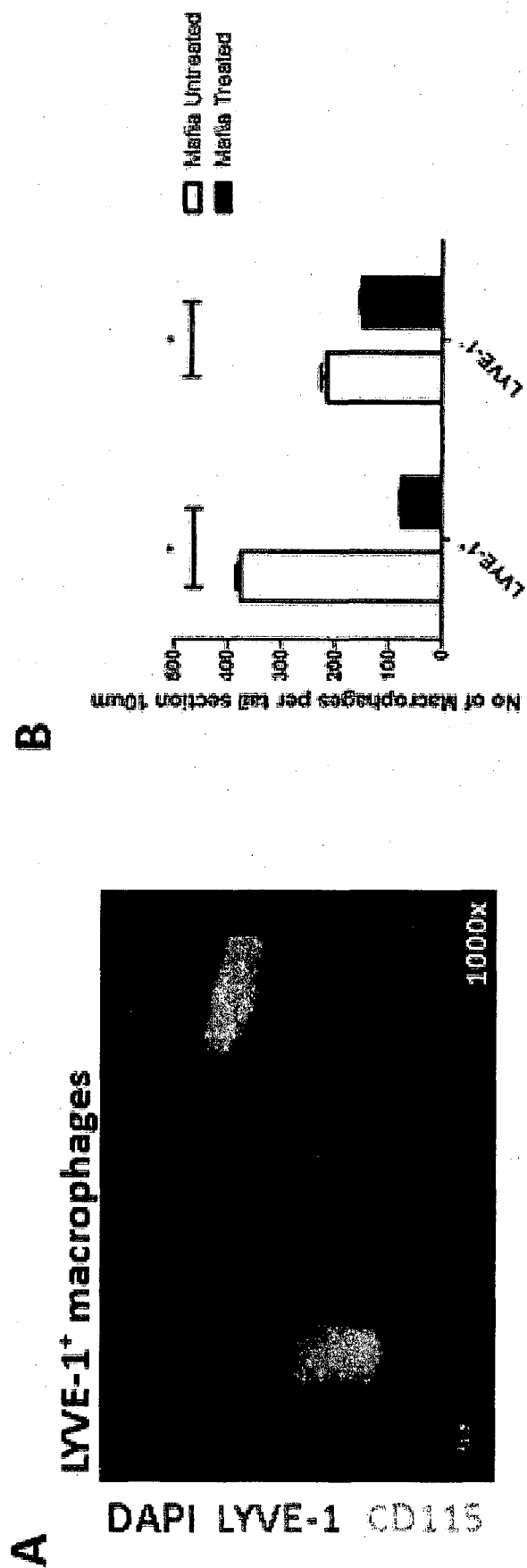
FAS LIGAND



Apoptosis of macrophages
that express CSF1R

Figure 8

RESULTS



MACROSCOPIC CHANGES

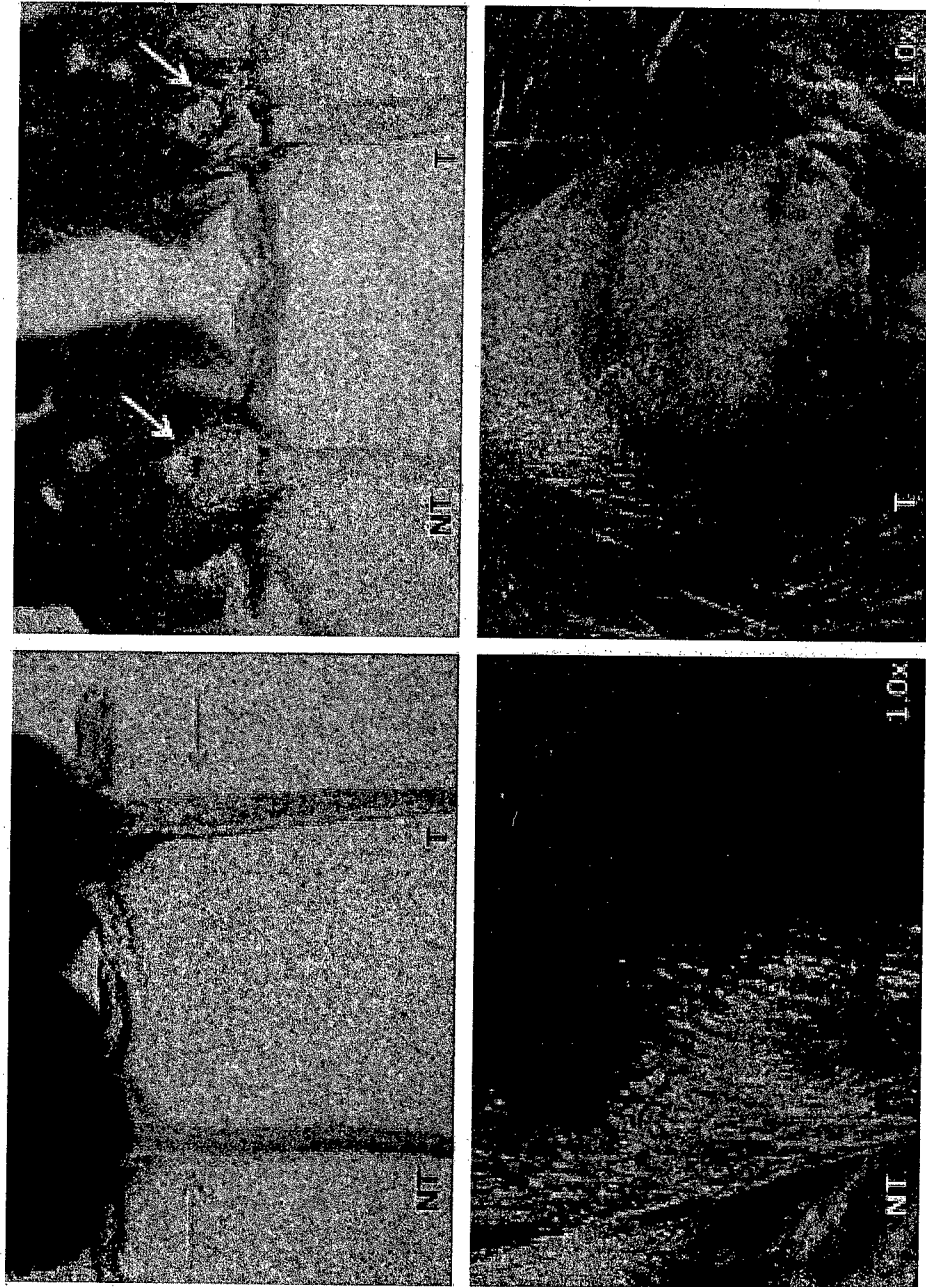


Figure 9

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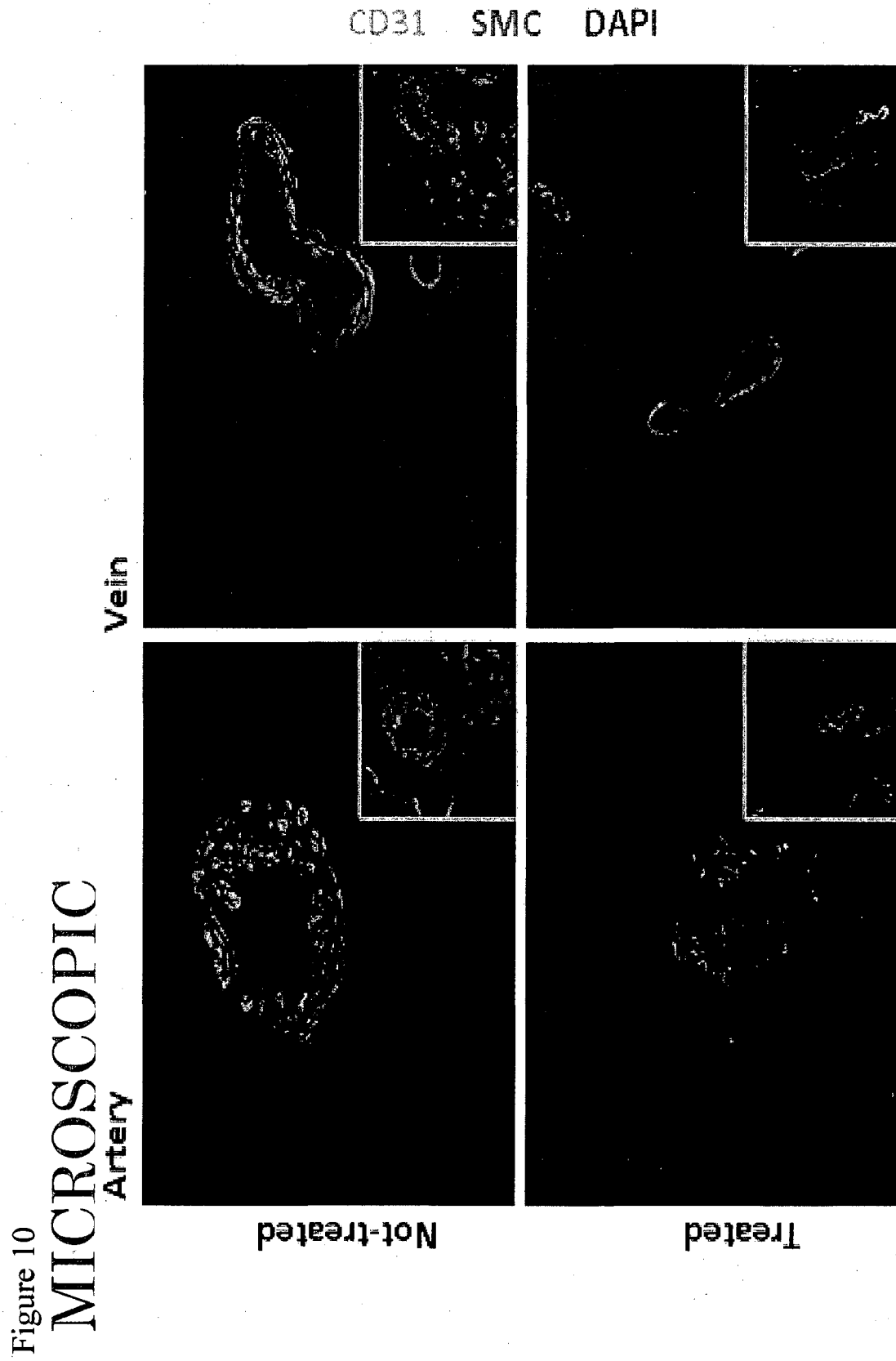


Figure 11

KI20227 tyrosine kinase inhibitor of MCSF-R

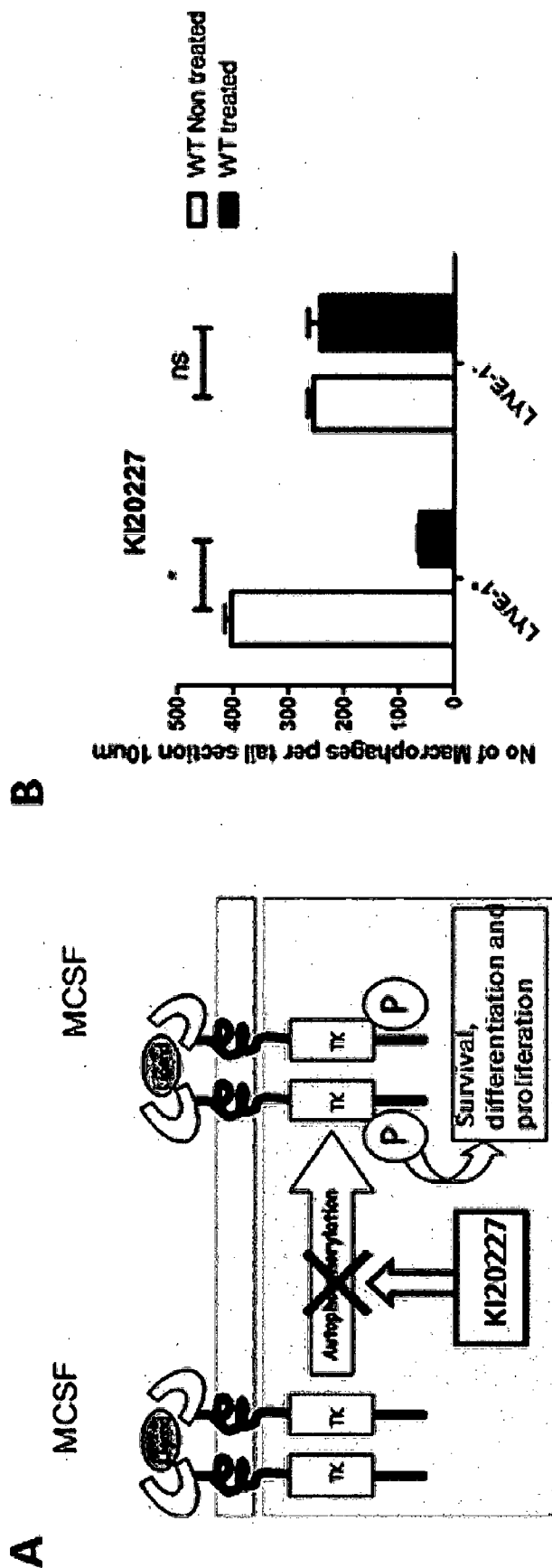
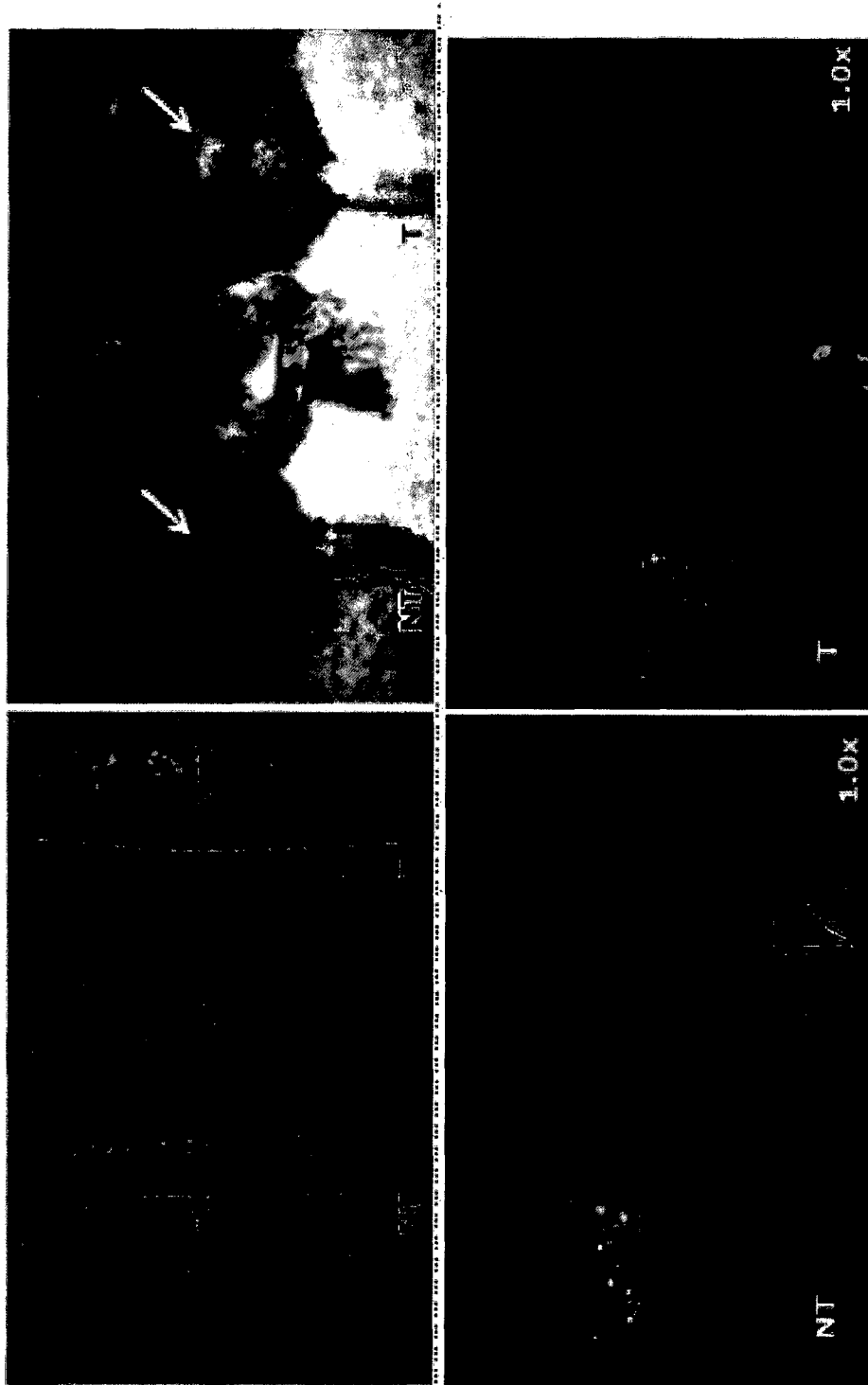


