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Effective siRNA delivery to inflamed primary vascular endothelial cells by anti-E-selectin and anti-VCAM-1 PEGylated SAINT-based lipoplexes

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ABSTRACT

The endothelium represents an attractive therapeutic target due to its pivotal role in many diseases including chronic inflammation and cancer. Small interfering RNAs (siRNAs) specifically interfere with the expression of target genes and are considered an important new class of therapeutics. However, due to their size and charge, siRNAs do not spontaneously enter unperturbed endothelial cells (EC). To overcome this problem, we developed novel lipoplexes for siRNA delivery that are based on the cationic amphiphilic lipid SAINT-C18. Antibodies recognizing disease induced cell adhesion molecules were employed to create cell specificity resulting in so-called antibody-SAINTargs. To improve particle stability, antibody-SAINTargs were further optimized for EC-specific siRNA-mediated gene silencing by addition of polyethylene glycol (PEG). Although PEGylated antibody-SAINTargs maintained specificity, they lost their siRNA delivery capacity. Coupling of antibodies to the distal end of PEG (so-called antibody-SAINTPEGargs), resulted in anti-E-selectin- and anti-vascular cell adhesion molecule (VCAM)-1-SAINTPEGarg that preserved their antigen recognition and their capability to specifically deliver siRNA into inflammation-activated primary endothelial cells. The enhanced uptake of siRNA by antibody-SAINTPEGargs was followed by improved silencing of the target gene VE-cadherin, demonstrating that antibody-SAINTPEGargs were capable of functionally delivering siRNA into primary endothelial cells originating from different vascular beds. In conclusion, the newly developed, physicochemically stable, and EC-specific siRNA carrying antibody-SAINTPEGargs selectively down-regulate target genes in primary endothelial cells that are generally difficult to transfect.

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1. Introduction

In recent years, the endothelium has become an attractive target for therapeutic intervention due to its role in the pathophysiology of many diseases and easy accessibility for intravenously administered therapeutic compounds (Ásgeirsdóttir et al., 2007), including small interfering RNAs (siRNAs). siRNAs are short double-stranded RNA molecules composed of 21–23 nucleotides. Each siRNA has a perfect or nearly perfect complementary sequence to its target messenger RNA (mRNA), leading to gene-specific degradation of the mRNA by RNA interference (RNAi) (Fire et al., 1998). Although RNAi is regarded as a conserved endogenous cellular mechanism that protects against viruses, RNAi can be exploited therapeutically by introducing chemically synthesized siRNA molecules in the cellular cytosol to suppress, e.g., disease-associated genes (Aagaard and Rossi, 2007).

Nowadays, siRNAs are considered an important new class of therapeutics and the first evidence of successful siRNA-mediated gene silencing in humans has recently been published (Davis et al., 2010). However, siRNAs do not spontaneously enter unperturbed cells due to their relatively high molecular weight (∼15 kDa), negative charge (∼40 phosphate groups), and high hydrophilicity (Whitehead et al., 2009). Moreover, upon systemic injection, rapid degradation by serum RNases and reticuloendothelial system (RES)-mediated clearance are inevitable (Li and Huang, 2009). Consequently, chemical modification of siRNAs, or formulation in a

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delivery system that protects the siRNA, is required for *in vivo* use. The latter approach has the advantage that it does not affect the pharmacological potential of the siRNA, contrary to various forms of chemical modification (Behlke, 2008). A drug delivery system could furthermore reduce side effects and immune stimulation (Jackson and Linsley, 2010).

To achieve targeted delivery of siRNA to diseased endothelial subsets, molecular determinants on the surface of activated endothelium, such as cell adhesion molecules and/or receptors associated with disease are employed (Kowalski et al., 2011). Ideally, target epitope expression should be restricted to diseased endothelial cells (EC) and the epitope should be internalized after ligand binding. E-selectin is one of the molecules that meets these criteria, as it is dramatically upregulated during inflammation *in vivo* (Ásgeirsdóttir et al., 2007) and shuttles antibody-bound cargo into the receptor-mediated endosomal pathway (Everts et al., 2002). Vascular cell adhesion molecule 1 (VCAM-1) is also an internalizing target (Muro, 2007), and although its expression is not restricted to endothelial cells at inflamed sites, VCAM-1 is still considered an attractive endothelial target due to its massive upregulation upon pro-inflammatory stimulation (van Meurs et al., 2008).

In a previous study, we successfully designed anti-E-selectin-SAINTargs for siRNA delivery to inflamed primary endothelial cells (Ásgeirsdóttir et al., 2010). The results demonstrated specific and efficacious siRNA delivery *in vitro*, which justified further optimization of this delivery device for *in vivo* application. In the early 1990s, it was demonstrated that the circulation half-life of liposomes could be prolonged by surface grafted polyethylene glycol (PEG) (Klibanov et al., 1990). PEG reduces the opsonization by serum proteins, thereby minimizing clearance by the RES, which leads to improved pharmacokinetic properties (Kesharwani et al., 2012; Tseng and Huang, 2009). Moreover, the stability of the carrier is promoted by incorporation of PEG in the lipid formulation (Wang et al., 2012). Since the RES, located mainly in the spleen and the liver, avidly takes up (charged) nanoparticles, the overall positively charged SAINT-based lipoplex should for *in vivo* application be coated with PEG to prevent aggregation and RES-mediated clearance.

The aim of the current study was to develop a PEGylated EC-specific SAINTarg-based drug delivery system, which promotes efficient siRNA delivery into inflammation-activated endothelial cells. We synthesized anti-E-selectin- and anti-VCAM-1-SAINTargs, based on the concept of the spatiotemporal expression of E-selectin and VCAM-1 on inflammation-activated endothelial cells *in vivo*, and modified these formulations by addition of PEG. To increase exposure of the antibody at the surface of the lipoplex, antibodies were coupled to the distal end of DSPE-PEG$_{2000}$-Mal, resulting in so-called anti-E-selectin- and anti-VCAM-1-SAINTPEGargs. The siRNA delivery effectiveness of the newly developed SAINT-based delivery systems was quantified by real-time RT-PCR analysis in primary endothelial cells from arterial, micro-vascular and venous origin. The results presented in the current study describe the development of a novel PEGylated targeted SAINT-based lipoplex with superior siRNA delivery in the presence of serum and gene silencing capacity in different primary endothelial cells *in vitro*.

2. Materials and methods

2.1. Materials

The lipids 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$]-maleimide (DSPE-PEG$_{2000}$-Mal) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-(polyethylene glycol)$_{2000}$ (DOPE-PEG$_{2000}$) were purchased from Avanti Polar lipids (Alabastar, AL, USA). SAINT-MIX applied as siRNA transfection system, consisting of the cationic amphiphilic lipid SAINT-C18 and the neutral helper-lipid DOPE in a 1:1 ratio, was purchased from Synvolux Therapeutics (Groningen, the Netherlands). N-succinimidyl-S-acethylthioacetate (SATA) was from Pierce (Rockford, IL, USA). siRNAs were supplied by Qiagen (Benelux, Venlo, the Netherlands). The sequence of the predesigned siRNA targeting the human VE-cadherin gene (Hs_CDH5_2_HP) was ACGTATTATTATCACAATAACGAA. AllStars negative control siRNA (cat. no. 1027281) with no homology to any known mammalian gene was used as a control for functional experiments, and for size and zeta-potential measurements. For siRNA delivery capacity studies, AllStars negative control siRNA was used, which was labeled at the 5′-end of the sense strand with Alexa$_{488}$ (flow cytometry; cat. no. 1027292) or Alexa$_{546}$ (microscopy; cat. no. 1027293). All chemicals used were at least Reagent Grade.

