Endothelial-specific delivery of siRNA by novel SAINT-based lipoplexes

Leus, Niek

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An in vitro and in vivo study

Printing of this thesis was financially supported by:

Rijksuniversiteit Groningen

Universitair Medisch Centrum Groningen

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Restaurants Louis XV en El Txoko te Groningen

RC TRITEC AG

Cover design: Nikki Vermeulen, Ridderprint BV, Ridderkerk, the Netherlands
Niek G.J. Leus

Lay-out: Rianne Jongman (rianne_jongman@hotmail.com)
Printed by: Ridderprint BV, Ridderkerk, the Netherlands

ISBN (printed): 978-90-367-7282-2
ISBN (digital): 978-90-367-7281-5

rijksuniversiteit
groningen

Endothelial-specific delivery of siRNA by novel SAINT-based lipoplexes

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Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 8 oktober 2014 om 14.30 uur

door

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geboren op 13 maart 1982
te Almelo

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Voor mijn lieve ouders

Paranimfen
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Dr. T.E. Woudenberg-Vrenken

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Chapter 1

Introduction and aim of the thesis

Chapter 1

Introduction

Endothelial cells

Endothelial cells form the inner cellular lining of all blood vessels, from the major arteries and veins to the smallest capillaries, regulating vascular physiology and homeostasis. Endothelial cells do not act as an inert lining of the blood vessels, instead they are active regulators of various processes. The endothelium is involved in most, if not all, disease processes, either as a primary determinant of patho(physio) logy or as a direct and/or indirect responder to an inciting stimulus. Endothelial cells are differentially regulated in the various (micro)vascular beds, giving rise to the phenomenon of endothelial cell heterogeneity [1, 2].

Structural and functional heterogeneity of endothelial cells is evident between arteries and veins, as well as between the capillaries in different organs. Not only do different endothelial subsets in one organ have different phenotypes and functions related to organ physiology, they also behave differently under pathophysiologic stress. As a consequence, endothelial cells in various (micro)vascular beds express different proteins upon pro-inflammatory stimuli [3]. This heterogeneity in the expression of epitopes on disease-associated endothelial subsets *in vivo* potentially allow them to be used for targeted drug delivery strategies [4, 5].

In the last two decades, the endothelium has become an attractive target for therapeutic interventions due to its important role in the pathophysiology of (chronic) inflammatory diseases and cancer. However, most drugs, including small interfering RNA (siRNA), lack selectivity for endothelial cells and give rise to adverse effects in non-target cells. Formulation of highly potent drugs in endothelial-specific drug delivery devices will be essential to provide these drugs with a potential for clinical application [6].

RNA interference

Initially, RNA was considered no more than just a passive intermediate carrying information between DNA and protein. The discovery of catalytic RNAs three decades ago had a tremendous impact on this paradigm [7]. RNAs changed after being unrecognized for a long time into a convenient tool for screening gene function. Almost a decade ago, Nobel Prize winners Andrew Fire and Craig Mellow discovered and described a technique in which they by injecting double stranded RNAs (dsRNAs) into the worm *Caenorhabditis elegans* observed down-regulation (silencing) of genes complementary to the dsRNA injected [8, 9]. This so-called RNA interference (RNAi) technique is a highly conserved process of post-transcriptional gene silencing

mediated by siRNAs. siRNAs, which are 21-23 nucleotides long, are produced by the cleavage of introduced long dsRNAs and recognize their complementary mRNA and subsequently guide degradation of the target mRNA. A few years later RNAi became even more interesting when Tuschl and colleagues showed that RNAi also occurs in mammalian cells [10]. Then RNAi and the application of siRNA evolved to a widely used approach for silencing gene expression in mammalian cells *(in vitro)* to study gene function and to elucidate molecular mechanisms underlying disease. Today, this technology has become a powerful tool in basic research and has huge potential to become an important class of therapeutics for selective intervention in disease [11]. Recently, the first evidence of successful siRNA-mediated gene silencing in humans has been published [12].

siRNA delivery

siRNAs can be easily produced synthetically and can be relatively easily introduced into cells *in vitro*. In theory, any target gene can be silenced when the siRNA is accurately designed, giving the application of RNAi with siRNA also a broad pharmacological potential. However, before widespread use of RNAi is possible in a clinical setting, important hurdles have to be overcome. The success of siRNA-based pharmacological interventions is dependent on the delivery system used, which modulates the pharmacokinetics and intracellular trafficking of siRNA. These systems should protect siRNA against degradation in the blood, reduce distribution to non-target sites, and facilitate cellular uptake by the target cells and release within the cytoplasm [13, 14].

In most cases chemically synthesized siRNA molecules are used to silence target gene expression, exploiting the endogenous RNAi processing machinery of the target cell, which results in complementary binding to its target mRNA. *In vivo*, these unmodified and uncomplexed siRNAs, so-called naked siRNAs, have a half-life in the blood of only a few minutes because they are rapidly eliminated by renal excretion, and/or degraded by serum RNases [15]. Moreover, due to their relatively large molecular weight and polyanionic nature, naked siRNAs will not passively cross the membrane of unperturbed cells and either need to be chemically modified or delivered by a delivery systems. The latter one has the advantage that it does not affect the pharmacological activity of the siRNA, in contrast to some forms of chemical modification [16, 17].

Various approaches for the delivery of siRNA have been proposed. In general two classes of delivery systems are distinguished, namely viral and non-viral. Although

viruses possess outstanding transfection efficacies, they have some inherent drawbacks, such as limited loading capacity, complicated large-scale production and, most meaningful, severe safety risks. Therefore viral siRNA delivery systems have limited potential for *in vivo* applications. Non-viral siRNA delivery systems are often based on cationic lipids or polymers, which do not have or may circumvent some of the shortcomings of viruses. They can be specifically tailored for the proposed application, easily produced at large-scale, and demonstrate adequate safety profiles. However, their siRNA delivery efficiency and efficacy is often limited and needs improvement [18, 19].

Most of the target cells e.g., (micro)vascular endothelial cells, can only be reached through systemic administration, in contrast to eye, skin and local tumors. Therefore, for siRNA delivery systems to reach the cytoplasm of endothelial cells *in vivo*, some physiological hurdles have to be overcome. Firstly, upon systemic injection, the siRNA delivery system has to circulate long enough to reach its target, while avoiding kidney filtration and liver clearance, uptake by macrophages, aggregation with serum proteins, and degradation by endonucleases [20, 21]. Secondly, the (micro) vascular endothelium does not avidly take up molecules larger than 5 nm in diameter, hence, the siRNA delivery system will remain in the circulation until clearance by the reticuloendothelial system (RES) [22]. For this reason, many siRNA formulations end up in the liver and spleen, since these organs harbor the largest population of resident macrophages and allow passage of particles up to 200 nm [23]. When an siRNA delivery system has been taken up by (micro)vascular endothelial cells, the carrier and/or the siRNA must escape the endosomes to enter the cytoplasm where RNAi occurs and to avoid degradation in the lysosomes. Additionally, when delivered in the endosomes, respectively cytoplasm, the siRNA faces the challenge of release from its carrier, after which it can bind to its target mRNA and induce gene silencing [24].

Endothelial-specific siRNA delivery presents a promising strategy for anti-inflammatory interventions aiming at silencing disease-associated genes. Non-targeted delivery prevents successful clinical development of siRNA-based therapeutics for disease-associated endothelial cells as it results in unwanted side effects in non-target cells. Molecular determinants expressed on the surface of activated (diseased) endothelial cells, like certain cell adhesion molecules and/or receptors involved in endocytosis, are excellent candidates to be applied as specific target epitopes for siRNA delivery to the endothelial cell surface and intracellular compartments to pharmacologically interfere in disease [6]. Yet, a targeted delivery

system, which efficiently and selectively delivers siRNA into the cytoplasm of disease-associated (micro)vascular endothelial cells, in therapeutically relevant doses, has not been developed.

Outline of the thesis

Since the discovery of RNAi in mammalian cells, there has been great interest in harnessing this pathway for the treatment of disease. So far, clinical successes of RNAi-based strategies to inhibit disease-associated genes have been limited due to lack of efficient drug delivery systems [25]. The research described in this thesis aimed to develop an efficient and endothelial-specific lipid-based siRNA delivery system, which ultimately can be applied *in vivo* to effectively silence disease-associated genes engaging in the pathophysiology of inflammatory diseases and cancer. The concept for the design of such a carrier was based on the ability of the cationic amphiphilic lipid 1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride (SAINT-C18), formulated with the neutral helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), to efficiently deliver siRNA, DNA and proteins into cells *in vitro* [26]. Lipoplexes, based on SAINT-C18, can be tailored on demand to introduce cell specificity, have a high drug payload, and can functionally deliver siRNA in the presence of serum without exerting significant cellular toxicity [26, 27]. Incorporation of anti-E-selectin and anti-VCAM-1 antibodies in the formulation enables selective siRNA delivery into activated (inflamed) endothelial cells, thereby enhancing their suitability for endothelial-specific delivery of siRNA *in vivo*. To achieve this a combination of research efforts in the field of endothelial cell biology and pharmaceutical sciences was essential.

In **Chapter 2**, we introduced general concepts of endothelial heterogeneity in relation to disease state and its consequences for targeted therapeutic interventions. Furthermore, we described novel approaches to interfere with endothelial cell engagement in disease focusing on siRNA-based therapeutics and currently used non-viral lipid and polymer-based siRNA delivery systems. The last part of this chapter addresses some technical issues that are essential in proving the concept of target mRNA knock-down or down-regulation in a vascular bed specific manner, and the further development of effective endothelial-specific drug delivery systems.

Based on the concept of the spatiotemporal expression of E-selectin and VCAM-1 by inflammation-activated endothelial cells *in vivo*, we developed anti-VCAM-1 -SAINTarg (anti-VCAM-1 antibody covalently conjugated to the SAINT-C18 lipid according to [27]), and anti-E-selectin- and anti-VCAM-1-SAINTPEGarg (antibodies

conjugated to the distal end of DSPE-PEG_{2000}-Mal prior to complexation with SAINT-DOPE and siRNA). The results presented in **Chapter 3** describe the characterization, and siRNA delivery capability of anti-VCAM-1-SAINTarg, and of PEGylated anti-E-selectin- and anti-VCAM-1-SAINTPEGarg (antibody-SAINTPEGarg/PEG2%) in primary endothelial cells from arterial, micro-vascular, and venous origin.

Chapter 4 describes the study on the *in vivo* behavior of anti-VCAM-1-SAINTPEGarg/PEG2% to deliver siRNA into inflammation-activated endothelium. The pharmacokinetic behavior, biodistribution, and effects of targeted anti-VCAM-1-SAINTPEGarg/PEG2% siRNA delivery were studied in control and TNFα-challenged mice. Vascular localization of anti-VCAM-1-SAINTPEGarg/PEG2% in lung tissue was determined using immunohistochemistry and confocal laser scanning microscopy. We furthermore analyzed the effect of targeted $siRNA_{VE\text{-}cadherin}$ delivery by anti-VCAM-1-SAINTPEGarg/PEG2% on VE-cadherin gene expression in liver, lungs, and kidney of TNFα-challenged mice.

In **Chapter 5** we finally investigated approaches to improve the siRNA delivery capability and effects of targeted siRNA delivery by antibody-SAINTPEGarg/PEG2% on gene expression in inflammation-activated endothelial cells. These experiments were performed *in vitro*, aimed to further guide the development of a more versatile application for siRNA delivery to disease-associated endothelial cells *in vivo*.

The results of the research described in this thesis are summarized and discussed in a broader context, and together with the future perspectives for endothelial-specific SAINT-based siRNA delivery systems, delineated in **Chapter 6**.

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Chapter 2

Targeted siRNA Delivery to Diseased Microvascular Endothelial Cells - Cellular and Molecular Concepts

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IUBMB LIFE. 2011 AUG;63(8):648-58.

Summary

Increased insight in the role of endothelial cells in the pathophysiology of cancer, inflammatory and cardiovascular diseases, has drawn great interest in pharmacological interventions aiming at the endothelium in diseased sites. Their location in the body makes them suitable targets for therapeutic approaches based on targeted drug delivery. Functional heterogeneity of the microvascular bed in normal organ homeostasis has been appreciated for a long time, and more recent studies have revealed heterogeneity in endothelial reactivity to inflammatory stimuli as well. Upon stimulation, each organ displays a vascular bed specific pattern of cell adhesion molecules providing challenging opportunities to deliver drugs or small RNAs to organ specific (micro)vascular endothelial subsets. In this review we introduce general concepts of endothelial heterogeneity in relation to disease state and its consequences for targeted therapeutic interventions. Furthermore, we will describe novel approaches to interfere with endothelial cell engagement in disease with a main focus on siRNA therapeutics and currently used non-viral lipid and polymer-based siRNA delivery systems. The last part of this review addresses some technical issues that are essential in proving the concept of target mRNA knock-down in a vascular bed specific manner, and the further development of effective endothelial cell specific drug delivery devices.

Introduction

The endothelium is the cell layer that forms the inner lining of blood vessels. It is a spatially distributed system that extends to all organs and tissues of the body. The endothelium is a key regulator of vascular homeostasis and functions not only as a barrier but also acts as an active signal transducer for metabolic, hemodynamic and inflammatory input that modifies the function and morphology of the vessel wall [1]. Moreover, the smallest blood vessels engage in angiogenic processes that accompany wound healing, tissue repair, and solid tumor growth [2]. Depending on the location in the body, endothelial cells (ECs) display their own molecular make up that drives basic behavior as well as responses to inciting stimuli [3]. Along the vascular tree, major differences are observed in EC phenotype, permeability, endocytosis and transcytosis capacities, and responsiveness to activation. For example, brain microvasculature is an integral part of the impermeable blood brain barrier, whereas liver sinusoidal endothelial cells form a densely fenestrated sieve with support of a discontinuous basement membrane and engage in clearance of a variety of molecular entities from the circulation [4, 5]. Furthermore, endothelial cells aligning the postcapillary venules are primarily responsible for mediating leukocyte trafficking, whereas arteriolar endothelial cells regulate vasomotor tone [2].

Being a keeper of internal homeostasis, ECs are continuously sensing and responding to changes in the extracellular environment. They are the first cells exposed to pro-inflammatory stimuli associated with systemic diseases such as atherosclerosis, sepsis, diabetes, vasculitis and other (chronic) inflammatory disorders. The pro-inflammatory stimulus leads to activation of ECs, the status of which varies according to the nature of the activating factor and the location of the vascular bed [6]. For example, significant differences in response to pro-inflammatory stimuli such as tumor necrosis factor (TNF)α are observed between venous and arteriolar endothelial cells [7]. Activated endothelium also accompanies the outgrowth of many tumors, where sustained formation of new blood vessels is one of the key factors leading to progression of the disease [8].

A variety of pharmacological approaches to counteract endothelial activation are already applied in the clinic, tested in clinical trials, or in preclinical development, including the potent inhibitors that affect receptor tyrosine kinase activation as well as specific kinases involved in the various signal transduction cascades [9]. Besides kinase inhibitors, drugs based on RNA interference (RNAi), i.c., small interfering RNAs (siRNAs), offer increased specificity and efficient gene silencing of disease-associated genes. Formulation of such drugs in a targeted delivery system would

create potentially powerful gene silencing therapeutics with diminished side effects, and hold a great promise for successful treatment of chronic inflammatory diseases and cancer. In the current review, we will focus on recent developments in the design of siRNA delivery approaches with the aim to therapeutically affect abnormal endothelium. We will introduce general consideration of endothelial heterogeneity in relation to disease state and its consequences for targeted therapeutic interventions. Next we will focus on drugs based on RNA interference, on their mechanisms of action and obstacles limiting application of siRNA in the clinic. We will provide an overview of currently used siRNA delivery systems designed to interfere with endothelial cell engagement in disease, with emphasis on non-viral approaches including lipids and polymers. In the last part, we will discuss the *in vivo* complexity of endothelial cell behavior and the difficulties encountered when attempting to mimic this in an *in vitro* setting. This calls for the use of new technologies that allow for endothelial gene expression analysis and studying targeted drug delivery devices in the complex environment of an organ.

Endothelial Heterogeneity and Abnormal ECs as Therapeutic Target

In recent years, the endothelium has become an attractive target for therapeutic intervention by virtue of its association with the pathophysiology of many diseases, its prevalence throughout the body, and its accessibility to intravenously administered agents [10]. Much effort has been dedicated to the development of drugs that inhibit endothelial cell activation to treat chronic inflammatory diseases, to disrupt tumor vasculature, or to halt angiogenesis. However, most of the drugs lack specificity for the endothelium, giving rise to adverse effects in other cells in the body. Formulation of highly potent drugs in EC-specific delivery devices will be essential to provide these drugs with a potential for future clinical application [9, 11]. Critical for success of these approaches is the identification of target epitopes on the diseased endothelial cells as well as choosing the proper drug target and concurrent molecular entity for therapeutic effects. This justifies an approach in which knowledge of microvascular endothelial cell biology and pharmaceutical sciences are combined, as we do in our own research as well as in outlining the content of this review.

Endothelial cells are differentially regulated in diverse (micro)vascular beds and in time, giving rise to the phenomenon of endothelial cell heterogeneity. Structural and functional heterogeneity of endothelial cells is evident between arteries and veins as well as between the capillaries in the different organs. Not only do different

endothelial cell subsets in one organ have a different phenotype and function related to organ physiology, they also behave differently under pathophysiologic stress. As a consequence, endothelial cells in various (micro)vascular beds express different proteins at different moments in time during disease initiation and development [4]. The differentially expressed determinants on the surface of disease-activated endothelium are excellent targets for drug delivery towards abnormal endothelium, and include molecules involved in leukocyte rolling and adhesion to the vascular wall during the inflammatory process, and in other disease–related processes [12]. It should be noted, however, that many of these proteins are not homogeneously expressed by the endothelium but rather are (micro)vascular bed specific. Moreover, the kinetics of disease-induced target epitope expression may spatiotemporally differ between endothelial cell subsets in the diseased sites [13]. This could have either an advantageous or a detrimental effect with regard to specificity and/or extent of local accumulation of the drug delivery formulation.

Endothelial Adhesion Molecules as Targets for Inflamed Endothelium

Their position in the body makes the ECs one of the first cells to be exposed to systemic pro-inflammatory stimuli such as bacterial endotoxin (lipopolysaccharide, LPS), or systemically released cytokines such as TNFα, interleukin (IL)-1, and IL-6. Exposure to these pro-inflammatory conditions leads to EC activation and expression of cell adhesion molecules and various other molecules associated with pro-inflammatory activation. Several of them can serve as molecular targets for siRNA delivery in pathologies such as atherosclerosis [vascular cell adhesion molecule-1 (VCAM-1), [14]], myocardial injury [P-selectin, [15], glomerulonephritis [E-selectin, (16)]; rheumatoid arthritis [$\alpha_v\beta_3$ integrin, E-selectin [17, 18]], and pulmonary inflammatory diseases [intercellular adhesion molecule-1 (ICAM-1), [19]].

Ideally, target epitope expression should be restricted to diseased endothelium, thereby preventing accumulation of a drug in non-diseased endothelium. E-selectin is one of the few molecules that meet this criterion. Moreover, it is not present on non-endothelial cells and its expression is dramatically upregulated during inflammation *in vivo*. The endothelial adhesion molecule expression induced by systemic stimuli varies between organs according to the nature of the stimulus and the origin of the vascular bed. Each organ displays a unique pattern of molecules providing further challenging opportunities to deliver drugs or small RNAs to organ specific (micro) vascular endothe lial subsets (Fig. 1A). For example, van Meurs et al., observed E-selectin expression primarily in glomerular ECs in a study on microvascular

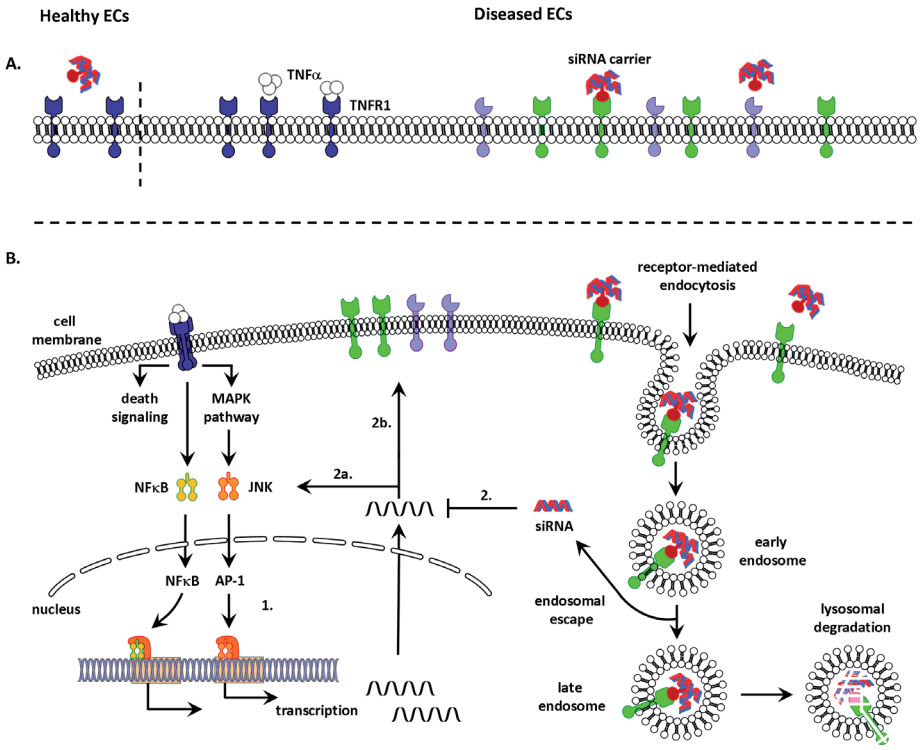

Fig. 1. Simplified scheme of disease-associated cellular events that lead to endothelial cell activation and provide challenging opportunities for targeted siRNA delivery.
(A) Microvascular endothelial cells (EC) are exposed to microenvironmental factors that differ from one vascular bed to the other, as a consequence of which their basic phenotype may vary. Exposure to pro-inflammatory conditions (e.g., TNFα or IL-1β) leads to activation and expression of a variety of cell adhesion molecules and other determinants associated with disease which may vary between endothelial subsets. (B) The differentially expressed determinants on the surface of disease-activated endothelium are excellent targets for drug delivery to abnormal endothelium. The targeted siRNA carriers (e.g., lipoplexes, polyplexes, or liposomes) interact with cell surface receptors and are taken up into the target cells via receptor-mediated endocytosis. The resulting endocytotic vesicles fuse to form early endosomes. These mature into late endosomes which ultimately become part of the lysosomes, where proteins and nucleic acids are degraded by acid hydrolases. To achieve target gene silencing, siRNAs need to be released from the carrier and escape from the endosomes into the cytoplasm, where it associates with the RNAi machinery and directs the cleavage of target mRNAs. 1. The activated intracellular signal transduction cascade controls transcription factor activation and pro-inflammatory gene expression. 2. Once escaped from the endosomes, the siRNA incorporates into the RNA-induced silencing complex, is subsequently unwound, and next guided to the complementary site in the target mRNA. This leads to specific gene silencing of, for example, (2a) transcription factors (TFs) NFκB or AP-1 to block downstream signaling pathways regulated by these TFs, or (2b) other determinants associated with disease activity.

activation following induction of systemic inflammation in a hemorrhagic shock mouse model. In contrast, VCAM-1 expression was induced in all vascular segments except in glomerular ECs [20].

Furthermore, it is important to note that target epitopes should reside at the exterior of the cell membrane of the target cells, that they are not avidly shed, and that they become internalized upon ligand binding and intracellularly processed when intracellular drug release is a prerequisite. E-selectin is an internalizing receptor that routes its ligands including antibodies and antibody modified-liposomes to the lysosomal compartment [21–23]. This feature substantially contributes to its outstanding quality as a target to be exploited for intracellular delivery of small RNAs. Also VCAM-1 and ICAM-1 are internalizing receptors [24] and although their expression is not restricted to endothelial cells in inflamed sites, they can still be considered attractive targets, provided that the therapeutic advantages outweigh the undesired side effects of delivery of the drug into non-diseased endothelium.

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are interesting molecules to consider for targeted drug delivery to ECs as they are upregulated in physiological as well as pathological angiogenic vessels, and possess proangiogenic functions [11, 25, 26]. For example, peptides with the arginine glycine aspartic acid (RGD) amino acid sequence show high affinity for the $\alpha_v\beta_3$-integrin, as do $\alpha_v\beta_3$-specific antibodies. During the past decade, RGD-peptides have become an established tool for the targeting of drugs and imaging agents to $\alpha_v\beta_3$-integrin expressing ECs [27].