Figure 12

MACROSCOPIC CHANGES



MICROSCOPIC CHANGES

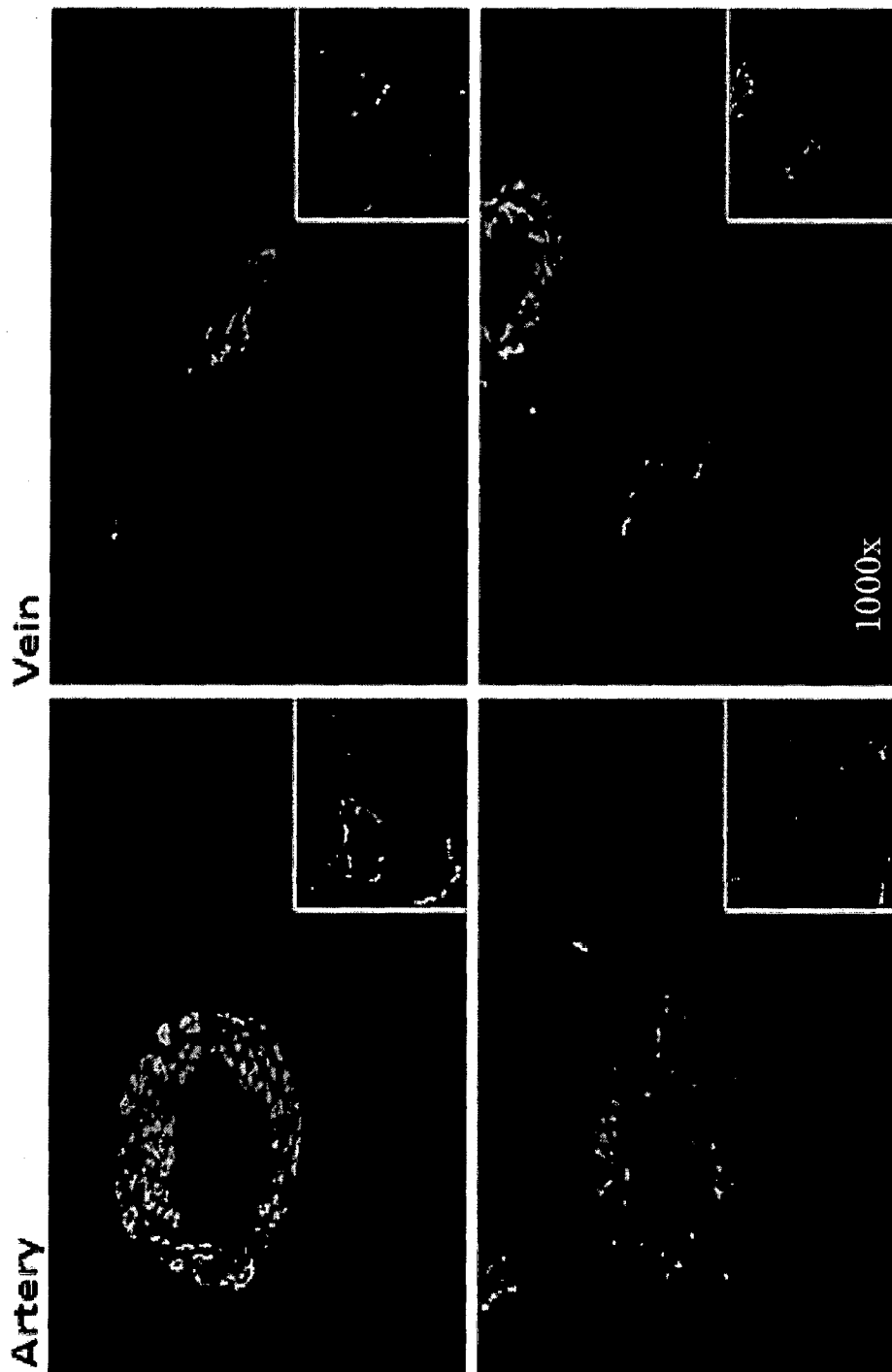


Figure 13

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Figure 14

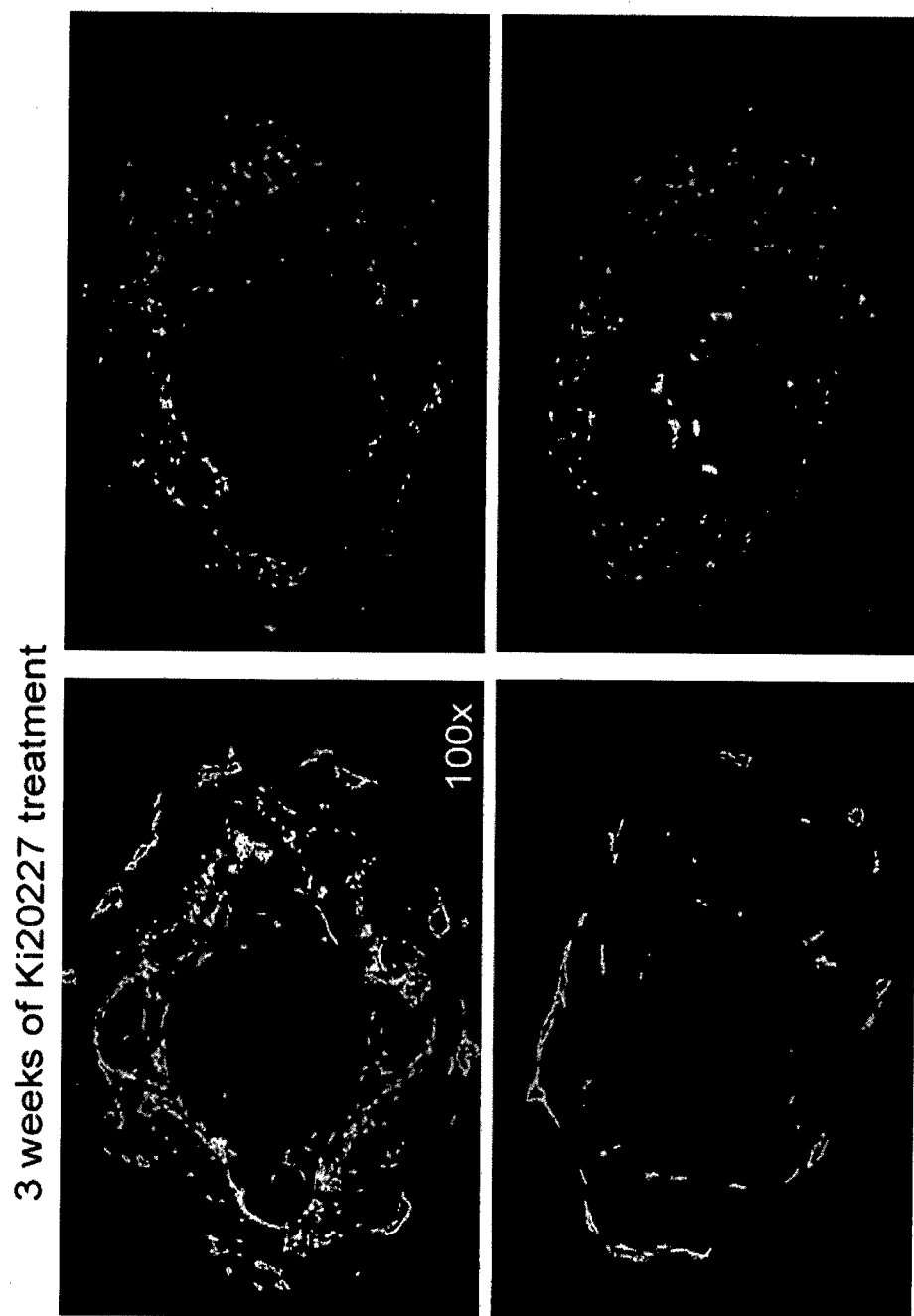


Figure 15 **Ki20227 treatment induces vascular leakage in aorta**

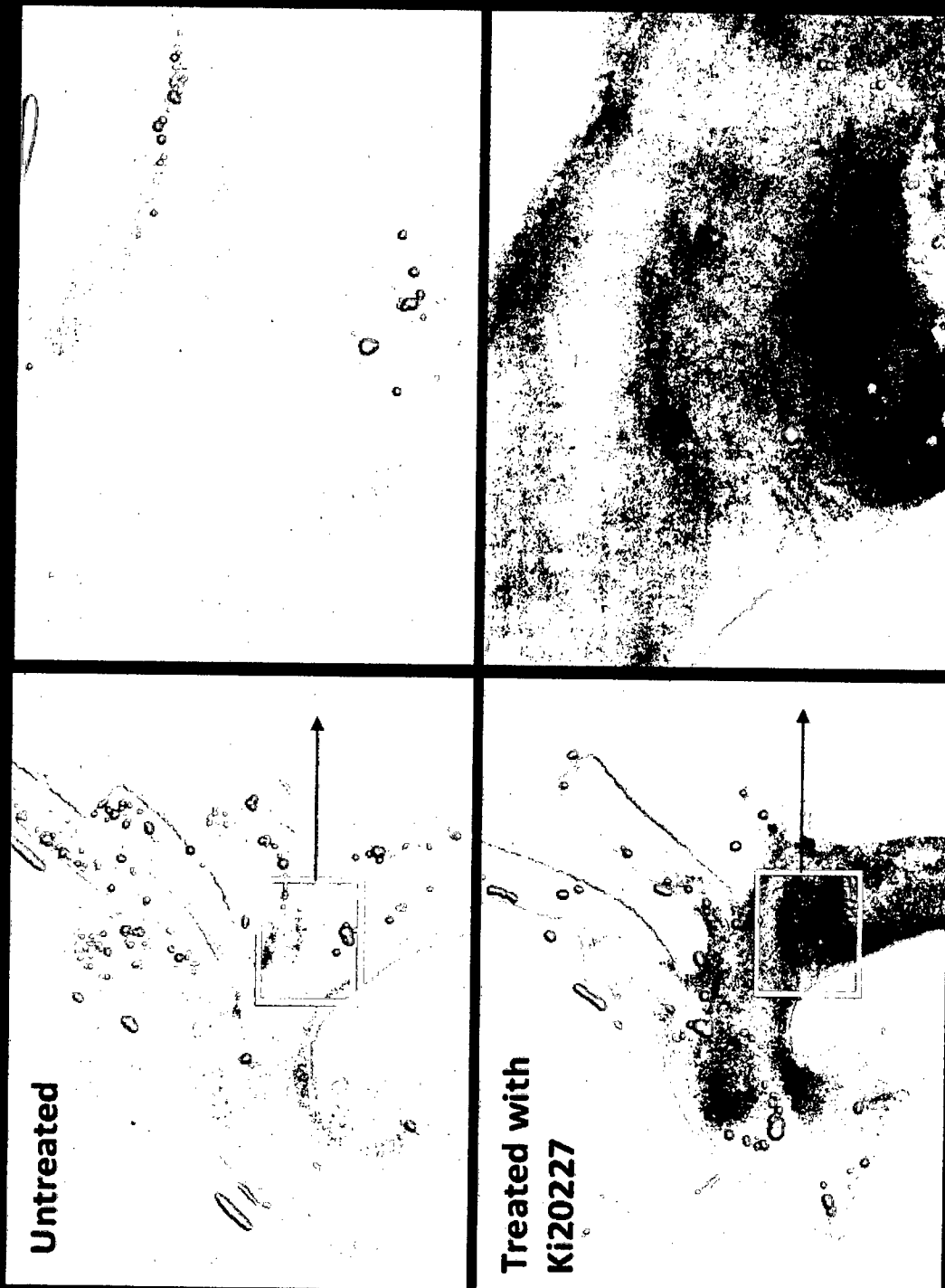


Figure 16

Loss of smooth muscle and poor lymphatic drainage are associated with a decrease in LYVE-1+ macrophages