2.2. Endothelial cell culture

Primary Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from the Endothelial Cell Facility UMCG (Groningen, the Netherlands). Cells were isolated from two umbilical cords to circumvent donor bias (Mulder et al., 1995) and cultured to confluence in 1% gelatin-precoated plastic tissue culture plates or flasks (Costar Europe, Badhoevedorp, the Netherlands) at 37 °C under 5% CO_2/95% air in EC-medium consisting of RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 20% (v/v) heat inactivated fetal calf serum (FCS, Hyclone, Logan, UT, USA), 2 mM L-glutamine (GIBCO-BRL), 5 U/ml heparin (Leo Pharma, Breda, the Netherlands), 100 IU/ml penicillin (Yamanouchi Pharma, Leiderdorp, the Netherlands), 100 μg/ml streptomycin (Radiumfarma-Fisiopharma, Milano, Italy), and 20 μg/ml endothelial cell growth factor (ECGF) extracted from bovine brain (Maciag et al., 1982). HUVEC were used between passage 1 and 4.

Primary Human Aortic Endothelial Cells (HAEC) were purchased from Cascade Biologics (Portland, OR, USA) and cultured at 37 °C under 5% CO_2/95% air in medium-200 containing 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and low serum growth supplement (LSGS; Cascade Biologics). LSGS supplement contained 2% fetal bovine serum (FBS), 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor and 10 μg/mL heparin. HAEC were used between passages 5 and 8.

Primary Human Hepatic Sinusoidal Endothelial Cells (HHSEC) were from Science Cell (Carlsbad, California, USA). HHSEC were cultured in endothelial cell medium supplemented with 5% FBS, endothelial cell growth supplements and antibiotics (Science Cell). HHSEC were used between passage 4 and 6. VE-cadherin gene expression in HAEC, HHSEC and HUVEC was not affected by tumor necrosis factor (TNF)α (Roche Diagnostics, Almere, the Netherlands) activation, whereas E-selectin and VCAM-1 were significantly upregulated (Supplementary Table 1).

2.3. Synthesis and characterization of SAINT- and PEG-conjugates

Mouse anti-human E-selectin (IgG2a) and anti-human VCAM-1 (IgG1) were purified from supernatant produced by hybridomas H18/7 and E1/6aa2, respectively, and conjugated to the amphiphilic lipid SAINT-C18 as previously described for mouse anti-human E-selectin (Ásgeirsdóttir et al., 2010). The monoclonal antibody-producing hybridomas H18/7 and E1/6aa2 were kindly provided by Dr. M. Gimbrone Jr. (Harvard Medical School, Boston, MA, USA).

Purified anti-human E-selectin and anti-human VCAM-1 were coupled to DSPE-PEG$_{2000}$-Mal via thiolation by means of SATA and coupling to the maleimide group at the distal end of the

polyethylene glycol chain. Briefly, SATA-modified antibodies containing free sulfhydryl groups (8.8 mol SH per mol antibody), were added to DSPE-PEG_{2000}-Mal at a molar ratio of 1:20 and incubated at room temperature for 1 h. Excess of free DSPE-PEG_{2000}-Mal was removed by Zeba™ Desalt Spin Columns, 7K MWCO (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentration of the DSPE-PEGlinker was determined by a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at a wavelength of 280 nm. The number of DSPE-PEGlinkers attached to the monoclonal antibodies was measured by MALDI-TOF mass spectrometry on a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems, Nieuwerkerk a/d Ijssel, the Netherlands).

2.4. siRNA delivery by SAINT-MIX and targeted delivery with SAINTarg or SAINTPEGarg

siRNA delivery experiments using either SAINT-MIX or SAINTarg were performed as described previously (Ásgeirsdóttir et al., 2010). Standard siRNA transfection experiments with SAINT-MIX were performed in a 6-well plate upon complexing 60 pmol/ml siRNA with 15 nmol/ml SAINT-MIX according to manufacturer's protocol. Targeted siRNA delivery with SAINTarg was performed by mixing either anti-E-selectin- or anti-VCAM-1-SAINT conjugate with SAINT-MIX in a fixed 1:2000 molar ratio. Subsequently, conjugate and lipids were vortexed for 5 s, mixed with siRNA, incubated for 5 min at room temperature and added to the culture medium supplemented with 20% FCS.

Formulation of antibody-SAINTPEGargs was performed in a similar manner as for antibody-SAINTargs. In this case, the antibody-SAINT conjugate was replaced by an antibody-PEG conjugate. Unless stated otherwise, the antibody-PEG conjugate:SAINT-MIX ratio was 1:2000 with a final lipid concentration of 15 nmol/ml. Where appropriate, DOPE-PEG_{2000} was added at indicated mol% at the cost of DOPE. Where higher siRNA concentrations were used, SAINT-MIX and antibody-SAINT or antibody-PEG conjugate were adjusted according to the ratios indicated above. Lipoplexes were prepared freshly prior to each experiment or measurement.

2.5. Particle size, zeta-potential and siRNA integrity

Mean particle size and zeta-potential were analyzed by dynamic light scattering using a Nicomp submicron particle analyzer (model 380 ZLS, Santa Barbara, CA, USA). For particle sizing, antibody-SAINTPEGargs (15 nmol/ml lipid, 7.5 pmol/ml antibody-PEG conjugate, 60 pmol/ml siRNA) were prepared freshly in Hepes buffer (supplied with SAINT-MIX transfection system; Synvolux Therapeutics) and, when indicated, formulated with 2 mol% DOPE-PEG_{2000}. Samples were briefly vortexed and the mean diameter was obtained from the number distribution curves produced by the particle analyzer. For zeta-potential measurements, antibody-SAINTarg and antibody-SAINTPEGarg formulations were prepared in water.

To study the ability of the lipids used in the formulations to protect the siRNA integrity in the presence of serum, 220 ng control siRNA was complexed with SAINT-MIX and incubated with 50% human serum (diluted in water) for different time periods at 37 °C. An equal amount of non-formulated control siRNA was incubated with human serum for the same period of time. At the end of the incubation, 1% (v/v) Triton X-100 and 6× gel loading dye (BioLabs, Leiden, the Netherlands) were added to the samples. Subsequently, samples were loaded on a 2.5% agarose gel containing ethidium bromide (60 μg/ml) and electrophoresed for 30 min at 100 V. Bands were visualized using the Chemidoc™ XRS system (Bio-rad, Veenendaal, the Netherlands).

To investigate the siRNA encapsulation efficiency upon PEGylation by addition of DOPE-PEG_{2000} to the formulation, freshly prepared SAINT-based lipoplexes formulated with or without 2 respectively 4 mol% DOPE-PEG_{2000} were analyzed to detect the presence of free, non-formulated siRNA. Samples treated with or without 1% Triton X-100 were electrophoresed as described above.

2.6. Cellular binding of SAINT- and PEG-conjugates by flow cytometric analysis

When indicated, HUVEC were activated with 10 ng/ml TNFα for 2 h or 12 h depending on the target, E-selectin or VCAM-1, respectively. Cells were detached by trypsin/EDTA (0.5/0.2 mg/ml in PBS), and equal cell numbers were transferred to FACS-tubes containing ice-cold FACS-buffer (PBS/1% FCS). Cells were incubated with antibody-SAINT conjugate, antibody-PEG conjugate or free parental antibodies at concentrations of 10, 1 and 0.1 μg/ml and incubated for 45 min on ice (to block the uptake via the endosomal pathway). Simultaneously, to validate endothelial activation status, cells were stained for ICAM-1 (clone hu5/3-2.1; kindly provided by Dr. M. Gimbrone Jr.) and the endothelial marker CD31 (clone JC/70A; DakoCytomation, Glostrup, Denmark). Non-specific staining was assessed by incubation of the cells with the appropriate mouse isotype control antibodies.

Cells were washed with ice-cold FACS-buffer and incubated with FITC-conjugated rabbit anti-mouse antibody (Jackson ImmunoResearch, PA, USA) for 45 min on ice. After incubation, cells were washed with FACS-buffer and fixed in PBS containing 1% paraformaldehyde. Fluorescence was acquired on a flow cytometer (Calibur, BD Biosciences, NJ, USA) and mean fluorescence intensity was calculated using the FlowJo software package (Tree Star Inc., OR, USA), version 7.6.5. Per sample 10,000 events were analyzed.