Small Interfering RNAs, a New Class of Therapeutics

Three decades ago, RNA was generally considered to be no more than just a passive intermediate transferring information from DNA to the protein-synthesizing machinery. The discovery of catalytic RNAs in the 1980s had a tremendous impact on this dogma. Small RNAs are key players in triggering post-transcriptional or translational gene silencing and nowadays they represent one of the most promising new classes of molecular target-specific therapeutics. There are two main groups of small RNAs, i.e., siRNA and micro RNA (miRNA), which are short double-stranded RNA (dsRNA) molecules generally composed of 21-23 (siRNA) or 18-25 (miRNA) nucleotides (nt). siRNA generally has perfect complementary sequence to its target messenger RNA (mRNA), leading to gene-specific degradation of the mRNA, in contrast miRNA has predominantly imperfect sequence complementarity in the 3′ untranslated region of the target mRNA which leads to translational silencing without mRNA degradation [28, 29]. Mostly chemically synthesized siRNA molecules

are used to silence target gene expression, exploiting the cells' endogenous RNAi processing machinery for further processing before hybridization with its target mRNA. Another method of mediating the RNAi effect involves exogenously administered vector-based short hairpin RNA (shRNA) which is transcribed in the nucleus, further processed and transported to the cytoplasm for silencing activity [30]. Although a vector-based shRNA system may have advantages such as robust and long term gene silencing in the transfected cell, its expression is hard to control with regard to length of time and efficiency. Moreover, vector-based shRNA require nuclear entry, which represents an additional hurdle in the overall mechanism of action. The use of exogenous siRNA results in direct gene silencing since it does not require additional processing. Furthermore, its effect is transient, which may be preferred in a therapeutic setting.

RNAi has become a widely used approach for silencing gene expression *in vitro* and *in vivo*, to study gene functions and elucidate molecular mechanisms in mammalian cells. Both endogenously produced siRNAs and chemically synthesized siRNAs become assembled within a multisubunit ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Subsequently, the sense strand of the siRNA is removed, leaving the antisense strand to guide the 'activated' RISC to its site of action where it hybridizes with its target mRNA resulting in mRNA cleavage by the RNAi endonuclease Argonaute 2 [31]. This type of silencing occurs when siRNA molecules perfectly match their complementary target mRNA. The RISC complex can also guide and incorporate partially homologous siRNA strands to target mRNAs, causing translational repression of the particular mRNA present in the cytoplasm. In this case the siRNA acquires miRNA activity, and can control the expression of numerous target mRNAs at the translational level, which remains a critical issue for therapeutic applications of RNAi [32, 33]. The siRNA-loaded RISC is recycled for additional rounds of gene silencing activity. The rate of target cell divisions determines the persistence of siRNA-mediated gene silencing and not of shRNA when stably incorporated in the DNA, since the siRNAs will be diluted after each cell division.

Apart from siRNA, gene silencing involving RNAi can also be achieved using so called DNA enzymes (deoxyribozymes) which can be perceived as molecular scissors containing a catalytic core of 15 deoxyribonucleotides that binds to and cleaves its target RNA [reviewed by [34]]. Moreover, interference with miRNA pathways is possible by means of antisense oligonucleotides, which are complementary to the sense strand of the target miRNA duplex and block its processing [reviewed

by [35]]. RNAi-based drugs may allow for specific silencing of a gene involved in downstream signaling of pro-inflammatory and pro-angiogenic stimuli, whereas interference with miRNA pathways may result in downregulation of multiple pro-inflammatory genes, both theoretically leading to suppression of inflammation. The recent discovery of miRNA involvement in tumor angiogenesis [36] furthermore paves the road for inhibitory miRNAs to be further developed for endothelium-specific therapy of cancer. For reasons of space limitations we will restrict ourselves in the next paragraphs to siRNA, although several of the concepts discussed may be applicable to other types of inhibitory RNAs as well.

siRNA Delivery Into Endothelial Cells

The first decade of targeted endothelial drug delivery research focused on the identification of molecular targets on the endothelial cells that are selectively expressed during disease development [27, 37]. Initially employed drugs include the cell death inducing molecules doxorubicin [38] and the proapoptotic heptapeptide dimer D(KLAKLAK)2 [39]. Moreover, anti-inflammatory enzymes that provide anti-oxidant protection [40] and corticosteroids that inhibit intracellular signaling and concomitant pro-inflammatory gene expression [16, 22] have been formulated and were shown to improve disease status. Also, targeted delivery of antisense oligonucleotides has been investigated in detail in the last 15 years [41]. The design of new siRNA delivery devices for future therapeutic application has benefitted and will continue to benefit from the knowledge gained by advancements in targeted delivery of these conventional drugs.

Unmodified and uncomplexed siRNAs (so-called naked siRNAs) have a half-life in the blood of only a few minutes which limits their usefulness as a drug *per se*. They are rapidly eliminated by renal excretion and are also degraded with a $t_{1/2} \sim 1$ h by serum RNases [42]. Due to its relatively large molecular weight (~ 13 kDa), polyanionic nature (~ 40 negatively charged phosphate groups), and high hydrophilicity, naked siRNA will furthermore not passively cross the membrane of unperturbed cells. To apply siRNA for *in vivo* gene silencing, it either needs to be chemically modified or formulated and delivered to protect it from rapid clearance and degradation by serum RNases, to prevent activation of the immune system and interactions with other non-target cells, and to allow cellular uptake, finally leading to participation in the RNAi pathway [43]. Formulation of siRNA in an advanced drug delivery system has the advantage that it does not affect the pharmacological potential of the siRNA, contrary to various forms of chemical modification.

Ideally, for *in vivo* application the siRNA needs to be efficiently formulated in carrier systems to contain sufficient amounts of siRNA and be stable to resist degradation or disassembly in the circulation. At the same time, carrier systems should allow efficient release of the cargo once the carrier arrives in the endocytotic vesicles and/or in the cytoplasm of the cells [discussed in more details by [44]]. Physicochemical characteristics of the carrier such as composition, size and surface charge can handicap pharmacokinetic behavior by engaging in interactions with serum proteins including serum albumin, lipoproteins and immunoglobulins, which leads to clearance by cells of the reticulo-endothelial system (RES) in the spleen and liver [45]. To limit these interactions and prolong circulation half-life, the surface charge can be masked by covering the carrier with a hydrophilic polymer such as (poly)ethylene glycol (PEG) [46]. Recent work revealed that lipid-based particles containing high levels of PEG (10 mol%) are not taken up by the liver after systemic administration [47]. Moreover, different types of polymers, such as pH-sensitive or diffusible PEG variants are available for shielding off the vesicle surface. Such alterations provide increased carrier responsiveness to low pH and enhanced cytoplasmic release of its cargo [48]. Furthermore, it is possible to control the circulation time of particles by time dependent diffusion-mediated release of PEG shielding which can reduce side effects of formulations containing toxic drugs [49, 50].

Size and shape of the carrier also determine its fate *in vivo*. Most of the systems used for targeting endothelial cells have size ranging between 100–200 nm to minimize clearance from the circulation by renal filtration and liver uptake [51, 52]. Moreover, several groups reported that nano-sized particles are more effective than microsized particles in targeting microcapillary sized vessels, where red blood cells (RBCs) preferentially line up in the center of the blood stream, thereby increasing nanoparticle contact with the vessel wall. They may, however, not be adequate for targeting to medium-to-large size blood vessels due to RBC-hindered margination which may decrease particle contact with the vessel wall [53, 54]. The shape of the carrier may also determine the extent and mode of interaction with the vascular wall and affect the rate of cellular uptake. Spherical nanoparticles are typically proposed for vasculature-targeted drug delivery by virtue of their relatively unrestricted capacity to navigate through the circulatory system bringing along minimal risk of vessel occlusion [10]. Furthermore, Muro et al., reported that *in vitro* endothelial ICAM-1-targeted 0.1 x 1 x 3 μm elliptical polystyrene disks exhibited four times slower uptake rates when compared to 0.1 and 5 μm diameter spheres [55].

To achieve specific delivery of siRNA to the desired endothelial subsets, carriers can be surface-modified with monoclonal antibodies, peptides, small-molecule ligands, or for example, aptamers, to recognize determinants on the cell surface. Antibodies and small antigen binding fragments have been studied most extensively for this purpose. A good ligand needs to be specific for a target expressed on the surface of ECs and should bind with sufficient capacity to promote internalization of drug loaded carrier to deliver therapeutically effective amounts of drug into the cells' interior. Furthermore, ligand-target molecule binding should avoid (prolonged) disruption or interference with normal functions of the target epitope [56]. Targeting endothelial cells can also be achieved by employing their basic functional heterogeneity without concomitant diseases activity. For example, liver sinusoidal endothelial cells, acting as scavengers specialized in the uptake of polyanionic macromolecules, could be efficiently reached by carriers conjugated with polyaconitylated human serum albumin, a ligand for scavenger receptors [57]. Moreover, pulmonary endothelial cells which function as a nonthrombogenic semipermeable barrier and provide a vast surface for gas exchange, readily express high levels of thrombomodulin and angiotensin-converting enzyme at the cell surface as compared to other ECs, allowing effective preferential targeting [58, 59]. In addition, delivery to endothelium does not always require harnessing the carrier with a targeting ligand. Santel et al. reported that systemic administration of an siRNA complexed with a lipid-based carrier, creating a so called lipoplex, led to significant uptake of siRNA by endothelial cells in different organs. siRNA-lipoplexes were extensively taken up by the vasculature of the heart, lung, and liver resulting in RNAi-mediated silencing of endothelial cell restricted genes CD31 and Tie2 [60].

Intracellular Fate of Endothelium Targeted Delivery Systems and Their Cargo

When a delivery system has reached the target endothelial cells, the carrier has to be internalized by the cell and release its content into the cytoplasm. Many carriers conjugated with ligands for extracellular receptors are internalized via endocytotic pathways leading to degradation, transcytosis, or sorting of internalized material to different cell compartments [58]. The type of pathway utilized depends on the target receptor, and the size and the nature of the drug carrier [44]. Clathrin-mediated uptake and caveolae-mediated uptake are two main mechanisms involved in endocytosis in ECs. The clathrin-mediated pathway mainly guides the delivery system to the endosomes with subsequent degradation in the lysosomes, whereas the caveolae-mediated pathway predominantly serves as an entry for transcytosis

through endothelial monolayers that usually avoids lysosomal compartments [58]. Carriers targeting plasma membrane proteins like selectins are mainly taken up via clathrin-mediated endocytosis guiding the internalized content to the lysosomal degradation pathway within 2–4 h [22, 61]. On the other hand, ligands that bind to ICAM-1 were shown to enter ECs by a non-classical endocytotic mechanism named cell adhesion molecule (CAM) mediated endocytosis. It requires formation of small multimeric complexes of the receptors and depends on target molecule clustering and size of the conjugates (100–300 nm). This mechanism also delivers materials to lysosomal compartments within 3 h [40, 62]. If the vesicular cargo enters the lysosomal degradation pathway, the initially formed early endosomes mature to more acidified (pH 5.0–6.0) late endosomes and eventually merge with lysosomes, rendering their content for degradation by lysosomal enzymes and low pH [63]. For siRNA delivery, however, endocytosis via the non-degradative route (e.g., caveolae-mediated pathway) likely leads to entrapment of the cargo in the endosomes [64].

We have shown that E-selectin targeted, lipid-based, conventional liposomes are extensively taken up by TNFα-activated HUVEC but also that they are degraded to a minor extent inside the endocytic vesicles of the endothelial cells [65]. Regardless of the entry pathway, a lack of endosomal escape generally leads to poor siRNA efficacy, thus carriers have to be able to release their siRNA before entering lysosomes and enable escape of intact siRNA from the endosomal compartment into the cytoplasm (Fig. 1B). To aid siRNA delivery, several mechanisms allowing penetration of the endosomal membrane before transfer to the lysosomal compartment have been proposed. Vesicle type carriers can be modified with pore forming peptides that are able to disturb the continuity of the bilayer by introducing a pore in the membrane, thereby facilitating release of endosomal contents. Those peptides are often based on naturally occurring toxins or venoms like diphtheria toxin or melittin, a major component of bee venom [64]. Cationic lipids can destabilize the endosomal membrane by inducing 'flipping' of anionic lipids in the endosomal bilayer, leading to formation of ion pairs which facilitates vesicle fusion with the endosomal membrane and release of the cargo into the cytoplasm [66, 67]. Moreover, addition of a helper lipid with fusogenic properties [e.g., 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)] to a carrier formulation can significantly improve content release and escape from the endosomes [68]. Furthermore, so called pH-sensitive carriers were developed by formulating DOPE with a pH-titrable lipid (displaying a pH-dependentcharge) such as cholesteryl hemisuccinate (CHEMS) or by combination of cationic and anionic lipids in one lipid membrane.

These formulations promoted content release by increased destabilization of the carrier in the endosomal compartment upon low pH [69]. In contrast, polymers like polyethylenimine (PEI) and polyamidoamine (PAM) can induce endosomolysis by the so-called proton sponge effect. These polymers have a strong buffering capacity due to protonation of amino nitrogens upon endosome acidification. This invokes a high chloride ion influx into the endosome, causing osmotic swelling of the endosome and eventually endosome lysis [70].

Endothelial Cell Targeted siRNA Delivery Systems

Lipid-based systems have been used for the delivery of nucleic acids for over 20 years, starting with studies by Felgner et al. [71]. Liposomes and lipoplexes are the two main categories of lipid-based systems, although novel types of carriers such as stabilized nucleic acid-lipid particles (SNALP), lipid polycation-DNA nanoparticles (LPD) and lipid like molecules called lipidoids [extensively reviewed by [72]] have entered the stage in recent years. For siRNA delivery, liposomes and lipoplexes are usually composed of a cationic lipid, helper lipid (e.g., DOPE and/or cholesterol) and a (poly)ethylene glycollipid [44]. Liposomes consist of an aqueous core enclosed in a phospholipid bilayer with nucleic acids mainly entrapped in the central aqueous compartment. Liposomes have generally stable physicochemical characteristics, while lipoplexes are spontaneously formed via interaction of positively charged lipids and negatively charged nucleic acids which makes them more unstable [73]. The advantages of lipid-based systems are their low toxicity (several liposomal formulations are FDA approved), easy sizing to below 200 nm, and great flexibility in tailoring them on demand with targeting ligands. Lipid structures can be easily modified by coupling targeting ligands to improve their delivery potential or by adding pH-sensitive or fusogenic moieties to aid intracellular release of siRNA [41].

So far only a few types of carriers suitable for systemic siRNA delivery into endothelial cells have been developed (Table 1). Successful siRNA delivery to tumor endothelial cells expressing integrin $\alpha_v\beta_3$ using RGD-based homing peptides has first been reported by Schiffelers et al. [74]. In this study, the siRNA that inhibited vascular endothelial growth factor receptor 1 (VEGFR2) expression was incorporated into self-assembling nanoparticles constructed with RGD-harnessed PEGylated polyethyleneimine, after it was shown that formulation into conventional liposomes did not lead to target gene silencing. We recently developed two novel lipid-based systems which show potential for systemic siRNA delivery to activated endothelial cells. The first system is based on a lipoplex composed of the cationic amphiphilic

Table 1. Examples of non-viral systems suitable for siRNA delivery to endothelial cells

Validated	Delivery system	Chemical composition	Target epitope	Pharmacological target	Endothelial subset	Ref.
In vivo	polyplex	PEGylated branched PEI	integrin $\alpha_V\beta_3$	VEGFR2	Tumor vasculature	[74]
		Chitosan nanoparticles	integrin $\alpha_V\beta_3$	PLXDC1	Tumor vasculature	[79]
	lipoplex	AtuPlex (PEG-DSPE: AtuFECT01:DPhyPE)	-	PECAM-1, PKN3	Tumor vasculature	[80, 60]
		DDAB:chol	-	Caveolin-1	Lung vasculature	[81]
In vitro	liposome	SAINT-O-Somes (SAINT:POPC:Chol:PEG-DSPE)	E-selectin	VE-cadherin	HUVEC	[65]
		POPC:DAP:PEG-DMA	-	GAPDH	HUVEC	[82]
	lipoplex	SAINTarg (SAINT:DOPE)	E-selectin	VE cadherin	HUVEC	[76]

PEI, polyethyleneimine; PEG (poly)ethylene glycol; DPhyPE, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DDAB, Dimethyldioctadecylammonium Bromide); SAINT, (1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride); DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DAP, 1,2-dioleoyl-3-dimethylammonium-propane; DMA, 2-dimethylamino ethyl methacrylate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Chol, Cholesterol

lipid SAINT (1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride), a well established delivery agent of nucleotides and proteins, and the helper lipid DOPE [75]. To achieve specificity towards activated endothelium, SAINT was covalently coupled to a monoclonal anti-E-selectin antibody forming a construct referred to as SAINTarg. With this anti-E-selectin-SAINTarg we were able to substantially enhance siRNA uptake, transfection specificity and efficacy of VE-cadherin down-regulation in activated endothelial cells, as compared to transfection with a non-targeted SAINT formulation [76].

In addition, we have developed a novel generation of liposomes called SAINT-O-Somes, based on formulation of conventional long circulating liposomes by the addition of the cationic amphiphilic lipid SAINT. These liposomes harnessed with anti-E-selectin antibodies showed specific uptake by activated endothelial cells, and displayed good size stability (100 nm in diameter) in the presence of serum, but were destabilized at lower pH as occurs in the endosomes of endothelial cells, thereby showing superior intracellular release of their content. We were able to efficiently encapsulate low molecular weight compounds such as doxorubicin and siRNA in these carrier systems, rendering this formulation an interesting candidate for systemic application [65].

Issues to be Addressed for Further Development of EC Specific Delivery Devices

Significant progress has been made in the last decade with regard to the development of endothelial cell specific drug delivery devices. Increased knowledge of the molecular changes within these cells during the onset and progression of disease has spurred identification of new potential targets on the cell membranes, while new molecular entities give rise to the design of novel carriers with important added value over conventional ones. Knowledge of the molecular control and pharmacology of microvascular endothelial cells remains, however, scarce. Basic heterogeneity in the control of EC behavior makes it highly likely that their responsiveness to 'drugs' is also microvascular bed dependent, although data to support this are only slowly emerging. Moreover, the loss of microenvironment driven EC behavior upon culturing the cells *in vitro* requires solid validation of *in vitro* molecular control or responsiveness to stimuli and pharmacological observations in the *in vivo* context (Fig. 2). This is only occasionally pursued, which is mostly due to the limited availability of methods to assess kinase activity, gene and protein expression in tissue biopsies, and to locate them specifically in the endothelium. As such, the choice of molecular target(s) allowing successful therapeutic interference

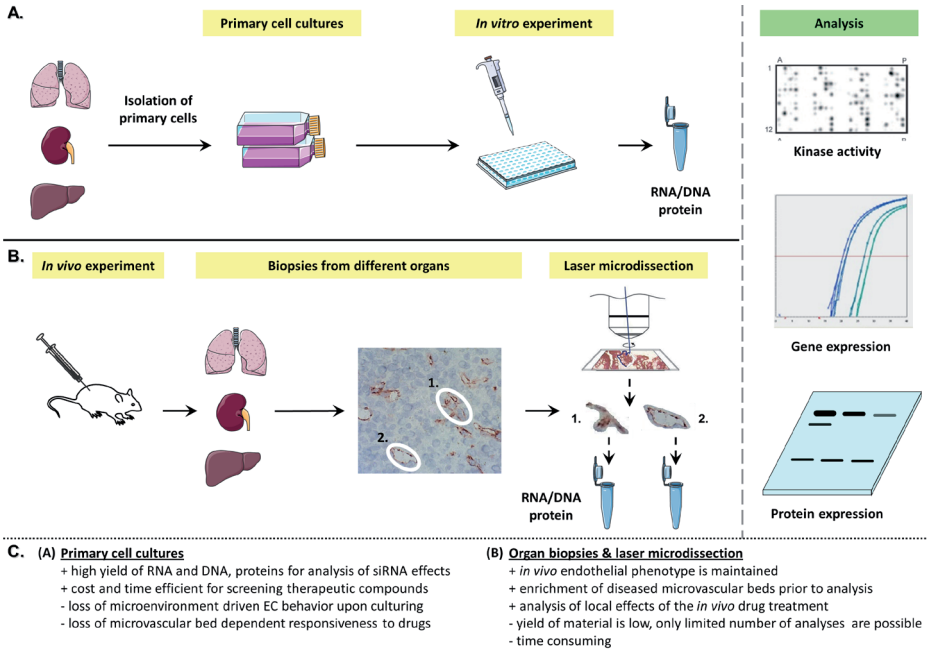

Fig. 2. Experimental approaches to study the selectivity, intracellular delivery capacity and efficacy of endothelial targeted siRNA delivery systems *in vitro* and *in vivo*.
Studying endothelial cell association, internalization and siRNA delivery using primary endothelial cell cultures (A) may be inadequate due to high rate of endothelial cell dedifferentiation upon *in vitro* culturing. This leads among others to loss of heterogenic behavior of ECs associated with the location of the microvascular bed *in vivo*. Laser microdissection (B) of ECs from specific microvascular segments in tissues is an essential technique to assist in the development of endothelial-specific siRNA delivery devices, as it allows validation of local target gene knock-down and studying downstream molecular consequences of gene knock-down. The advantages and disadvantages of both approaches are summarized in (C).

by means of targeted siRNA approaches remains somewhat elusive.

After a drug delivery system carrying siRNA has been developed and properly optimized and sufficient siRNA can be delivered inside the target cell leading to the desired down-regulation of the disease-associated target gene, novel technologies should be used to bring siRNA as therapeutic tool closer toward clinical application. Laser microdissection (LMD) of endothelial cells from specific (micro)vascular segments in tissues allows compartmentalized analysis of gene expression and hence the examination of local effects of the targeted drug treatment [77]. LMD can be applied to both animal and human tissues and it allows for enrichment of endothelial cells from (micro)vascular segments which can be prior assessed histologically for disease activity. Combining targeted drug delivery systems carrying siRNA to selectively down-regulate disease-associated genes in restricted

microvascular segments with LMD-based validation of gene silencing in the target endothelial compartment represents not just a powerful, but rather an essential strategy to provide proof of concept of *in vivo* siRNA delivery studies (Fig. 2).

Another technology that can significantly assist in further development of novel targeted delivery systems toward clinical application is precision-cut tissue slices. They closely resemble the organ from which it is prepared, with all cell types present in their original tissue-matrix configuration [78]. The circumstance that in tissue slices the architecture of the original organ is retained makes them an attractive tool for drug delivery studies. Using this system, we showed that anti-E-selectin-SAINTarg specifically associated with activated endothelial cells in human kidney tissue slices subjected to inflammatory conditions, exactly following the expression pattern of E-selectin [76].

Furthermore, one should take into account the differences in behavior between primary endothelial cells and endothelial cell lines. Endothelial cell lines, for example, are easier to transfect and hence are often chosen for protein overexpression studies, yet they display quite some differences in phenotype and responsiveness compared to primary cells. Moreover, their internalization machinery is often more tumor cell like and less relevant to the uptake features of primary cells. Since primary endothelial cells are closest, though not identical, to endothelial cells *in vivo*, they should at one point in the design of targeted drug delivery systems be used to further validate binding, internalization and intracellular drug release characteristics.

Conclusions

Increased insight in the role of endothelial cells in the pathophysiology of cancer, inflammatory and cardiovascular diseases, has drawn great interest in the design of pharmacological interventions aiming at the endothelium in diseased sites. The effectiveness of drugs intended to affect diseased endothelium is however limited which is likely partly due to the existence of endothelial subset specific responsiveness to pro-inflammatory cytokines [6]. This heterogeneity on the other hand provides an opportunity for identification of disease-associated target epitopes expressed by vascular segment restricted endothelial cells as well as for selection of the proper drug target and concurrent drugs for therapeutic effects. Emerging therapeutic strategies based on RNAi have a great potential for therapeutic application. siRNAs can, however, not be directly applied for *in vivo* treatment of diseased endothelium due to a short half-life of the molecules, in the circulation, inability to pass the

cellular membrane, high toxicity, and low cell selectivity. Formulation of these therapeutic molecules into delivery devices such as lipid-based or polymer-based systems targeted to diseased endothelial cells can provide them with a potential for further clinical application. Target determinants like E-selectin, VCAM-1, ICAM-1, and integrins with expression restricted to endothelial cells and upregulated during inflammation or angiogenesis may help to achieve specific and safe delivery of drugs into EC subsets involved in diseases, thereby improving pharmacological efficacy. Widespread use of RNAi therapeutics for endothelial diseases requires a clinically suitable, safe and effective delivery vehicle. This can be achieved by developing new formulations and bio-materials (e.g., novel cationic lipids, polymers or pH-sensitive PEG) to avoid detection by cells of the RES and improve intracellular siRNA release properties of existing lipid or polymer-based systems. Combined research efforts in the field of microvascular endothelial cell biology and pharmaceutical sciences are crucial to achieve the final goal, that is, the development of an efficient and cell-specific siRNA drug delivery system that can be applied in the clinic to effectively silence endothelial cell engagement in the patho(physio)logy of disease.

Acknowledgements

This research was partly funded by the European Fund for Regional Development (EFRO), project number 068/073-Drug Delivery and Targeting. The authors declare no competing financial interests. M.H.J. Ruiters is CEO of Synvolux Therapeutics.

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Chapter
2

Chapter 3

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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INT J PHARM. 2014 JAN 1;459(1-2):40-50.

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.

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 [1], 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) [2]. 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 [3].

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 [4]. 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 [5]. Moreover, upon systemic injection, rapid degradation by serum RNases and reticuloendothelial system (RES)-mediated clearance are inevitable [6]. Consequently, chemical modification of siRNAs, or formulation in a 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 [7]. A drug delivery system could furthermore reduce side effects and immune stimulation [8].