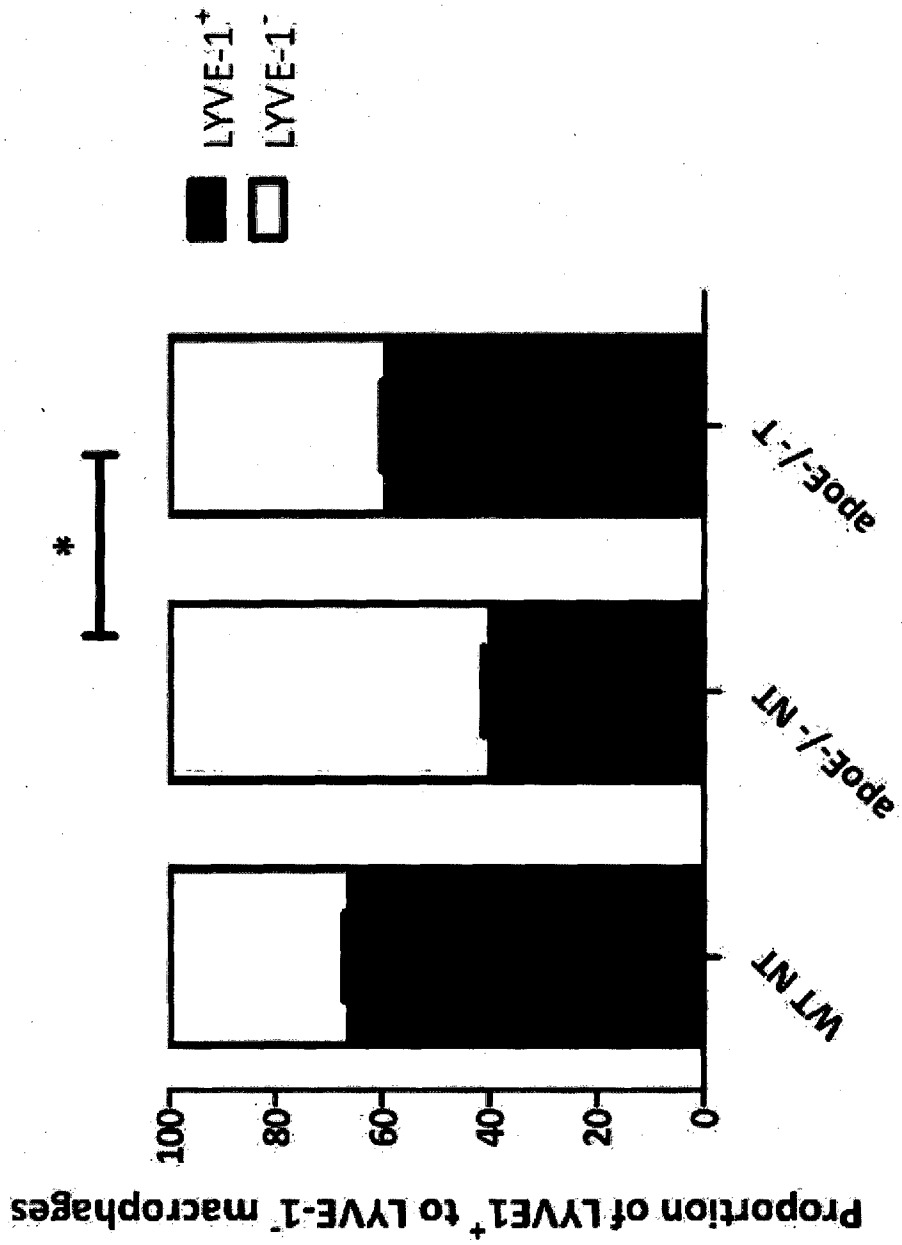
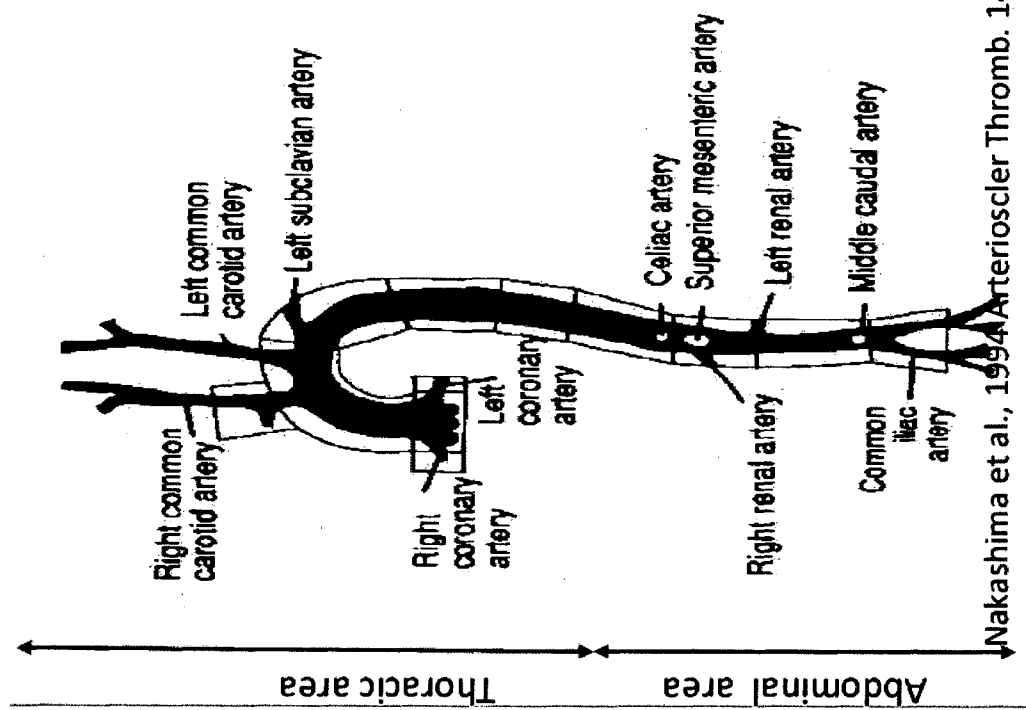
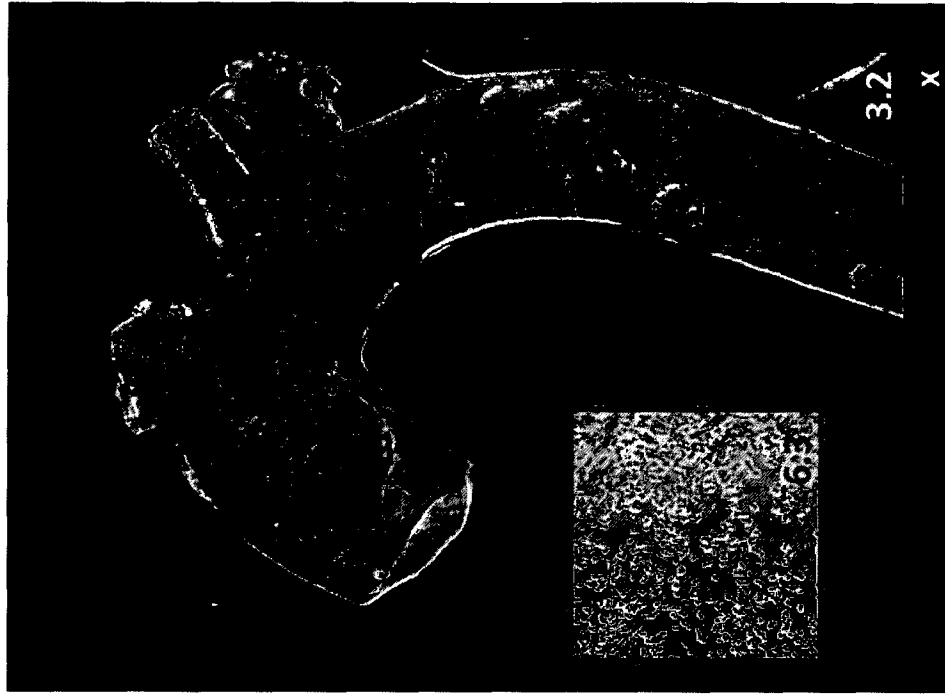


Figure 17

The aorta (adventitia) is fully covered with lyve-1+ macrophages



Nakashima et al., 1994 Arterioscler Thromb. 14(1):133-40.



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The vascular wall: intima, media and adventitia

Figure 18

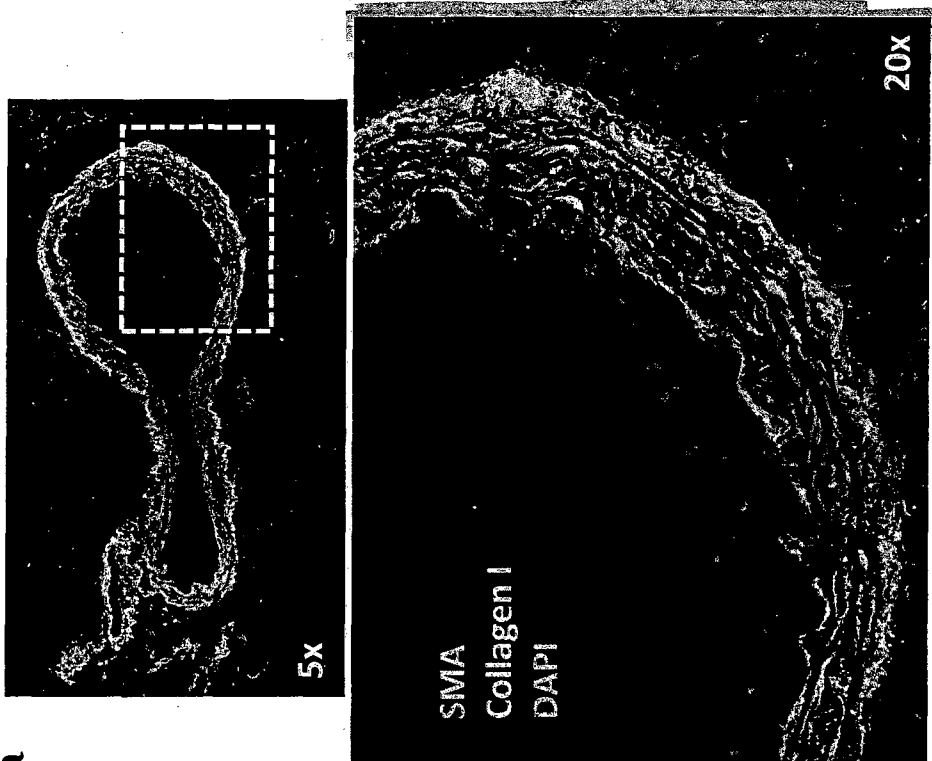
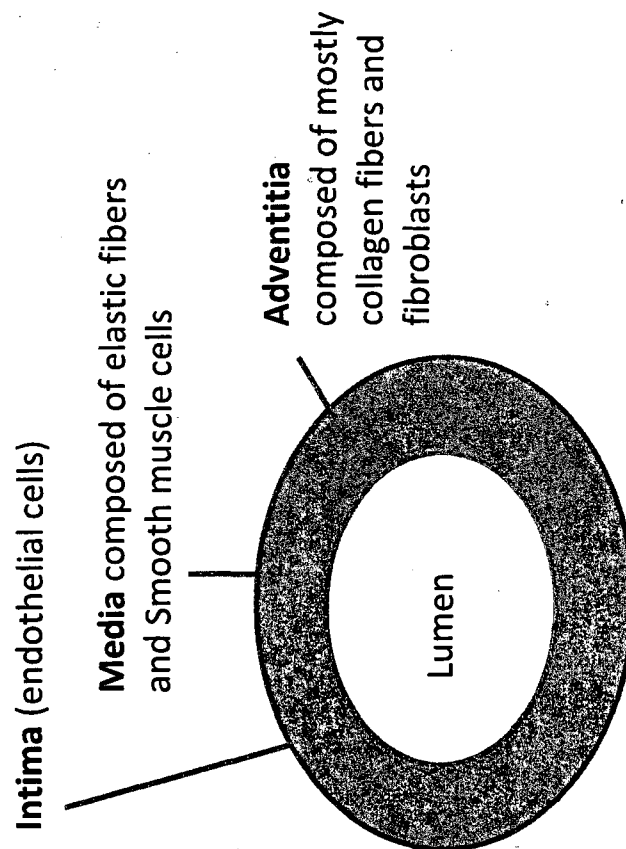
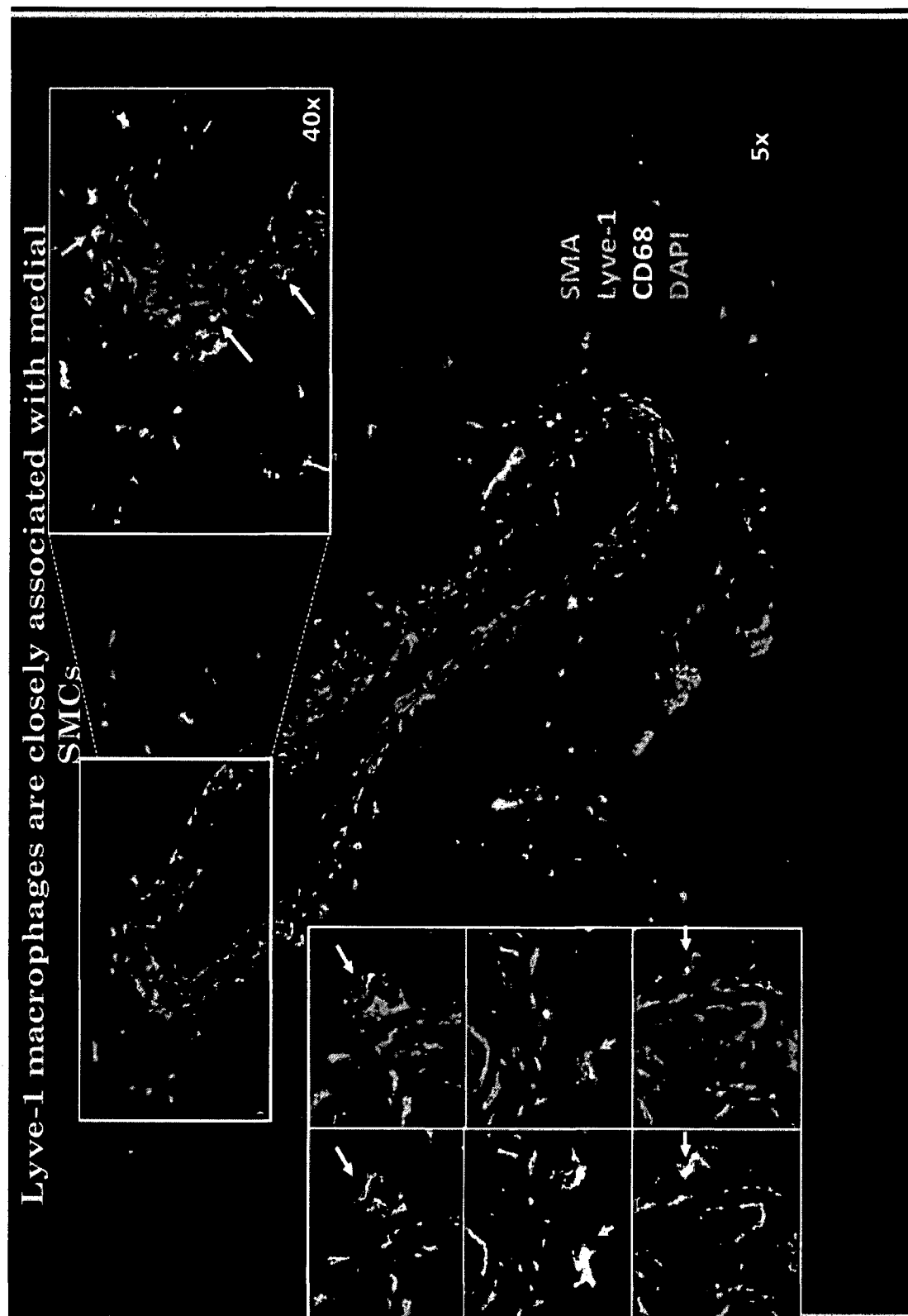