2.7. Flow cytometric and microscopic analysis of the association of SAINT-based lipoplexes in HUVEC and HAEC

To obtain identical cell density at the start of the experiments, HUVEC and HAEC were seeded at 15,000 cells/cm^2 and 17,500 cells/cm^2, respectively, one day prior to the experiment in 12-well tissue plates (Costar, San Diego, CA, USA). Resting and TNFα-activated cells were incubated with SAINT-MIX, antibody-SAINTarg or antibody-SAINTPEGarg containing $Alexa_{488}$-tagged control siRNA in the absence or presence of additional DOPE-PEG_{2000} at the indicated mol%. After 4 h, cells were trypsinized, washed with FACS-buffer and fixed in PBS containing 1% paraformaldehyde. When competing antibodies were used, experiments were performed in the presence of 100-fold excess (750 pmol/ml) of specific or irrelevant antibodies. Fluorescence was acquired on a flow cytometer and analyzed as described.

For fluoromicroscopic analysis, HUVEC were seeded in Lab-Tek chambers (Nunc, Rochester, NY, USA) and siRNA delivery experiments were performed as described above. Subsequently, cells were washed twice with ice-cold PBS, fixed at room temperature for 10 min in 4% paraformaldehyde and nuclei were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Roche Diagnostics). Slides were mounted with citifluor (Agar Scientific, Stansted, UK) and analyzed by fluorescence microscope (DM RXA, Leica Microsystems AG, Wetzlar, Germany), and Leica Q600 Qwin software V01.06.

2.8. Gene expression analysis by real-time RT-PCR

HUVEC were seeded at 15,000 cells/cm^2, HAEC and HHSEC at 17,500 cells/cm^2, in 12-well plates one day prior to experiments. Where indicated, endothelial cells were activated with TNFα for 2 h or 12 h. Antibody-SAINTargs or antibody-SAINTPEGargs containing VE-cadherin specific siRNA or control siRNA were added to the cells at an siRNA concentration of 60 pmol/ml, unless stated otherwise,

and incubated for 48 h. Next, lipoplexes were removed, cells were washed and RNA was isolated using the RNeasy mini plus kit (Qiagen) according to the protocol of the manufacturer. RNA integrity was analyzed by gel electrophoresis and consistently found intact. Subsequently, RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) and random hexamers (Promega, Leiden, the Netherlands). 10 ng of cDNA was applied for each real-time PCR on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The primers for VE-cadherin (Hs00174344_m1), CD31 (Hs00169777_m1), Tie2 (Hs00176096_m1) and GAPDH (Hs99999905_m1) were purchased as Assay-on-Demand (Applied Biosystems). For each sample, the real-time PCR was performed at least in duplicate and the averages of the obtained C_t values were used for further calculations. GAPDH was used as housekeeping to normalize gene expression levels giving the ΔC_t value. Relative mRNA levels were calculated by $2^{-\Delta Ct}$.

2.9. Statistical analysis

Statistical analysis of the results was performed by a two-tailed unpaired Student's *t*-test. Analysis of differences between multiple groups was analyzed with one-way ANOVA followed by Tukey *post hoc* analysis. *p* values <0.05 were considered to be significant. Data were analyzed with Graphpad prism (Graphpad software 5.0b, San Diego CA, USA).

3. Results

3.1. Anti-VCAM-1-SAINTargs specifically deliver siRNA into activated primary endothelial cells and effectively down-regulate VE-cadherin expression

Because of the (over)expression of VCAM-1 on inflammation-activated endothelial cells *in vivo*, VCAM-1 was employed as molecular determinant to create cell specificity and facilitate carrier-mediated siRNA uptake. For that reason, monoclonal anti-VCAM-1 antibodies were covalently conjugated to the amphiphilic lipid SAINT-C18. During this study anti-VCAM-1-SAINT conjugate was synthesized 7 times with protein yield ranging from 10 to 25%, based on the antibody input. On average, 1.5 SAINT-aminolinkers were attached to one anti-VCAM-1 antibody. Based on these results, and on the ones described previously for anti-E-selectin-SAINT conjugate (Ásgeirsdóttir et al., 2010), a 1:2000 ratio of anti-VCAM-1-SAINT conjugate:SAINT-MIX was used in the experiments.

Flow cytometric analysis demonstrated concentration dependent binding of anti-VCAM-1-SAINT conjugate to TNFα-activated HUVEC (Fig. 1), which was comparable to the binding of the parental anti-VCAM-1 antibody. No binding was observed to quiescent endothelial cells.

To investigate whether anti-VCAM-1-SAINTargs enhanced the siRNA delivery capability and specificity for TNFα-activated HUVEC, cells were incubated with anti-VCAM-1-SAINTargs or non-targeted lipoplexes. Delivery with anti-VCAM-1-SAINTarg resulted in considerably higher siRNA accumulation in activated HUVEC compared to non-targeted SAINT-MIX, as demonstrated by fluorescence microscopy (Fig. 2).

We next analyzed the VE-cadherin gene silencing capacity of anti-VCAM-1-SAINTarg formulated with VE-cadherin specific siRNA. In resting and TNFα-activated HUVEC, VE-cadherin gene expression was down-regulated to the same extent as with SAINT-MIX (Fig. 3). However, anti-VCAM-1-SAINTarg was significantly more effective in reducing VE-cadherin gene expression in TNFα-activated than in resting HUVEC. Both lipoplexes, when containing control siRNA, did not alter VE-cadherin expression. The expression of an unrelated gene, Tie-2, was not altered by VE-cadherin siRNA delivered by either anti-VCAM-1-SAINTarg or SAINT-MIX (Supplementary Figure 1).

3.2. Effect of PEGylation on the siRNA encapsulation capacity of SAINT-based lipoplexes and delivery capacity of anti-E-selectin- and anti-VCAM-1-SAINTarg

To reduce the non-specific siRNA delivery by antibody-SAINTargs in endothelial cells and to increase the stability of the lipoplex, the PEG-conjugated lipid DOPE-PEG_{2000} was included in the lipoplex formulation to coat the surface of the lipoplex. Firstly, we studied if PEGylation influences the siRNA encapsulation

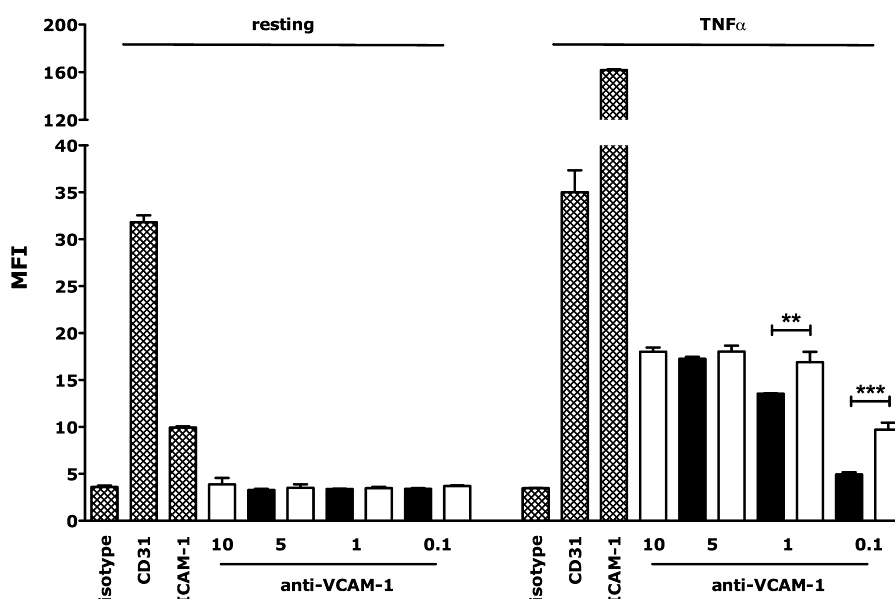

Fig. 1. Anti-VCAM-1 antibody preserves its antigen recognition after conjugation to SAINT-C18. Anti-VCAM-1-SAINT conjugate (black bars) and parental anti-VCAM-1 antibody (open bars) bind specifically to TNFα-activated HUVEC. In resting cells MFI (mean fluorescent intensity) is identical to the isotype control. Controls are shown by hatched bars and include incubation with an isotype matched non-specific antibody, and expression of CD31 and ICAM-1. The endothelial cell marker CD31 is constitutively expressed by HUVEC, while ICAM-1 expression is induced by TNFα. Data are presented as mean values ± SD, $n = 3$. $^{**}p < 0.01$ and $^{***}p < 0.001$.