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 [9]. 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* [1] and shuttles antibody-bound cargo into the receptor-mediated endosomal pathway [10]. Vascular cell adhesion molecule 1 (VCAM-1) is also an internalizing target [11], 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 [12].

In a previous study, we successfully designed anti-E-selectin-SAINTargs for siRNA delivery to inflamed primary endothelial cells [13]. 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) [14]. PEG reduces the opsonization by serum proteins, thereby minimizing clearance by the RES, which leads to improved pharmacokinetic properties [15, 16]. Moreover, the stability of the carrier is promoted by incorporation of PEG in the lipid formulation [17]. 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*.

Materials and Methods

Materials

The lipids 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$]-maleimide (DSPE-PEG_{2000}-Mal) and (1,2-diacyl-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.

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 [18] 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 [19]. 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).

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 [13]. 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).

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 [13]. 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 1:2,000 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 1:2,000 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.

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 B.V.) 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 6x gel loading dye (BioLabs, Leiden, 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.

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.

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.

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 reaction 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 reactions were performed at least in duplicate and the averages of the obtained C_t values were used for further calculations. GAPDH was used as reference gene to normalize gene expression levels giving the ΔC_t value. Relative mRNA levels were calculated by $2^{-\Delta Ct}$.

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).

Results

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 [13], a 1:2,000 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

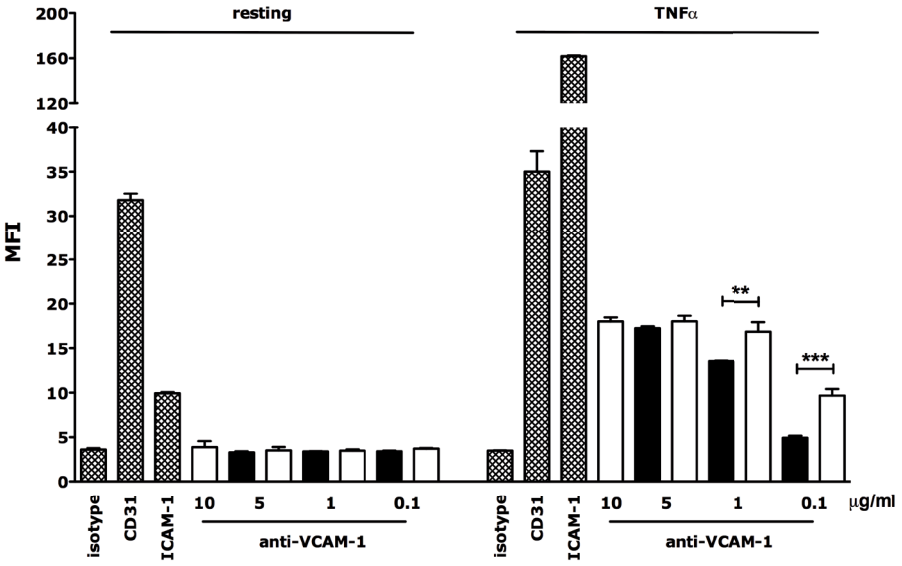

Fig. 1. Anti-VCAM-1 antibody preserves its antigen recognition after conjugation to SAINT-C18. Anti-VCAM-1-SAINT conjugate (black bars) and parental anti-VCAM-1 antibody (open bars) bind specifically to TNFα-activated HUVEC. In resting cells MFI (mean fluorescent intensity) is identical to the isotype 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 ***, $p < 0.001$.

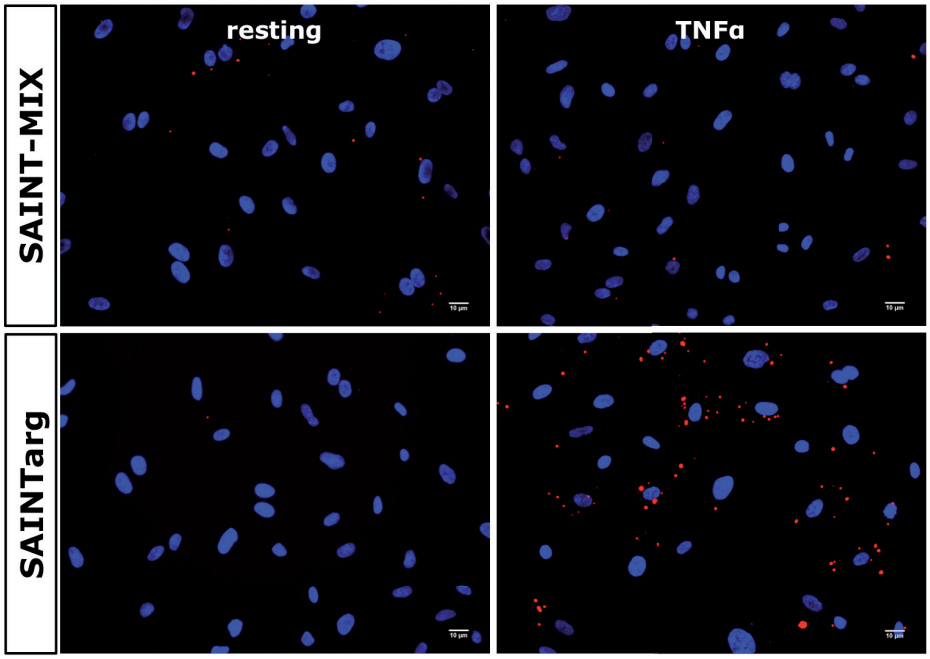

Fig. 2. Anti-VCAM-1-SAINTargs increases siRNA delivery capacity and specificity for activated primary endothelial cells. Delivery of $Alexa_{546}$-tagged siRNA was enhanced by targeted delivery with SAINTarg in TNFα-activated HUVEC, as demonstrated by fluorescence microscopy. HUVEC were incubated for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

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).

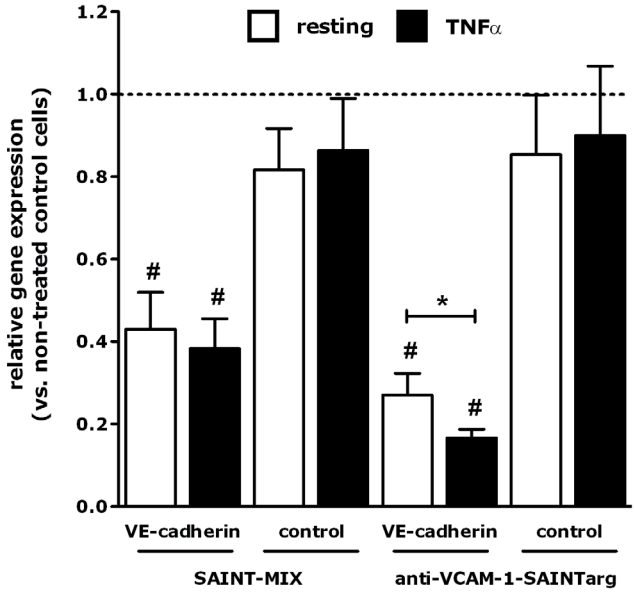

Fig. 3. Down-regulation of VE-cadherin gene expression in HUVEC by non-targeted SAINT-MIX and anti-VCAM-1-SAINTarg. VE-cadherin down-regulation is enhanced by targeted siRNA delivery with anti-VCAM-1-SAINTarg in TNFα-activated HUVEC, while down-regulation in resting HUVEC was similar for SAINT-MIX and 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.

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 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).

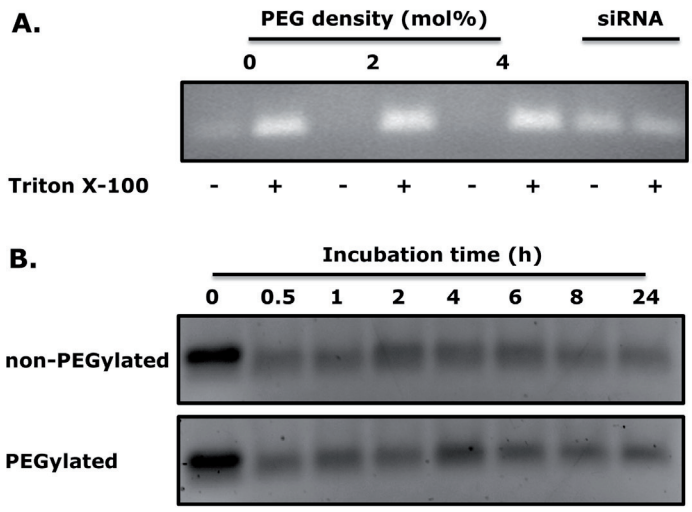

Fig. 4. SAINT-based lipoplexes protect the siRNA integrity and the siRNA encapsulation capacity is not affected in presence of PEG. (A) The influence of increasing concentrations of PEG on the siRNA encapsulation was investigated by gel electrophoresis. Freshly prepared SAINT-based lipoplexes formulated 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 'Materials and Methods'.

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 figure 5.

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 1:2,000 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.

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 [13]. Antibody-SAINTPEGargs (antibody-PEG conjugate:SAINT-MIX:siRNA in a molar ratio

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 ± 0.1
anti-E-selectin-SAINTPEGarg + 2 mol% PEG	141 ± 21	36 ± 15	
anti-VCAM-1-SAINTPEGarg	194 ± 20	37 ± 30	1.1 ± 0.1
anti-VCAM-1-SAINTPEGarg + 2 mol% PEG	146 ± 15	42 ± 34	

Data are presented as means of 4-5 preparations ± SD

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).

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

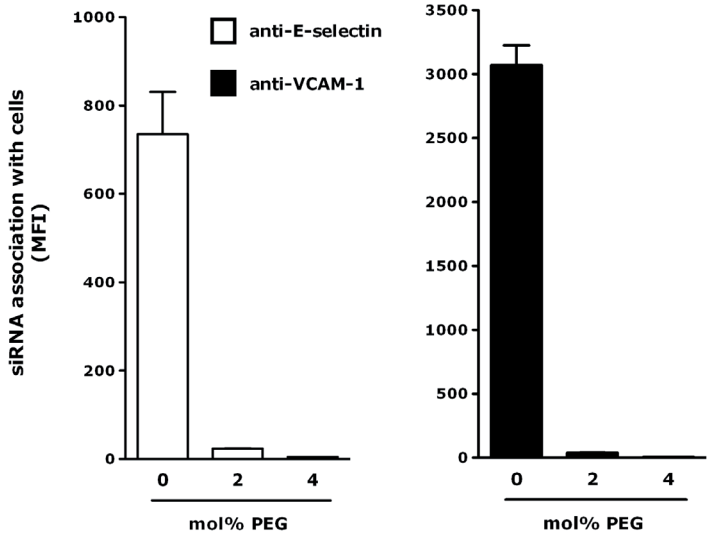

Fig. 5. PEGylation of antibody-SAINTargs leads to loss of siRNA delivery capacity in primary endothelial cells. TNFα-activated HUVEC were incubated for 4 h with anti-E-selectin-SAINTarg or anti-VCAM-1-SAINTarg formulated in absence or presence of 2 respectively 4 mol% PEG. Endothelial cell siRNA association (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.

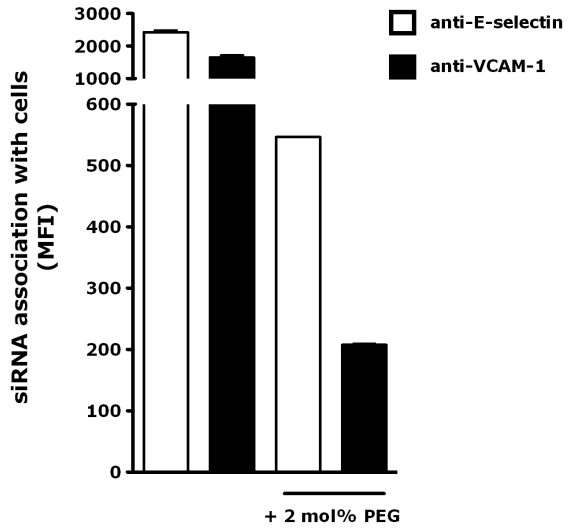

Fig. 6. PEGylated anti-E-selectin- and anti-VCAM-1-SAINTPEGargs retained their siRNA delivery capacity in activated HUVEC. TNFα-activated HUVEC were incubated for 4 h with antibody-modified SAINTPEGarg formulated in absence or presence of 2 mol% PEG. Endothelial cell siRNA association was determined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

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 and co-authors showed that SAINT-O-Somes targeted to VCAM-1 and E-selectin specifically deliver siRNA into TNFα-activated HAEC [20].

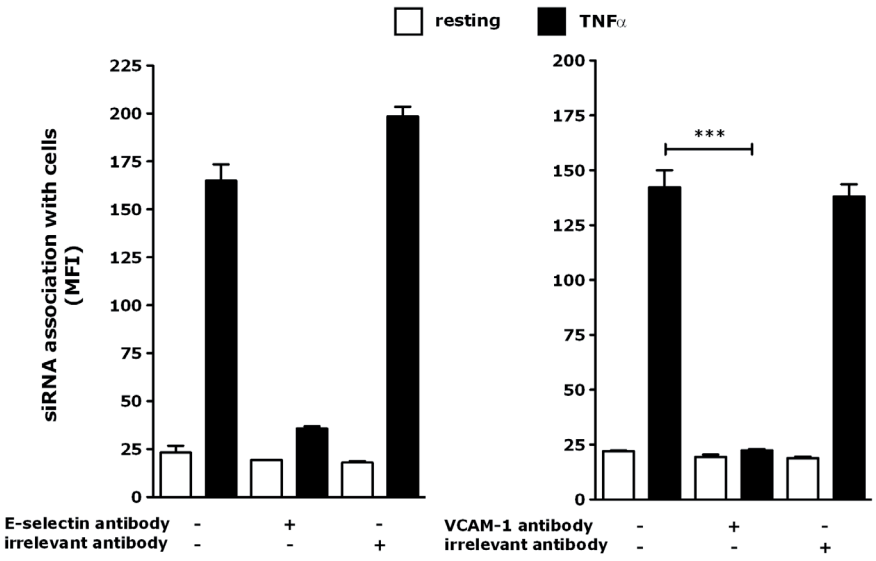

Fig. 7. antibody-SAINTPEGarg-mediated siRNA delivery in TNFα-activated HUVEC was completely blocked by excess of free parental antibodies. Resting or activated HUVEC were incubated for 4 h with antibody-modified SAINTPEGarg formulated with 2 mol% PEG. Specificity of binding was determined by co-incubation 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. ***, $p < 0.001$.

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 [21] 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 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

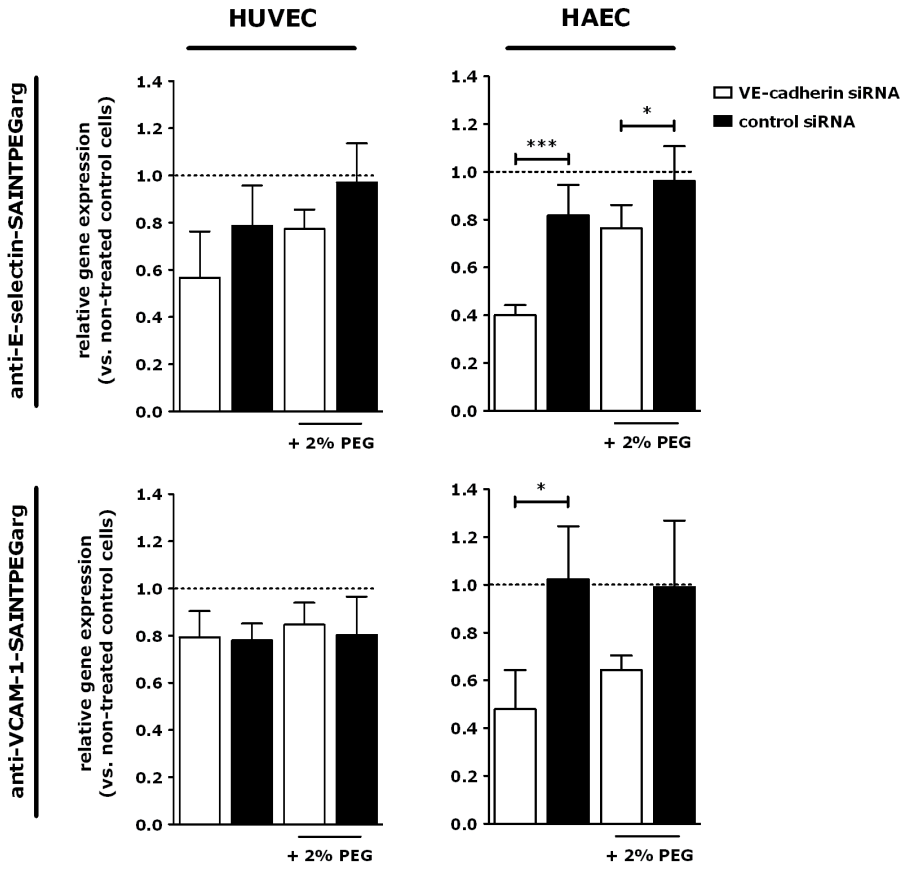

Fig. 8. Down-regulation of VE-cadherin by antibody-SAINTPEGargs containing $siRNA_{VE\text{-}cadherin}$ in HUVEC and HAEC. Antibody-SAINTPEGargs with or without 2 mol% PEG containing VE-cadherin siRNA or control siRNA were incubated at 60 nM with TNFα-activated HUVEC or HAEC for 4 h. Subsequently, particles 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 ***, $p < 0.001$.

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-

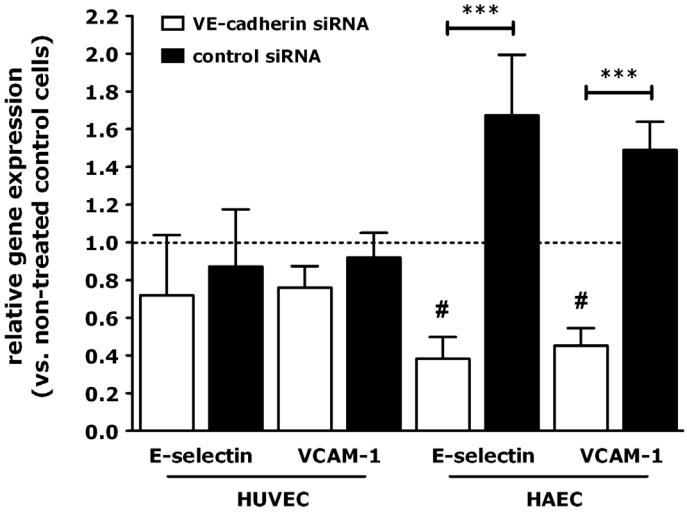

Fig. 9. Down-regulation of VE-cadherin gene expression by antibody-SAINTPEGargs formulated with 2 mol% PEG containing 300 nM siRNA. TNFα-activated HUVEC and HAEC were incubated with antibody-SAINTPEGargs containing VE-cadherin siRNA or control siRNA for 4 h. In HAEC, VE-cadherin gene expression was down-regulated, 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.

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 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.

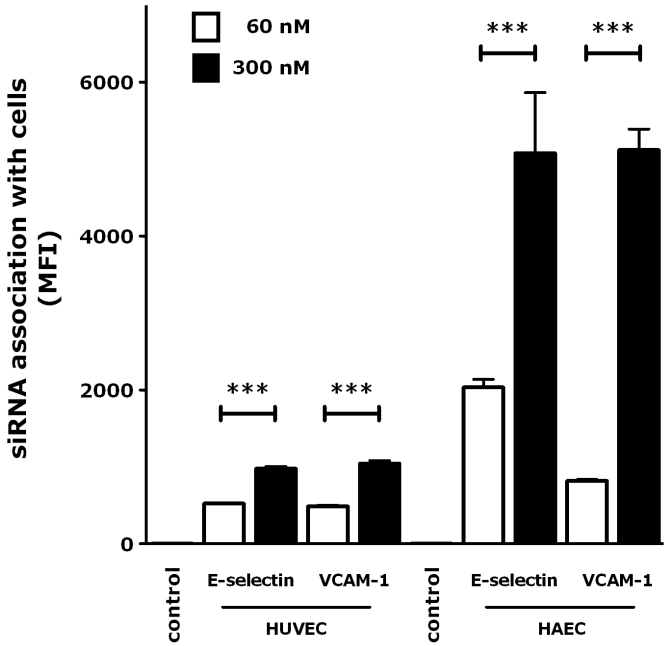

Fig. 10. Association of antibody-SAINTPEGargs in HUVEC and HAEC as function of siRNA concentration. TNFα-activated HUVEC and HAEC were incubated with anti-E-selectin- and anti-VCAM-1-SAINTPEGargs containing $Alexa_{488}$-tagged siRNA. The extent of siRNA uptake by HUVEC and HAEC is different when treated 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. ***, $p < 0.001$.

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 [22, 23]. 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

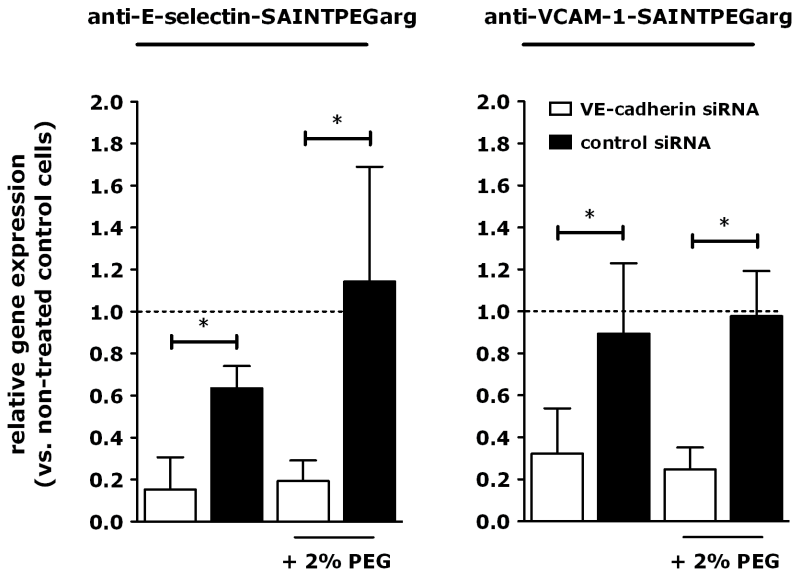

Fig. 11. Down-regulation of VE-cadherin in HHSEC by antibody-SAINTPEGargs containing 60 nM $siRNA_{VE\text{-}cadherin}$. TNFα-activated HHSEC were incubated with antibody-SAINTPEGargs containing VE-cadherin siRNA or control siRNA for 4 h. VE-cadherin gene expression was down-regulated, while control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

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 - 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 [24] is a highly effective transfection agent when complexed with nucleotides or proteins [25] that is not affected by the presence of serum [26]. 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 [13]. Apart from E-selectin [27], also VCAM-1 [28] has been described as an attractive target for specific drug delivery strategies to activated endothelium. To achieve specificity towards 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. 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 and co-authors 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* [29]. 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 [30]. 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 [13]. 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 [31]. Since heterogenic endothelial cell behavior is the backbone of endothelial subset specific targeted drug delivery, this issue wil be further investigated

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*

Acknowledgements

We thank Henk Moorlag, Mariëlle Voortman and Henriëtte Morselt for excellent technical assistance. This research was supported by the European Fund for Regional Development (EFRO) from the European Union, project NTS 068 and 073 Drug Delivery and Targeting. The authors declare no competing financial interests. M.H.J. Ruiters is CEO of Synvolux Therapeutics.