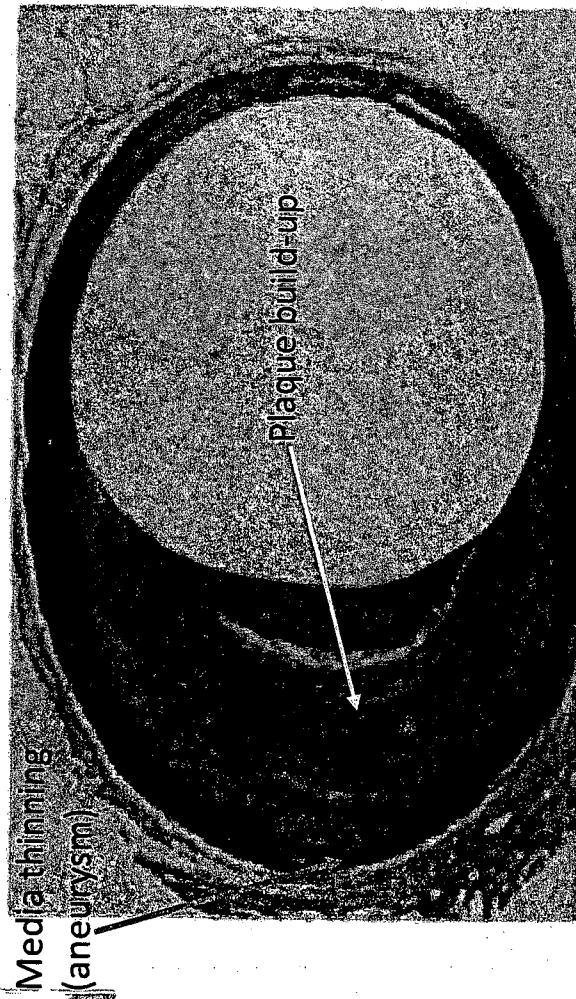
Figure 19



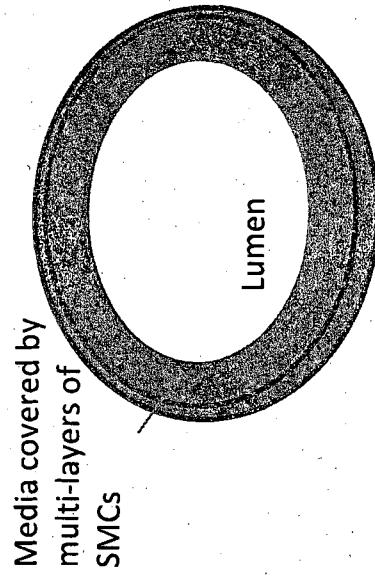
20/44

Atherosclerosis

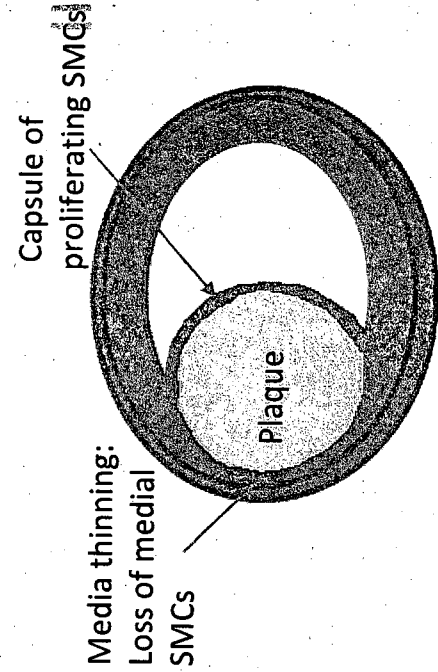
Figure 20



Normal blood vessel



What happens to SMCs during Atherosclerosis



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Atherosclerosis

1. Aortic root
2. Ascending aorta
3. Carotid 1 (innominate/
Brachiocephalic artery)

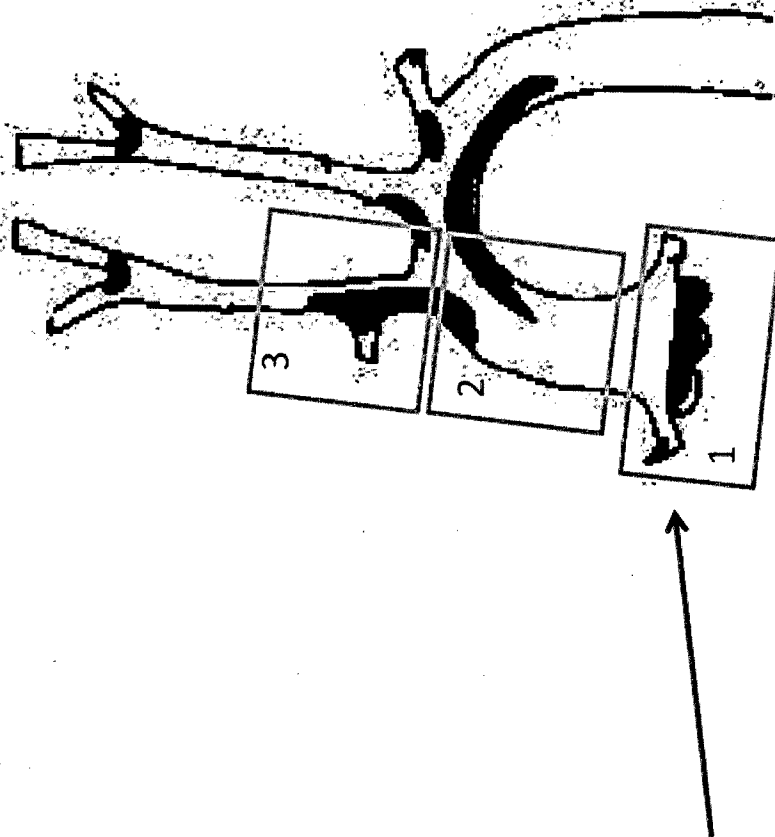


Figure 21

Figure 22

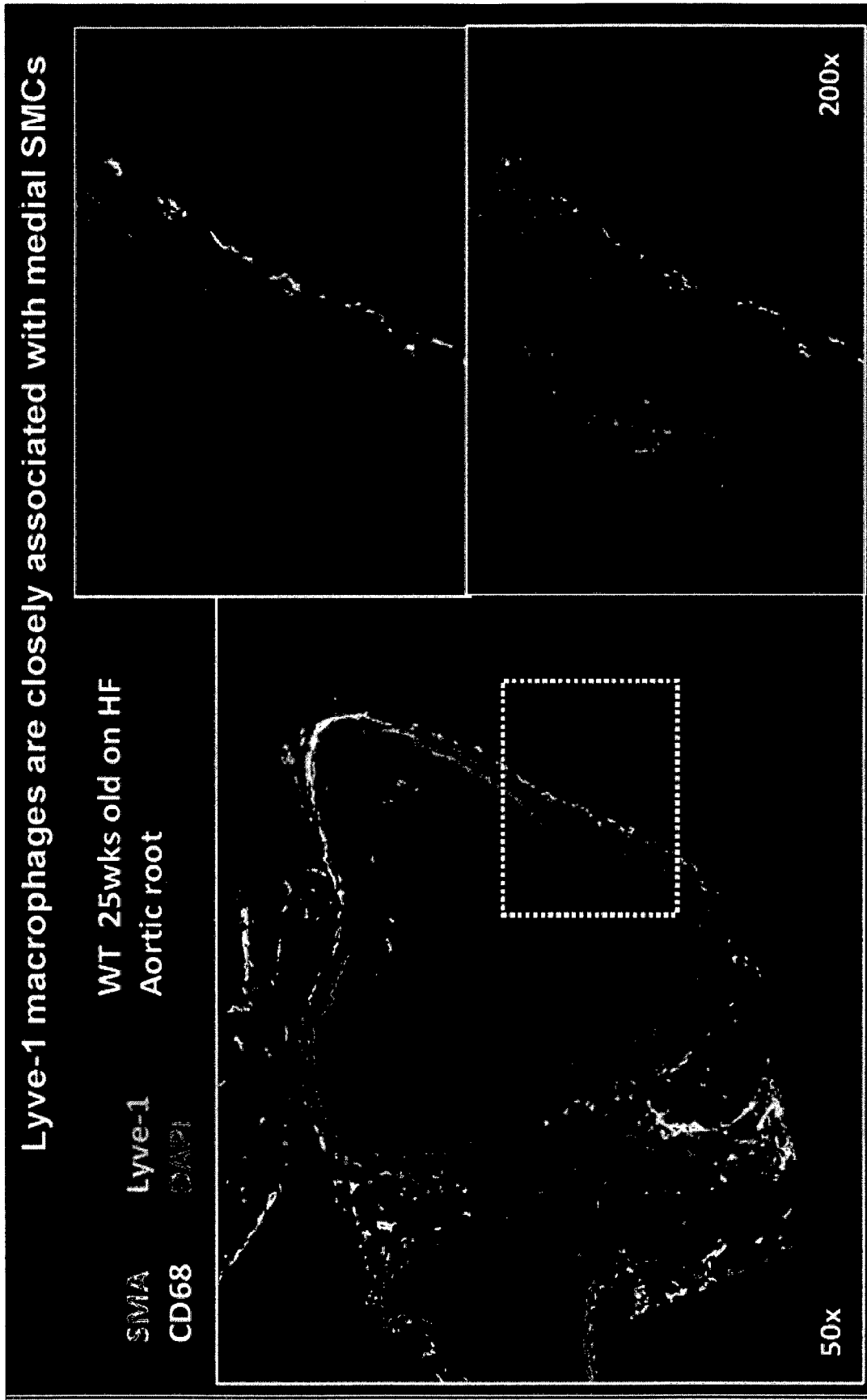


Figure 23

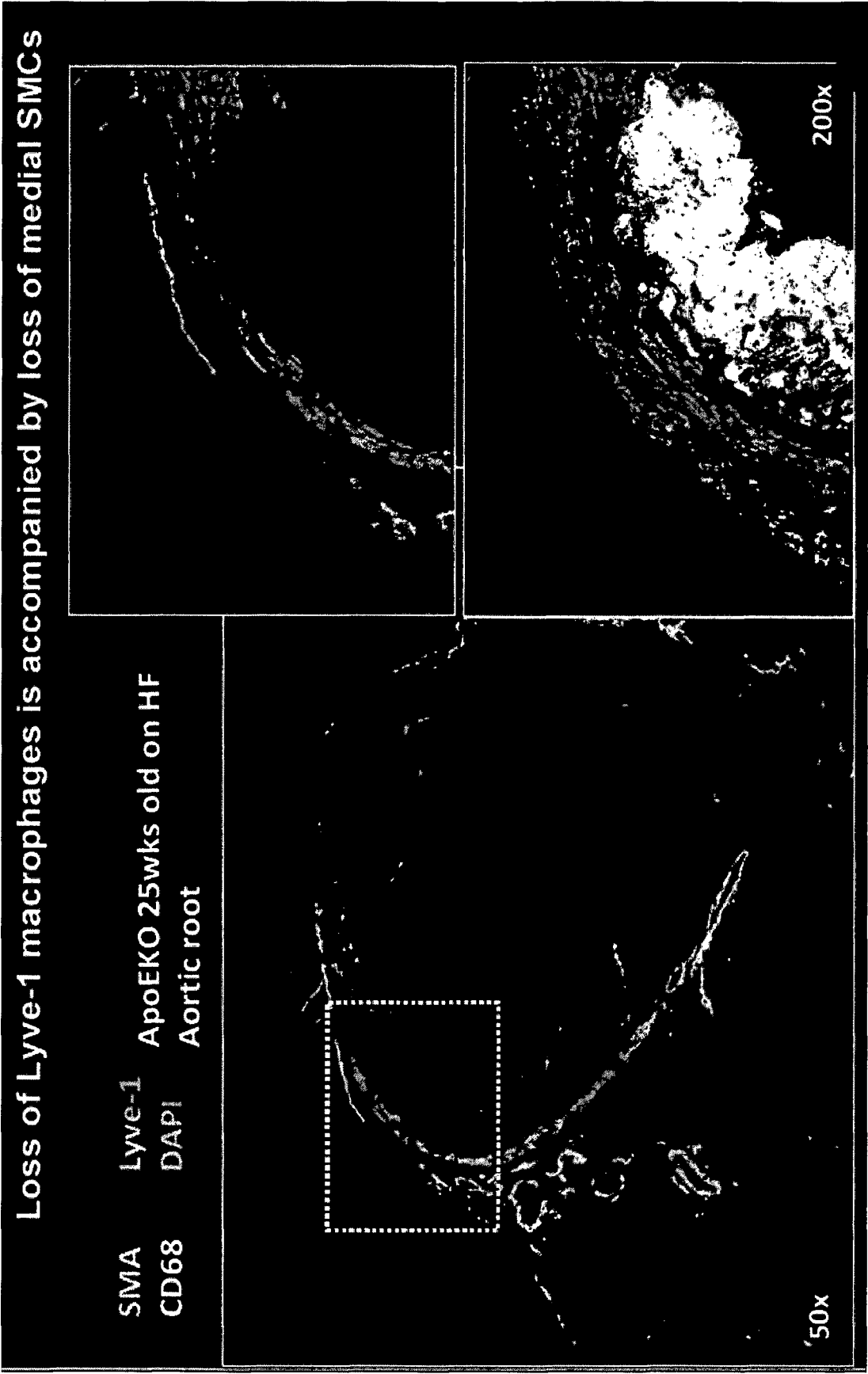
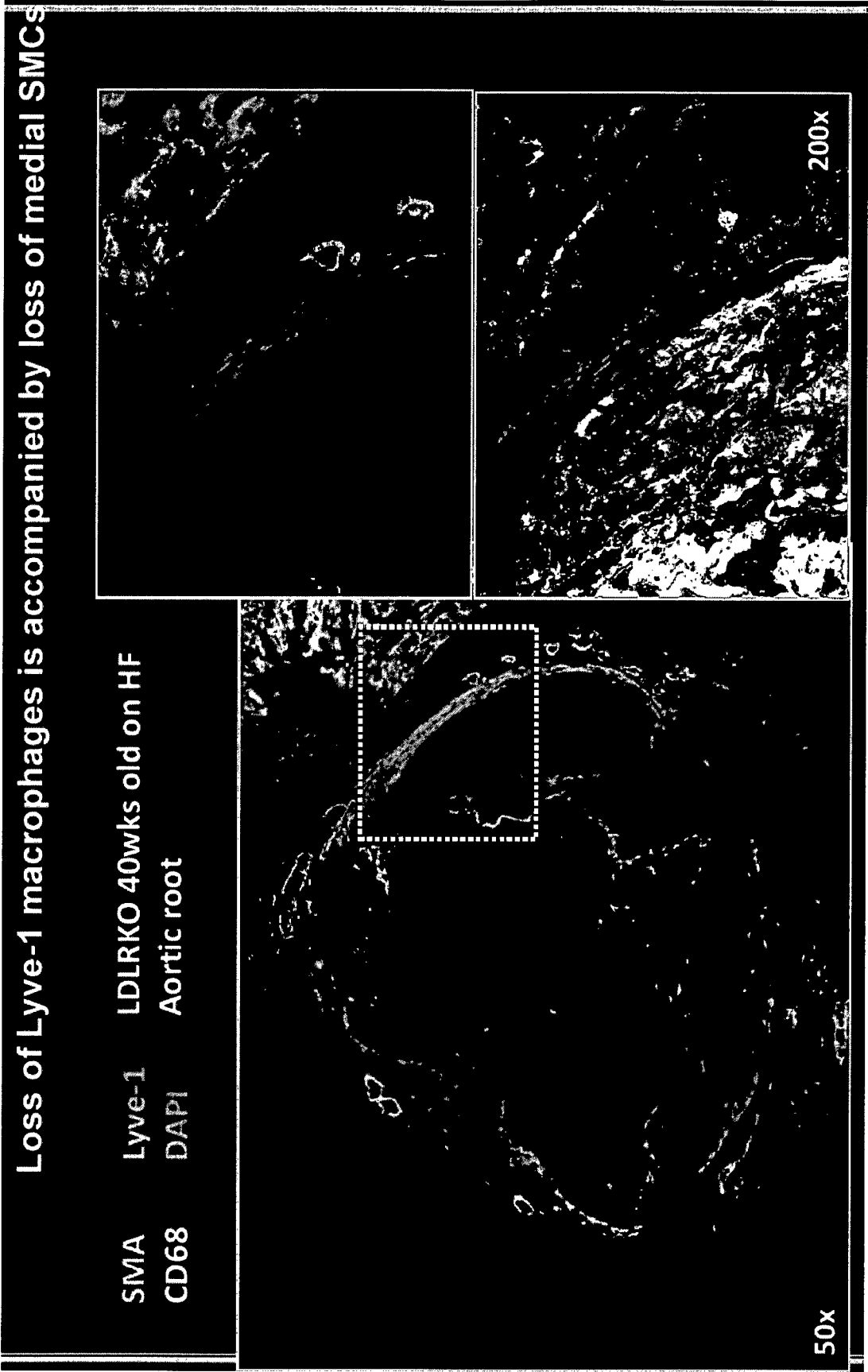


