Oxidized LDL-induced angiogenesis involves sphingosine-1-phosphate. Prevention by anti-S1P antibody.

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SUPPORTING INFORMATIONS

Supporting information - Figure S1

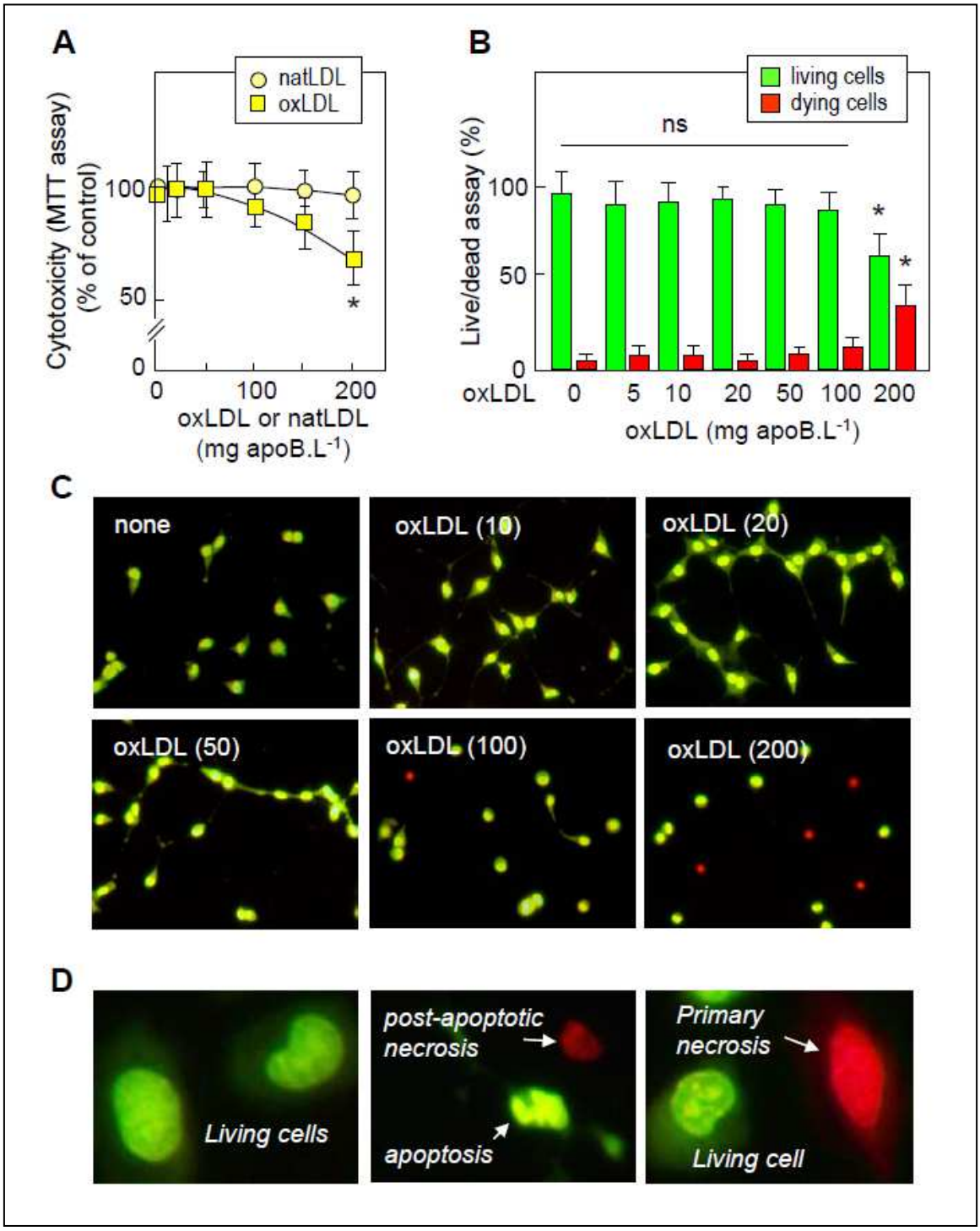

Figure S1. Toxicity of increasing concentration of oxLDL in HMEC-1 grown on Matrigel.
HMEC-1, grown on Matrigel, were incubated for 24h with increasing concentration of oxLDL or native LDL (natLDL). The cytotoxicity was evaluated (A) by the MTT assay, expressed as % of the untreated (0) control and (B,C) by the live/dead assay. Cells were stained by two fluorescent intercalating compounds, the permeant green-colored syto13 and the non permeant red colored propidium iodide (PI) (under conditions where the red fluorescence prevails over the green fluorescence when the two fluorescent probes enter the nucleus, i.e. cell membrane permeabilized). Living and dying cells were counted on the basis of staining and morphological features. In A,B, mean ± SEM of 6 to 10 separate experiments. Comparison to the untreated control by one way Anova and Holm-Sidak * $p<0.05$; ns, not significant. D. Higher magnification showing the structure of the nucleus. Left panel. Living cells exhibit a loose green colored chromatin (PI does not enter the normal cell). Middle panel. Primary apoptosis is characterized by condensed pyknotic or fragmented nucleus stained green/yellow by Syto13 (at this stage, apoptotic cells are not permeable to PI). During post-apoptotic necrosis, the nucleus exhibits similar apoptotic morphology but is stained red by PI (due to plasma membrane permeabilization in a late step of apoptosis). Right panel. Primary necrosis is characterized by an early permeabilization of the plasma membrane allowing PI to enter the cell and stain red the loose chromatin of the nucleus.