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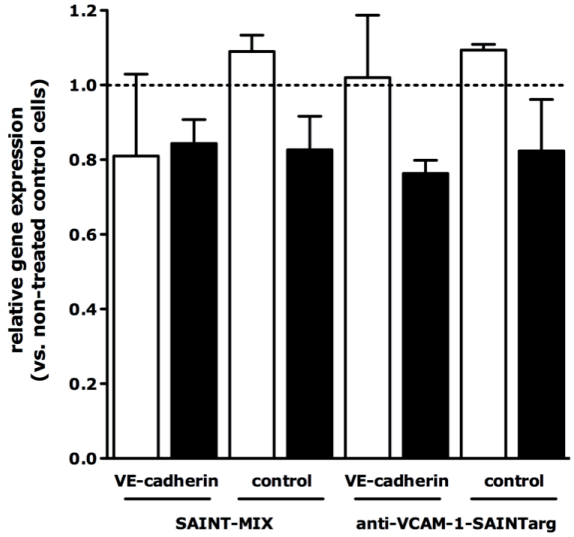
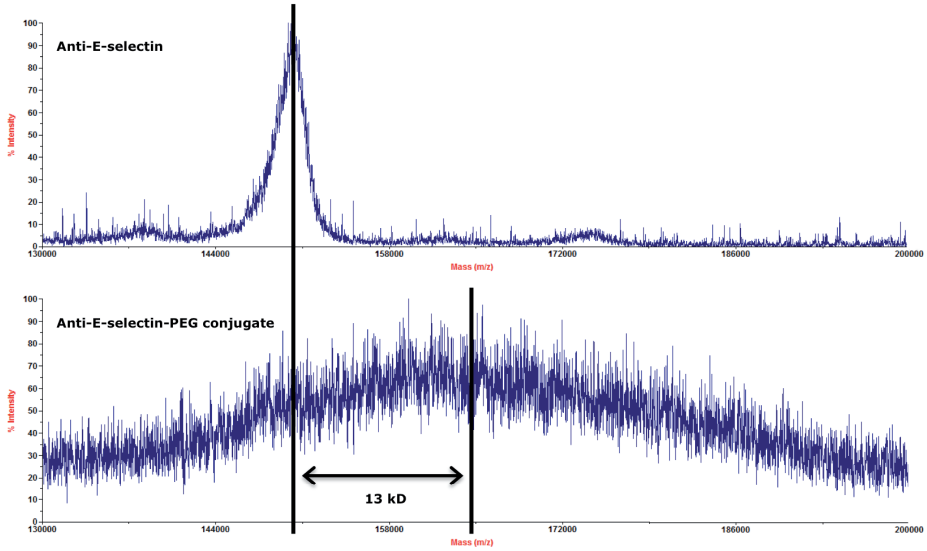
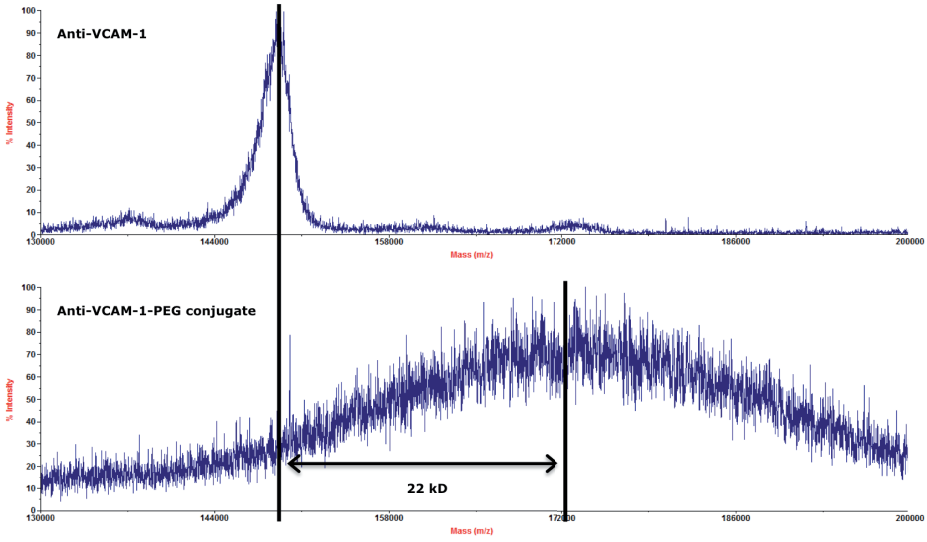
Supplementary figures



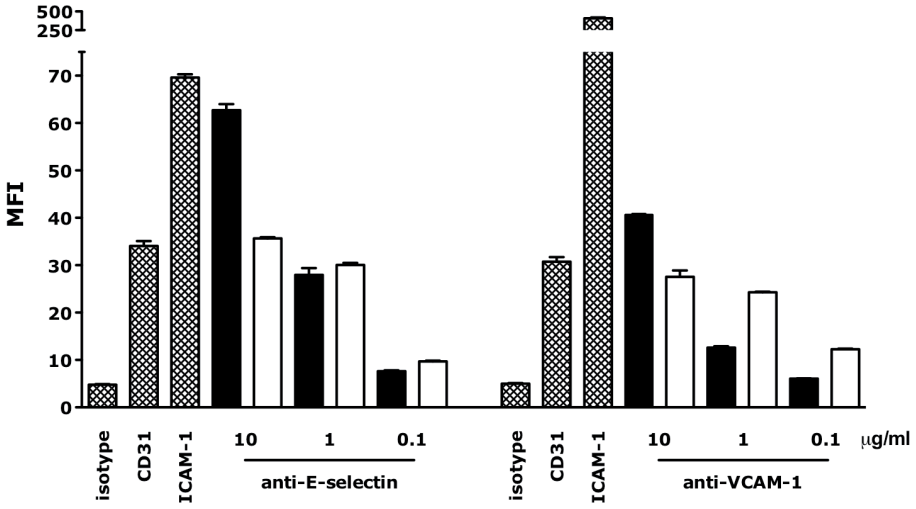
Supplementary figure 1. Tie2 gene expression was statistically unaffected in resting (open bars) and TNFα-activated (black bars) HUVEC incubated with either non-targeted SAINT-MIX or anti-VCAM-1-SAINTarg. Anti-VCAM-1-SAINTargs containing VE-cadherin or control siRNA were incubated at 60 nM for 48 h. Data are presented as relative gene expression ± SD compared to non-treated control cells, n=3.



Supplementary figure 2A. MALDI-TOF mass spectra of anti-E-selectin-PEG conjugate and parental anti-E-selectin antibody demonstrating an increase in mass from 151 kD to 164 kD after conjugation with DSPE-PEG_{2000}-Mal, corresponding to 4 DSPE-PEG_{2000}-Mal molecules conjugated to one anti-E-selectin antibody.



Supplementary figure 2B. MALDI-TOF mass spectra of anti-VCAM-1-PEG conjugate and parental anti-VCAM-1 antibody demonstrating an increase in mass from 151 kD to 173 kD after conjugation with DSPE-PEG_{2000}-Mal, corresponding to 7 DSPE-PEG_{2000}-Mal molecules conjugated to one anti-VCAM-1 antibody.



Supplementary figure 3. Antigen recognition of anti-E-selectin and anti-VCAM-1 antibodies is preserved after coupling to DSPE-PEG_{2000}-Mal. Antibody-PEG conjugates (black bars) and parental antibodies (open bars) bind specifically to TNFα-activated HUVEC. Controls are shown by hatched bars. Data depict a representative result of 3-4 independent experiments. Values are presented as mean ± range of duplicate samples.

Supplementary table 1. Gene expression of E-selectin, VCAM-1 and VE-cadherin in resting and TNFα-activated cells.

		HUVEC	HAEC	HHSEC
E-selectin	T0	$3.3 \times 10^{-4} \pm 3.6 \times 10^{-5}$	$3.6 \times 10^{-5} \pm 5.3 \times 10^{-5}$	$1.2 \times 10^{-3} \pm 1.5 \times 10^{-3}$
	T2	0.29 ± 0.04	0.16 ± 0.02	0.26 ± 0.01
	T5	0.19 ± 0.02	0.15 ± 0.02	0.24 ± 0.01
VCAM-1	T0	$1.2 \times 10^{-4} \pm 2.1 \times 10^{-5}$	$8.8 \times 10^{-6} \pm 1.1 \times 10^{-4}$	$9.5 \times 10^{-5} \pm 1.1 \times 10^{-4}$
	T2	0.0157 ± 0.0028	0.0177 ± 0.0048	0.0167 ± 0.0008
	T5	0.026 ± 0.004	0.023 ± 0.001	0.038 ± 0.002
VE-cadherin	T0	0.25 ± 0.02	0.09 ± 0.01	0.27 ± 0.04
	T2	0.32 ± 0.03	0.08 ± 0.01	0.25 ± 0.01
	T5	0.30 ± 0.01	0.08 ± 0.01	0.27 ± 0.01

Gene expression levels relative to GAPDH ± SD, n=3. T0, resting cells; T2/T5, activated for 2 h and 5 h respectively, with 10 ng/ml TNFα.

Chapter
3

Chapter 4

VCAM-1 specific PEGylated SAINT-based lipoplexes deliver siRNA to activated endothelium in vivo but do not attenuate target gene expression

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INT J PHARM. 2014 JUL 20;469(1):121-31.

Abstract

In recent years much research in RNA nanotechnology has been directed to develop an efficient and clinically suitable delivery system for short interfering RNA (siRNA). The current study describes the *in vivo* siRNA delivery using PEGylated antibody-targeted SAINT-based-lipoplexes (referred to as antibody-SAINTPEGarg/PEG2%), which showed superior siRNA delivery capacity and effective down-regulation of VE-cadherin gene expression *in vitro* in inflammation-activated primary endothelial cells of different vascular origins. PEGylation of antibody-SAINTPEGarg resulted in more desirable pharmacokinetic behavior than that of non-PEGylated antibody-SAINTPEGarg. To create specificity for inflammation-activated endothelial cells, antibodies against vascular cell adhesion molecule-1 (VCAM-1) were employed. In TNFα-challenged mice, these intravenously administered anti-VCAM-1-SAINTPEGarg/PEG2% homed to VCAM-1 protein expressing vasculature. Confocal laser scanning microscopy revealed that anti-VCAM-1-SAINTPEGarg/PEG2% co-localized with endothelial cells in lung postcapillary venules. Furthermore, they did not exert any liver and kidney toxicity. Yet, lack of *in vivo* gene silencing as assessed in whole lung and in laser microdissected lung microvascular segments indicates that *in vivo* the internalization and/or intracellular trafficking of the delivery system and its cargo in the target cells are not sufficient, and needs further attention, emphasizing the essence of evaluating siRNA delivery systems in an appropriate *in vivo* animal model at an early stage in their development.

Introduction

Endothelial cells have obtained considerable attention as targets for selective drug delivery in disease. Not only by virtue of their direct contact with the blood and consequent easy accessibility for systemically administered therapeutic compounds, but also because of their active engagement in inflammatory processes and pivotal role in the pathophysiology of many diseases, e.g. chronic inflammation and cancer [1-3]. Small interfering RNA (siRNA) specifically interferes with the expression of a target gene and is considered an important new class of therapeutics [4]. The physicochemical properties of siRNAs enforce their formulation in a delivery system [5]. Consequently, interest in siRNA delivery technologies has increased in recent years in recognition of their potential for applications in nanomedicine [6]. Most drug delivery systems, however, have no affinity for endothelial cells, leading to adverse effects or sub-optimal effectiveness when not being taken up by diseased endothelium [7]. Selective and functional delivery still represents the major obstacle for the utility of siRNA to target endothelial cells *in vivo*, which limits their usefulness as a therapeutic *per se*. It requires a delivery system that is avidly internalized by disease-associated endothelium and releases its cargo (siRNA), as intracellular drug release is a prerequisite for the effect of RNA interference [3].

Selective delivery of siRNAs using targeting moieties that specifically bind to epitopes (over)expressed on the endothelial cells involved in disease provides an exciting gateway to functionally deliver siRNAs to organ specific (micro)vascular endothelial subsets. By this means, only in the target cell sufficient intracellular siRNA concentrations will be reached that are appropriate for pharmacological effects, while unwanted side effects elsewhere in the body are circumvented. Inflammation-related cell adhesion molecules, including E-selectin and vascular cell adhesion molecule-1 (VCAM-1), are attractive candidates as target epitopes on activated endothelium because of their increased (over)expression during inflammation, interorgan and intraorgan vascular differences in expression, and predominant endothelial localization [8-10]. For example, VCAM-1 is massively upregulated at sites of pathological inflammation in chronic diseases such as atherosclerosis [11], rheumatoid arthritis [12], inflammatory bowel disease [13], and on tumor vasculature [14]. Endothelial-specific drug delivery strategies involving lipid-based drug carriers (reviewed by [15]) and molecular targets that internalize upon carrier binding (reviewed by [3] could improve the efficiency of current treatment of diverse (inflammatory) diseases.

Lipoplexes, complexes of cationic lipids and (poly)anionic nucleotides including siRNA, are high-capacity drug carriers that, when harnessed with monoclonal antibodies, can be targeted to a specific target epitope of choice [16]. We developed a novel lipid-based siRNA delivery device called SAINTPEGarg by combining the transfection ability of the cationic amphiphilic lipid SAINT-C18 (1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride), a well established potent delivery agent of nucleotides and proteins [17, 18], with antibody-based endothelial cell specificity [19]. Targeted siRNA delivery into endothelial cells *in vivo*, however, is hampered by the lack of stability of lipoplexes. PEGylation by polyethylene glycol (PEG), incorporated in the formulation, could stabilize and prolong the circulation half-life of newly developed antibody-SAINTPEGarg [20]. PEG has been shown to reduce the opsonization by serum proteins, thereby minimizing clearance by the RES leading to improved pharmacokinetic properties [21]. Recently, we demonstrated that these newly developed PEGylated antibody-SAINTPEGarg (referred to as antibody-SAINTPEGarg/PEG2%) are effective devices for specific and functional delivery of siRNA into inflammation-activated endothelial cells of different vascular origins *in vitro* [20], and thus provide an opportunity for further studies for their applicability in specific disease-associated endothelial cell subsets *in vivo*.

The current paper describes the studies on VCAM-1 targeting using anti-VCAM-1-SAINTPEGarg formulated with additional 2 mol% DOPE-PEG_{2000} (anti-VCAM-1-SAINTPEGarg/PEG2%) to deliver siRNA into inflammation-activated endothelium *in vivo*. We studied the pharmacokinetic behavior, biodistribution, and effect of anti-VCAM-1-SAINTPEGarg/PEG2% on liver and kidney function in TNFα-challenged mice. Homing and cellular localization of anti-VCAM-1-SAINTPEGarg/PEG2% in lung tissue was studied using immunohistochemistry and confocal laser scanning microscopy (CLSM). Effectiveness of $siRNA_{VE\text{-}cadherin}$ delivery by anti-VCAM-1-SAINTPEGarg/PEG2% on VE-cadherin gene expression was determined in whole-lung isolates and laser microdissected postcapillary venules.

Materials and Methods

Preparation and characterization of targeted lipoplexes

Targeted siRNA delivery with antibody-SAINTPEGarg was performed by mixing either rat-IgG- or anti-VCAM-1-PEG conjugate with SAINT-MIX (SAINT-C18:1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); molar ratio 1:1; Synvolux Therapeutics, Groningen, the Netherlands) in a fixed 1:2,000 molar ratio. Subsequently, conjugate and lipids were vortexed for 5 s, mixed with siRNA, incubated for 5 min and intravenously (i.v.) injected. Lipoplexes were freshly prepared in 5% (w/v) glucose prior to each experiment and where appropriate, PEGylated by addition of 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$] (DOPE-PEG$_{2000}$; Avanti Polar lipids Alabastar, AL, USA) to the formulation at indicated mol% at the cost of DOPE. Mean particle size of antibody-SAINTPEGargs/PEG2% was analyzed by dynamic light scattering using a Nicomp submicron particle analyzer (model 380 ZLS, Santa Barbara, CA, USA). For particle sizing, antibody-SAINTPEGarg/PEG2% was prepared freshly in Hepes buffer (supplied with SAINT-MIX transfection system; Synvolux Therapeutics B.V.). Samples were briefly vortexed and the mean diameter was obtained from the number distribution curves produced by the particle analyzer. Where indicated antibody-SAINTPEGargs contained a trace amount (0.017 µCi/nmol of total lipid) of ^{3}H-SAINT-C18 (RC Tritec, Teufen, Switzerland). The monoclonal rat anti-mouse VCAM-1 (IgG1) was purified from supernatant produced by hybridoma M/K-2.7 (American Type Culture Collection (ATCC), Manassas, VA, USA). Rat anti-mouse VCAM-1 and rat-IgG (Sigma-Aldrich, Zwijndrecht, the Netherlands) were coupled to the maleimide group at the distal end of the polyethylene glycol chain (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$]-maleimide) (DSPE-PEG$_{2000}$-Mal; Avanti Polar lipids) after thiolation of the antibody by means of N-succinimidyl-S-acethylthioacetate (SATA; Pierce, Rockford, IL, USA). The number of DSPE-PEG$_{2000}$-Mal molecules attached to the SATA-modified antibodies was measured by MALDI-TOF mass spectrometry on a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands), as described previously [20]. The sequence of the predesigned siRNA targeting mouse VE-cadherin gene (Mm_CDH5_2_HP) was AAGGATCAAGTCCAATCTAAA (Qiagen Benelux, Venlo, the Netherlands). AllStars negative control siRNA (Qiagen) with no homology to any known mammalian gene was used as control for functional experiments.

Animals

C57bl/6 male mice (weight 18-22 g; age 7-9 weeks) were purchased from Harlan (Zeist, the Netherlands). Animals were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12 h light/dark cycle. All animal experiments were performed according to national guidelines and upon approval of the experimental procedures by the local Animal Care and Use committee of Groningen University, DEC number 6150A. Mice were randomly assigned to the experiments as described below.

Examination of liver and kidney function parameters

Liver and kidney function parameters were measured in heparin plasma from peripheral blood of mice used in the functional study of antibody-SAINTPEGarg/PEG2%. 2 h prior to injection of targeted lipoplexes, systemic endothelial activation was induced by i.v. injection of 200 ng recombinant mouse (rm)TNFα (Invitrogen, Breda, the Netherlands) per 20 gram body weight. Next, TNFα-challenged mice were i.v. injected with targeted SAINTPEGargs (250 nmol total lipid (TL) containing 0.033 mg siRNA/20 g body weight) or vehicle (Hepes buffer; Synvolux Therapeutics). Liver function was analyzed by determining the levels of liver enzymes ALAT (alanine aminotransferase), ASAT (aspartate aminotransferase) and ALP (alkaline phosphatase), kidney function by urea and creatinine measurements. All analyses were performed using assays from Roche Diagnostics (Mannheim, Germany), and were measured using a Hitachi automatic analyzer (Modular Analytics, Roche Diagnostics).

Pharmacokinetics and biodistribution of antibody-SAINTPEGargs

Mice were anesthetized by 2.5% isoflurane/O_2 inhalation. A single dose of 30 nmol ^{3}H-SAINT-C18-labelled IgG-SAINTPEGargs containing 120 pmol AllStars negative control siRNA was i.v. injected via the penis vein (in all *in vivo* experiments) into C57bl/6 mice (n=3-4/group). For all *in vivo* experiments, lipoplexes were prepared in 5% (w/v) glucose to ensure stability of the ^{3}H-labelled antibody-SAINTPEGargs during the experimental timeline as previously shown for SAINT-MIX [22]. Blood samples were taken at different time-points after injection as indicated. Serum was harvested and ^{3}H radioactivity was measured as described before [23]. Pharmacokinetic parameters were calculated using the Interactive Two-Stage Bayesian program MultiFit [24]. At 4 h, mice were sacrificed and liver, spleen, lungs, kidneys, heart, and brain were removed and processed for measurement of radioactivity. Briefly, tissues

were homogenized using a Potter-Elvehjem homogenizer and ^{3}H radioactivity was determined after solubilization of 0.4 ml of the tissue homogenate in 100 µl 10% SDS and 3.5 ml liquid scintillation cocktail (Ultima Gold XR; PerkinElmer, Waltham, MA, USA). Radioactivity was measured by a Packard Tri-Carb 2500 TR liquid scintillation analyzer (PerkinElmer). Recoveries were calculated from the individual values of the organs taken out for further examination, and the blood values.

Immunohistochemical detection of VCAM-1 protein and localization of PEGylated anti-VCAM-1-SAINTPEGarg in mouse tissue

2 h after i.v. injection of rmTNFα, anti-VCAM-1-SAINTPEGarg/PEG2% or rat-IgG-SAINTPEGarg/PEG2% (150 nmol TL containing 0.020 mg control siRNA/20 g body weight) were i.v. injected. 2 h after injection of the antibody-SAINTPEGarg/PEG2%, mice were sacrificed and major organs collected, snap-frozen on liquid nitrogen, and stored at -80°C.

Cryostat sections (5 µm) of organs were fixed in acetone for 10 min and non-specific binding was blocked by 30 min incubation with PBS containing 1% BSA (Bovine Serum Albumin; Sigma-Aldrich) and 2% FCS (Fetal Calf Serum; Thermo Scientific HyClone, Cramlington, UK). Thereafter, endogenous biotin was blocked by Biotin Blocking System according to the manufacturer's guidelines (DakoCytomation, Glostrup, Denmark). VCAM-1 protein was detected by incubation with rat anti-mouse monoclonal VCAM-1 (10 µg/ml) in the presence of 1% BSA/2% FCS for 1 h. Subsequently, sections were incubated for 30 min with rabbit anti-rat antibodies (10 µg/ml; AI-4001, Vector, Burlingame, CA, USA) in the presence of 5% normal mouse serum (NMS, in PBS; Sanquin, Amsterdam, the Netherlands) followed by incubation for 30 min with anti-rabbit polymer-HRP (EnVision kit (EnVision + System-HRP), DAKO, Carpinteria, CA, USA). Sections were counterstained with Mayer's Hematoxylin (Klinipath, Duiven, the Netherlands). Staining with isotype-matched control antibodies (purified rat IgG1, Antigenix America, NY, USA, detected as described above) was consistently found to be devoid of staining.

Immunohistochemical detection of i.v. injected anti-VCAM-1-SAINTPEGarg/PEG2% was performed as described above, except for the fact that sections were not incubated with primary rat anti-mouse VCAM-1 (n=3/group).

VE-cadherin gene knock-down by anti-VCAM-1-SAINTPEGarg/PEG2% containing siRNA$_{VE\text{-}caherin}$ in TNFα-challenged mice

A single dose (250 nmol TL containing 0.033 mg siRNA$_{VE\text{-}cadherin}$/20 g body weight) of anti-VCAM-1-SAINTPEGarg/PEG2% (n=6 mice), rat-IgG-SAINTPEGarg/PEG2% (n=6 mice) or vehicle (n=5 mice) was injected i.v. into C57bl/6 mice 2 h after TNFα challenge as described above. At 48 h, mice were sacrificed and major organs were removed, snap-frozen on liquid nitrogen and stored at -80°C. The left lung was filled with a 60:40 Tissue-Tek/PBS solution (Sakura, Alphen aan den Rijn, the Netherlands) to maintain lung morphology for laser microdissection of postcapillary venules. Total RNA was isolated from twenty-five 10 µm lung cryosections using the RNeasy Plus Mini 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, which was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The primers for VE-cadherin (Mm00486938_m1), Tie2 (Mm00443242_m1), ICAM-1 (Mm00516023_m1) and GAPDH (Mm99999915_g1) were purchased as Assay-on-Demand (Applied Biosystems). For each sample, the real-time PCR reactions were performed in duplicate and the averages of the obtained C_t values were used for further calculations. GAPDH was used as reference gene to normalize gene expression levels resulting in the ΔC_t value. Relative gene expression levels were calculated by $2^{-\Delta Ct}$.

Laser Dissection Microscopy of lung venules

Serial cryosections (9 µm) from mouse lung tissue were mounted on membrane-covered slides (PEN membrane; PALM Microlaser Technologies, Bernried, Germany), fixed in acetone, and stained with Mayer's Hematoxylin. Subsequently, sections were washed with DEPC-treated tap water and air-dried. Seventy-five to one hundred fifty postcapillary venules (1.0-2.5 x 10^6 µm^2 of tissue per mouse) from the anti-VCAM-1-SAINTPEGarg treated group (n=4 mice) and the control group (n=3 mice) were laser microdissected using the Laser Robot Microbeam System (LMD6000; Leica, Wetzlar, Germany). Venules were identified based on size and morphology. Extraction of total RNA was carried out according to the protocol of RNeasy Plus Micro Kit (Qiagen). cDNA synthesis and real-time PCR were performed as described above. To correct for contaminations of adjacent non-endothelial cells, VE-cadherin gene expression was normalized to the expression of the pan-endothelial marker gene CD31

(Mm00476702_m1), which was not influenced by the experimental procedures.

(Co)-localization of anti-VCAM-1-SAINTPEGarg with vWF and CD45.2 in mouse lung tissue

Cryostat sections were fixed and blocked for non-specific binding as described above. Immunofluorescence double staining for anti-VCAM-1-SAINTPEGargs with either the endothelial specific marker Von Willebrand factor (vWF) or the leukocyte common antigen CD45.2 was performed as follows. vWF was detected with rabbit anti-human (cross-reacts with mouse) vWF (1 h incubation, 20 µg/ml; A0082, DAKO), followed by 30 min incubation with goat anti-rabbit $AlexaFluor_{488}$ (20 µg/ml; A-11008, Molecular Probes). CD45.2 was detected with biotin-labelled anti-mouse CD45.2 (1 h incubation, 20 µg/ml; clone 104, eBioscience, San Diego, CA, USA), followed by 30 min incubation with $AlexaFluor_{488}$-conjugated streptavidin (20 µg/ml; S-11223, Molecular Probes). Anti-VCAM-1-SAINTPEGargs were detected with $AlexaFluor_{546}$-conjugated goat anti-rat antibodies (1 h incubation, 20 µg/ml; A11081, Molecular Probes, Leiden, the Netherlands). Endogenous biotin was blocked by Biotin Blocking System (DakoCytomation). All incubation steps were carried out in the presence of 5% NMS. Sections were mounted with Citi-Fluor AF1 (Citifluor Ltd, London, UK) and fluorescence was examined using a confocal laser scanning microscope (CLSM) equipped with true confocal scanner (TCS; SP8 Leica, Heidelberg, Germany), using a 63x oil immersion lens. Sequential scans were obtained to avoid bleed through. $AlexaFluor_{488}$ was excited using the 488 nm blue laser line, $AlexaFluor_{546}$ was excited using the 552 nm green laser line. All images were recorded in the linear range, avoiding local saturation, at an image resolution of 2048x2048 pixels and with a pinhole size of 1 Airy unit. Differential interference contrast (DIC) images were taken to more clearly illustrate the intra-pulmonary co-localization of the antibody-SAINTPEGarg/PEG2%. Presented images show a single z-scan. Images were further processed using ImageJ V1.48d (National Institute of Health, USA).