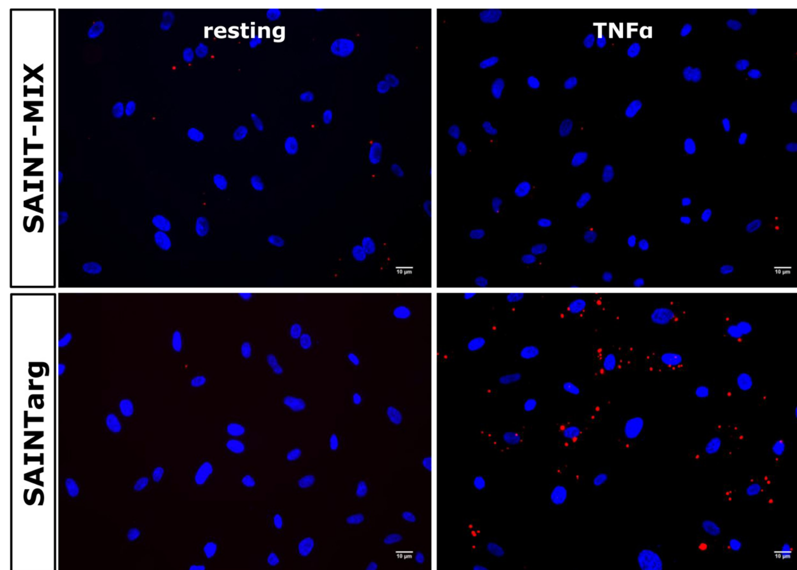

Fig. 2. Anti-VCAM-1-SAINTargs increases siRNA delivery capacity and specificity for activated primary endothelial cells. Delivery of $Alexa_{546}$-tagged siRNA was enhanced by targeted delivery with SAINTarg in TNFα-activated HUVEC, as demonstrated by fluorescence microscopy. HUVEC were incubated for 4 h with SAINT-MIX (non-targeted control), or with anti-VCAM-1-SAINTargs. Nuclei were stained blue by DAPI. Original magnification 200×.

capacity, SAINT-based lipoplexes formulated with or without 2 or 4 mol% DOPE-PEG_{2000} were subjected to agarose gel electrophoresis to detect non-complexed siRNA. At PEGylation densities of 0, 2 and 4 mol%, no free siRNA was detected demonstrating that the siRNA encapsulation capacity was not affected by additional DOPE-PEG_{2000} (Fig. 4A). To determine whether SAINT-based lipoplexes can protect the siRNA against serum RNases, the lipoplexes were incubated in 50% human serum at 37 °C. Non-complexed siRNA was entirely degraded in serum within 1 h (data not shown), whereas SAINT-based lipoplexes protected the siRNA integrity for up to 24 h (Fig. 4B). No significant difference in siRNA integrity was observed between non-PEGylated and PEGylated SAINT-based lipoplexes indicating that the siRNA is protected by both lipoplexes.

Secondly, we investigated if PEGylation affected anti-E-selectin- and anti-VCAM-1-SAINTargs siRNA delivery capacity. TNFα-activated HUVEC were incubated with anti-E-selectin- or anti-VCAM-1-SAINTarg formulated in the presence or absence of 2 respectively 4 mol% PEG. While non-PEGylated anti-E-selectin- or anti-VCAM-1-SAINTarg delivered significant amounts of siRNA into activated HUVEC, PEGylated anti-E-selectin- and anti-VCAM-1-SAINTarg lost their siRNA delivery capability irrespective of the concentrations of PEG used in the formulations, as shown in Fig. 5.

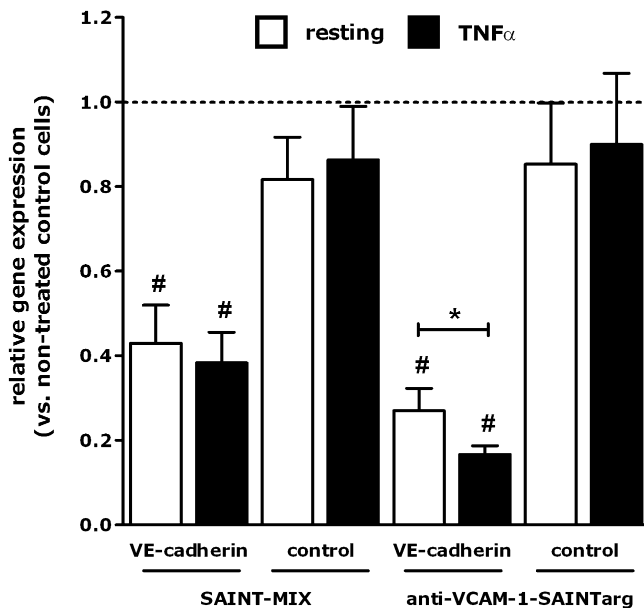

Fig. 3. Down-regulation of VE-cadherin gene expression in HUVEC by non-targeted SAINT-MIX and anti-VCAM-1-SAINTarg. VE-cadherin down-regulation is enhanced by targeted siRNA delivery with anti-VCAM-1-SAINTarg in TNFα-activated HUVEC, while down-regulation in resting HUVEC was similar for SAINT-MIX and anti-VCAM-1-SAINTarg. Anti-VCAM-1-SAINTargs containing VE-cadherin or control siRNA were incubated at 60 nM for 48 h. The expression of VE-cadherin was measured by real-time RT-PCR as described. Data are presented as relative gene expression ± SD, $n = 3$. Expression levels of non-treated control cells are arbitrarily set at one. $^{*}p < 0.05$ and $^{\#}p < 0.05$ compared to non-treated control cells.

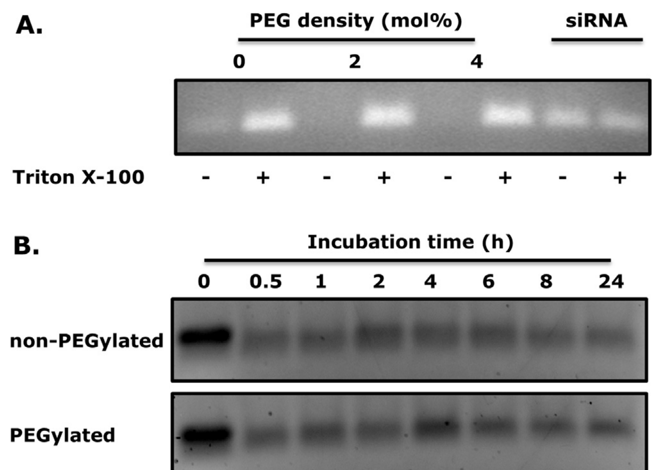

Fig. 4. SAINT-based lipoplexes protect the siRNA integrity and the siRNA encapsulation capacity is not affected in presence of PEG. (A) The influence of increasing concentrations of PEG on the siRNA encapsulation was investigated by gel electrophoresis. Freshly prepared SAINT-based lipoplexes formulated in the absence or presence of 2 respectively 4 mol% DOPE-PEG_{2000} were electrophoresed to detect the presence of free, non-formulated siRNA. (B) Maintenance of siRNA integrity after complexation into SAINT-based lipoplexes formulated in the presence or absence of 2 mol% PEG was analyzed after incubation in 50% human serum for 0.5, 1, 2, 4, 6, 8, and 24 h at 37 °C. Samples were subjected to gel electrophoresis as described in Section 2.