Supporting information - Figure S2

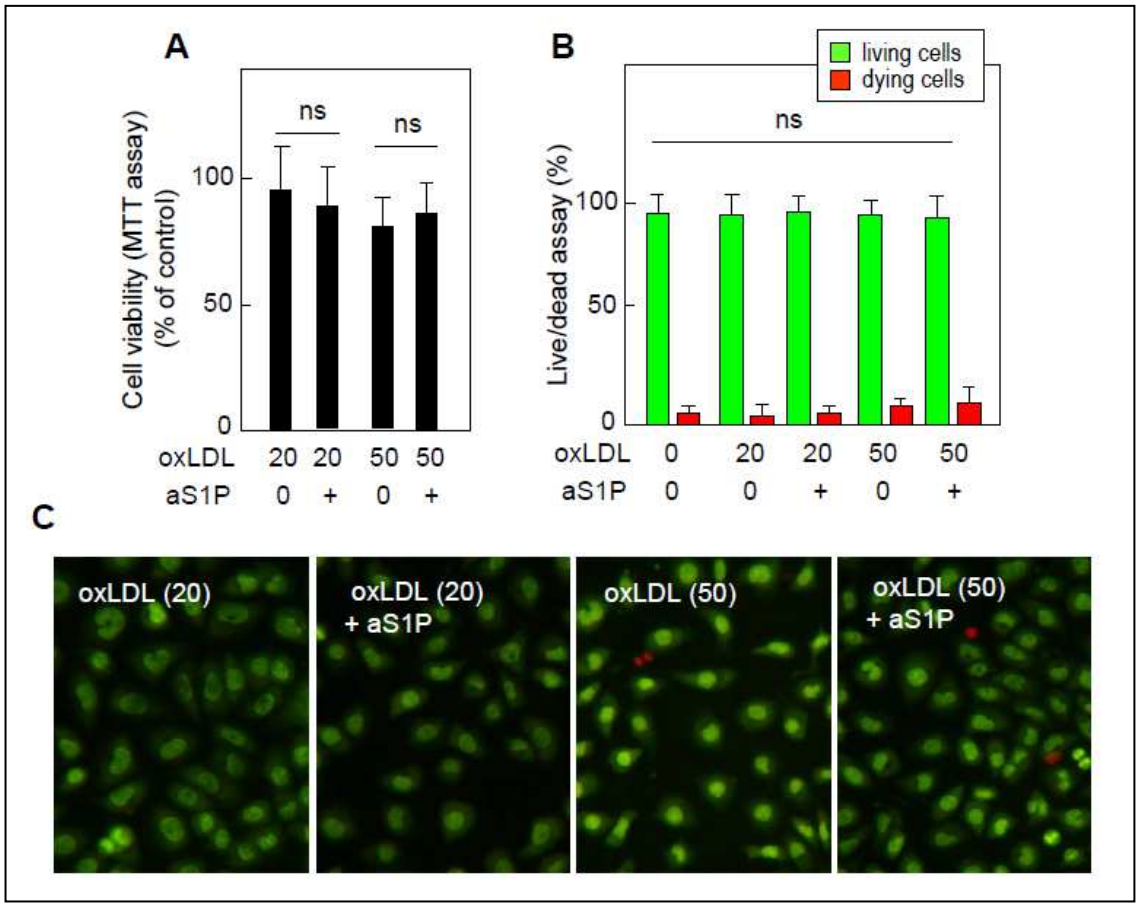

Figure S2. Anti-S1P mAb is not toxic to HMEC-1 treated with low concentration of oxLDL.
HMEC-1 were grown in 12 multiwell plates in MCDB-131 medium containing 10% FCS, then starved in serum poor (0.1% FCS) medium for 24h before addition of oxLDL at the indicated concentrations (20 or 50 mg apoB.L^{-1}) and anti-S1P mAb (aS1P, 10 mg.L^{-1}). In A, evaluation by the MTT assay of the whole toxicity of oxLDL (20 and 50 mg.L^{-1}) and aS1P. In B,C, live/dead assays using syto-13 (green) and PI (red) DNA probes, as in A, on HMEC-1 incubated with oxLDL and aS1P. In B, counting of living and dying cells. C, pictures representative of HMEC-1 viability in the presence of oxLDL (at the indicated concentrations) and aS1P. Mean ± SEM of 8 separate experiments, * $p<0.05$; ns, not significant.