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).

Table 1. Characterization of antibody-SAINTPEGarg/PEG2%

Sample	Mean diameter (nm)	pmol antibody/15 nmol lipid
IgG-SAINTPEGarg/PEG2%	148 ± 11	1.5 ± 0.1
anti-VCAM-1-SAINTPEGarg/PEG2%	152 ± 16	1.1 ± 0.1

Data are presented as mean values of 3 preparations ± SD.

Results

PEGylation changes the pharmacokinetic behavior of antibody-SAINTPEGargs

Initial studies in the 1990s revealed that surface grafted PEG improves the *in vivo* pharmacokinetic properties of liposomes [25, 26]. First, antibody-SAINTPEGarg/PEG2% was characterized by a mean diameter of approximately 150 nm (table 1), which is comparable to our previous study [20]. Next, we studied the pharmacokinetic behavior of the recently developed antibody-SAINTPEGargs and PEGylated antibody-SAINTPEGargs (referred to as antibody-SAINTPEGarg/PEG2%). The blood concentration of ^{3}H-SAINT-C18-labelled IgG-SAINTPEGarg and IgG-SAINTPEGarg/PEG2% decreased in a biphasic pattern and significant differences were observed

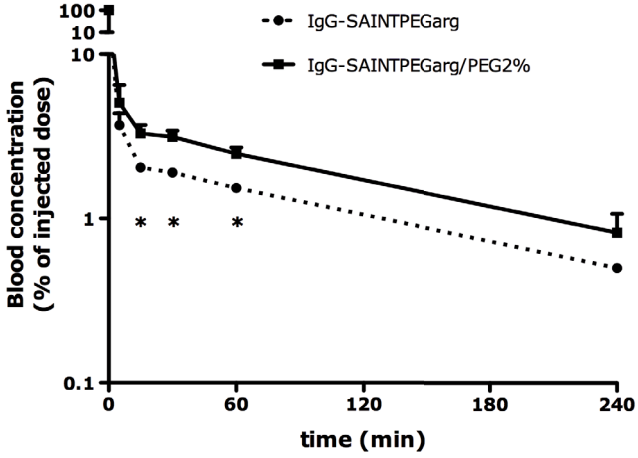


Fig. 1. Co-formulation of 2 mol% PEG affects the blood disappearance of IgG-SAINTPEGarg.
30 nmol 3H-SAINT-C18-labelled IgG-SAINTPEGargs containing 120 pmol siRNA and formulated in the presence or absence of 2 mol% were i.v. administered to healthy mice. A significant difference was observed in blood disappearance between IgG-SAINTPEGarg and IgG-SAINTPEGarg/PEG2%. Values are presented as mean ± SD; n=3-4 mice/group. *, p <0.05 compared to IgG-SAINTPEGarg.

Table 2. Pharmacokinetic parameters of IgG-SAINTPEGarg and IgG-SAINTPEGarg/PEG2% in healthy mice

Parameter	IgG-SAINTPEGarg	IgG-SAINTPEGarg/PEG2%
CL, ml/min/g	0.0118 ± 0.0011	0.0074 ± 0.0010*
V, ml/g	1.64 ± 0.05	1.06 ± 0.14*
$t_{1/2}$ (2), min	97 ± 3	100 ± 20

Values represent mean values ± SD of 3-4 mice/group. CL, plasma clearance; V, volume of distribution; $t_{1/2}$ (2), elimination half-life. *, p <0.05 compared to IgG-SAINTPEGarg.

between the two siRNA delivery systems (fig. 1). Plasma clearance and volume of distribution of IgG-SAINTPEGarg/PEG2% were significantly lower than those of IgG-SAINTPEGarg (table 2). PEGylation of IgG-SAINTPEGarg furthermore did not affect the elimination half-life ($t_{1/2}$ (2)) of the lipoplex compared to its non-PEGylated counterpart. The initial half-life ($t_{1/2}$ (1)) could not be calculated due to lack of data-points.

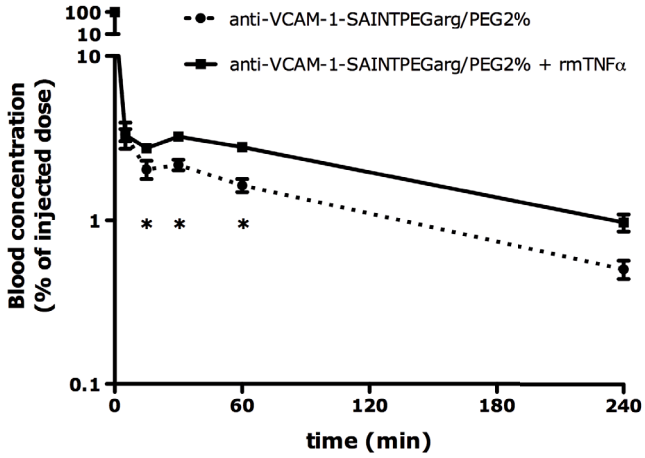


Fig. 2. Effect of TNFα-challenge on the blood disappearance of anti-VCAM-1-SAINTPEGarg formulated with 2 mol% PEG.
30 nmol 3H-SAINT-C18-labelled anti-VCAM-1-SAINTPEGarg/PEG2% containing 120 pmol siRNA were administered to either healthy control mice or TNFα-challenged mice. Significant differences were observed between the blood disappearance of the two siRNA carriers. Values are presented as mean ± SD; n=4 mice/group. *, p <0.05 compared to healthy control mice.

Since PEGylation of IgG-SAINTPEGarg improved the pharmacokinetic behavior of the lipoplex, we set out to investigate the *in vivo* behavior of antibody-SAINTPEGarg/PEG2% equipped with anti-VCAM-1 antibody as a targeting moiety in TNFα-challenged mice. TNFα is a well-characterized cytokine and a potent activator of the inflammatory response in the vasculature. Intravenous injection of TNFα results in a pronounced acute VCAM-1 expression (2 h post challenge) in different endothelial subsets of major organs, including in lungs, as compared to healthy control mice [27]. Like IgG-SAINTPEGargs, ^{3}H-SAINT-C18-labelled anti-VCAM-1-SAINTPEGarg/PEG2% in control mice and anti-VCAM-1-SAINTPEGarg/PEG2% in TNFα-challenged mice disappeared from the blood in a biphasic manner and significant differences between the blood disappearances were observed (fig. 2). Strikingly, plasma clearance and volume of distribution of anti-VCAM-1-SAINTPEGarg/PEG2% were significantly lower in TNFα-challenged mice than in healthy control mice (table 3), which was paralleled by an increased circulation half-life.

Biodistribution of antibody-SAINTPEGargs/PEG2%

To study if antibody-SAINTPEGargs/PEG2% displayed increased accumulation in organs of TNFα-challenged mice, reflected by an increased targeting ratio (% of injected dose (ID)/gram organ of anti-VCAM-1-SAINTPEGarg/PEG2% divided by % of ID/gram organ of IgG-SAINTPEGarg/PEG2%), ^{3}H-labelled antibody-SAINTPEGargs/PEG2% were i.v. injected in control and TNFα-challenged mice. At 4 h, accumulation of anti-VCAM-1-SAINTPEGarg/PEG2% in TNFα-activated lungs was 2.5 times higher than that of IgG-SAINTPEGarg/PEG2%, displaying the highest targeting ratio of all organs analyzed (fig. 3). Accumulation in liver and spleen (% of ID/gram organ) was the highest of all organs irrespective the state of activation, however, in these organs

Table 3. Pharmacokinetic parameters of PEGylated anti-VCAM-1-SAINTPEGarg in healthy and TNFα-challenged mice

Parameter	anti-VCAM-1-SAINTPEGarg/PEG2%	anti-VCAM-1-SAINTPEGarg/PEG2% + rmTNFα
CL, ml/min/g	0.0115 ± 0.0008	0.0066 ± 0.0006*
V, ml/g	1.62 ± 0.10	1.26 ± 0.02*
$t_{1/2}$ (2), min	97 ± 6	133 ± 7*

Values represent mean values ± SD of 4 mice/group. CL, plasma clearance; V, volume of distribution; $t_{1/2}$ (2), elimination half-life. *, $p < 0.05$ compared to healthy mice.

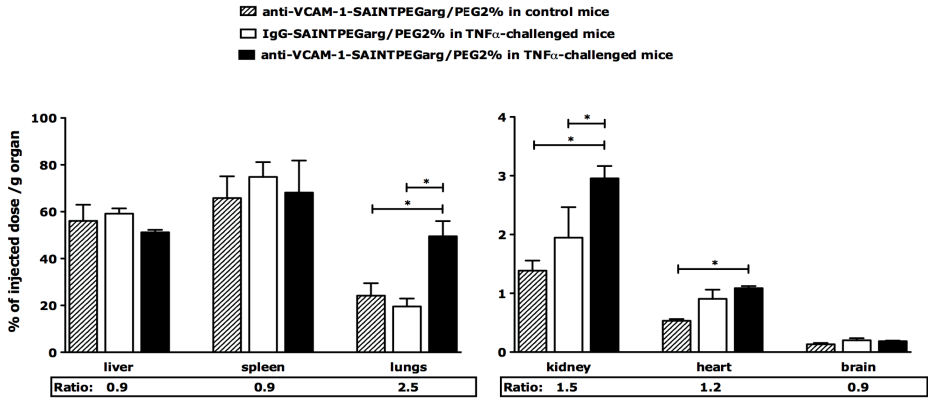


Fig. 3. Increased accumulation of anti-VCAM-1-SAINTPEGarg/PEG2% in lungs and kidney of TNFα-challenged mice.
3H-SAINT-C18-labelled anti-VCAM-1-SAINTPEGarg/PEG2% and control IgG-SAINTPEGarg/PEG2% were administered to either control mice or to TNFα-challenged mice. At 4 h, the tissue distribution of 3H-SAINT-C18-labelled anti-VCAM-1-SAINTPEGarg/PEG2% and control IgG-SAINTPEGarg/PEG2% was analyzed. Ratio indicates targeting ratio in TNFα-challenged mice (%ID anti-VCAM-1-SAINTPEGarg/PEG2% / %ID IgG-SAINTPEGarg/PEG2%). Values are presented as mean ± SD; n=3-4 mice/group. *, p <0,05.

anti-VCAM-1-SAINTPEGarg/PEG2% did not provoke additional accumulation. Upon TNFα challenge, accumulation of anti-VCAM-1-SAINTPEGarg/PEG2% in kidney and heart was significantly higher when compared to healthy control mice, though, when compared to control IgG-SAINTPEGarg/PEG2% the differences in heart accumulation were not found to be significant. Accumulation of both antibody-SAINTPEGarg/PEG2% was low in the brain (≤ 0.2% ID/g organ) irrespective the state of activation. Recoveries (% of ID) of the ^{3}H-labelled antibody-SAINTPEGarg/PEG2% were consistent throughout the experiments and ranged from 71.6 ± 5.4% to 85.1 ± 5.7% (mean ± SD).

Antibody-SAINTPEGarg/PEG2% administration does not affect liver and kidney function parameters

Analysis of the biochemical blood parameters revealed that no loss in liver function was observed as a consequence of administration of antibody-SAINTPEGarg/PEG2%, as the liver enzymes ASAT, ALAT and ALP were not increased compared to those of vehicle treated mice (table 4). In addition, no differences in plasma levels of creatinine and urea were observed in mice subjected to either vehicle or antibody-SAINTPEGarg/PEG2%, indicating maintenance of regular kidney function. Furthermore, no changes in the weight of the animals were observed (data not shown).

Table 4. Effect of antibody-SAINTPEGarg/PEG2% administration on liver and kidney function parameters

Treatment	ASAT (U/l)	ALAT (U/l)	ALP (U/l)	Creatinine (µmol/l)	Urea (mmol/l)
Healthy control	41.6 ± 11.6	21.5 ± 4.5	70.0 ± 11.4	13.4 ± 5.2	9.7 ± 0.8
Vehicle	31.0 ± 2.5	21.8 ± 1.9	75.8 ± 6.1	14.8 ± 1.1	9.5 ± 0.7
IgG-SAINTPEGarg/PEG2% *siVE-cad*	30.0 ± 2.5	20.8 ± 1.9	71.2 ± 7.4	14.2 ± 2.1	9.4 ± 1.0
anti-VCAM-1-SAINTPEGarg/PEG2% *siVE-cad*	32.5 ± 8.2	22.5 ± 9.1	70.7 ± 8.5	14.2 ± 1.5	9.1 ± 0.5
anti-VCAM-1-SAINTPEGarg/PEG2% *siControl*	31.0 ± 8.2	19.7 ± 5.4	66.8 ± 7.3	11.5 ± 2.2	8.6 ± 1.1

Blood samples were taken from healthy control and TNFα-challenged mice i.v. injected with either vehicle or antibody-SAINTPEGarg/PEG2% and analyzed for blood levels of the enzymes alanine aminotransaminase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), creatinine, and urea. Values are presented as mean ± SD; n=5-6 mice/group. siVE-cad; siRNA against mouse VE-cadherin, siControl; negative control siRNA.

Anti-VCAM-1-SAINTPEGarg/PEG2% home to VCAM-1 positive cells in TNFα-challenged mice and co-localize with vWF

To determine the intra-organ localization of antibody-SAINTPEGarg/PEG2%, immunohistochemical analysis was performed on lung tissue of TNFα-challenged mice 2 h after intravenous administration of the targeted and control lipoplexes (fig. 4A). Lungs were chosen as the organ of interest based on the biodistribution study, which showed an accumulation of the VCAM-1 targeted lipoplexes of 50% of ID/g lung. Immunohistochemical staining demonstrated that i.v. injected anti-VCAM-1-SAINTPEGarg/PEG2% predominantly homed to VCAM-1 positive postcapillary venules (fig. 4B), whereas IgG-SAINTPEGarg/PEG2% was not unambiguously associated with VCAM-1 positive venules. Immunohistochemistry did not reveal any indication of aggregation of antibody-SAINTPEGarg/PEG2% in lung tissue. Comparable homing of anti-VCAM-1-SAINTPEGarg/PEG2% in relation to IgG-SAINTPEGarg/PEG2% was observed in kidney glomeruli of TNFα-challenged mice (supplementary fig. 1).

Confocal laser scanning microscopy (CLSM) was performed to study the intra-pulmonary co-localization of anti-VCAM-1-SAINTPEGarg/PEG2% with vWF positive endothelial cells in TNFα-challenged mice. CLSM revealed that anti-VCAM-1-SAINTPEGarg/PEG2% co-localized with endothelial cells positive for vWF (fig. 5A) in postcapillary venules of TNFα-challenged mice, 2 hours after i.v. administration. Since TNFα was used to induce systemic inflammation and leukocytes (including monocytes/macrophages) are among the most abundant cells in the lungs [28], we studied whether leukocytes played a role in the accumulation of antibody-

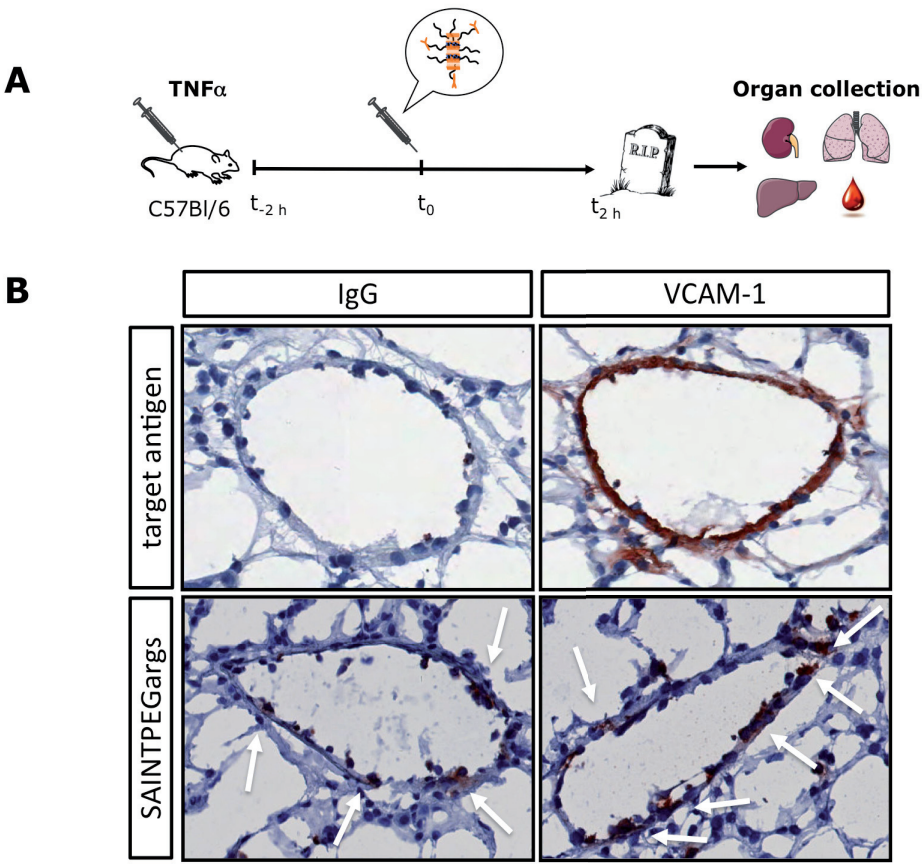
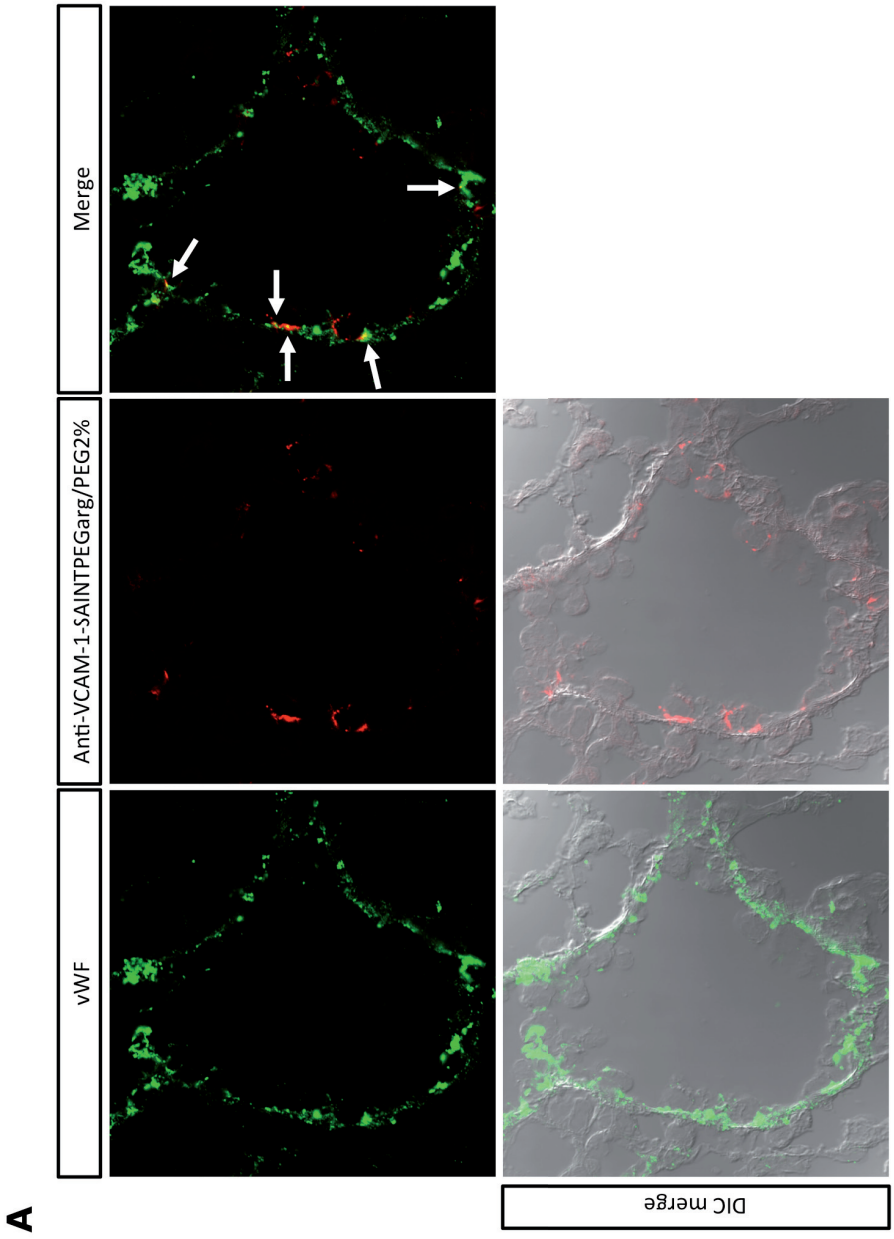
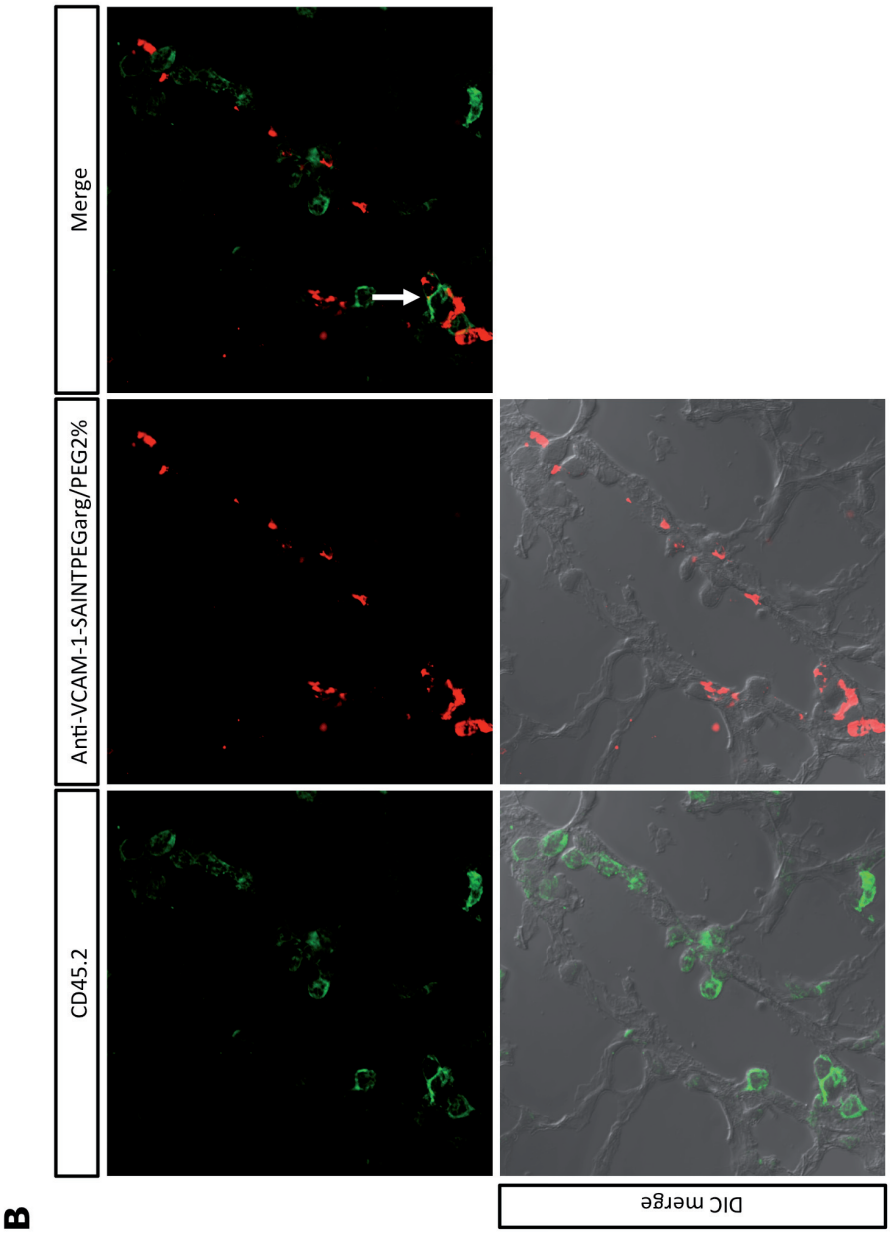


Fig. 4. The intra-pulmonary localization of anti-VCAM-1-SAINTPEGarg/PEG2% corroborates the expression pattern of VCAM-1 protein *in vivo* in lungs of TNFα-challenged mice.
(A) Representation of the experimental setup and timeline. (B) Immunohistochemical detection of anti-VCAM-1-SAINTPEGargs formulated with 2 mol% PEG after i.v. injection into TNFα-challenged mice. White arrows point at side of localization. Original magnification 200x. Representative images from 3 mice/group.

SAINTPEGarg/PEG2% in lungs. Some co-localization of anti-VCAM-1-SAINTPEGarg/PEG2% with leukocytes was observed (fig. 5B top), which is more clearly illustrated by the DIC merge (fig. 5B bottom). Anti-VCAM-1-SAINTPEGargs/PEG2% administration did not affect the overall picture of CD45.2 positive cells that infiltrate in lung venules of TNFα-challenged mice (fig. 5C).