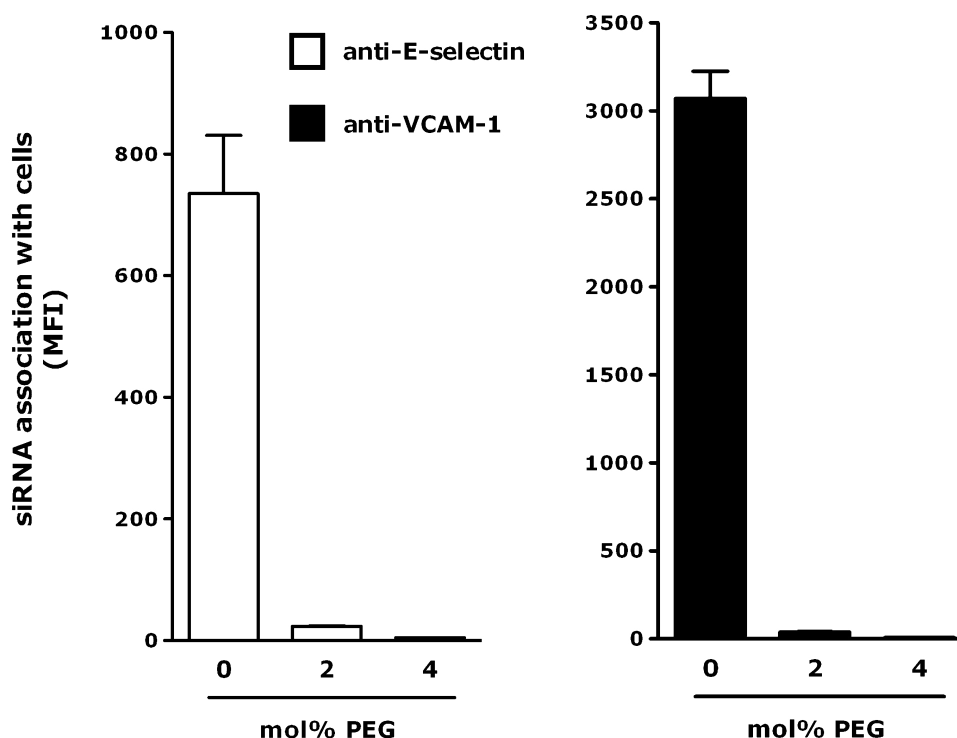

Fig. 5. PEGylation of antibody-SAINTargs leads to loss of siRNA delivery capacity in primary endothelial cells. TNFα-activated HUVEC were incubated for 4 h with anti-E-selectin-SAINTarg or anti-VCAM-1-SAINTarg formulated in absence or presence of 2 respectively 4 mol% PEG. Endothelial cell siRNA association (binding and uptake; $Alexa_{488}$-tagged siRNA) was determined by flow cytometric analysis. Data depict representative graphs of 3 independent experiments. Values are presented as mean ± range of duplicate samples.

3.3. *Synthesis and binding specificity of antibody-PEG conjugates*

A possible reason for PEGylated anti-E-selectin- and anti-VCAM-1-SAINTarg losing their siRNA delivery capability is steric hindrance of antibody-antigen recognition capacity by PEG molecules. We therefore modified the delivery system by coupling the antibodies to the distal end of DSPE-PEG_{2000}-Mal, resulting in antibody-PEG conjugates. Based on antibody input, overall protein yield coupled to DSPE-PEG_{2000}-Mal ranged from 70 to 85%. MALDI-TOF mass spectrometry demonstrated that, on average, 4 DSPE-PEG_{2000}-Mal molecules were attached to one anti-E-selectin monoclonal antibody (Supplementary Figure 2A) and 7 DSPE-PEG_{2000}-Mal molecules to one anti-VCAM-1 monoclonal antibody (Supplementary Figure 2B).

Cell binding studies with anti-E-selectin- and anti-VCAM-1-PEG conjugates demonstrated specific and concentration dependent binding of both conjugates to TNFα-activated HUVEC (Supplementary Figure 3). The binding capacity of both conjugates to HUVEC followed a pattern comparable to that of the parental antibody, while no binding was observed to resting cells (data not shown). At a protein concentration of 10 μg/ml, the binding of the anti-E-selectin-PEG conjugate and anti-VCAM-1-PEG conjugate was 75 and 47%, respectively, higher than that of the parental antibody (Supplementary Figure 3). This might be caused by non-specific interactions of the lipid part of the antibody-PEG conjugate with the cell membrane. Optimizing the antibody-PEG conjugate:SAINT-MIX ratio revealed an optimal protein concentration of 1.13 μg/ml (equivalent of 1:2000 ratio) at which no non-specific binding to the cells was observed (data not shown). This antibody-PEG conjugate:SAINT-MIX ratio was used in all (PEGylated) antibody-SAINT-MIX formulations, the so-called antibody-SAINTPEGargs.

3.4. *Formulation and characterization of (PEGylated) antibody-SAINTPEGargs*

Just prior to cell binding and siRNA delivery experiments, antibody-SAINTPEGargs were formulated by mixing antibody-PEG conjugate with SAINT-MIX followed by addition of siRNA, resulting in a comparable complex as previously published (Ásgeirsdóttir et al., 2010). Antibody-SAINTPEGargs (antibody-PEG conjugate:SAINT-MIX:siRNA in a molar ratio of 7.5:15,000:60) were characterized by a mean diameter of approximately 200 nm and a positive zeta-potential ranging from 27–42 mV (Table 1). When 2 mol% additional PEG was added, the mean diameter of antibody-SAINTPEGargs was decreased to 145 nm. The zeta-potential was not altered by the addition of 2 mol% PEG (Table 1).

Table 1
Characterization of antibody-SAINTPEGargs.

Sample	Mean diameter (nm)	Zeta-potential (mV)	pmol antibody/15 nmol lipid
Anti-E-selectin-SAINTPEGarg	223 ± 35	27 ± 10	1.8
Anti-E-selectin-SAINTPEGarg + 2 mol% PEG	141 ± 21	36 ± 15	± 0.1
Anti-VCAM-1-SAINTPEGarg	194 ± 20	37 ± 30	1.1
Anti-VCAM-1-SAINTPEGarg + 2 mol% PEG	146 ± 15	42 ± 34	± 0.1

Data are presented as means of 4–5 preparations ± SD.

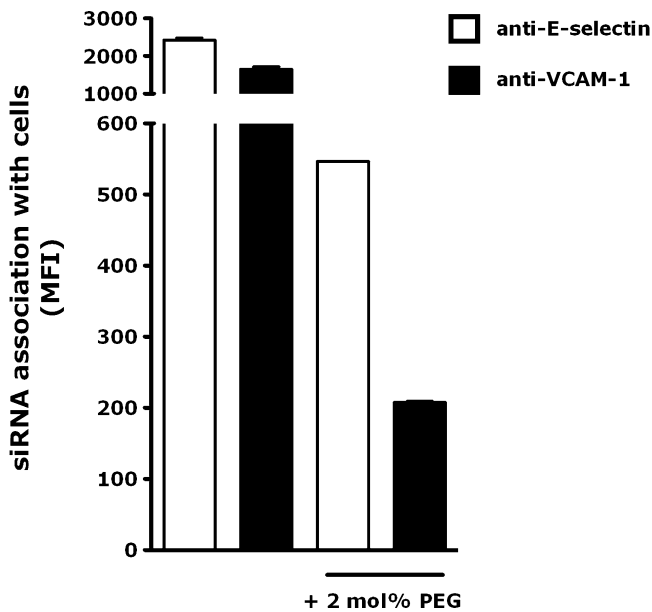

Fig. 6. PEGylated anti-E-selectin- and anti-VCAM-1-SAINTPEGargs retained their siRNA delivery capacity in activated HUVEC. TNFα-activated HUVEC were incubated for 4 h with antibody-modified SAINTPEGarg formulated in absence or presence of 2 mol% PEG. Endothelial cell siRNA association was determined by flow cytometric analysis. Data depict representative results of 3–4 independent experiments. Values are presented as mean ± range of duplicate samples.