Supporting information - Figure S3

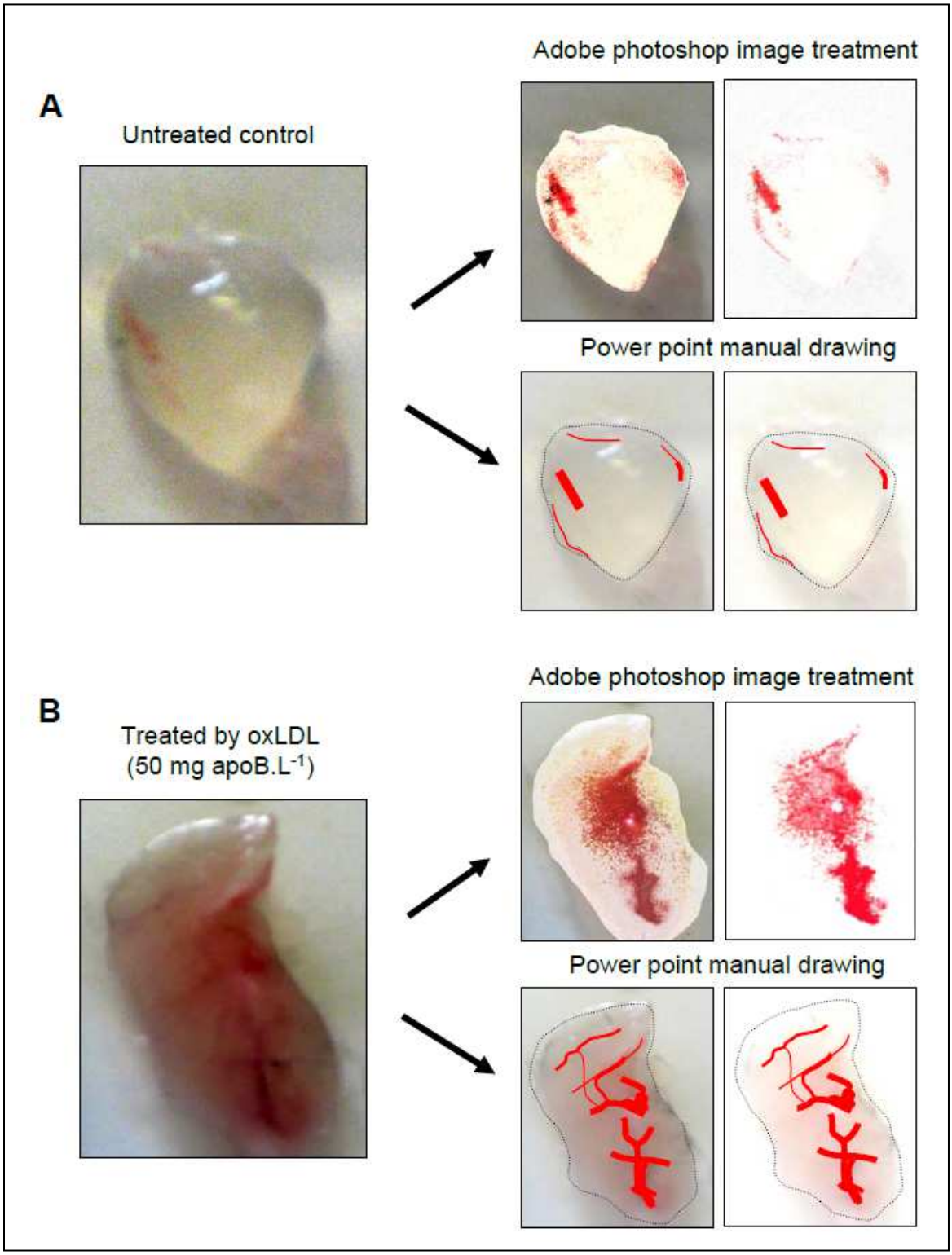

Figure S3. Human oxLDL stimulate angiogenesis in vivo in the Matrigel plug model.
A,B. Angiogenesis in Matrigel plugs. C57/BL6 mice were injected subcutaneously with 0.4 mL Matrigel containing on one flank PBS and on the other flank human oxLDLs. Plugs were removed after 2 weeks and photographied. Representative macrophotographies of plugs containing PBS (control) or oxLDLs (50 mg apoB.L^{-1}). Angiogenesis was quantified by image analysis of the red blood color using Adobe photoshop software (upper right panel in A and B) or by manual drawing followed by image analysis (lower right panels in A and B).