A
vWF
Anti-VCAM-1-SAINTPEGarg/PEG2%
Merge
DIC merge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CD45.2
Anti-VCAM-1-SAINTPEGarg/PEG2%
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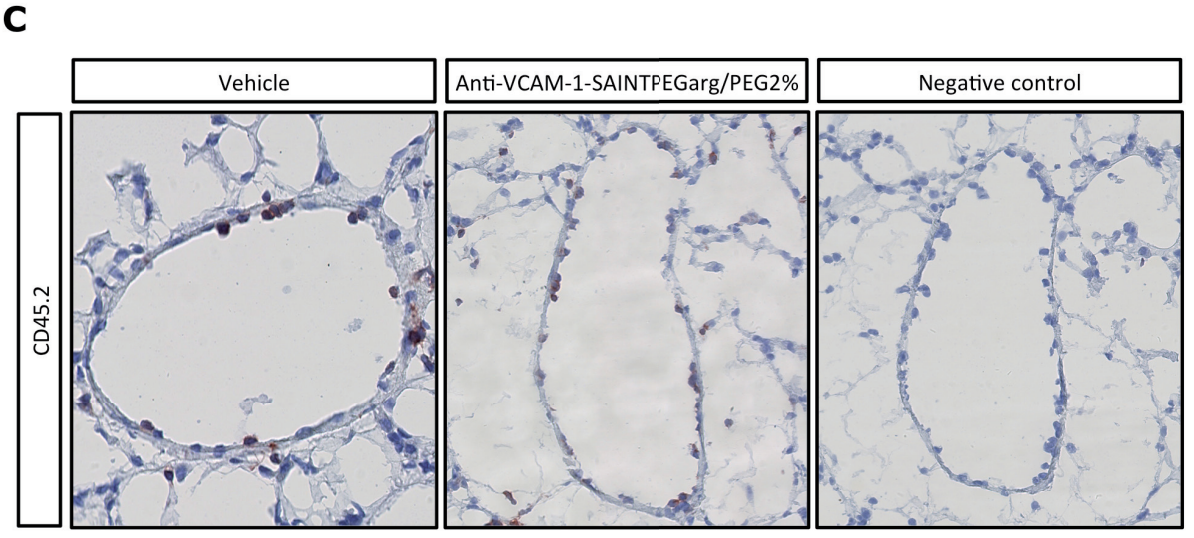

Fig. 5. PEGylated anti-VCAM-1-SAINTPEGarg localize in lung postcapillary venules of TNFα-challenged mice.
Confocal microscopy images of double immunofluorescent staining for: (A) endothelial marker vWF (green) and anti-VCAM-1-SAINTPEGarg/PEG2% (red), (B) leukocyte common antigen CD45.2 (green) and anti-VCAM-1-SAINTPEGarg/PEG2% (red), both 2 h after i.v. injection of anti-VCAM-1-SAINTPEGarg/PEG2%. White arrows point at site of co-localization. Data sets show representative images from 2 mice/group. (C) Anti-VCAM-1-SAINTPEGarg/PEG2% did not affect the overall picture of CD45.2 positive cells that infiltrate *in vivo* in lungs of TNFα-challenged mice, 2 h after i.v. injection of either vehicle or anti-VCAM-1-SAINTPEGarg/PEG2%. Presented data sets show representative images from 3 mice/group.

Anti-VCAM-1-SAINTPEGarg/PEG2% does not attenuate VE-cadherin gene expression in lung tissue

To investigate whether the delivery of siRNA by anti-VCAM-1-SAINTPEGarg/PEG2% results in *in vivo* down-regulation of the endothelial-restricted target gene VE-cadherin, antibody-SAINTPEGarg/PEG2% carrying VE-cadherin or negative control siRNA were i.v. administered in TNFα-challenged mice. Lungs were harvested 48 h later and VE-cadherin mRNA levels were analyzed. Neither formulation significantly down-regulated VE-cadherin gene expression in lungs (fig. 6A). The expression of an unrelated gene, Tie-2, and the endothelial-restricted gene CD31 were analyzed as control genes and were also not altered by anti-VCAM-1-SAINTPEGarg/PEG2%. In mice treated with IgG-SAINTPEGarg/PEG2%, comparable effects on gene expression were observed (fig. 6A). To correct for possible differences in the number of (micro) vessels present in the examined lung tissues, we normalized VE-cadherin gene expression to the constitutive expression of CD31. The normalization procedure did not alter the outcome that the siRNA-carrier devices did not affect target gene expression (fig. 6B).

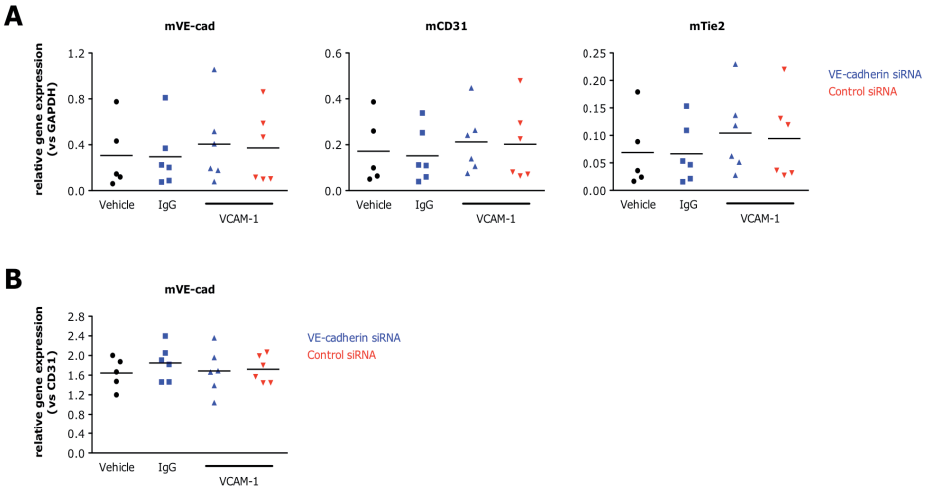

Fig. 6. Effects of single i.v. administration of antibody-SAINTPEGarg/PEG2% containing siRNA$_{VE\text{-}cadherin}$ on VE-cadherin gene expression in lungs.
Control IgG and anti-VCAM-1 conjugated SAINTPEGarg/PEG2% containing siRNA$_{VE\text{-}cadherin}$ or control siRNA were i.v. injected (250 nmol TL containing 0.033 mg siRNA/20 g body weight) into TNFα-challenged mice. 48 h later, mice were sacrificed and gene expression was assessed by real-time PCR. (A) Gene expression was analyzed in whole lung isolates. (B) VE-cadherin gene expression was normalized to the expression of the endothelial-restricted gene CD31 to correct for endothelial context of the sample. Data are presented as mean values ± SD. n=5-6 mice/group.

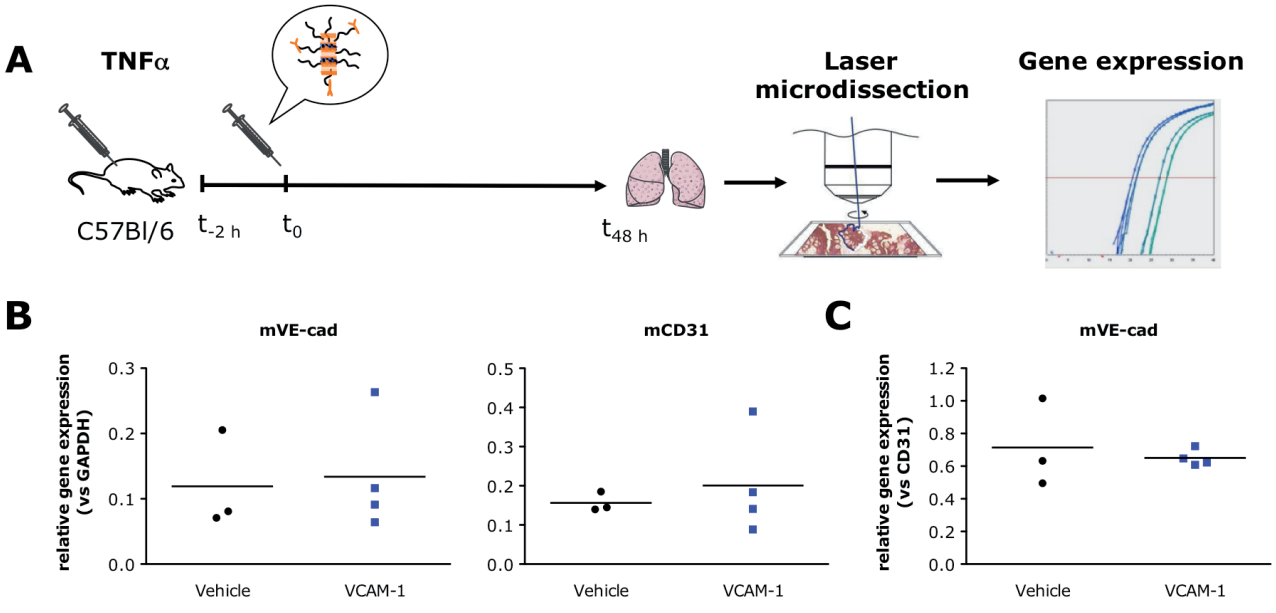

Fig. 7. VE-cadherin gene expression in laser microdissected lung postcapillary venules after administration of anti-VCAM-1-SAINTPEGarg/PEG2%.
Anti-VCAM-1-SAINTPEGarg/PEG2% containing VE-cadherin siRNA were injected (250 nmol TL containing 0.033 mg siRNA/20 g body weight) into TNFα-challenged mice. After 48 h, mice were sacrificed and gene expression was assessed by real-time PCR. (A) Representation of the experimental setup and timeline. (B) VE-cadherin gene expression normalized to GAPDH. (C) Normalization to the endothelial-restricted gene CD31 to correct for the endothelial context of the laser microdissected samples. Data are presented as mean values ± SD. n=3-4 mice/group.

As venules positive for anti-VCAM-1-SAINTPEGarg/PEG2% are numerically underrepresented compared to the capillaries, endothelial subset-specific delivery and down-regulating capabilities of anti-VCAM-1-SAINTPEGarg/PEG2% could be masked in whole lung mRNA isolates by an excess of non-targeted endothelial cell derived mRNA. To overcome this, venules were laser microdissected based on morphology in Hematoxylin stained sections since antigen-based protocols did not allow for proper visualization with maintenance of RNA integrity (fig 7A). Yet, also in laser microdissected lung venules no differences in VE-cadherin gene expression were observed (fig. 7B), whether or not the samples were normalized to endothelial enrichment as assessed by CD31 mRNA analysis (fig. 7C).

Discussion

Endothelium is an attractive target for anti-inflammatory therapy due to its active engagement and pivotal role in the pathophysiology of inflammatory diseases. The interest to therapeutically target inflammatory processes using siRNA as pharmacological compound is triggered by a devastating increase in widespread chronic inflammatory diseases [29]. A selective drug delivery system is essential to circumvent targeting non-diseased endothelial cells. In a recent study, we designed a novel generation of targeted PEGylated SAINT-based lipoplexes, so-called antibody-SAINTPEGargs, which showed superior siRNA delivery capacity and effective down-regulation of VE-cadherin gene expression *in vitro* in inflammation-activated endothelial cells of different vascular origins [20]. This provided an opportunity for further studies of PEGylated antibody-SAINTPEGarg for siRNA delivery to specific disease-associated endothelial cell subsets *in vivo*. In the present study we thus describe the *in vivo* behavior of siRNA containing PEGylated antibody-SAINTPEGarg, equipped with antibodies recognizing the inflammatory adhesion molecule VCAM-1, so-called anti-VCAM-1-SAINTPEGarg/PEG2%. These anti-VCAM-1-SAINTPEGarg PEG2% showed increased accumulation in lung and kidney of TNFα-challenged mice, without affecting liver and kidney function parameters. Lungs were next chosen as the organ of further study based on the high accumulation of anti-VCAM-1-SAINTPEGarg/PEG2%. Immunohistochemical staining demonstrated homing of i.v. injected anti-VCAM-1-SAINTPEGarg/PEG2% to VCAM-1 protein expressing vascular beds, and co-localization with endothelial cells in lung postcapillary venules of TNFα-challenged mice. This selective accumulation of anti-VCAM-1-SAINTPEGarg/PEG2% carrying VE-cadherin siRNA did however not significantly affect VE-cadherin gene expression.

From the above we conclude that anti-VCAM-1-SAINTPEGarg/PEG2% are not suitable for effective siRNA delivery to inflamed vascular lung endothelium *in vivo*. Despite, we recently showed that antibody-SAINTPEGarg/PEG2% were capable of functionally delivering siRNA *in vitro* into primary endothelial cells. Differences in the intracellular trafficking and processing in endothelial cells *in vitro* and *in vivo* possibly underlie the absence of down-regulation of VE-cadherin mRNA levels *in vivo*. This emphasizes the importance of evaluating siRNA delivery systems in an appropriate *in vivo* animal model at an early stage in their development. It furthermore calls for development of innovative techniques that enable quantification of intracellular trafficking of siRNA delivery systems in (micro)vascular endothelial cells.

Low mol% of PEG-conjugated lipids in siRNA-lipoplex formulations are sufficient to reduce unspecific toxic side effects *in vivo*, and to maintain siRNA-mediated gene silencing [30]. Furthermore, we showed that exogenous addition of 2 mol% PEG has a positive effect on their physicochemical properties [20] and pharmacokinetic behavior (this study). The lower blood disappearance of IgG-SAINTPEGarg/PEG2% is in line with initial reports that PEG reduces opsonization by serum proteins, thereby minimizing clearance by the RES [25, 26]. The effect of PEGylation on the pharmacokinetic behavior is very modest and most likely of no real biological significance since PEGylation of IgG-SAINTPEGarg did not affect the elimination half-life ($t_{1/2}$ (2)) of the SAINT-based lipoplex compared to its non-PEGylated counterpart.

Based on prior observations that VCAM-1 was heterogeneously upregulated in various organs 2 h post TNFα challenge, we investigated the *in vivo* behavior of anti-VCAM-1-SAINTPEGarg/PEG2% in TNFα-challenged mice. Strikingly, plasma clearance and volume of distribution of anti-VCAM-1-SAINTPEGarg/PEG2% was significantly lower in TNFα-challenged mice than in healthy control mice, which was paralleled by an increased elimination half-life. These findings are contradictory to the results reported by Ásgeirsdóttir *et al.* [23]. In this study, anti-E-selectin equipped liposomes demonstrated an increased blood disappearance compared to control liposomes. Whether the physicochemical difference in delivery system formulation (liposomes vs. lipoplexes), or the specificity of the targeting moiety (E-selectin vs. VCAM-1) plays a role in the here observed lower blood clearance in TNFα-challenged mice and its biological relevance, needs to be elucidated.

After a single dose (250 nmol TL containing 0.033 mg siRNA/20 g body weight) of antibody-SAINTPEGarg/PEG2% to TNFα-challenged mice, no changes in parameters of liver and kidney function were observed. The absence of toxic effects of antibody-SAINTPEGarg/PEG2% is likely a consequence of the presence of the non-toxic

cationic amphiphilic lipid SAINT-C18 [18], as toxicity is often attributed to the type and the content of cationic lipid in the formulation. Moreover, the neutral helper lipid DOPE (present in the antibody-SAINTPEGarg/PEG2% formulation) has been reported to play an important role in preventing toxicity [31, 32].

Pulmonary drug delivery employing lipid-based nanoparticles containing cationic lipids is often mediated by serum-induced aggregation of lipid-based nanoparticles. Subsequently, the large-size aggregates become entrapped in the pulmonary microvasculature since the lung is the first large capillary bed the particles encounter [33]. Immunohistochemistry demonstrated that no aggregation of antibody-SAINTPEGarg/PEG2% was observed in the pulmonary microvasculature indicating that the 50% of ID/g lung was most likely not caused by mechanical uptake e.g., aggregation. Yet, this phenomenon cannot be completely excluded.

VCAM-1 is specifically upregulated at sites of pathological inflammation though the approximate copies of VCAM-1 on the endothelial surface *in vivo* are to our knowledge not known. Other targets successfully used for targeting anti-inflammatory agents are intercellular adhesion molecule-1 (ICAM-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) [34]. Endothelial cells constitutively expresses approximately 1.5-2.5×10^5 copies of ICAM-1 [35] and 0.5-1.5×10^6 copies of PECAM-1 [36], respectively, on the surface of one cell. When comparing gene expression levels in lung, VCAM-1 expression is considerably lower than ICAM or PECAM. Upon injection of TNFα the expression of VCAM-1 and ICAM-1 mRNA were upregulated in the same range [37]. In lung VCAM-1 was clearly (over)expressed in arterioles and venules, while ICAM-1 differences could scarcely be visualized due its high expression levels under basal conditions indicating more specificity [37, 38]. Therefore, the increased accumulation and the localization of anti-VCAM-1-SAINTPEGarg/PEG2% in the lungs is likely to be attributed to specific antigen-antibody interaction, which is in line with the TNFα-induced VCAM-1 protein expression on lung endothelium.

The presence of IgG-SAINTPEGarg/PEG2% in lung venules may be attributed to passive accumulation at the site of inflammation due to an increase in local inflammation-associated vascular permeability, as described for tumor vasculature [39]. With CLSM we showed that anti-VCAM-1-SAINTPEGarg/PEG2% co-localized with endothelial cells in lung venules of TNFα-challenged mice and that leukocytes most likely do not contribute to the increased accumulation, as anti-VCAM-1-SAINTPEGarg/PEG2% were hardly co-localizing with CD45.2 positive cells.

Based on the increased homing of anti-VCAM-1-SAINTPEGarg/PEG2% to VCAM-1

positive lung vasculature and its co-localization with endothelial cells, anti-VCAM-1-SAINTPEGarg/PEG2% was applied as siRNA carrier to explore endothelial-specific gene down-regulation. The endothelial-restricted gene VE-cadherin was selected as a target gene for the siRNA since its expression is not influenced by TNFα stimulation [40]. PEGylated anti-VCAM-1-SAINTPEGarg containing VE-cadherin specific siRNA could however not achieve down-regulation of VE-cadherin mRNA in lungs of TNFα-challenged mice. This was an unexpected result considering the high (50% ID/g) and endothelial-specific association of anti-VCAM-1-SAINTPEGarg/PEG2% in lungs. However, this could also be attributed to inadequate siRNA concentrations at the side of interest since many delivery systems designed for *in vivo* application show silencing in μM range [41, 42]. In the kidney and liver the carriers were also devoid of VE-cadherin down-regulation (data not shown). Laser microdissection in combination with quantitative RT-PCR enabled analysis of vascular bed-specific pharmacological effects, which could be otherwise masked in whole lung mRNA isolates. Yet, we did not observe down-regulation of VE-cadherin gene expression in laser microdissected lung isolates either. However, recently we demonstrated that antibody-SAINTPEGarg/PEG2% are effective devices for specific and functional delivery of siRNA into inflammation-activated primary endothelial cells *in vitro* [20]. Therefore, differences in the internalization and intracellular trafficking and processing in cells *in vitro* and *in vivo* possibly underlie the absence of siRNA-mediated VE-cadherin down-regulation *in vivo*. Masson and co-authors demonstrated that by incorporating acid-sensitive PEG-orthoester-lipid conjugates into lipoplexes a significant higher delivery efficiency was achieved than with pH-insensitive analogs [43]. Recently, orthoester-based pH-sensitive lipoplexes demonstrated efficient nucleic acid delivery both in cultured cells and *in vivo* using a cationic and acid-labile lipid [44]. Incorporation of these acid-sensitive PEG analogs could improve the siRNA release of PEGylated antibody-SAINTPEGargs. In addition, the size of the positively charged antibody-SAINTPEGarg/PEG2%, may be increased in the circulation through non-specific electrostatic interactions with e.g., blood components. It has been shown that particle size has a profound impact on the internalization pathway [45, 46]. Furthermore, when lipoplexes reach their sites of action, a too tight complex can be a problem if not de-packaged appropriately allowing bioavailability of the cargo at therapeutic sites. This "package and release dilemma" accounts for the lack of effectiveness of many drug delivery systems [47] and will need further attention for the targeted lipoplexes reported here.

Conclusion and future perspectives

RNAi holds enormous potential both as a tool in molecular biology and as a powerful therapeutic agent. Currently, however, questions remain with regard to the applicability of RNAi in medicine due to difficulties in delivering the drug to the cells of interest while maintaining its activity and avoiding toxicity. In this study we examined the potential of recently designed PEGylated SAINT-based lipoplexes called anti-VCAM-1-SAINTPEGarg/PEG2% for *in vivo* siRNA delivery into inflammation-activated endothelial cells. We showed increased homing of anti-VCAM-1-SAINTPEGarg/PEG2% to lung postcapillary venules, which followed the increased *in vivo* VCAM-1 protein expression in this particular vascular segment. However, despite detectable accumulation in VCAM-1 positive venules, anti-VCAM-1-SAINTPEGarg/PEG2% did not affect VE-cadherin gene expression. More information on the molecular mechanisms underlying the *in vivo* intracellular trafficking and processing of anti-VCAM-1-SAINTPEGarg is needed to shed more light on the absence of detectable gene silencing, thereby allowing formulation optimization and improvement of effective *in vivo* siRNA delivery to silence target genes involved in the inflammatory process.

Acknowledgements

We thank Arjen H. Petersen and Henk Moorlag for excellent technical assistance. We thank Eduard G. Talman for providing high quality SAINT-C18 suitable for tritium-labelling and Dr. Hans Proost (Department of Pharmacokinetics and Drug Delivery, Groningen Research Institute for Pharmacy, University of Groningen) for advice with regard to the pharmacokinetic analysis. Microscopic fluorescence imaging was performed at the UMCG Microscopy & Imaging Center (UMIC) that is supported by the Netherlands Organization for Health Research and Development (ZonMW grant 40-00506-98-9021). This research was supported by the European Fund for Regional Development (EFRO) from the European Union (project NTS 068 and 073 Drug Delivery and Targeting), and the J.K. de Cock foundation. The authors declare no competing financial interests. M.H.J. Ruiters is CEO of Synvolux Therapeutics.

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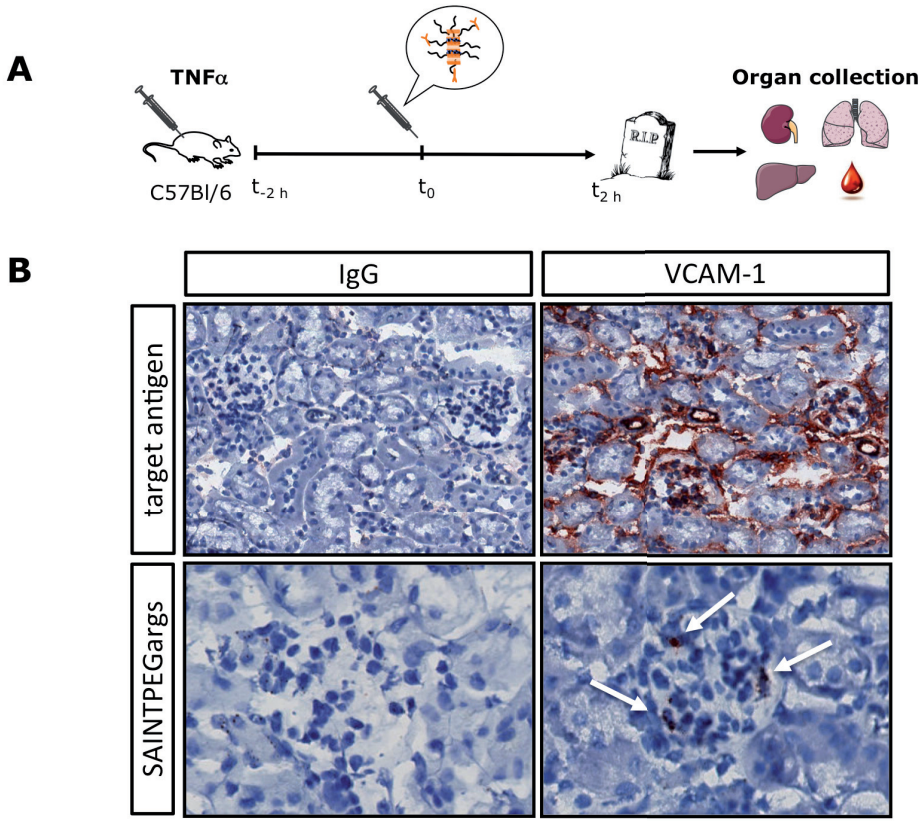
Supplementary figures



Supplementary Fig. 1. The intra-organ localization of anti-VCAM-1-SAINTPEGarg/PEG2% in kidney of TNFα-challenged mice.
(A) Representation of the experimental setup and timeline. (B) Immunohistochemical detection of anti-VCAM-1-SAINTPEGarg/PEG2% after i.v. injection into TNFα-challenged mice. White arrows point at site of localization. Original magnification 200x. Representative images from 3 mice/group.

Chapter 5

SAINT-based siRNA lipoplexes: how to improve their efficiency?