3.5. PEGylated SAINTPEGargs targeted to E-selectin or VCAM-1 specifically deliver siRNA into activated primary endothelial cells

We next studied whether PEGylated antibody-SAINTPEGargs exhibited selectivity for target cells and whether siRNA delivery was maintained. For this purpose, resting and TNFα-activated HUVEC were incubated with antibody-SAINTPEGargs containing $Alexa_{488}$-tagged control siRNA formulated in the presence or absence of 2 mol% PEG. PEGylated anti-E-selectin- or anti-VCAM-1-SAINTPEGarg showed a decrease in siRNA delivery capacity compared to non-PEGylated antibody-SAINTPEGarg (Fig. 6). However, PEGylated anti-E-selectin- or anti-VCAM-1-SAINTPEGarg were 25-fold more efficient in delivering the siRNA at the target cells than PEGylated antibody-SAINTargs (Fig. 5). At the same time, no siRNA delivery was observed in resting cells (data not shown). These findings demonstrate that the use of antibody-PEG combined with DOPE-PEG_{2000} co-formulated with SAINT-MIX resulted in regaining the desired specificity for siRNA delivery into activated primary endothelial cells.

To demonstrate that PEGylated SAINT-lipoplexes mediate siRNA delivery into TNFα-activated endothelial cells through E-selectin respectively, VCAM-1, cells were co-incubated with the antibody-SAINTPEGargs in the presence of a 100-fold excess of the appropriate parental antibody. This resulted in a complete blockage of lipoplex binding, hence siRNA delivery into activated cells (Fig. 7). Irrelevant antibody was devoid of such an effect, indicating that lipoplex association (binding and uptake) into activated endothelial cells was E-selectin and VCAM-1 specific. Recently, Kowalski et al. (2013) showed that SAINT-O-Somes targeted to VCAM-1 and E-selectin specifically deliver siRNA into TNFα-activated HAEC.

3.6. Down-regulation of VE-cadherin gene expression by antibody-SAINTPEGargs in different primary endothelial subsets

To investigate whether the delivery of siRNA by the PEGylated anti-E-selectin- and anti-VCAM-1-SAINTPEGargs translates into down-regulation of the target gene, HUVEC, HAEC and HHSEC, representing different vascular beds present in different tissues, were used to determine the pharmacological effects of the developed siRNA delivery systems. For this purpose the endothelial restricted gene VE-cadherin (Lampugnani et al., 1992) was selected as a target gene for the siRNA since VE-cadherin gene expression is not influenced by TNFα stimulation (Supplementary Table 1). Neither PEGylated anti-E-selectin- nor PEGylated anti-VCAM-1-SAINTPEGargs loaded with 60 pmol VE-cadherin specific siRNA were capable of down-regulating VE-cadherin mRNA in activated HUVEC. In contrast, in HAEC VE-cadherin gene

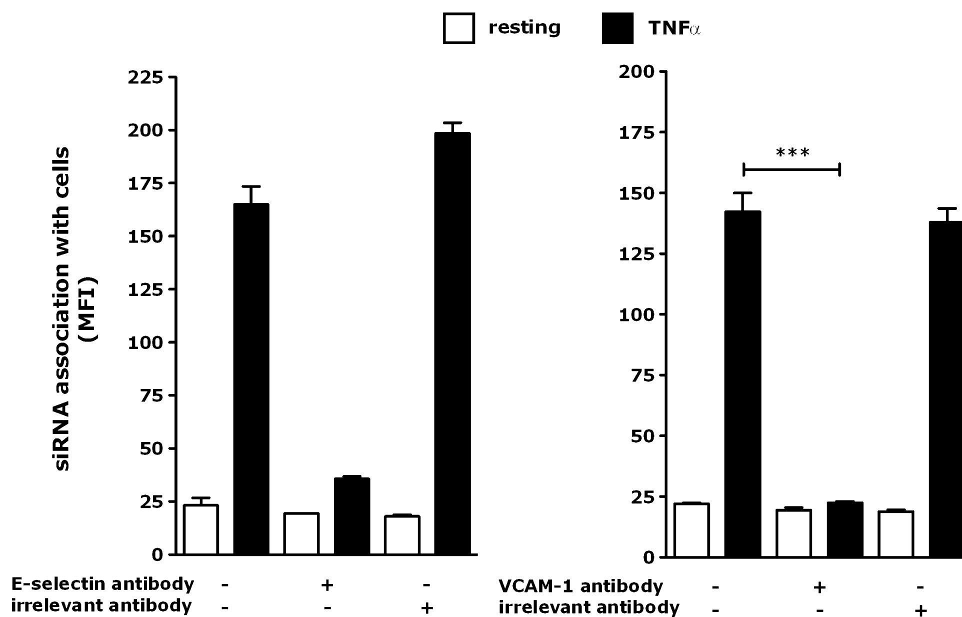

Fig. 7. antibody-SAINTPEGarg-mediated siRNA delivery in TNFα-activated HUVEC was completely blocked by excess of free parental antibodies. Resting or activated HUVEC were incubated for 4 h with antibody-modified SAINTPEGarg formulated with 2 mol% PEG. Specificity of binding was determined by co-incubation with 100-fold excess of free parental or irrelevant antibody. siRNA association was measured by flow cytometric analysis. Data of anti-E-selectin-SAINTPEGarg depict a representative result of 2 independent experiments, mean ± range of duplicate samples. Data of anti-VCAM-1-SAINTPEGarg are presented as mean values ± SD, $n = 3$. $^{***}p < 0.001$.