Supporting information - Figure S4

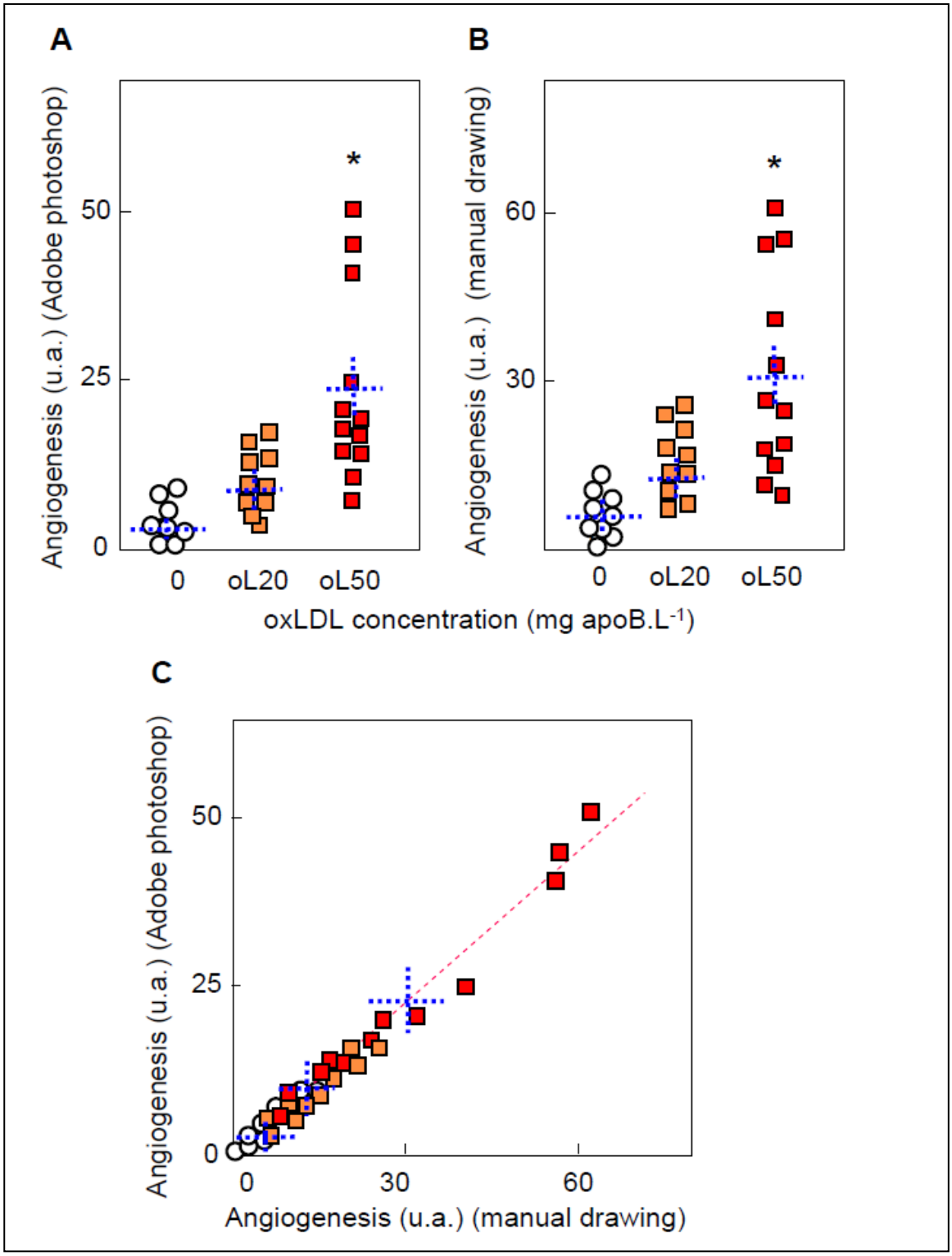

Figure S4. Comparison of the two methods of quantification of angiogenesis on macrophotographies.

A and B, Quantification of angiogenesis in Matrigel plugs obtained by image analysis of blood colored vessels using Adobe photoshop software (A) and by manual drawing followed by image analysis (B). In C, comparison of the data by the two methods.

Supporting information - Figure S5

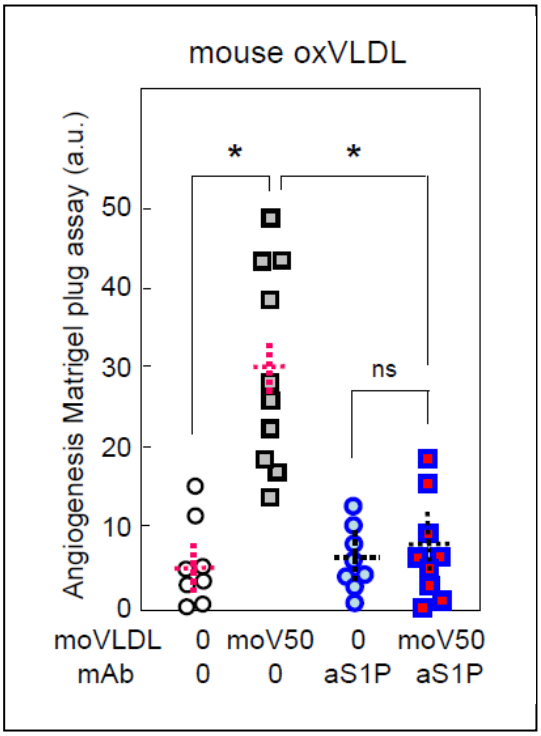

Figure S5. Effect of anti-S1P mAb on mouse oxVLDL (moV)-induced angiogenesis in the murine Matrigel plug assay.
Mice were injected with Matrigel containing 0 or 50 mg apoB.L^{-1} murine oxVLDL (moV50). Mice were intraperitoneously injected every 3 days with (or without) the anti-S1P mAb (50 mg.kg^{-1} body weight) for 2 weeks before removing the plugs and quantification of angiogenesis. Each point represents the angiogenesis score in one plug. Mean ± SEM are indicated by the doted line. Comparison of groups was performed by one way Anova followed Holm-Sidak test (SigmaStat sofware) * $p < 0.05$; ns, not statistically significant.