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WORK IN PROGRESS

Abstract

Adhesion molecules like E-selectin and vascular cell adhesion molecule-1 (VCAM-1) are mainly expressed after endothelial activation, which makes them suitable target epitopes for targeting drugs to inflamed endothelial cells. Moreover, they are internalized upon ligand binding. By conjugating antibodies recognizing these adhesion molecules to siRNA containing lipoplexes so-called antibody-SAINTPEGargs will result. These anti-E-selectin- and anti-VCAM-1-SAINTPEGarg were shown to be specifically internalized into inflammation-activated endothelial cells, yet there effectiveness was limited. To improve the siRNA delivery efficacy of antibody-SAINTPEGargs into inflammation-activated endothelial cells, Calf Thymus-DNA (CT-DNA) was incorporated in the formulation. CT-DNA allows condensation into small nanometric particles when it is complexed with a cationic component like SAINT-C18. Additionally, incorporation of CT-DNA could result in a facilitated release of siRNAs. Although anti-E-selectin- and anti-VCAM-1-SAINTPEGargs were reduced in size upon CT-DNA incorporation, no beneficial effects of this adaptation in siRNA association (binding and uptake) and gene down-regulation were observed. As a second modification, we equipped SAINTPEGargs with both anti-E-selectin and anti-VCAM-1 antibodies in various ratios. We hypothesized that targeting multiple, transiently-upregulated cell adhesion molecules on endothelial cells could enhance binding specificity and cellular uptake of siRNA delivery systems. Yet, also with these systems, no improved siRNA association was observed compared to single targeted antibody-SAINTPEGargs. A temporal targeting approach was explored with anti-E-selectin- and anti-VCAM-1-SAINTPEGarg, the target cell of interest, E-selectin or VCAM-1, respectively, was targeted in time. However, the association of siRNA with TNFα-activated endothelial cells was not increased by antibody-SAINTPEGarg using such a temporal targeting approach. Taken together, neither antibody-SAINTPEGargs containing CT-DNA nor the applied dual targeting or temporal targeting approach was capable of functional improving the delivery of siRNA into TNFα-activated endothelial cells *in vitro*

Introduction

Nucleic acids such as short interfering RNAs (siRNAs) are a promising new class of therapeutics facilitating specific interference with (disease-associated) gene expression [1]. However, uncomplexed or unmodified siRNAs are subjected to rapid clearance from the circulation by the liver and renal filtration, and are rapidly degraded by serum RNases, which limits their potential for *in vivo* applications [2]. The potential of siRNA to silence disease-associated genes ensures future RNA interference (RNAi)-based therapeutic interventions. Though RNAi is not yet an established therapeutic modality because of delivery-related problems. RNAi demands targeted delivery systems, which efficiently and selectively deliver and release siRNA into the cytoplasm of diseased target cells.

The pivotal role of endothelial cells in the patho(physio)logy of inflammatory diseases and cancer along with the (increased) expression of disease-associated molecular targets (e.g. E-selectin, VCAM-1, ICAM-1, $\alpha_v\beta_3$-integrins) on diseased endothelium raised interest in the development of siRNA delivery systems for selective therapeutic intervention in the diseased endothelium [3, 4]. We recently demonstrated specific delivery of siRNA into inflammation-activated endothelial cells using two types of lipoplexes based on the cationic amphiphilic lipid 1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride (SAINT-C18), called antibody-SAINTargs and antibody-SAINTPEGargs [5, 6]. For *in vivo* application addition of polyethylene glycol (PEG) to an siRNA carrier is often essential to avoid rapid clearance. Yet, PEGylation of these SAINT-based lipoplexes significantly reduces their siRNA delivery capacity *in vitro* [6]. Moreover, PEGylated anti-VCAM-1-SAINTPEGargs were not able to functionally deliver siRNA into inflammation-activated lung postcapillary venules [7]. In order to improve the siRNA delivery capacity and effectiveness of antibody-SAINTPEGargs into inflammation-activated endothelial cells, we here studied the effects of two modifications of antibody-SAINTPEGargs. We describe the modification of antibody-SAINTPEGargs by: 1) incorporation of Calf Thymus-DNA (CT-DNA) in the formulation and 2) harnessing antibody-SAINTPEGargs with both anti-E-selectin and anti-VCAM-1 antibodies. Furthermore, a temporal delivery strategy consisting of consecutive targeting E-selectin and VCAM-1, which both internalize antibody-SAINTPEGargs, was examined.

siRNA molecules are small, strong and rigid molecules, which might prevent complete condensation of the nucleic acid. This could lead to degradation of the formulations by macromolecules present in the biological environment, which subsequently results in reduced gene silencing efficacy. DNA can improve core

compaction resulting in smaller particles [8, 9]. siRNA and DNA share similar characteristics: 1) they are both double stranded, 2) they both have phosphodiester backbones with the same anionic charge to nucleotide ratio, and 3) they interact electrostatically with cationic components. The presence of DNA in cationic lipoplexes resulted in a homogeneous blend between nucleic acids and lipids resulting in a better availability and enhanced gene silencing effect of siRNA in a murine melanoma cell line [10]. Additionally, it was reported that incorporation of DNA in the lipoplex formulation provides a higher efficiency in gene silencing in melanoma cells with fewer lipids and less toxicity [11]. CT-DNA is a well-characterized kind of DNA [12] and has been used in other studies to improve cargo release. Since CT-DNA might improve core compaction and improve cargo release, we incorporated CT-DNA in the antibody-SAINTPEGarg formulation to improve its siRNA delivery capacity and effectiveness.

The spatiotemporal expression of cell adhesion molecules on endothelial cells (ECs) presents a targeting opportunity for inflamed endothelium [3]. E-selectin, VCAM-1, and ICAM-1 are upregulated on ECs upon activation with inflammatory cytokines [13]. Each of these molecules has been investigated for use in targeted drug delivery systems [14-16]. Limited research has been performed on targeted drug delivery systems equipped with multiple antibodies, while in theory, targeting multiple, transiently-upregulated cell adhesion molecules on ECs may increase binding specificity and cellular uptake of drug delivery systems. Moreover, targeting multiple receptors may modulate the *in vivo* biodistribution properties of the drug delivery system to prevent non-desired accumulation in regions of the body associated with low target expression [17]. It has been shown that liposomes harnessed with both anti-VCAM-1 and anti-E-selectin antibodies, exerted increased uptake by endothelial cells [18]. Microbubble contrast agents conjugated with dual targeting antibodies against VCAM-1- and P-selectin bound to cells *in vitro* under flow conditions almost twice as effectively as single targeting microbubbles [19]. Nanocarriers equipped with both anti-ICAM-1 and anti-TfR showed higher accumulation in liver, compared to single targeted nanocarriers [20]. Harnessing antibody-SAINTPEGargs with both anti-E-selectin and anti-VCAM-1 antibodies may enhance the siRNA delivery capacity of antibody-SAINTPEGargs and its effectiveness into inflammation-activated endothelial cells.

In a previous study, we successfully developed a novel PEGylated targeted SAINT-based lipoplex displaying siRNA delivery and gene silencing capacity in various primary endothelial cell types *in vitro* under physiological conditions [6]. From

studying *in vivo* siRNA delivery by these PEGylated anti-VCAM-1-SAINTPEGarg we concluded that anti-VCAM-1-SAINTPEGargs were not able to functionally delivery siRNA into inflamed vascular lung endothelium [7]. Since these anti-VCAM-1-SAINTPEGargs were effective in silencing VE-cadherin gene expression in primary endothelial cells *in vitro* and co-localized with endothelial cells in VCAM-1 positive lung postcapillary venules *in vivo*, improvement of the siRNA delivery capacity and the intracellular siRNA release (into cytoplasm) by antibody-SAINTPEGargs should be the main emphasis in further research. The aim of the presented work was thus to investigate approaches that could improve the siRNA delivery and effectiveness of antibody-SAINTPEGarg in inflammation-activated endothelial cells *in vitro* and provide an opportunity for further application of siRNA delivery to disease-associated endothelial cells *in vivo*.

Materials and Methods

Materials

SAINT-MIX consisting of the cationic amphiphilic lipid 1-methyl-4-(cis-9-dioleyl) methyl-pyridiniumchloride (SAINT-C18) and the neutral helper-lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in equimolar amounts, was purchased from Synvolux Therapeutics (Groningen, the Netherlands). 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). Antibody-PEG conjugates were synthesized and characterized as described previously [6]. 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 for size measurements. For siRNA delivery capacity studies, AllStars negative control siRNA labeled at the 5'-end of the sense strand with Alexa$_{488}$ (cat. no. 1027292) was used. Deoxyribonucleic acid from Calf Thymus-DNA (CT-DNA; cat. no. D-4522) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). All chemicals used were of the highest purity grade.

Endothelial cells

Primary Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from the Endothelial Cell Facility UMCG (Groningen, the Netherlands) and cultured in flasks (greiner bio-one) as described previously [6]. In all experiments HUVEC were used between passage 1 and 4.

Preparation of targeted lipoplexes and size measurement

Antibody-SAINTPEGarg containing siRNA were prepared as described previously [6]. Shortly, anti-E-selectin- or anti-VCAM-1-PEG conjugate was mixed with SAINT-MIX (15 nmol/ml) in a fixed 1:2,000 molar ratio. Subsequently, conjugate and lipids were vortexed for 5 s, complexed with siRNA (0.9 µg/ml) or CT-DNA/siRNA (0.9 µg/ml), incubated for 5 min at room temperature and added to the endothelial cell culture medium or subjected to size measurements.

CT-DNA and siRNA were mixed (w/w) without changing the total amount of nucleic acids (CT-DNA + siRNA; 0.9 µg/ml). The CT-DNA/siRNA ratios used were 0, 0.25, 0.50, 0.75 and 1. When higher CT-DNA/siRNA concentrations were used, SAINT-MIX and antibody-PEG conjugate were used according to the molar ratios indicated above.

Antibody-SAINTPEGargs equipped with various ratios of anti-E-selectin-/anti-VCAM-1-PEG conjugate were formulated in a similar manner as used for single targeted antibody-SAINTPEGargs. Antibody-PEG conjugates were mixed in ratios anti-E-selectin/anti-VCAM-1 of 1:0, 0:1, 1:1, 2:1, 4:1, 8:1, 1:4 and 1:2. The antibody-PEG conjugate:SAINT-MIX molar ratio was 1:2,000 with a final lipid concentration of 15 nmol/ml. Targeted lipoplexes were freshly prepared prior to each experiment and where appropriate, PEGylated by addition of DOPE-PEG_{2000} to the formulation at indicated mol% at the cost of DOPE.

Mean particle size of antibody-SAINTPEGargs containing various ratios CT-DNA/siRNA was analyzed by dynamic light scattering using a Nicomp submicron particle analyzer (model 380 ZLS, Santa Barbara, CA, USA). For this, antibody-SAINTPEGargs (15,000 pmol/ml lipid; 7.5 pmol/ml antibody-PEG conjugate; 0.9 µg/ml CT-DNA/siRNA) were prepared freshly in Milli-Q. Samples were briefly vortexed and the mean diameter was obtained from the volume distribution curves produced by the particle analyzer. After size measurements, the prepared antibody-SAINTPEGargs were stored at 4°C and particle size was measured again at day 3 and day 7.

Flow cytometric analysis of the association of targeted SAINT-based lipoplexes with HUVEC

To obtain identical cell densities at the start of the experiments, HUVEC were seeded at 15,000 cells/cm^2 one day prior to the experiment in gelatin coated 12-well tissue plates (Costar, San Diego, CA, USA). Resting and TNFα-activated cells were incubated with antibody-SAINTPEGarg containing Alexa$_{488}$-tagged control siRNA in the absence or presence of DOPE-PEG$_{2000}$ at the indicated mol%. After 3 h, cells were trypsinized and the cell suspension was transferred to 4 ml tubes (greiner bio-one). Subsequently, cells were washed with ice-cold FACS-buffer and fixed in PBS containing 1% paraformaldehyde. Fluorescence was acquired by flow cytometry (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.

Temporal targeting of anti-E-selectin- and anti-VCAM-1-SAINTPEGargs was performed as follows, half the amount of siRNA was encapsulated by anti-E-selectin-SAINTPEGarg and applied 2 h after TNFα-activation. The residual amount was encapsulated by anti-VCAM-1-SAINTPEGarg and supplied 6 h after initial TNFα-activation. Yet, after incubation with anti-E-selectin-SAINTPEGarg (3 h in total) the cells were washed with PBS to remove non-cell associated lipoplexes and supplied with fresh EC-medium, allowing ECs to process the anti-E-selectin-SAINTPEGarg preceding incubation (3 h in total) with anti-VCAM-1-SAINTPEGarg. Subsequently, siRNA association was evaluated by flow cytometry as described above.

Gene expression analysis by real-time RT-PCR

HUVEC were seeded at 15,000 cells/cm^2 in gelatin coated 12-well plates one day prior to experiments. Endothelial cells were activated with TNFα for 2 h (E-selectin targeting) or 6 h (VCAM-1 targeting). Antibody-SAINTPEGargs containing VE-cadherin specific siRNA were added to the cells at a total nucleic acid concentration of 0.9 µg/ml, unless stated otherwise, and incubated for 48 h. Next, lipoplexes were removed, cells were washed and RNA was isolated using the RNeasy Plus Mini 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 reaction on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The primers for VE-cadherin

(Hs00174344_m1), and GAPDH (Hs99999905_m1) were purchased as Assays-on-Demand (Applied Biosystems). For each sample, the real-time PCR reactions were performed at least in duplicate and the averages of the obtained C_t values were used for further calculations. GAPDH was used as reference gene to normalize gene expression levels giving the ΔC_t value. Relative mRNA levels were calculated by $2^{-\Delta Ct}$

Results

Effect of incorporating CT-DNA on antibody-SAINTPEGarg particle size

To study the influence of CT-DNA on antibody-SAINTPEGarg particle size, anti-E-selectin-SAINTPEGarg and anti-VCAM-1-SAINTPEGarg containing varying CT-DNA/siRNA ratios were prepared, and characterized by mean particle size. Anti-E-selectin- and anti-VCAM-1-SAINTPEGargs were freshly prepared and as expected, a higher ratio of CT-DNA to total nucleic acids resulted in a lower mean particle size, indicating an increase in complex condensation (fig. 1; day 1). Whereas CT-DNA alone (CT-DNA/siRNA ratio of 1) complexed with lipids into nanosized complexes, siRNA alone (CT-DNA/siRNA ratio of 0) did not, which illustrates the effect of (carrier) DNA for the complexation of lipids and nucleic acids into nanosized particles. Anti-E-Selectin-SAINTPEGargs with varying CT-DNA/siRNA ratios showed sizes between ± 250 nm (ratio 1) and 1500 nm (ratio 0), whereas anti-VCAM-1-SAINTPEGargs showed sizes between ± 250 nm (ratio 1) and 400 nm (ratio 0). A CT-DNA to nucleic acid ratio of 0.5 resulted in complexes with mean particle sizes of 300 nm for both anti-E-selectin and anti-VCAM-1 equipped SAINTPEGargs. Since the total amount of siRNA in each complex is preferred to be as high as possible, this CT-DNA to nucleic acid ratio of 0.5 was considered optimal for efficient and functional siRNA delivery. Yet, this still has to be validated by determining the *in vitro* siRNA association (consisting of binding and uptake) capability and effectiveness in inflammation-activated endothelial cells.

To study whether the prepared anti-E-selectin- and anti-VCAM-1- SAINTPEGargs were stable over time, size measurements were performed on day 3 and 7. Both antibody-SAINTPEGargs containing varying CT-DNA/siRNA ratios were stable in size for up to 7 days (fig. 1).

siRNA association capability of antibody-SAINTPEGargs containing different CT-DNA/siRNA ratios in HUVEC

To investigate whether CT-DNA affected the siRNA association capability, resting and TNFα-activated primary endothelial cells were incubated with anti-E-selectin-

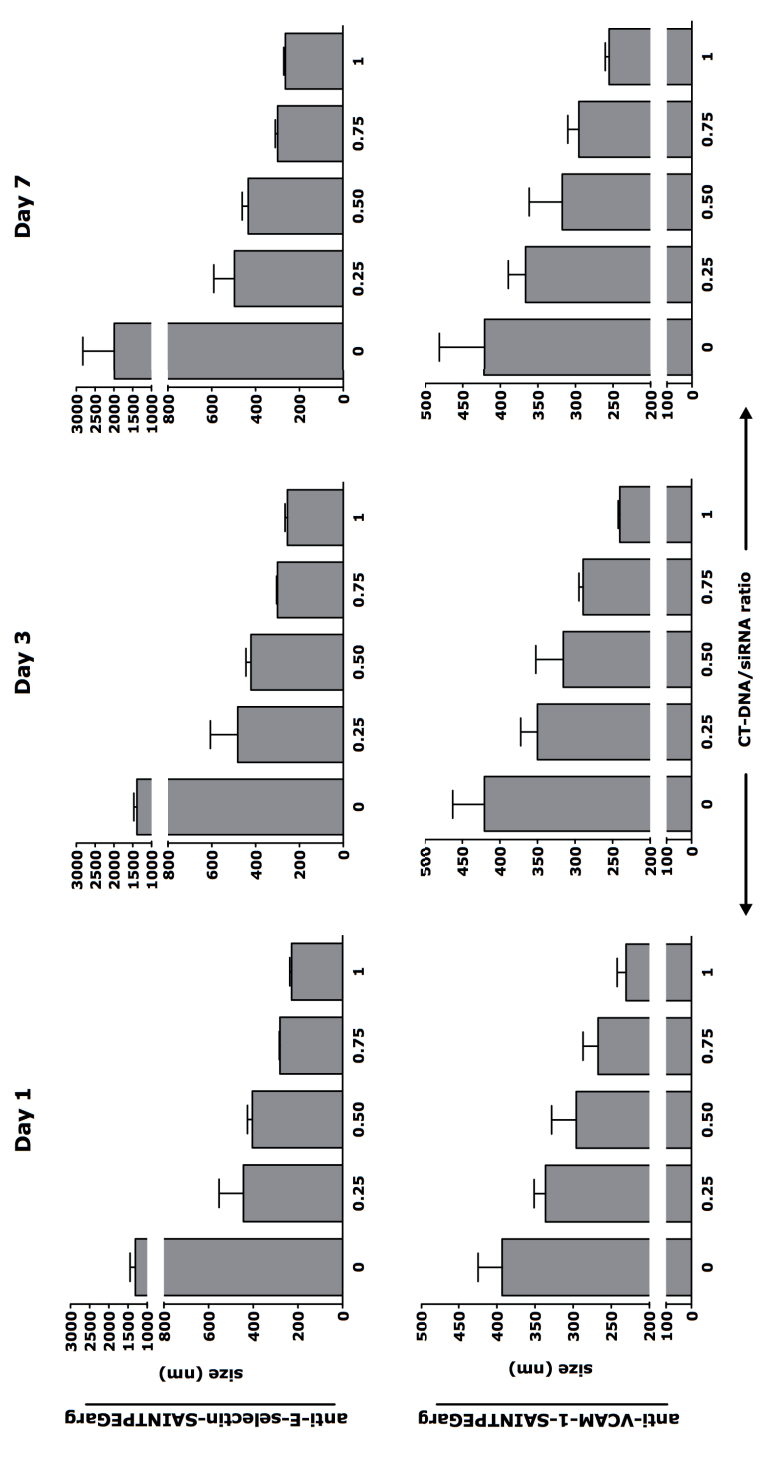

Fig. 1. Size of anti-E-selectin-SAINTPEGargs and anti-VCAM-1-SAINTPEGargs containing different CT-DNA/siRNA ratios upon preparation and in time. Particles were prepared in Milli-Q and particle size (nm) was measured on day 1, and during storage at 4°C on days 3 and 7. Data are presented as mean values ± range of 2 individual preparations.

and anti-VCAM-1-SAINTPEGargs containing varying ratios of CT-DNA to total nucleic acids. Anti-E-selectin- and anti-VCAM-1-SAINTPEGarg formulated with these varying CT-DNA/siRNA ratios showed a decrease in siRNA association capacity compared to antibody-SAINTPEGarg formulated with siRNA only (fig. 2). The siRNA association capacity of anti-E-selectin-SAINTPEGarg containing varying CT-DNA/siRNA ratios was enhanced when normalized to association by the siRNA only formulation (ratio 0), indicating that anti-E-selectin-SAINTPEGargs formulated with CT-DNA were more efficient than anti-E-selectin-SAINTPEGargs without CT-DNA (fig. 2 inset). In contrast, with anti-VCAM-1-SAINTPEGarg no increase in siRNA association was observed when the association of siRNA was normalized to association by the siRNA alone formulation (fig. 2 inset). No siRNA association was observed in the presence of a 100-fold excess of the appropriate parental antibody indicating that both antibody-SAINTPEGarg formulations are specifically bound and/or internalized by either E-selectin or VCAM-1, which is in line with previous observations [6].

Down-regulation of VE-cadherin gene expression in primary endothelial cells by antibody-SAINTPEGargs containing different CT-DNA/siRNA ratios

To investigate whether the improved siRNA association by anti-E-selectin-SAINTPEGarg containing different CT-DNA/siRNA ratios translates into down-

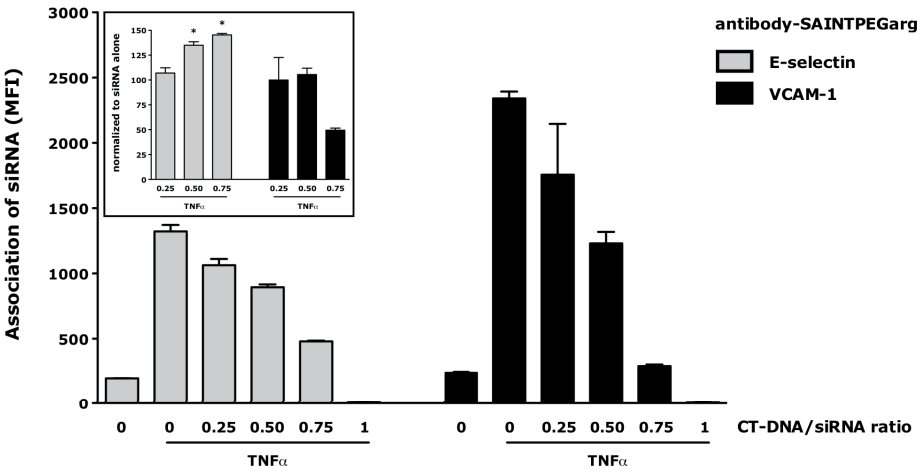

Fig. 2. siRNA association capacity of antibody-SAINTPEGargs containing different CT-DNA/siRNA ratios with HUVEC. Resting and TNFα-activated HUVEC were incubated for 3 h with anti-E-selectin-, respectively, anti-VCAM-1-SAINTPEGarg containing AlexaFluor$_{488}$ siRNA. The association of siRNA with the cells was quantified by flow cytometric analysis. Data are presented as mean florescence intensity (MFI) values ± range, n=2-3. Inset: siRNA association capacity of antibody-SAINTPEGargs containing different CT-DNA/siRNA ratios when normalized to siRNA alone (ratio 0).

regulation of the gene of interest, resting and TNFα-activated HUVEC were incubated with the respective antibody-SAINTPEGargs CT-DNA/siRNA formulations. The endothelial restricted gene VE-cadherin [21] was selected as a target gene for the siRNA since VE-cadherin gene expression is not influenced by TNFα stimulation [6]. Neither anti-E-selectin- nor anti-VCAM-1-SAINTPEGarg loaded with a total CT-DNA/VE-cadherin siRNA concentration of 1.8 µg/ml were capable of down-regulating VE-cadherin mRNA in activated HUVEC (fig. 3). This indicates that there is no beneficial effect of CT-DNA in gene silencing capacity. It should be kept in mind, though, that we recently showed that antibody-SAINTPEGargs containing only siRNA as cargo were not able to down-regulate VE-cadherin gene expression in HUVEC at siRNA concentrations of 4.5 µg/ml ≅ 300 pmol/ml [6]. It is highly likely that the CT-DNA/VE-cadherin siRNA concentrations used in this study were either too low to achieve down-regulation or that HUVEC are not the appropriate endothelial cell type to address this particular question.

siRNA association by antibody-SAINTPEGargs equipped with multiple ligands or association by a temporal targeting approach did not result in enhanced VE-cadherin down-regulation

Based on heterogeneous expression of E-selectin and VCAM-1 by inflammation-activated endothelial cells *in vivo*, dual targeting of both E-selectin and VCAM-1 by SAINTPEGargs could in theory result in increased siRNA uptake, thereby enhancing the siRNA-mediated gene silencing. To investigate the influence of the use of multiple

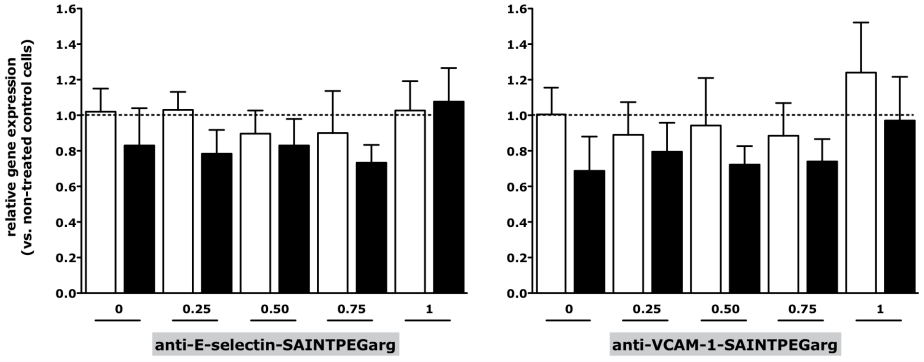

Fig. 3. Down-regulation of VE-cadherin gene expression is not enhanced by incorporation of CT-DNA in the antibody-SAINTPEGarg formulation. Antibody-SAINTPEGargs containing different CT-DNA/siRNA ratios were incubated for 48 h in resting (open bars) or TNFα-activated (black bars) HUVEC. The expression of VE-cadherin was measured by real-time RT-PCR as described. Data are presented as relative gene expression ± SD, n=3-4.

ligands on the siRNA association capability, SAINTPEGargs formulated with 2 mol% PEG were equipped with various ratios anti-E-selectin and anti-VCAM-1 antibodies. TNFα-activated primary endothelial cells were next incubated with these antibody-SAINTPEGargs and siRNA association was evaluated by flow cytometry as a function of the anti-E-selectin:anti-VCAM-1 ratio. In contrast to a previous publication showing that conjugating liposomes with both anti-VCAM-1 and anti-E-selectin antibodies, increased the uptake of immunoliposomes into endothelial cells compared to single targeted immunoliposomes [18], no differences in siRNA association were observed between SAINTPEGargs modified with anti-E-selectin:anti-VCAM-1 ratios when compared to anti-E-selectin-SAINTPEGarg (ratio 1:0, fig 4).