Figure 24



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Atherosclerosis

1. Aortic root
2. Ascending aorta
3. Carotid 1 (innominate/
Brachiocephalic artery)

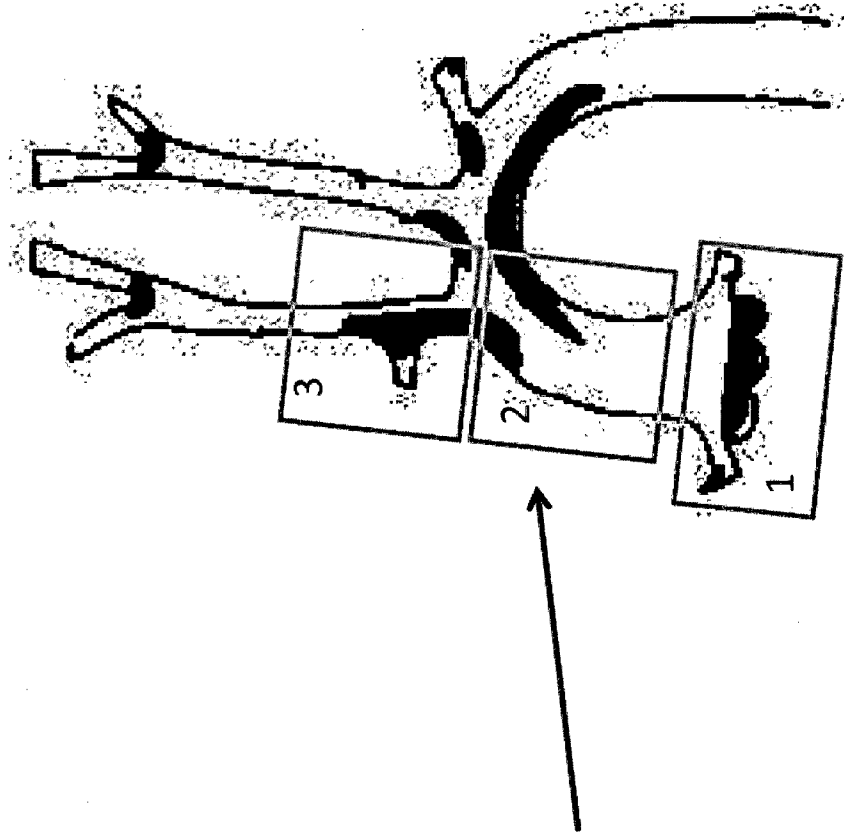


Figure 25

Figure 26

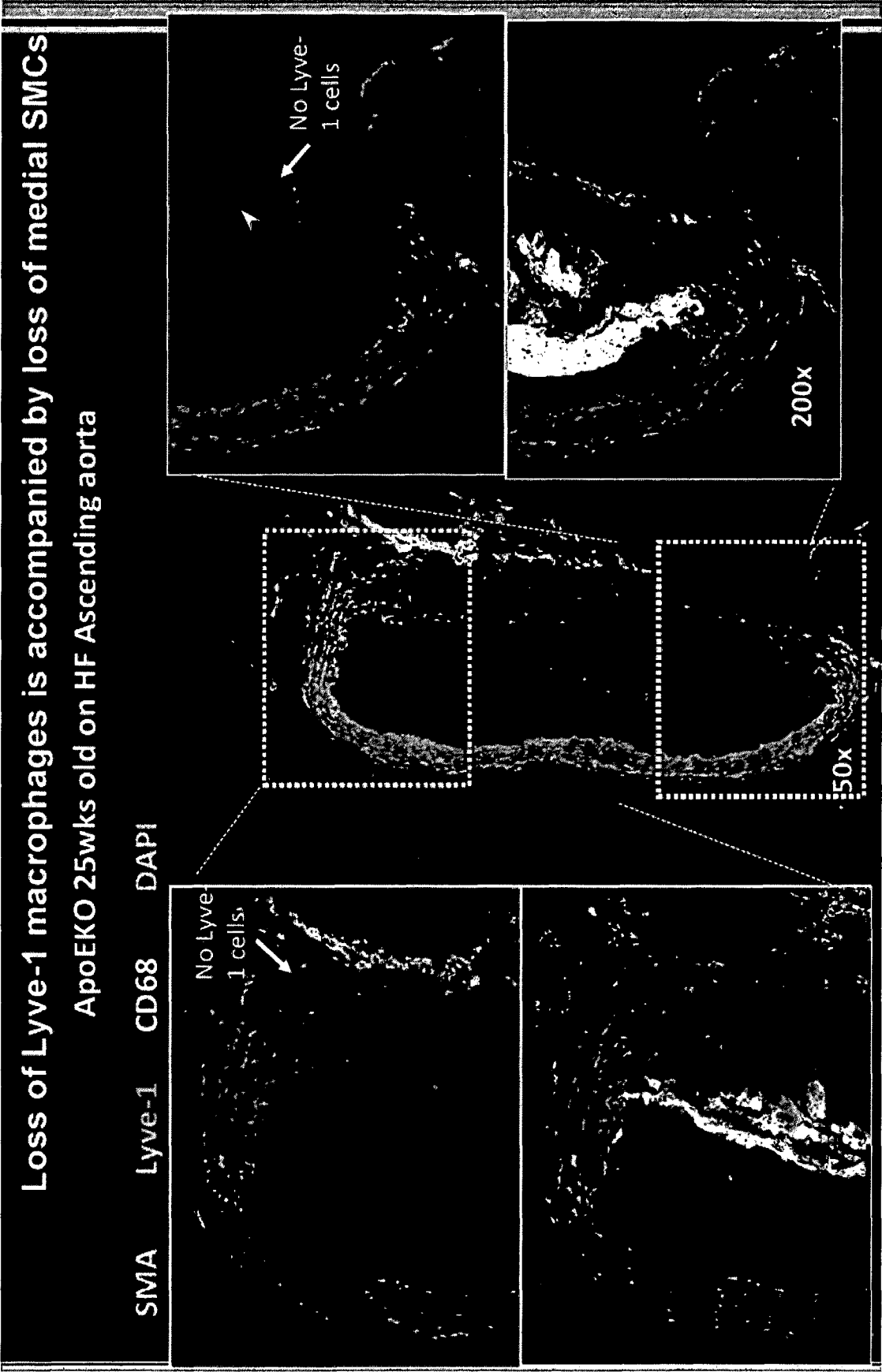
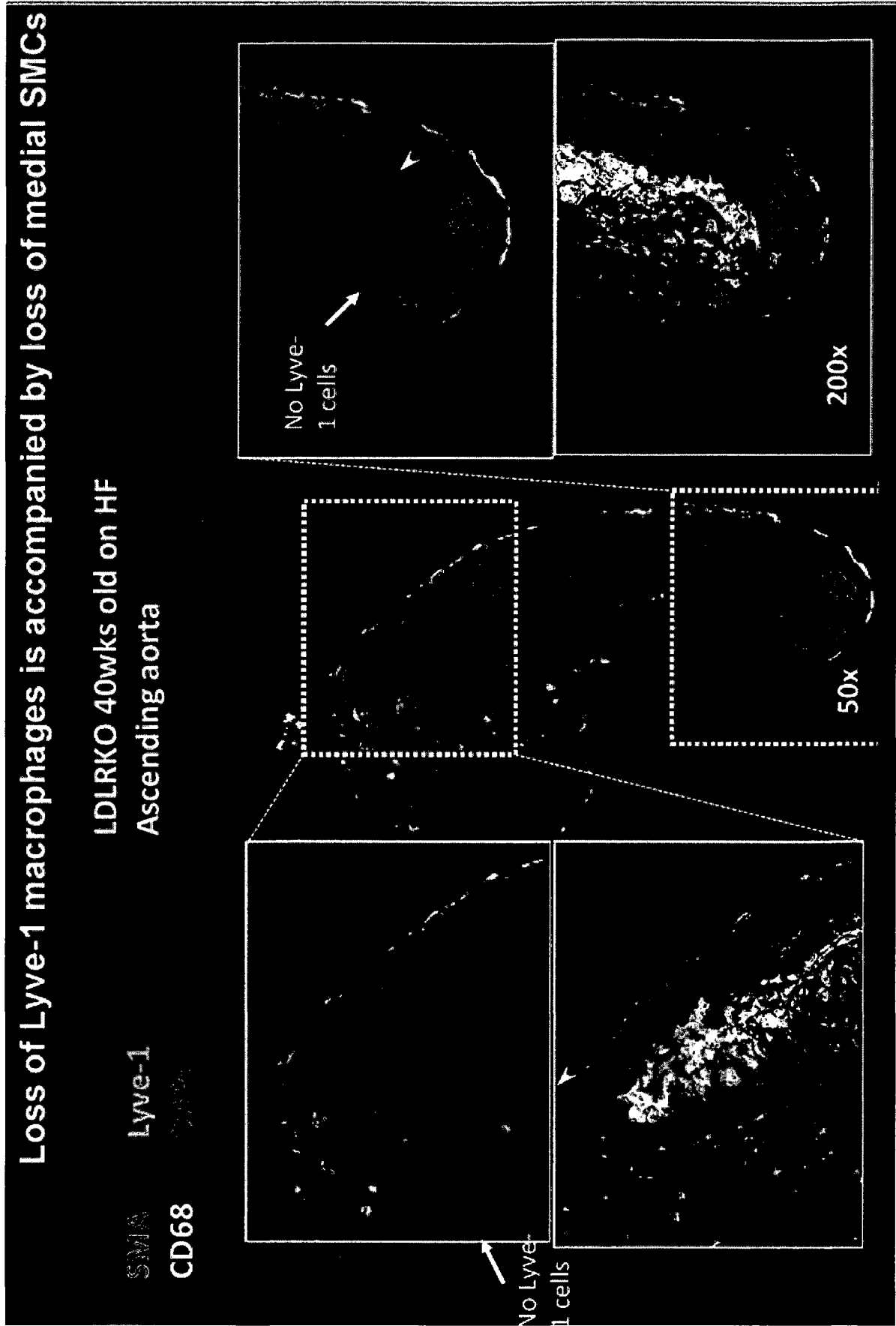