Supporting information - Figure S6

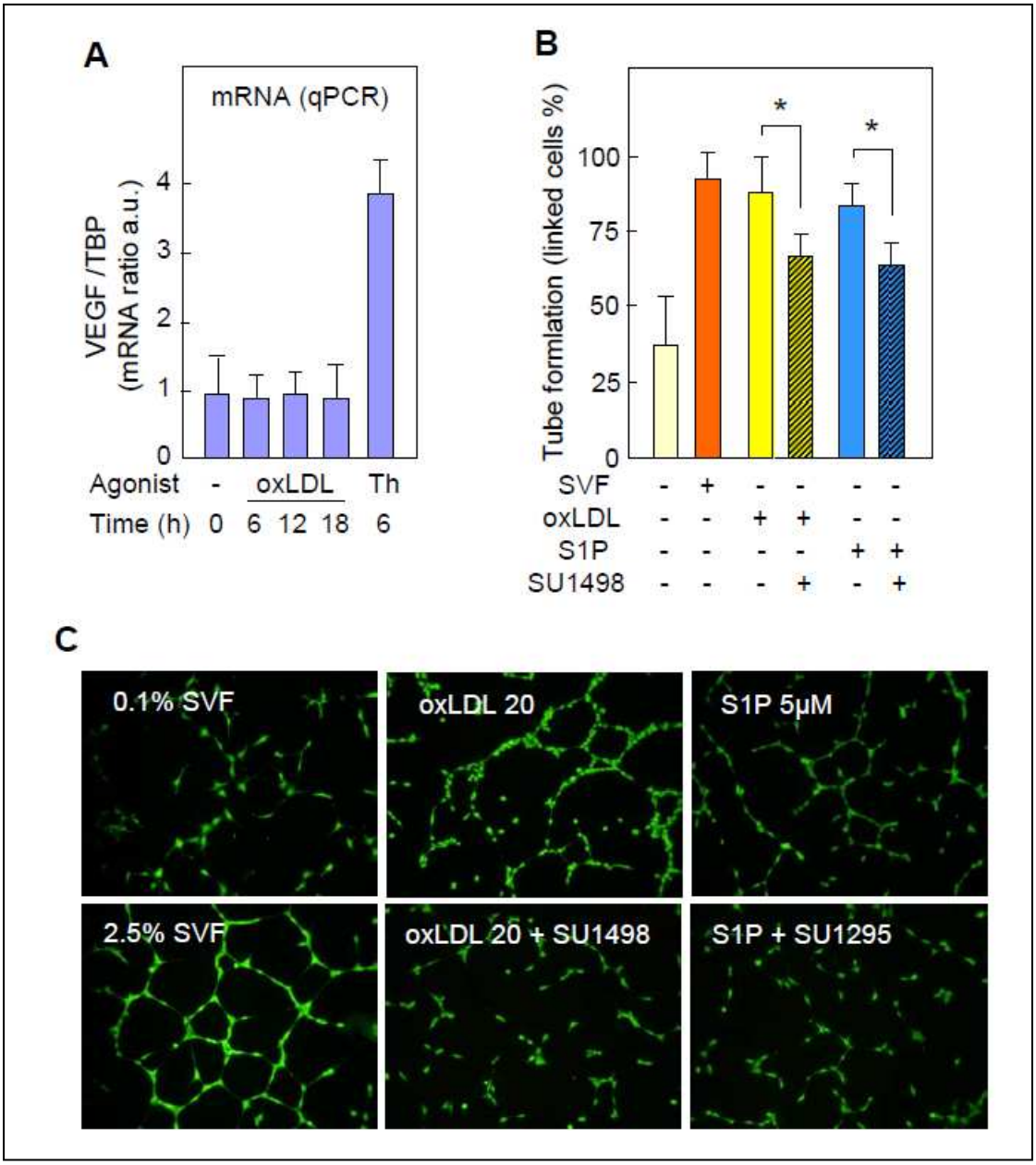

Figure S6. Effect of oxLDL on VEGF expression (A) and effect of the VEGFR2 inhibitor SU1295 on angiogenesis elicited by oxLDL.

A. Effect of oxLDL on VEGF expression in HMEC-1. Cells were incubated in the presence of oxLDL (20 mg apoB.L^{-1}) or thapsigargin (5 µmol.L^{-1}) at the indicated time. VEGF mRNA level was evaluated by qPCR (normalized to TBP mRNA). B and C, Effect of the VEGFR2 inhibitor SU1498 on angiogenesis induced by oxLDL (20 mg apoB.L^{-1}) or by S1P (5 µmol.L^{-1}). In A and B, mean ± SEM of 6 separate experiments, * $p<0.05$; ns, not significant. In C, representative microphotographs of cells stained as in Fig.1.