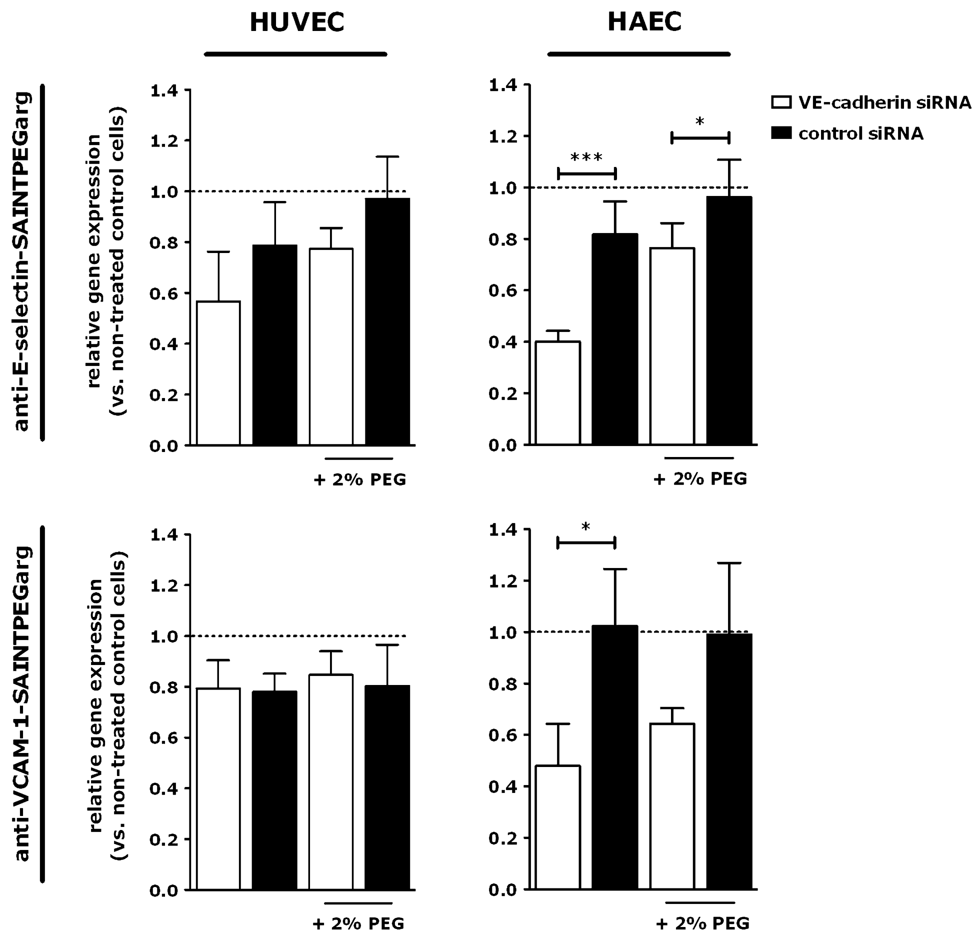

Fig. 8. Down-regulation of VE-cadherin by antibody-SAINTPEGargs containing siRNA$_{VE\text{-}cadherin}$ in HUVEC and HAEC. Antibody-SAINTPEGargs with or without 2 mol% PEG containing VE-cadherin siRNA or control siRNA were incubated at 60 nM with TNFα-activated HUVEC or HAEC for 4 h. Subsequently, particles were removed and cells were maintained for another 45 h. VE-cadherin gene expression was down-regulated in HAEC, while in HUVEC it remained unaffected. Control siRNA did not significantly affect VE-cadherin expression levels. Expression levels of non-treated control cells are arbitrarily set at one. Data are presented as relative expression ± SD compared to non-treated control cells, $n = 3–4$. $^{*}p < 0.05$ and $^{***}p < 0.001$.

expression was down-regulated up to 60% by antibody-SAINTPEGargs and their PEGylated formulations, except for PEGylated anti-VCAM-1-SAINTPEGarg (Fig. 8). When loaded with 300 pmol siRNA, PEGylated antibody-SAINTPEGargs caused up to 60% down-regulation of VE-cadherin mRNA in HAEC (Fig. 9), while in HUVEC VE-cadherin mRNA remained unaffected. A 1.5-fold elevation of VE-cadherin gene expression was observed in HAEC when antibody-SAINTPEGargs loaded with control siRNA were applied (Fig. 9), which we did not observe in HUVEC.

Differences in siRNA uptake capacity between HUVEC and HAEC might explain the observed differences in VE-cadherin down-regulation. To address this, resting and TNFα-activated HUVEC and HAEC were treated with PEGylated antibody-SAINTPEGargs containing 60 or 300 pmol Alexa-tagged siRNA. Flow cytometric analysis demonstrated that approximately 5-fold more siRNA was associated to activated HAEC than to activated HUVEC when using PEGylated antibody-SAINTPEGarg (Fig. 10), while minor uptake was observed in resting cells (data not shown). Anti-VCAM-1-SAINTPEGargs containing 60 pmol siRNA, on the other hand, delivered comparable amounts of siRNA into activated HUVEC and HAEC, thereby not elucidating the molecular basis for the observed differences in down-regulation.

Remarkably, in primary HHSEC, targeted delivery with (PEGylated) antibody-SAINTPEGargs caused up to 85% down-regulation of VE-cadherin mRNA at a VE-cadherin siRNA concentration

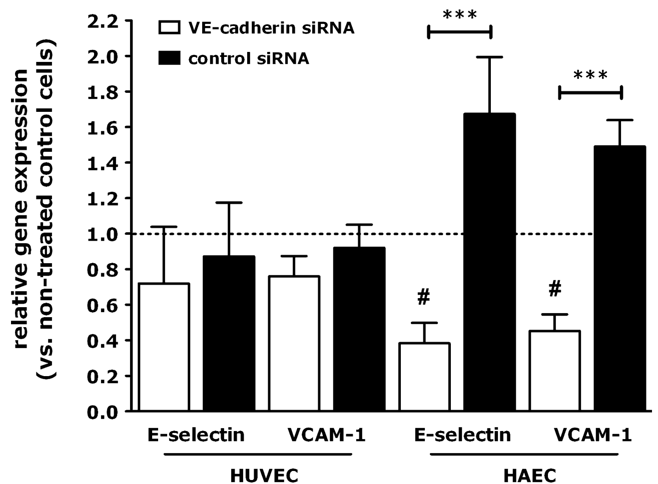

Fig. 9. Down-regulation of VE-cadherin gene expression by antibody-SAINTPEGargs formulated with 2 mol% PEG containing 300 nM siRNA. TNFα-activated HUVEC and HAEC were incubated with antibody-SAINTPEGargs containing VE-cadherin siRNA or control siRNA for 4 h. In HAEC, VE-cadherin gene expression was down-regulated, while HUVEC were not significantly affected. Data are presented as relative expression ± SD compared to non-treated control cells, $n = 3–4$. $^{***}p < 0.001$ and $^{\#}p < 0.05$ compared to non-treated control cells.

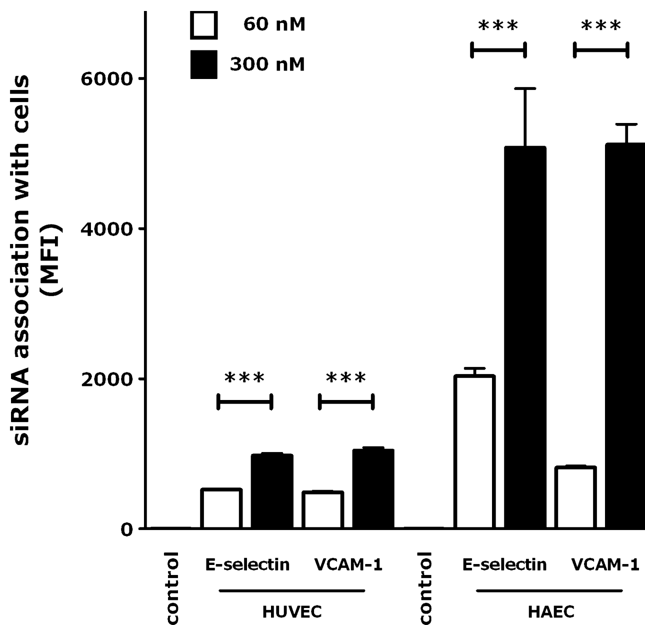

Fig. 10. Association of antibody-SAINTPEGargs in HUVEC and HAEC as function of siRNA concentration. TNFα-activated HUVEC and HAEC were incubated with anti-E-selectin- and anti-VCAM-1-SAINTPEGargs containing $Alexa_{488}$-tagged siRNA. The extent of siRNA uptake by HUVEC and HAEC is different when treated with equal doses siRNA. HUVEC and HAEC were incubated for 3 h with antibody-SAINTPEGargs formulated in the presence of 2 mol% PEG. Data are presented as mean values ± SD, $n = 3$. $^{***}p < 0.001$.

as low as 60 nM, while anti-E-selectin- and anti-VCAM-1-SAINTPEGargs containing control siRNA did not affect VE-cadherin gene expression (Fig. 11). Noteworthy is the observation that both anti-E-selectin- and anti-VCAM-1-SAINTPEGarg showed a comparable extensive gene down-regulation, which was not affected by the addition of PEG.