Figure 27



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Atherosclerosis

1. Aortic root
2. Ascending aorta
3. Carotid 1 (innominate/
Brachiocephalic artery)

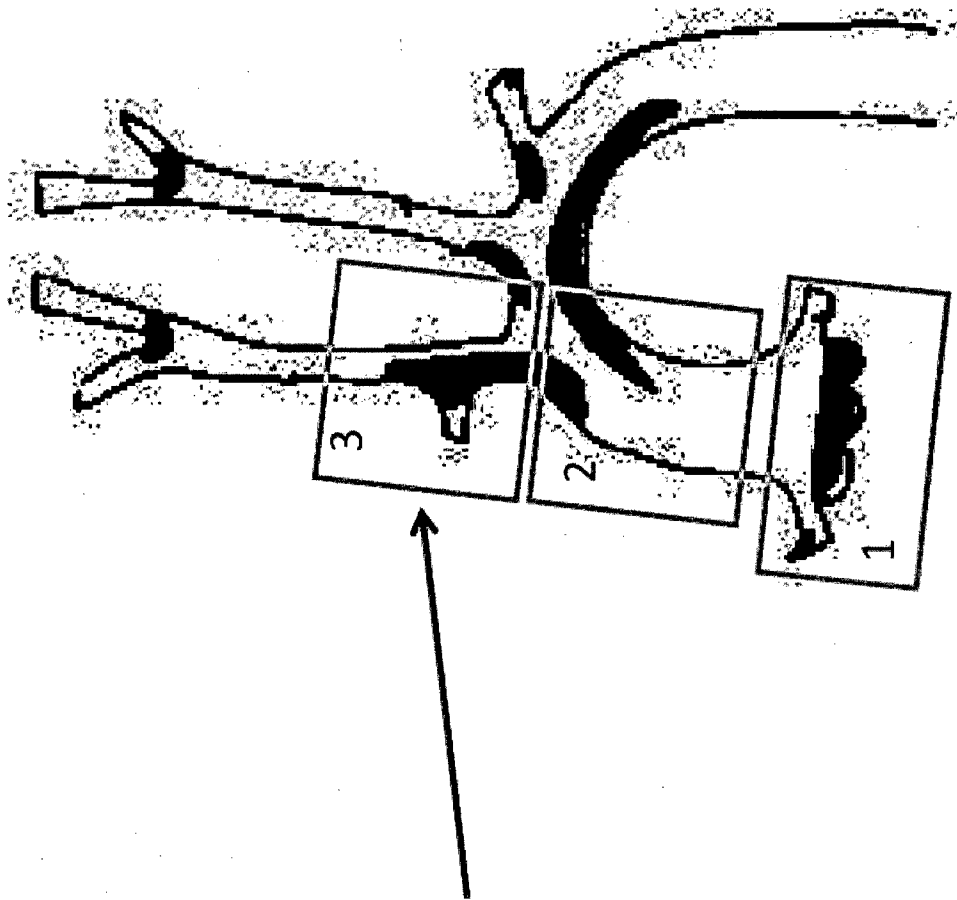


Figure 28

Figure 29

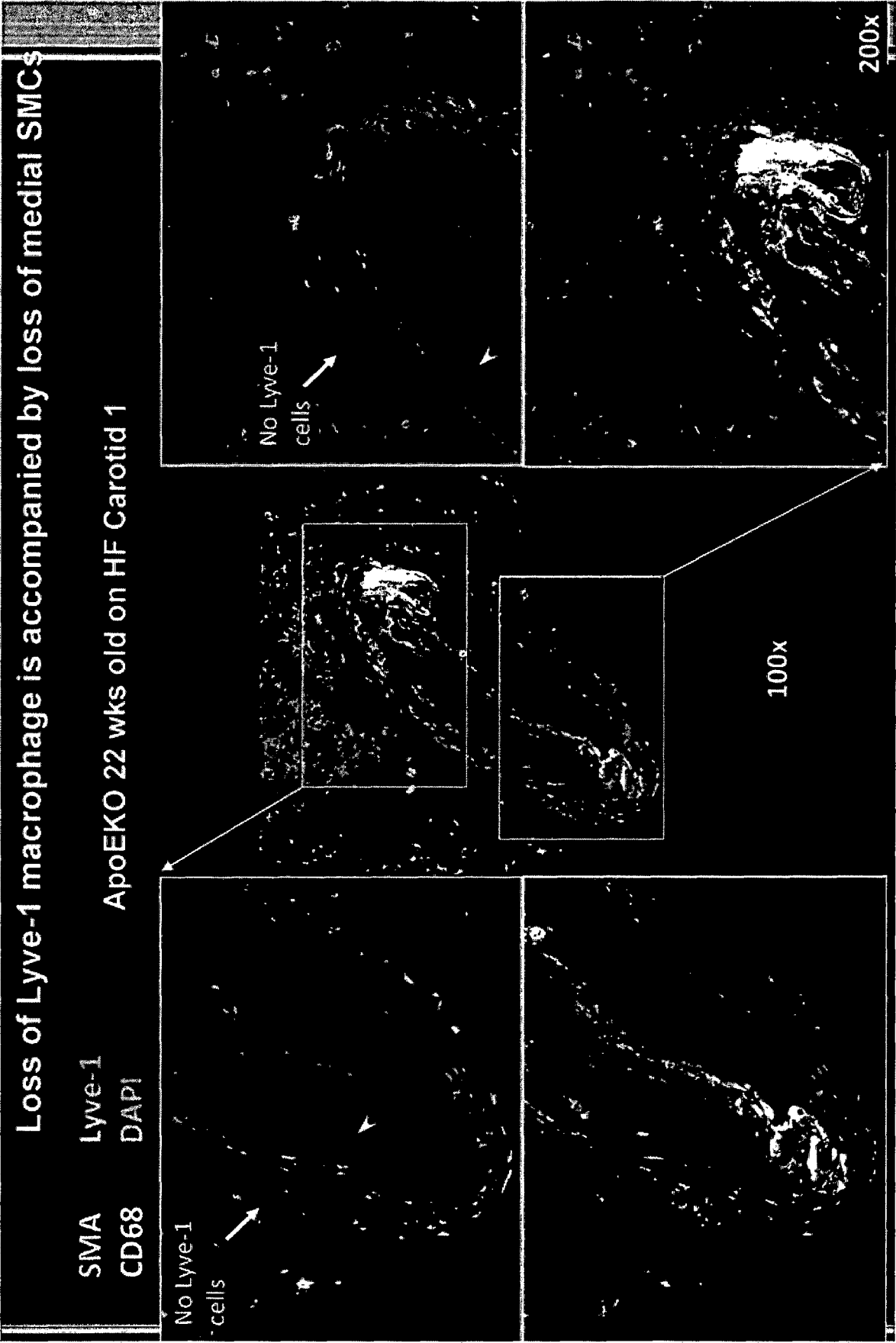
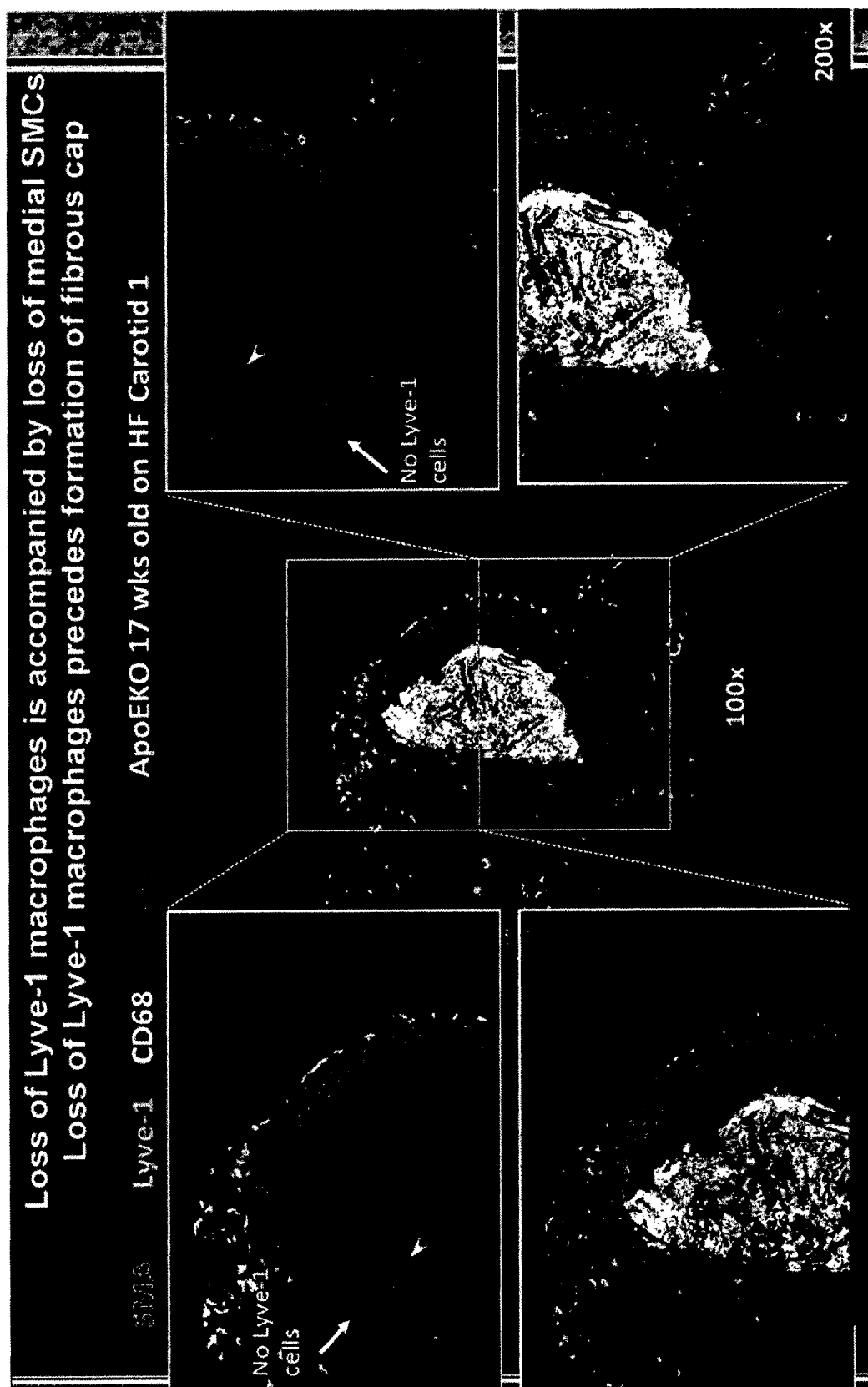
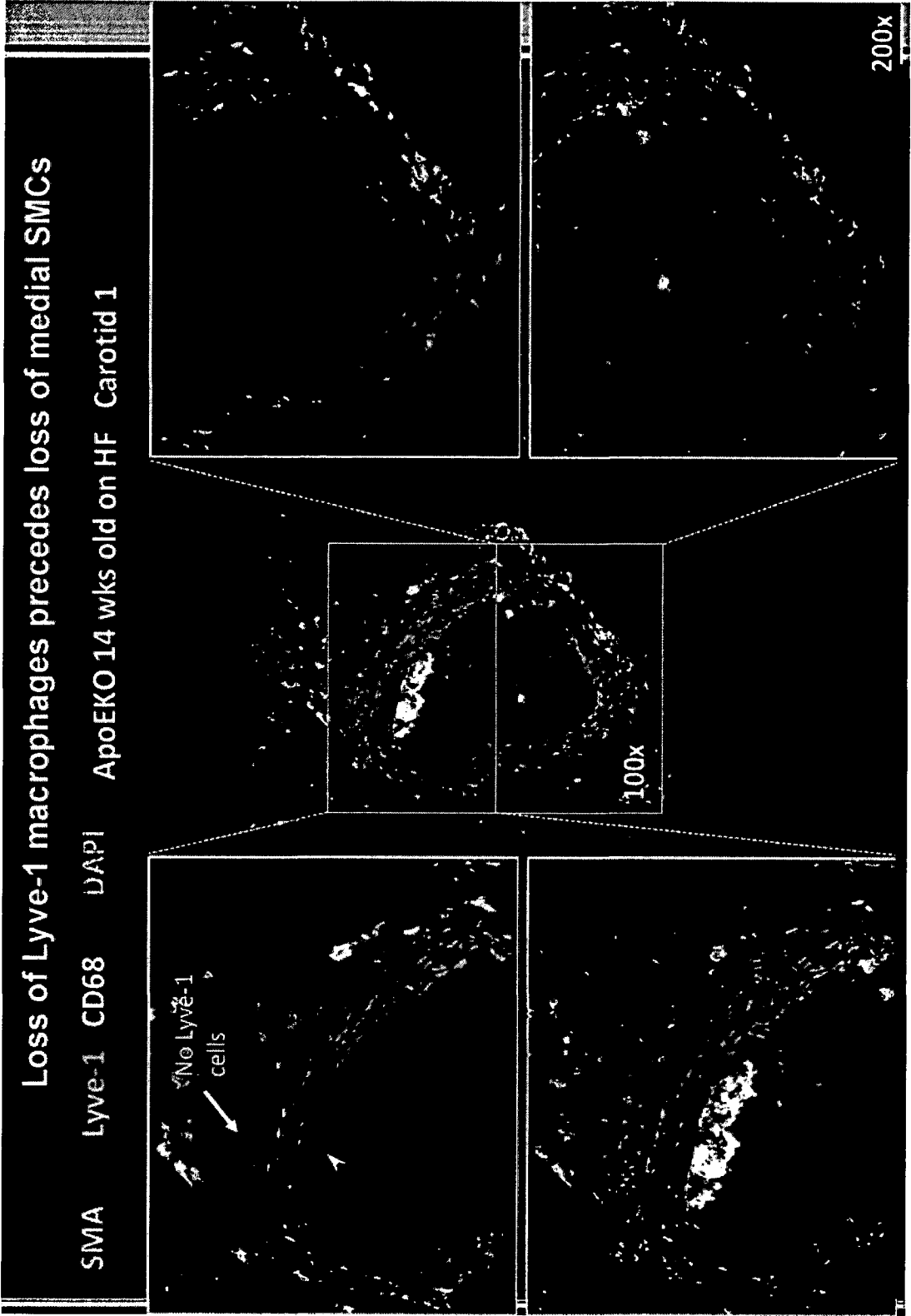