Supporting information - Figure S7

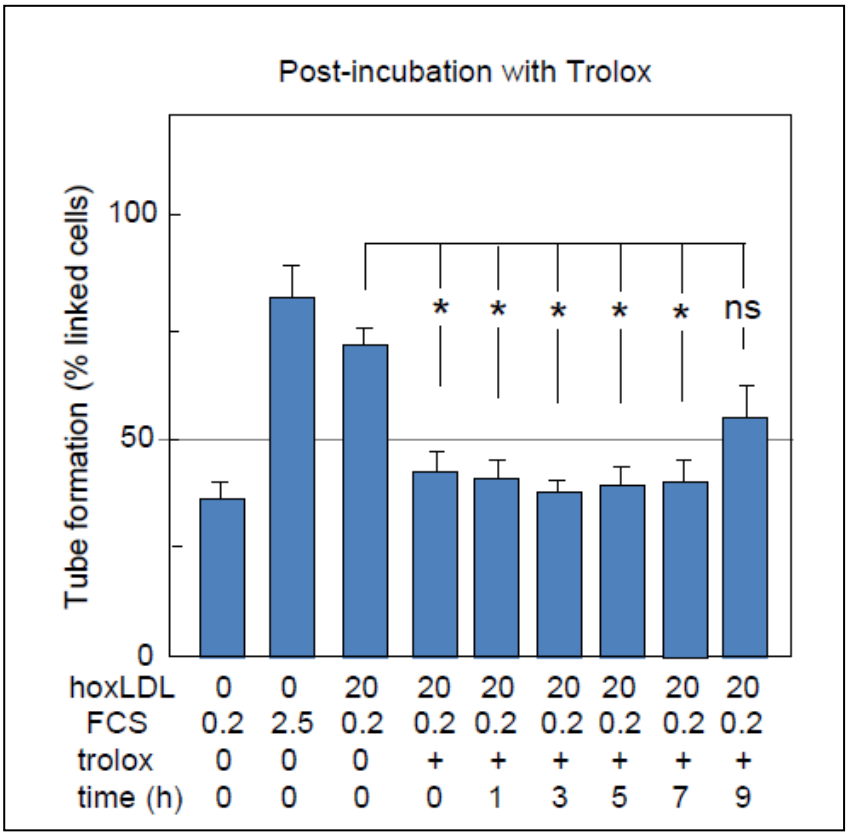

Figure S7. Time course of inhibition of tube formation by trolox added after oxLDL.

HMEC-1 were incubated with or without hoxLDL (20 mg apoB.L^{-1}, added at time 0) and trolox (10 µmol.L^{-1}), was added at the indicated time (either at time 0, or 1, 3, 5, or 9 h after oxLDL. Then, tube formation was evaluated at time 18h (as in Fig.1,3,4). Note that, under the used conditions, trolox added up to 7h, inhibited tube formation, thus suggesting that ROS generated up to 7h are required for tube formation. Mean ± SEM of 6 separate experiments. One way Anova followed by Holm-Sidak *post-hoc* test. * $p < 0.05$,

Supplemental Table S1 and S2

Table S1: Evaluation of ROS induced by oxLDL (20 mg apoB.L^{-1}) using various ROS specific probes (expressed as percent of the unstimulated control).

Probes	Agonist	Fluorescence
H2DCFDA-AM (HPO)	oxLDL	135 ± 5
MitoPY1 (H_2O_2)	oxLDL	133 ± 3
DAF (NO)	oxLDL	112 ± 2

Table S2: Effects of inhibitors on ROS generation elicited by oxLDLs.

oxLDL (mg.L^{-1})	Pharmacological agents	ROS (%)
0	none (unstimulated control)	100 ± 5
20	none (stimulated control)	131 ± 8
20	VAS (5 µmol.L^{-1})	89 ± 9 *
20	DPEI (1 µmol.L^{-1})	95 ± 7 *
20	Myxothiazol (10 µmol.L^{-1})	93 ± 6 *
20	NDGA (60 µmol.L^{-1})	88 ± 8 *
20	PEG-catalase (2.5 µmol.L^{-1})	87 ± 4 *
20	Ketoconazole (2 µmol.L^{-1})	116 ± 17
20	L-NAME (100 µmol.L^{-1})	118 ± 20
20	Indomethacin (10 µmol.L^{-1})	122 ± 11
20	Allopurinol (100 µmol.L^{-1})	126 ± 16

ROS were evaluated using H2DCFDA-AM and expressed as % of the fluorescence of the unstimulated control. Mean ± SEM of 6 separate experiments. Statistical analysis by one way Anova followed by Holm-Sidak *post-hoc* test (comparison to the stimulated control, i.e. assay containing oxLDL). * $p < 0.05$.