4. Discussion

Increased insight in the role of endothelial cells in the pathophysiology of cancer, inflammatory and cardiovascular diseases, has in recent years drawn great interest in pharmacological interventions aiming at the endothelium in diseased sites. Most of the drugs lack specificity for endothelial cells and give rise to adverse effects in non-target cells. Formulation of highly potent drugs, including siRNA, in endothelial-specific drug delivery devices will be essential to provide these drugs with a potential for future clinical application to treat chronic inflammatory diseases or to halt disease-associated angiogenesis (Kuldo et al., 2005; Molema, 2008). PEG-conjugated lipids are commonly used to increase the *in vivo* applicability of drug delivery devices. In the present study we describe the design and characterization of PEGylated SAINT amphiphile-based lipoplexes for the delivery of functionally active siRNA into inflammation-activated primary endothelial cells. We showed that the regular addition of PEG hampered the association of the PEGylated lipoplexes to endothelial cells. Incorporation of antibody-PEG conjugates in PEGylated SAINT-based lipoplexes, on the other hand, resulted in siRNA delivery systems that were fully capable of siRNA delivery into activated primary endothelial cells from various (micro)vascular origins, with concomitant target gene down-regulation. Furthermore, the physicochemical characteristics of the siRNA delivery system, i.e., size ranging between 140 and 225 nm, and protection of the siRNA integrity in the presence of serum at 37 °C, meet the requirements for future *in vivo* application.

The cationic amphiphilic lipid SAINT-C18 (van der Woude et al., 1997) is a highly effective transfection agent when complexed with nucleotides or proteins (van der Gun et al., 2007) that is not affected by the presence of serum (Audouy et al., 2000). A novel generation of targeted SAINT-based lipoplexes, so-called anti-E-selectin-SAINTargs, was previously designed and reported to show effective down-regulation of VE-cadherin in activated primary endothelial cells (Ásgeirsdóttir et al., 2010). Apart from E-selectin (Everts et al., 2003), also VCAM-1 (Voinea et al., 2005) has been described as an attractive target for specific drug delivery strategies to activated endothelium. To achieve specificity toward activated endothelium, we conjugated monoclonal anti-E-selectin respectively anti-VCAM-1 antibodies covalently to SAINT-C18. These antibody-SAINTargs, like other lipoplexes, are susceptible to aggregation upon intravenous injection due to interactions with blood proteins, which reduce their *in vivo* applicability. To overcome this problem, PEG-conjugated lipids such as DSPE-PEG_{2000} or DOPE-PEG_{2000} are commonly used to coat the surface of nanoparticles.

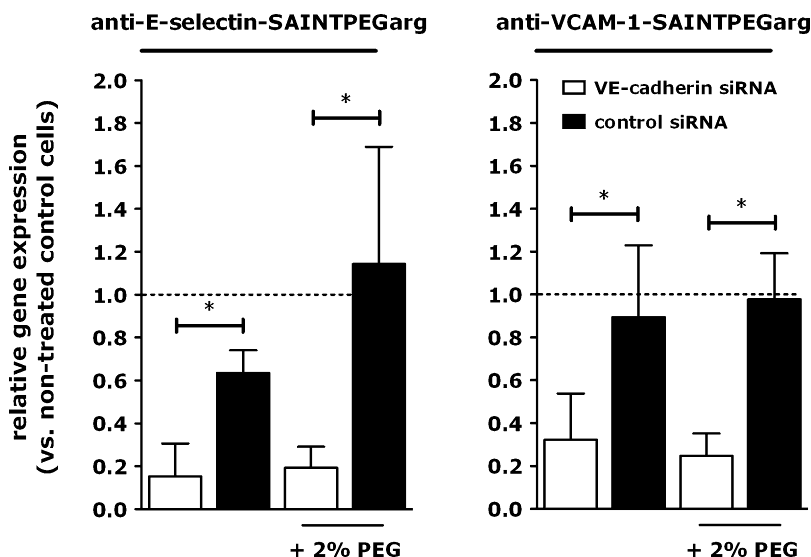

Fig. 11. Down-regulation of VE-cadherin in HHSEC by antibody-SAINTPEGargs containing 60 nM $siRNA_{VE\text{-}cadherin}$. TNFα-activated HHSEC were incubated with antibody-SAINTPEGargs containing VE-cadherin siRNA or control siRNA for 4 h. VE-cadherin gene expression was down-regulated, while control siRNA did not alter VE-cadherin expression. Data are presented as relative expression ± SD compared to non-treated control cells, $n = 3–4$. $^{*}p < 0.05$.

This creates a steric barrier that limits protein adsorption to the overall positively charged SAINT-based lipoplexes, thereby preventing aggregation and RES-mediated clearance upon injection *in vivo*. Santel et al. (2006) showed that low mol% of PEG-conjugated lipids in siRNA-lipoplex formulations are sufficient to reduce unspecific toxic side effects *in vivo* without a severe loss in RNAi efficacy *in vitro*. When anti-E-selectin- and anti-VCAM-1-SAINTarg were PEGylated using 2 or 4 mol% DOPE-PEG_{2000}, respectively, the *in vitro* siRNA delivery capability of these devices was significantly reduced. We hypothesized that steric hindrance by the PEG molecules prevented binding of antibody-SAINTargs to the endothelial surface. Therefore monoclonal anti-E-selectin and anti-VCAM-1 antibodies were coupled to DSPE-PEG_{2000}-Mal to improve availability of the antibodies at the surface of the lipoplex, resulting in so-called antibody-SAINTPEGargs. Antibody-SAINTPEGargs with additional 2 mol% DOPE-PEG_{2000} maintained the antigen recognition capacity of the parental antibody allowing specific siRNA uptake in HUVEC. We propose that this is a result of the restored antibody-antigen binding capacity through the use of antibody-PEG conjugate in the lipoplex formulation.

Upon systemic pro-inflammatory challenge, E-selectin and VCAM-1 are not homogeneously expressed by the endothelium in the different vascular beds but rather are heterogeneous distributed in a (micro)vascular bed specific manner (van Meurs et al., 2009). Therefore, in the current work, not only primary endothelial cells from venous (HUVEC) origin were studied but also primary endothelial cells from arterial (HAEC) and micro-vascular (HHSEC) origin. Anti-E-selectin- and anti-VCAM-1-SAINTPEGarg did not induce down-regulation of VE-cadherin mRNA in HUVEC neither at low nor higher siRNA concentrations. In contrast, down-regulation of VE-cadherin mRNA was achieved in HAEC partly in an siRNA concentration dependent manner. Binding experiments with antibody-SAINTPEGargs showed that approximately 5-fold as much siRNA was associated to HAEC than to HUVEC. Possibly, this quantitative difference is directly related to the difference in target gene down-regulation, as was previously described for HUVEC and HMEC-1 (Ásgeirsdóttir et al., 2010). Although theoretically differences in VE-cadherin down-regulation between HUVEC and HAEC might also be attributed to differences in VE-cadherin basal mRNA expression (Supplementary Table 1), the extensive down-regulation in HHSEC, which express VE-cadherin at comparable level as HUVEC, does not support such a relation. More likely, limited delivery device processing capabilities of HUVEC might explain the lack of down-regulation of VE-cadherin mRNA levels (Adrian et al., 2010). Since heterogenic endothelial cell behavior is the backbone of endothelial subset specific targeted drug delivery, this issue wil be further investigated.

5. Conclusion

In this study we demonstrated the potential of newly designed PEGylated SAINT-based lipoplexes called antibody-SAINTPEGargs for siRNA delivery in inflammation-activated endothelial cells. These PEGylated anti-E-selectin- and anti-VCAM-1-SAINTPEGargs are devoid of non-specific association with non-target cells, exert specificity for activated endothelial cells due to preservation of antigen recognition by the antibody, and have the capacity to functionally deliver siRNA into primary endothelial cells to down-regulate their target gene. Because of its PEGylation, the siRNA delivery system meets important requirements for future *in vivo* application, including defined particle size, improved stability and protection of siRNA integrity. Taken together, we demonstrated that these newly developed PEGylated antibody-SAINTPEGarg are effective devices for specific delivery of siRNA into inflammation-activated endothelial cells of different vascular origins, and thus provide an opportunity for further application for siRNA delivery to specific disease-associated endothelial cell subsets *in vivo*.

Conflict of interest

The authors declare no competing financial interests. M.H.J. Ruiters is CEO of Synvolux Therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.11.008.

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