Figure 30





Blood and bone marrow monocytes do not express LYVE-1

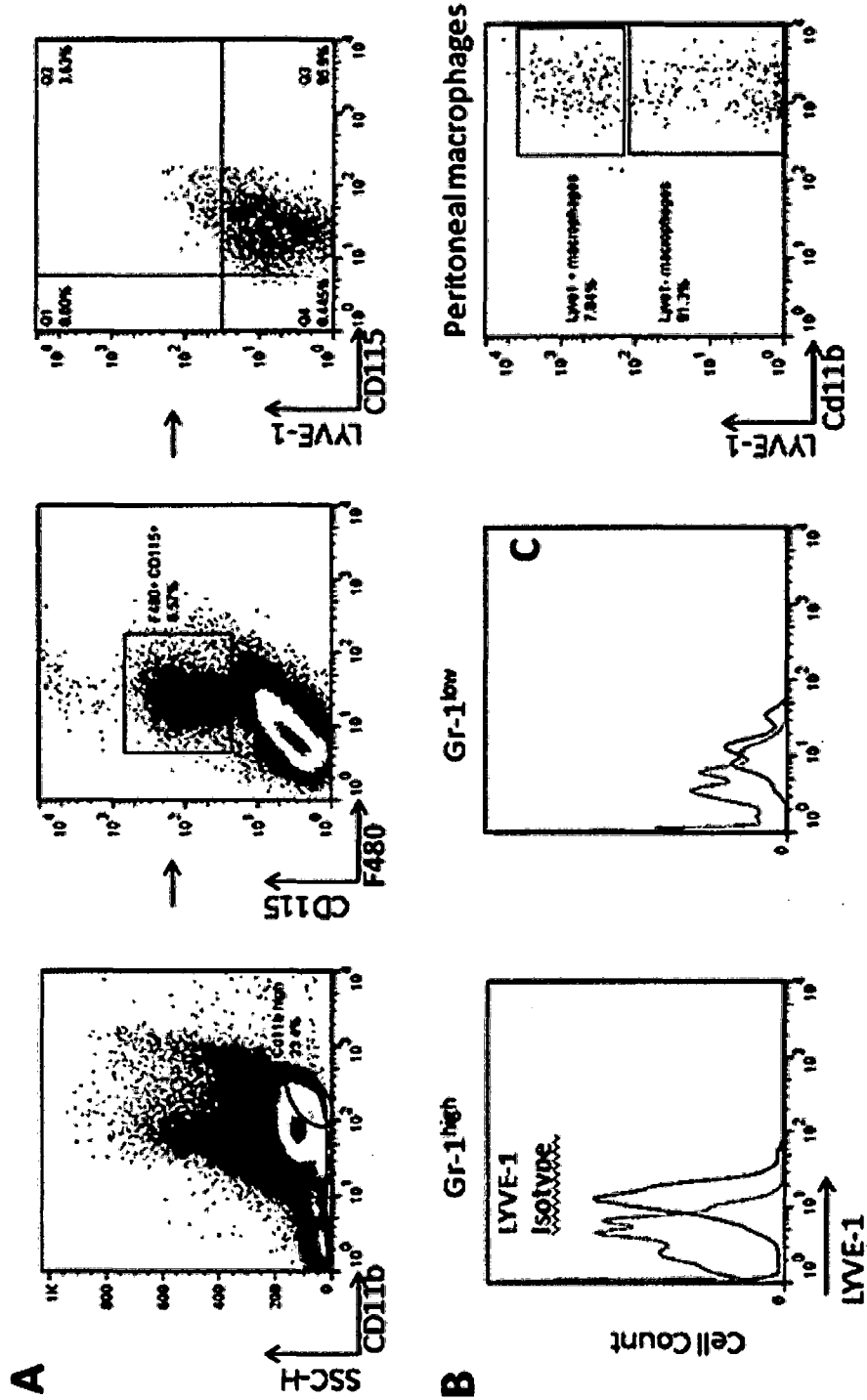


Figure 32

Figure 33

LYVE-1
a small population of bone marrow derived macrophage express

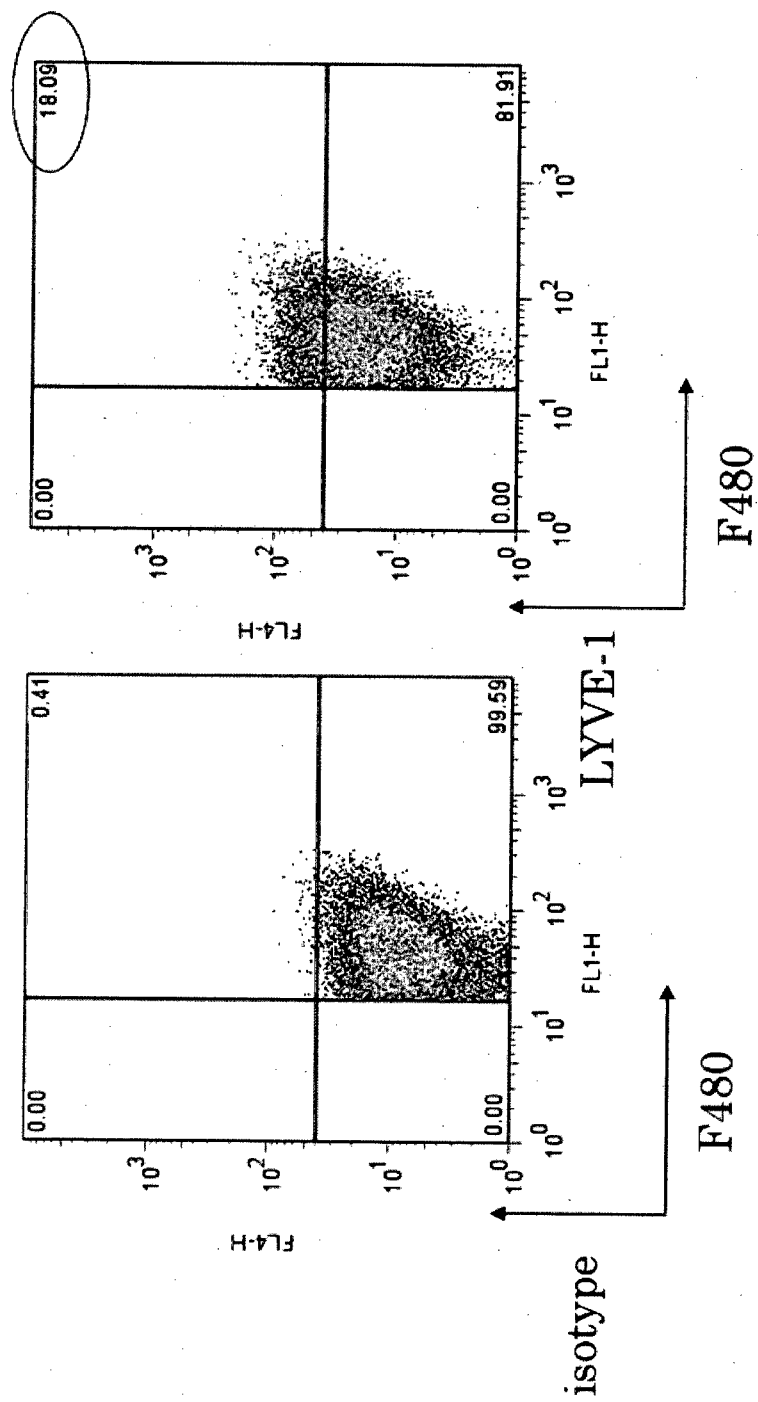


Figure 34

Adipose tissue from mouse contains a large population of LYVE-1⁺ macrophages

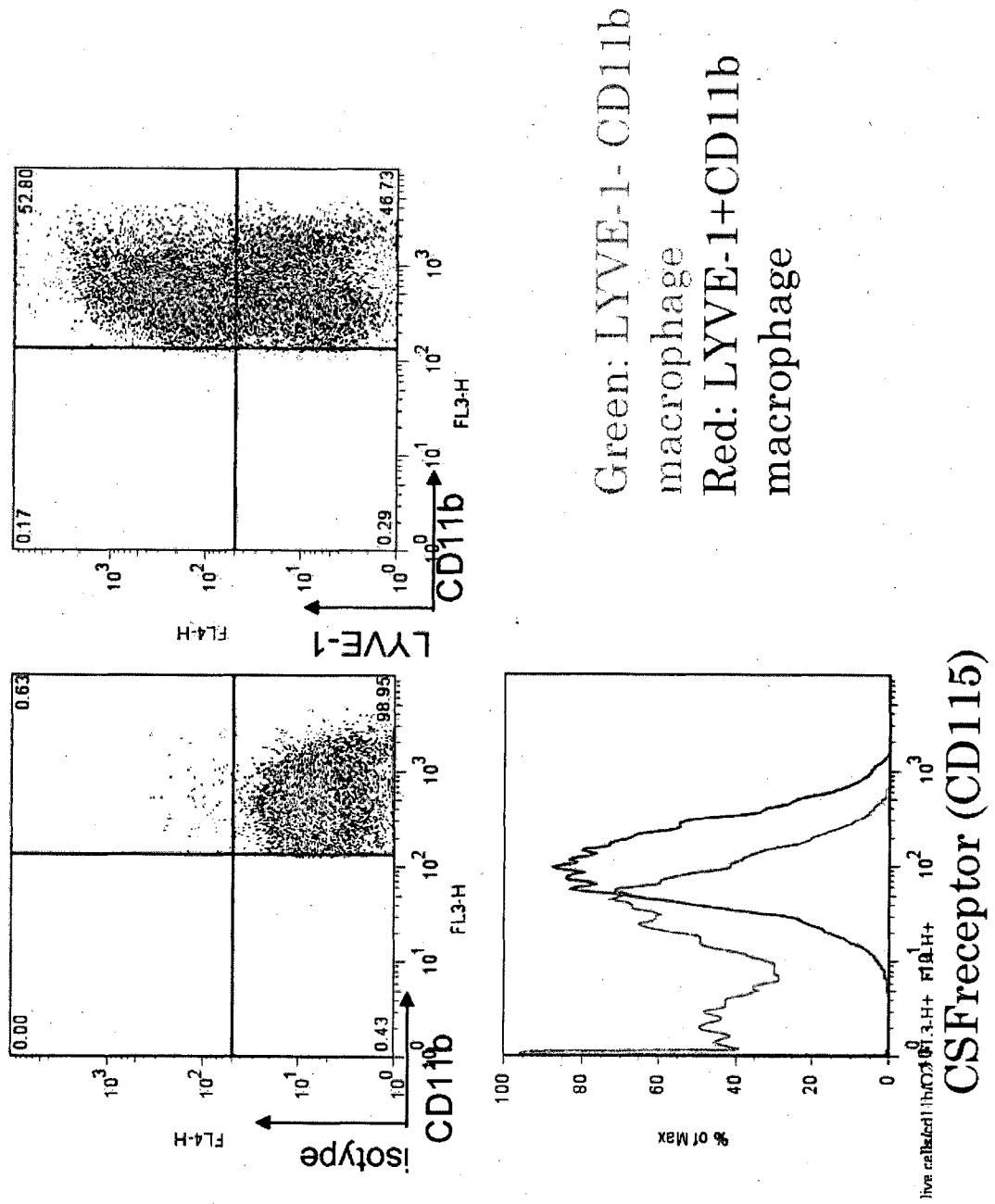


Figure 35

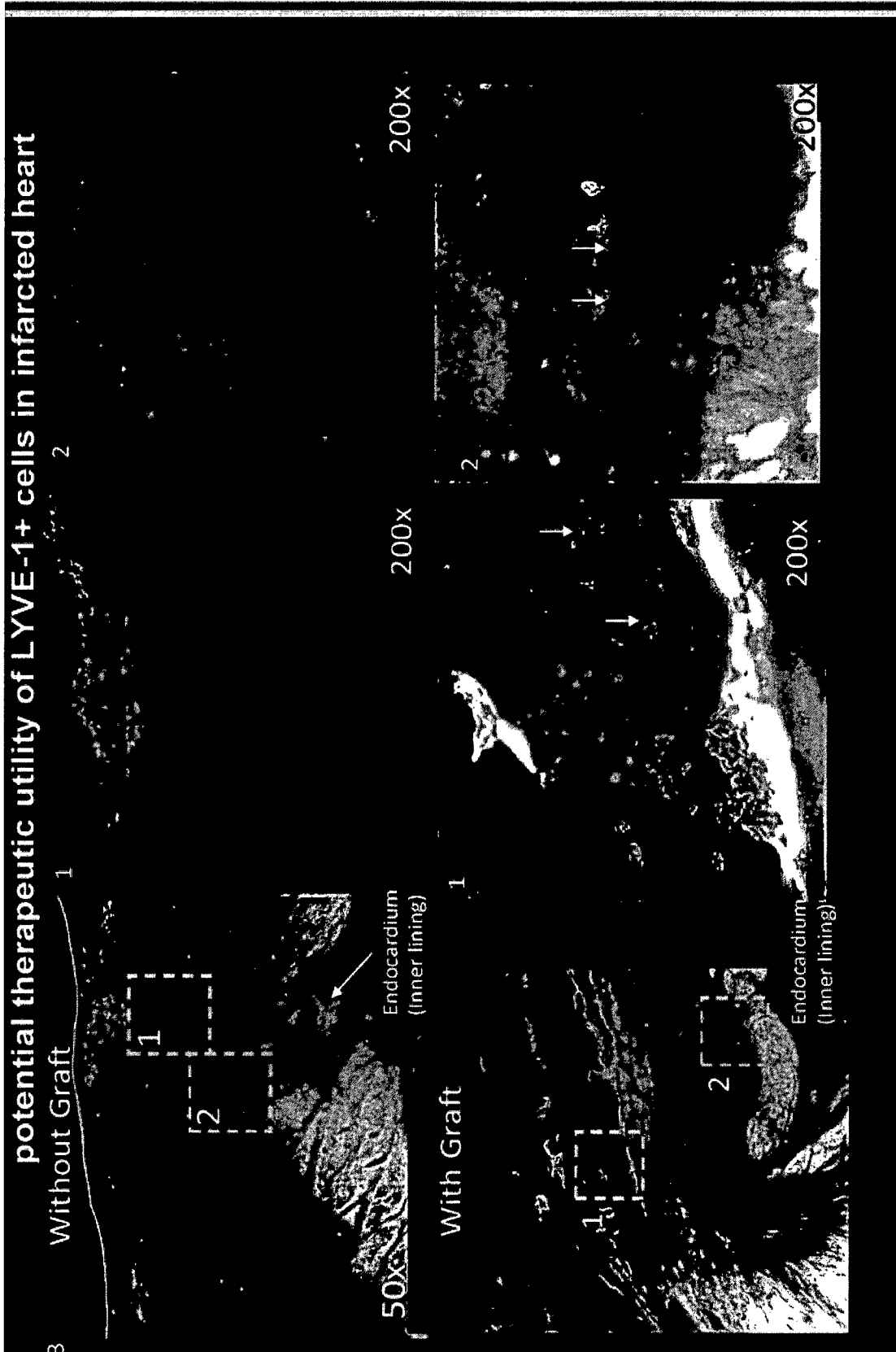


Figure 36

Depletion of LYVE-1⁺ cells in MAFIA treated mice induces aortic stiffness (1)

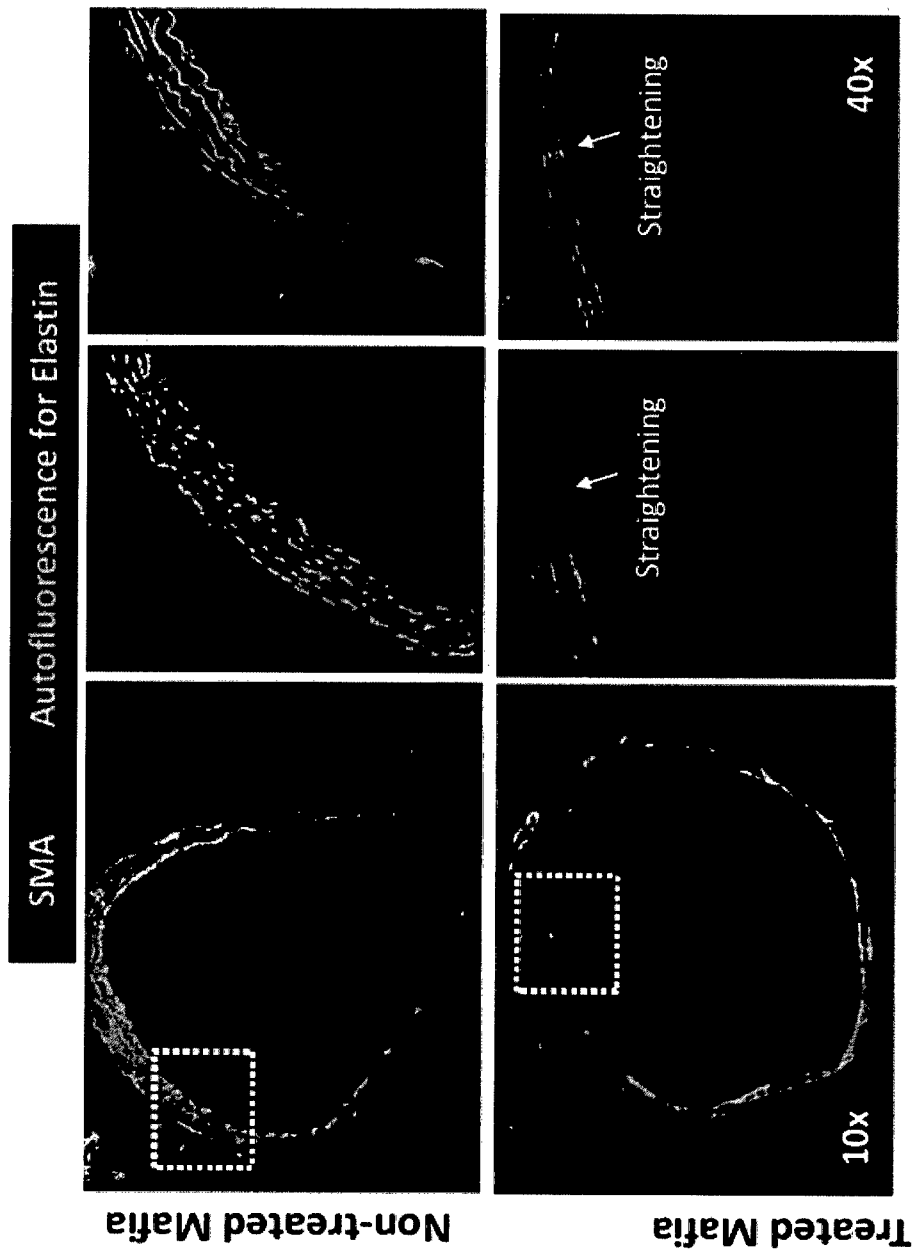


Figure 37

Depletion of LYVE-1⁺ cells induces aortic stiffness (2)

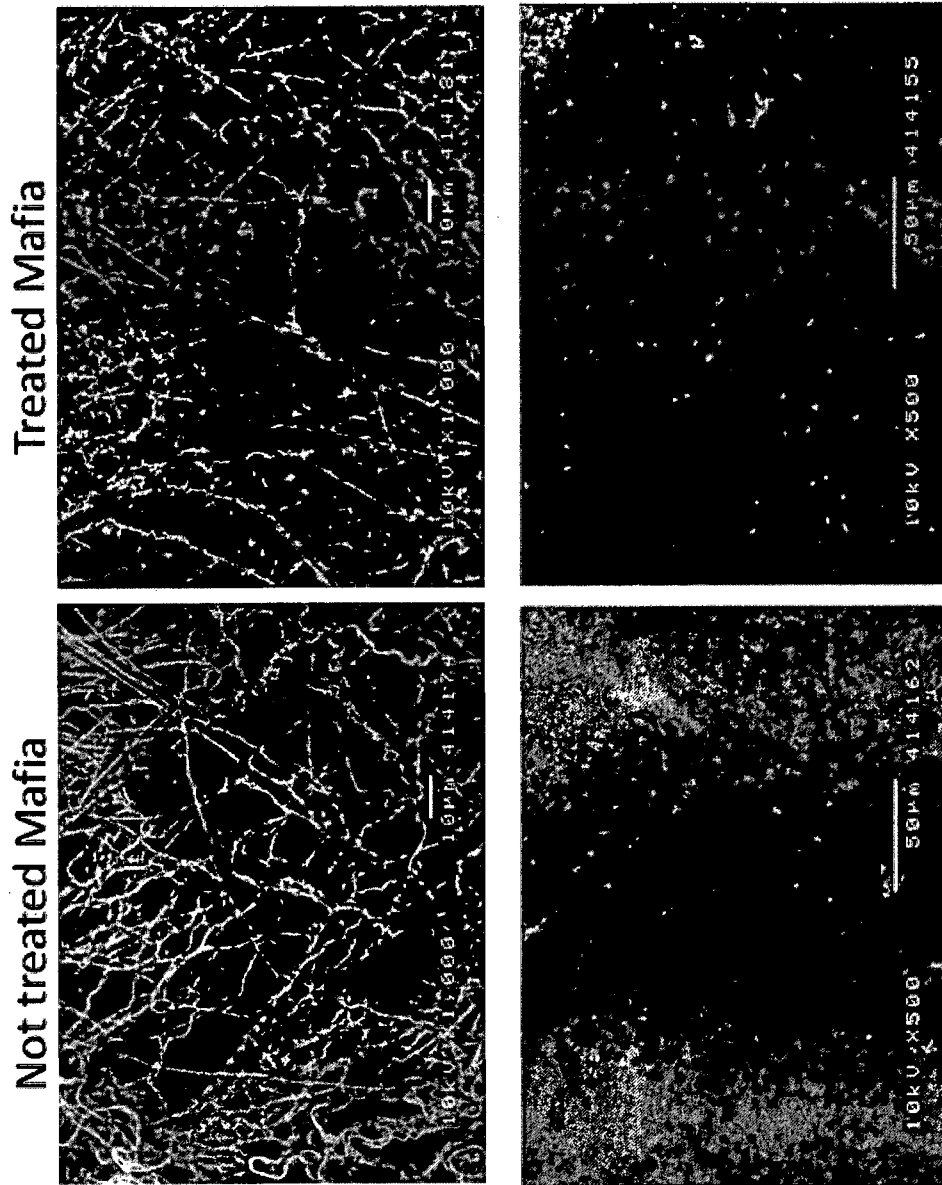
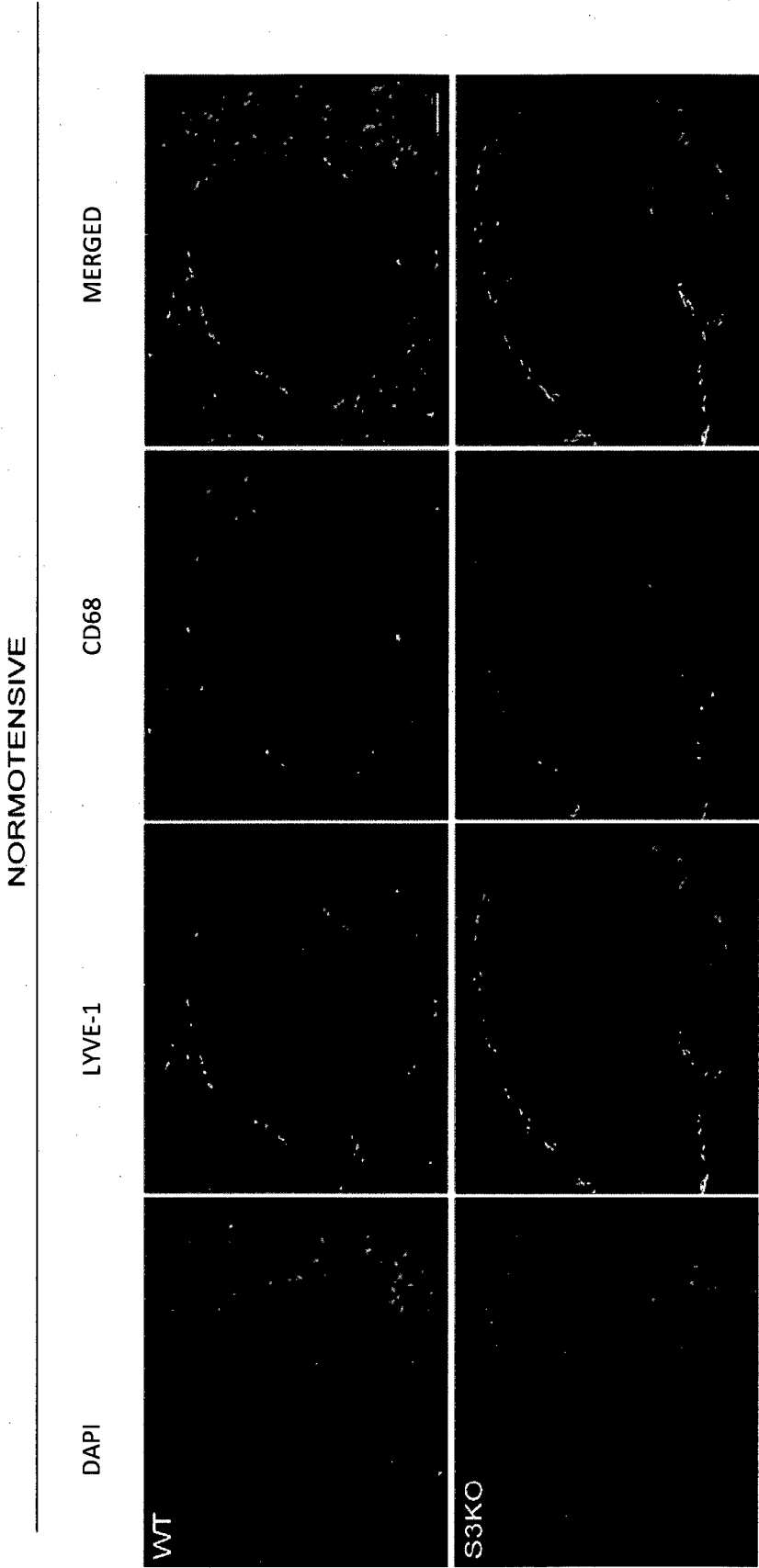


Figure 38A

Loss of LYVE-1⁺ cells in hypertensive mouse



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Figure 38B

HYPERTENSIVE

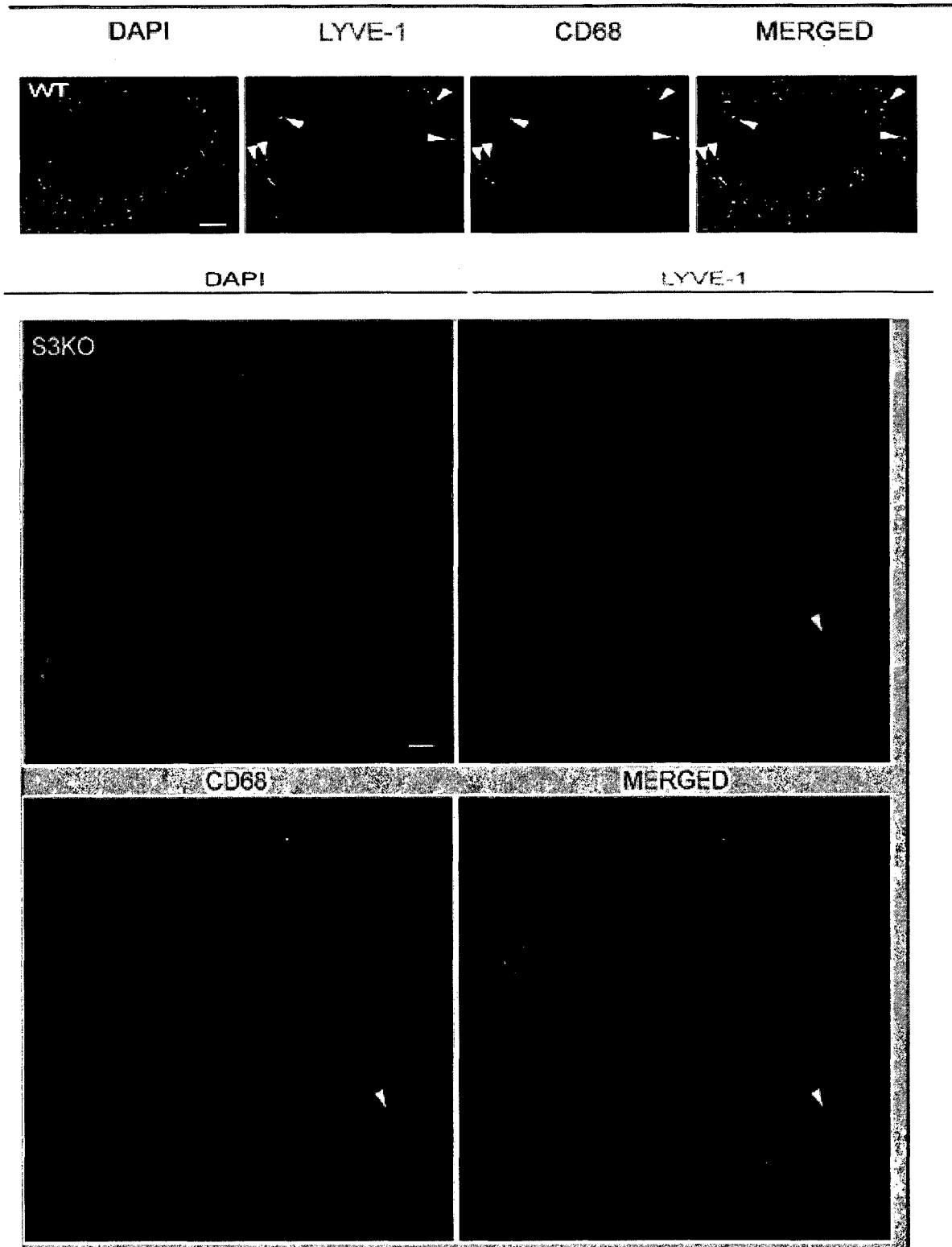
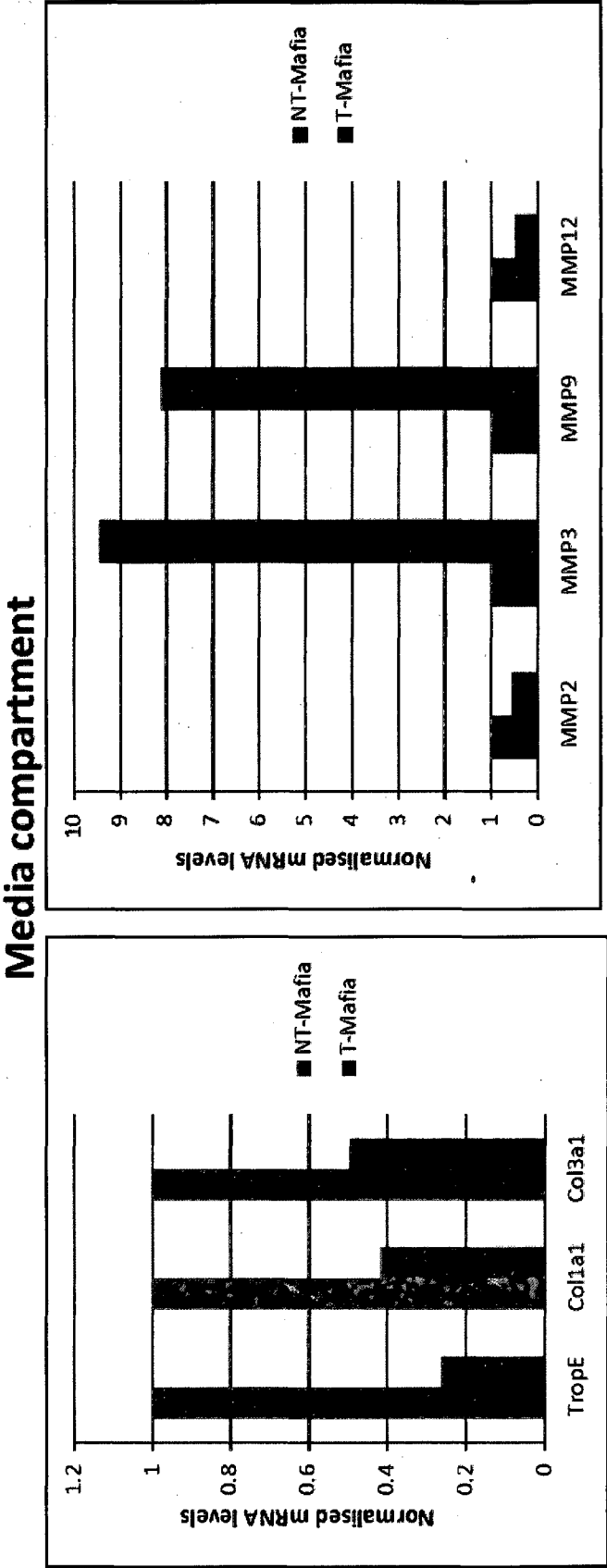


Figure 39

Depletion of LYVE-1⁺ cells induces aortic stiffness (3)



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LYVE-1⁺ macrophages express TGFbeta



Figure 40

Figure 41

High expression of MCSF in aortic media from WT mice at steady state

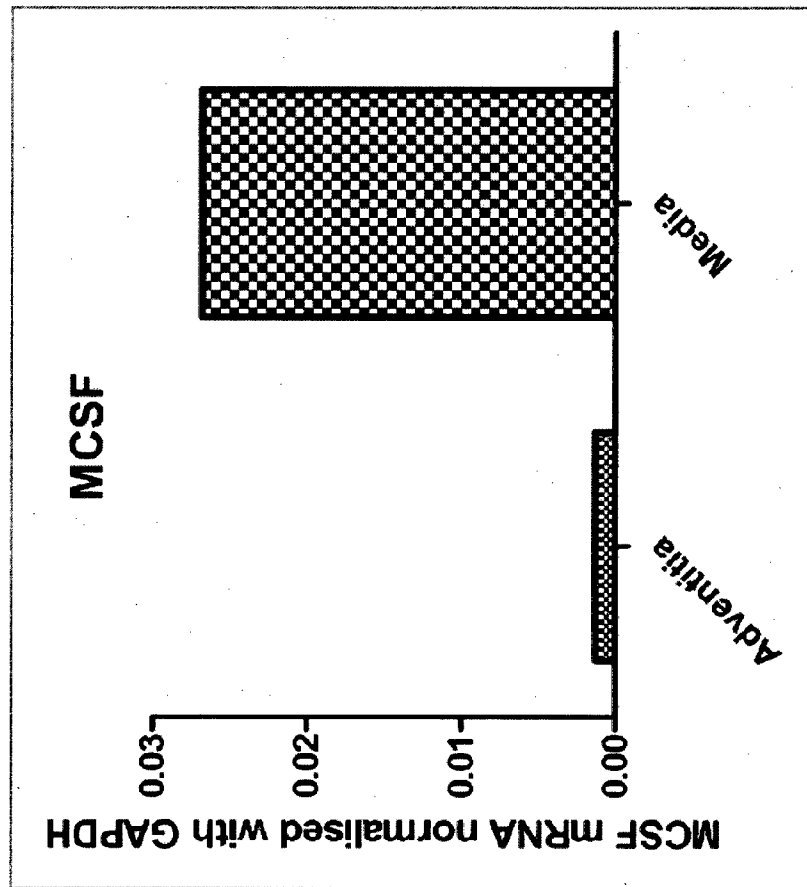
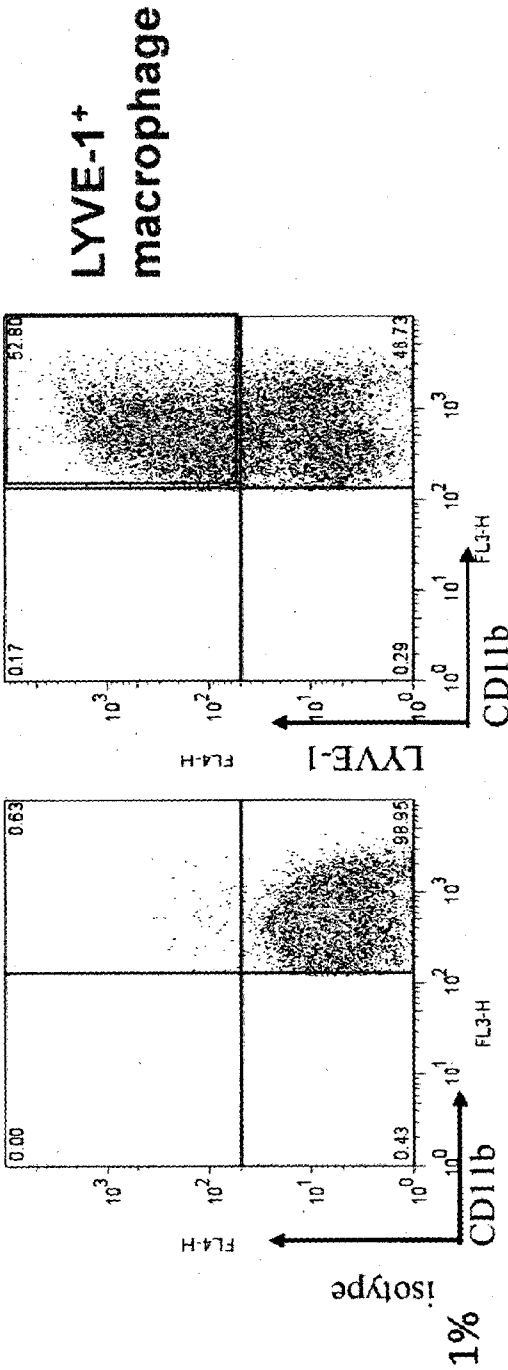


Figure 42

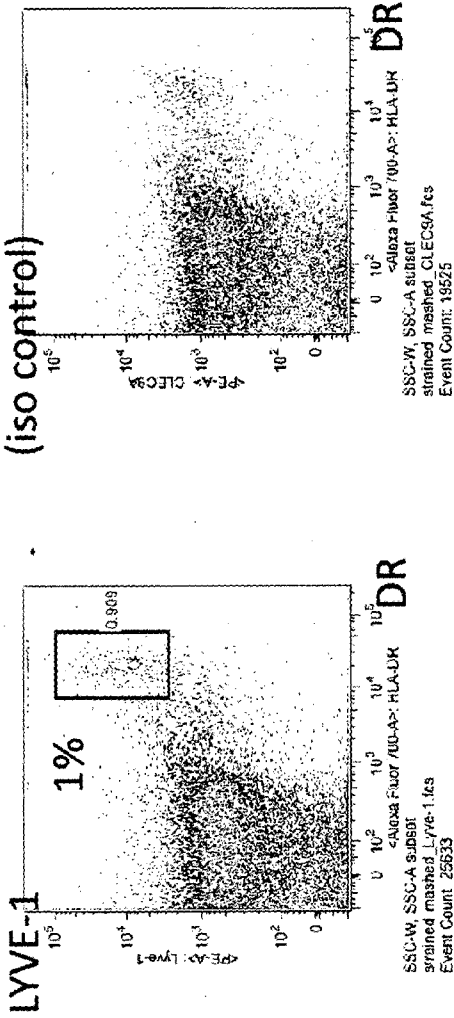
LYVE-1⁺ cells can be isolated from mice and human fat

MICE



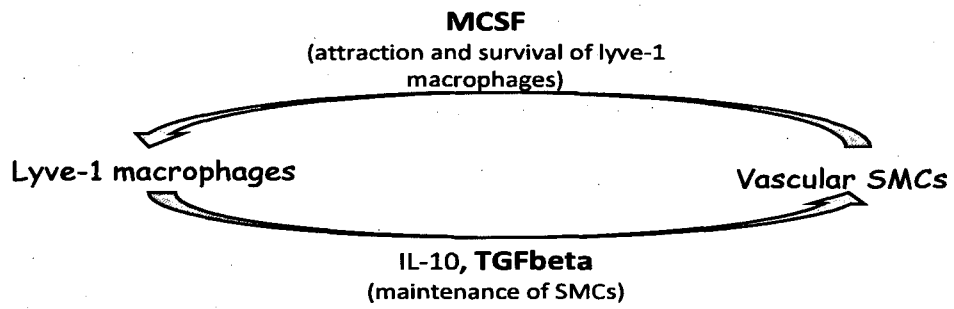
Human

LYVE-1⁺ macrophage



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Figure 43



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2012/000405

A. CLASSIFICATION OF SUBJECT MATTER

A61P 9/00 (2006.01) A61P 9/10 (2006.01) A61K 35/12 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS, EPODOC, MEDLINE. Keywords: Lymphatic vessel endothelial hyaluronic acid receptor-1, LYVE-1, macrophage, blood vessel, artery, vein, aorta, atherosclerosis, isch[a]emia, vascular+, macrophage stimulating growth factor, M-CSF.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search
2 January 2013Date of mailing of the international search report
02 January 2013

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2012/000405
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Kubota, Y. et al. "M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis." The Journal of Experimental Medicine, 11 May 2009. vol. 206, no. 5, pages 1089-1102. (see whole document)	
A	Watanabe, Y. "Effect of macrophage colony stimulating factor on the advanced atherosclerosis in Watanabe heritable hyperlipidemic rabbits." Hormone and Metabolic Research, October 1997. vol. 29, no. 10, pages 507-509. (see whole document)	
A	WO 2001/087314 A1 (GENETIX PHARMACEUTICALS, INC) 22 November 2001	
A	WO 1994/005334 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 17 March 1994	
A	Mozes, G. "Adenovirus-mediated gene transfer of macrophage colony stimulating factor to the arterial wall in vivo." Arteriosclerosis Thrombosis and Vascular Biology, July 1998. vol. 18, no. 7, pages 1157-1163. (see whole document)	

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2012/000405	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2001/087314 A1	22 Nov 2001	AU 6173701 A	26 Nov 2001
		US 2002034501 A1	21 Mar 2002
		WO 0187314 A1	22 Nov 2001
WO 1994/005334 A1	17 Mar 1994	AU 4845093 A	29 Mar 1994
		WO 9405334 A1	17 Mar 1994
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			