Secondly, a temporal targeting approach was explored with anti-E-selectin- and anti-VCAM-1-SAINTPEGargs, in which HUVEC were activated with TNFα for 2 h or 6 h depending on the target molecule of interest, E-selectin or VCAM-1, respectively. TNFα-activated primary endothelial cells were incubated with siRNA containing anti-E-selectin- or anti-VCAM-1SAINTPEGarg only at the indicated time-point, or with half the amount of siRNA encapsulated in anti-E-selectin-SAINTPEGarg and

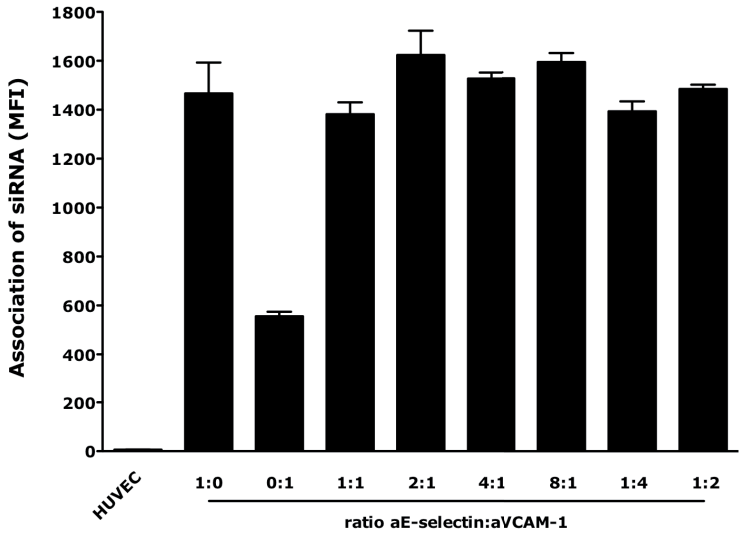

Fig. 4. HUVEC association of antibody-SAINTPEGargs chemically modified with various ratios of anti-(a)E-selectin and anti-(a)VCAM-1 antibodies. TNFα-activated HUVEC were incubated for 3 h with antibody-SAINTPEGargs formulated with 2 mol% PEG and conjugated with various ratios of anti-(a) E-selectin and anti-(a)VCAM-1 antibodies containing $AlexaFluor_{488}$ siRNA. Flow cytometry was performed to determine the cellular association. Data are presented as mean florescence intensity (MFI) values ± range of 2 individual experiments.

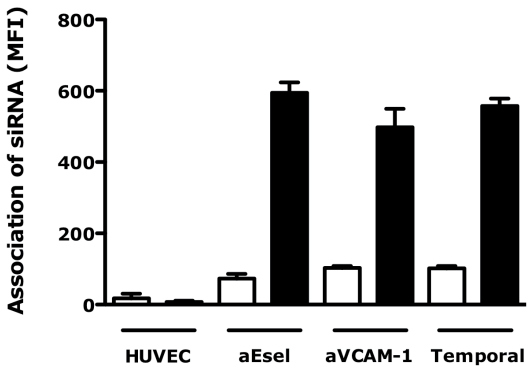

Fig. 5. Association of siRNA was not increased when delivered by antibody-SAINTPEGarg using a temporal targeting approach. Resting (open bars) and TNFα-activated (black bars) HUVEC were incubated for 3 h with antibody-SAINTPEGargs formulated with 2 mol% PEG containing $AlexaFluor_{488}$ siRNA as described in 'Materials and Methods'. With the temporal strategy, half the encapsulated amount of siRNA was delivered by anti-(a)E-selectin-SAINTPEGarg 2 h after TNFα-activation and the residual amount by anti-(a)VCAM-1-SAINTPEGarg at 6 h after TNFα-activation. Flow cytometry was performed to determine the cellular association. Data are presented as mean florescence intensity (MFI) values ± SD, n=3.

applied 2 h after TNFα-activation and with the other half formulated in anti-VCAM-1-SAINTPEGarg and supplied 6 h after initial TNFα-activation. The association of siRNA with TNFα-activated HUVEC was not increased by antibody-SAINTPEGarg using such a temporal targeting approach (fig. 5).

When studying the effects of either multiple ligands or temporal targeting on the siRNA association capability, neither the siRNA association by antibody-SAINTPEGargs containing various ratios of anti-E-selectin and anti-VCAM-1 antibodies nor temporal targeting enhanced VE-cadherin gene down-regulation in primary endothelial cells (fig. 6).

Discussion and conclusion

In this study we explored 2 approaches to improve the siRNA association capacity and effectiveness of antibody-SAINTPEGarg in inflammation-activated endothelial cells *in vitro*. Previously, we showed that the extent of VE-cadherin down-regulation by antibody-SAINTPEGargs in HUVEC could not be improved by increasing the amount of siRNA formulated in the delivery system. Therefore, we aimed to study whether optimization of antibody-SAINTPEGargs by the incorporation of CT-DNA or equipping the nanocarriers with multiple ligands would have added value.

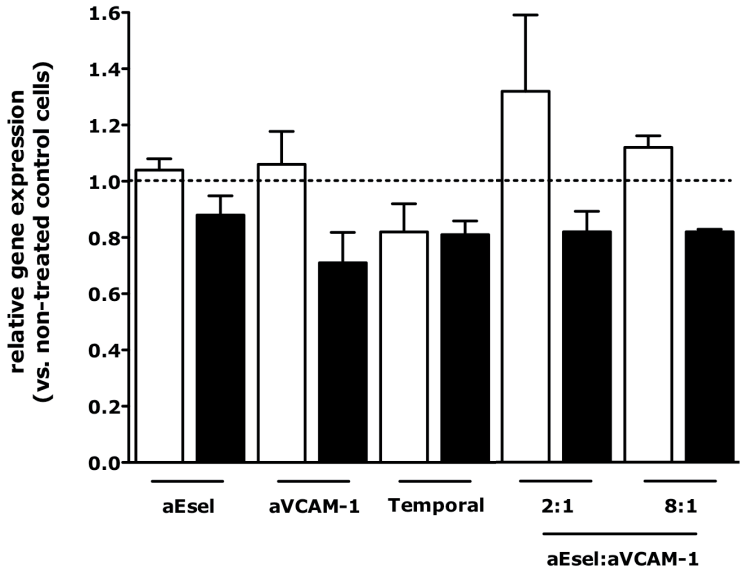

Fig. 6. Temporal siRNA delivery by antibody-SAINTPEGargs or delivery with antibody-SAINTPEGargs containing various ratios of anti-(a)E-selectin and anti-(a)VCAM-1 antibodies did not affect VE-cadherin gene expression. Antibody-SAINTPEGargs formulated with 2 mol% PEG and containing VE-cadherin siRNA (7.5 µg/ml) were incubated for 48 h with resting (open bars) or TNFα-activated (black bars) HUVEC. The expression of VE-cadherin was measured by real-time RT-PCR as described and data are presented as relative gene expression ± range of 2 individual experiments.

Besides a dual targeting strategy, also a temporal delivery strategy consisting of consecutive targeting two receptors that both internalize antibody-SAINTPEGargs, was examined.

Firstly, to improve the efficiency of anti-E-selectin- and anti-VCAM-1-SAINTPEGargs, CT-DNA was included in the antibody-SAINTPEGarg formulation. We hypothesized that co-complexation of siRNA with CT-DNA leads to smaller, more compact formulations, resulting in efficient uptake of anti-E-selectin- and anti-VCAM-1-SAINTPEGargs into activated endothelium. The addition of CT-DNA resulted in smaller anti-E-selectin- and anti-VCAM-1-SAINTPEGargs, which remained stable for up to at least 7 days upon storage at 4°C. The decreasing sizes at higher CT-DNA/siRNA ratios, suggests that CT-DNA is capable of complexing the 'rigid' siRNA, which corroborates results of earlier studies [9, 10]. Smaller size of anti-E-selectin- and anti-VCAM-1-SAINTPEGargs are more favorable for *in vivo* application, because larger complexes will be rapidly cleared from the blood circulation by the liver and spleen [22]. We did not measure the zeta-potentials of antibody-SAINTPEGargs containing

varying CT-DNA/nucleic acids ratios in this study. Since positively charged lipoplexes are desired, because they can improve cellular association by ionic interactions with the negative charged cell surface [23], zeta potential measurements of antibody-SAINTPEGargs may be helpful for interpretation of our results.

The decrease in size at higher CT-DNA/siRNA ratios could, however, affect the release of siRNA into the cytoplasm due to increased particle stability, which might be (partially) overcome by incorporation of protamine. Protamine, a small cationic protein, promotes nucleic acid condensation and has attracted much attention as nucleic acid delivery enhancer. It was employed by Huang and coworkers to improve the efficiency of siRNA delivery systems [24]. These so-called liposome-polycation-DNA (LPD) nanocarriers consist of siRNA and carrier DNA complexed with protamine and lipids [25]. Moreover, LPD nanocarriers could be grafted with up to 20 mol% PEG [25, 26]. Therefore, incorporation of protamine (allowing increased surface grafting with PEG) into the antibody-SAINTPEGarg formulation could improve the short blood circulation times of antibody-SAINTPEGargs *in vivo* due to low (2 mol%) amount of PEG [7], since higher mol% PEG significantly reduces their siRNA delivery efficiency *in vitro* [6].

In an *in vivo* inflammatory situation the expression of adhesion molecules varies in the course of time. At early times, E-selectin is highly expressed in activated ECs, whereas VCAM-1 expression increases steadily and achieves maximum at later times [27]. This differential expression as a function of time gives the opportunity to use these two receptors as complementary target for delivering siRNAs using antibody-SAINTPEGargs. Two different antibodies attached to one drug delivery vehicle were studied previously and showed beneficial effects in case of liposomes [18], hydrogels [28] and microspheres [29] as it ensured increased adhesion (1.7-fold for TNFα and 3.7-fold for IL-1α; [18]) to the surface of activated endothelial cells *in vitro*. We adapted this concept with the aim to increase the targeted siRNA delivery capacity of antibody-SAINTPEGargs. We hypothesized that lipoplexes with a surface presentation of antibodies complementary to the expression patterns of adhesion molecules on the activated endothelial surface may enhance lipoplex binding and thereby uptake. However, this was not what we observed, neither the siRNA association capacity nor effectiveness of antibody-SAINTPEGarg in inflammation-activated endothelial cells *in vitro* was improved. The shape of the delivery system (lipoplex; this study versus liposome; [18]) and the difference in lipid composition could affect the nanocarrier-cell interaction [30], which may underlie the absence of improved binding by complementary targeting E-selectin and VCAM-1. Moreover, *in*

vivo, flow conditions are always present in the normal vasculature and cause shear stress on ECs, which does not only change the morphology and function, but also the expression and cellular handling of E-selectin and other cell adhesion molecules [31]. The absence of flow might affect the expression of other genes as well upon pro-inflammatory stimulus (TNFα/LPS), which could have major consequences for interpretation of research on drug delivery into endothelial cells under static conditions and extrapolation to the *in vivo* situation. For example, it could affect molecular mechanisms underlying the internalization, intracellular trafficking and processing of antibody-SAINTPEGargs. Consequently, these experiments should in the future also be performed under flow conditions, to better mimic the *in vivo* situation.

In this work we examined the siRNA formulation potential of newly designed antibody-SAINTPEGargs, one approach dealing with varying ratios of CT-DNA to total nucleic acids, and the other dealing with a dual targeting approach. We studied their capacities to functionally deliver siRNA into inflammation-activated endothelial cells. Neither the newly developed antibody-SAINTPEGargs containing CT-DNA nor the applied dual targeting approaches were capable of functionally delivering siRNA into primary endothelial cells and down-regulate target gene expression. Since endothelial cell behavior is heterogenic and E-selectin and VCAM-1 are not homogeneously expressed by the endothelium in the different vascular beds upon a pro-inflammatory challenge, in future work a number of issues should be further explored. The molecular mechanisms underlying the absence of internalization, intracellular trafficking and processing of antibody-SAINTPEGargs should be studied in more detail like previously done for the SAINT-O-Somes (liposomal siRNA carrier) [32]. Moreover, not only primary endothelial cells from venous (HUVEC) origin should be studied but also primary endothelial cells from arterial and micro-vascular origin [7].

Acknowledgements

We thank Henk Moorlag for excellent technical assistance regarding endothelial cell culture and Eduard G. Talman for providing high quality SAINT-C18. This research was supported by the European Fund for Regional Development (EFRO) from the European Union, project NTS 068 and 073 Drug Delivery and Targeting, and the JK Cock foundation. The authors declare no competing financial interests. M.H.J. Ruiters is CEO of Synvolux Therapeutics.

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Chapter 6

Summary, conclusions and future perspectives

Summary and Conclusions

Since its discovery, RNA interference (RNAi) has emerged as one of the most powerful tools to study gene regulation and function, and has great potential to become an effective therapeutic strategy for various diseases [1], including (chronic) inflammatory diseases and cancer. Yet, siRNAs are large, negatively charged molecules and are therefore not able to readily cross cellular membranes. Furthermore, they are rapidly cleared from the body and are susceptible to degradation by serum RNases [2]. For therapeutic application, siRNA needs to be protected during circulation and delivered to the desired tissue and into the target cell in order to achieve therapeutic effects. Progress towards clinical application of siRNA-based interventions is still hampered by inadequate and ineffective delivery into target cells or tissues [3]. This thesis focuses on the development of cationic amphiphilic SAINT-C18-based lipoplexes suitable for efficient and endothelial-specific siRNA delivery *in vivo*. E-selectin and vascular cell adhesion molecule-1 (VCAM-1) were exploited as molecular targets to facilitate selective siRNA delivery into inflammation-activated endothelial cells *in vitro* and *in vivo*. Mice treated with tumor necrosis factor alpha (TNFα) were used as an inflammation model to evaluate the *in vivo* pharmacokinetic properties of SAINT-based lipoplexes and the potential of targeted SAINT-based lipoplexes to selectively deliver siRNA into inflamed endothelial cells. Finally, we investigated the effects of *in vivo* siRNA delivery on gene expression, assessed in whole organ RNA isolates and in laser microdissected vascular subsets.

Microvascular endothelial cells at the site of inflammation are both active regulators of, and participants in acute and chronic inflammatory processes. Therefore, endothelial-specific siRNA delivery presents a promising strategy for anti-inflammatory interventions aiming at silencing disease-associated genes involved in the pathophysiology of acute or chronic inflammation. In **chapter 2**, we reviewed novel approaches to interfere with endothelial cell engagement in disease focusing on non-viral lipid and polymer-based delivery systems for siRNA-based therapeutics. We introduced general concepts of endothelial heterogeneity in relation to disease state and their consequences for targeted therapeutic interventions. The impediments limiting clinical application of siRNA-based therapeutics for treatment of chronic inflammatory diseases, including (micro)vascular bed dependent heterogeneity upon pro-inflammatory stimulation and the intracellular obstacles affecting the fate of targeted delivery systems are discussed. Upon pro-inflammatory stimulation, the different vascular beds within an organ and between organs display heterogeneous expression of pro-inflammatory genes, including the

adhesion molecules E-selectin, VCAM-1 and ICAM-1 [4]. These adhesion molecules can be employed to selectively deliver siRNAs to inflamed endothelial cells, resulting in limited uptake by non-target cells, thereby increasing the effectivity of the therapeutic strategy. Yet, knowledge about the intracellular processing of siRNA carriers internalized via such endothelial adhesion molecules is still limited. The last part of **chapter 2** addresses novel technical approaches, including the use of tissue slices and laser microdissection (LMD), the latter enables analysis of vascular bed-specific pharmacological effects by enrichment of endothelial subsets prior to analysis [5], which can be otherwise masked in whole organ mRNA isolates. Tissue slices enable analysis of the effectiveness of the siRNA carrier in down-regulating genes in the complexity and natural environment of the whole organ [6].

In **chapter 3** we described the development and validation of targeted PEGylated lipoplexes based on the cationic amphiphilic lipid 1-methyl-4-(cis-9-dioleyl) methyl-pyridiniumchloride (SAINT-C18) [7], formulated with the neutral helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). These lipoplexes have been shown to efficiently deliver proteins, DNA and siRNA into cells *in vitro* in the presence of serum without exerting cellular toxicity [8]. Based on the *in vivo* concept of spatiotemporal expression of E-selectin and VCAM-1 by inflammation-activated endothelial cells, SAINT-based lipoplexes were harnessed with antibodies recognizing these inflammation-induced cell adhesion molecules to create cell specificity, resulting in so-called antibody-SAINTargs [9, 10]. Polyethylene glycol (PEG)-conjugated lipids were included in the lipid formulation of the antibody-SAINTarg-based drug delivery system to improve the requirements for *in vivo* application, including defined particle size, particle stability and protection of siRNA integrity. When anti-E-selectin- and anti-VCAM-1-SAINTarg were PEGylated using DOPE-PEG_{2000}, the *in vitro* siRNA delivery capability of these devices was significantly reduced. Coupling of antibodies to the distal end of PEG (so-called antibody-SAINTPEGargs), resulted in anti-E-selectin- and anti-VCAM-1-SAINTPEGarg, which preserved their antigen recognition and siRNA integrity. This study demonstrated that antibody-SAINTPEGargs formulated with additional 2 mol% DOPE-PEG_{2000} (antibody-SAINTPEGarg/PEG2%) were efficacious devices for specific delivery of siRNA into inflammation-activated endothelial cells of different vascular origins. These data thus provided an opportunity for further studies on siRNA delivery based target gene knock-down in specific disease-associated endothelial cell subsets *in vivo*.

The findings from **chapter 3** encouraged studying anti-VCAM-1-SAINTPEGarg/PEG2% as a means to deliver siRNA into specific disease-associated endothelial subsets *in vivo*. In **chapter 4** we first demonstrated that co-formulation of antibody-SAINTPEGarg with DOPE-PEG_{2000} resulted in more desirable pharmacokinetic behavior than that of non-PEGylated antibody-SAINTPEGarg. Moreover, antibody-SAINTPEGarg did not exert any liver or kidney toxicity as determined by function parameters. We next showed that i.v. administered anti-VCAM-1-SAINTPEGarg/PEG2% selectively homed to VCAM-1 protein expressing vasculature in specific (micro)vascular segments in organs of TNFα-challenged mice, predominantly in lungs. Confocal laser scanning microscopy revealed that anti-VCAM-1-SAINTPEGarg/PEG2% co-localized with endothelial cells in lung postcapillary venules. Yet, lack of *in vivo* gene silencing as assessed in whole lung RNA isolates and in RNA from laser microdissected lung microvascular segments indicates that *in vivo* the internalization and/or intracellular trafficking of the delivery system and its cargo in the target cells are not effective, and need further attention. As such, this outcome emphasizes the importance of evaluating siRNA delivery systems in *in vivo* animal models at an early stage in their development. It furthermore calls for development of innovative techniques that enable quantification of intracellular trafficking of siRNA delivery systems in (micro)vascular endothelial cells *in vivo*.

Lastly in **chapter 5** we describe the modification of antibody-SAINTPEGargs by incorporating Calf Thymus-DNA (CT-DNA). CT-DNA possesses a molecular topography that allows condensation into small nanometric particles when it is complexed with a cationic component like SAINT-C18. The incorporation of CT-DNA was based on the ability of CT-DNA to provide a higher efficiency in gene silencing with fewer lipids while exerting less toxicity [11]. Thus we aimed to improve the siRNA delivery efficacy of antibody-SAINTPEGargs into inflammation-activated endothelial cells. Although anti-E-selectin- and anti-VCAM-1-SAINTPEGargs were reduced in size upon CT-DNA incorporation, no beneficial effects of the incorporation of CT-DNA on gene down-regulation was observed. As a second modification compared to the SAINT-based delivery systems studied in chapter 3 and chapter 4, we equipped antibody-SAINTPEGargs with both anti-E-selectin and anti-VCAM-1 antibodies in various ratios. We hypothesized that targeting multiple, transiently-upregulated cell adhesion molecules on endothelial cells could amplify binding specificity and cellular uptake of SAINT-based siRNA delivery systems as was previously shown by Gunawan et al. for E-selectin and VCAM-1 dual targeted liposomes [12]. Yet, no improved siRNA delivery was observed between dual targeted and single targeted

antibody-SAINTPEGargs. Taken together, neither antibody-SAINTPEGargs containing CT-DNA nor the applied dual targeting approach was capable of increasing the siRNA delivery capacity into TNFα-activated endothelial cells *in vitro*.

In summary, many efforts have been undertaken to implement RNAi as a novel therapeutic tool to target disease-associated genes for therapeutic interventions. Endothelium is regarded as an attractive target for therapeutic intervention due to its role in the pathophysiology of many diseases. Most of today's used drug delivery systems are based on liposomal formulations. A number of liposomal formulations are on the market, and many more are in the pipeline [13]. So far, a few lipid-based systems for *in vivo* siRNA delivery into diseased-endothelium [14, 15] and other diseased target tissues [16] are in pre-clinical development. The studies described in this thesis have yielded novel SAINT-based carriers including anti-VCAM-1-SAINTarg, anti-E-selectin- and anti-VCAM-1-SAINTPEGarg that all showed functional *in vitro* siRNA delivery, and that we optimized for *in vivo* siRNA delivery into inflammation-activated endothelial cells. Even though anti-VCAM-1-SAINTPEGarg/PEG2% containing VE-cadherin specific siRNA selectively and extensively accumulated in VCAM-1 expressing postcapillary venules in lungs of TNFα-challenged mice, no down-regulation of VE-cadherin gene expression was found. Optimization of antibody-SAINTPEGargs by the incorporation of CT-DNA or dual ligands did not improve the siRNA delivery capacity into TNFα-activated endothelial cells *in vitro.* Consequently, the current antibody-SAINTPEGarg formulation still has to be improved in order to enable *in vivo* applications.

Future perspectives

In the last decade, an exceptional growth in innovative nanoparticle-based drug delivery systems for the treatment of numerous (inflammatory) diseases was observed. This expansion was largely driven by the discovery of RNAi and progress in the design of new materials (lipids and polymers) and bioengineering tools needed to deliver RNAi-based therapeutics. The potential of RNAi to silence disease-associated genes ensures future RNAi-based therapeutic interventions though RNAi is not yet an established therapeutic modality because of delivery-related problems. RNAi demands targeted delivery systems, which efficiently and selectively deliver and release siRNA into the cytoplasm of diseased target cells, in therapeutically relevant doses.

Endothelial cells are an attractive target for therapeutic intervention by virtue of their active engagement in the patho(physio)logy of many (inflammatory) diseases. However, most of the drugs lack specificity for 'diseased' endothelial cells, giving rise to adverse effects in other cells in the body. This study provided evidence that inflammation-activated endothelial cells of different vascular origin could be specifically employed as a target *in vitro* by antibody-SAINTPEGargs. A proof of concept study *in vivo* using anti-VCAM-1-SAINTPEGarg/PEG2%, however, showed no down-regulation of target gene expression in those endothelial cells in which the anti-VCAM-1-SAINTPEGarg/PEG2% accumulated.

HUVEC are used in many endothelial drug delivery studies as cell model to examine the association (uptake and binding) of the designed delivery system. One could however ask the question if HUVEC are the proper endothelial cell type for studying endothelial drug delivery, as they are not 'naturally' occurring in the human body and have limited delivery device processing capacity [17]. We showed that when 5-fold higher siRNA concentrations (encapsulated in antibody-SAINTPEGargs) were applied to TNFα-activated HUVEC *in vitro*, only 2-fold more siRNA was associated with activated HUVEC. When studying this in endothelial cells of different origin, binding experiments with antibody-SAINTPEGargs showed that approximately 5-times as much siRNA was associated with activated HAEC than with activated HUVEC. In HAEC the VE-cadherin gene expression was down-regulated up to 60% by siRNA containing antibody-SAINTPEGargs [10]. Either the quantitative difference in uptake between endothelial cells [9] or limited delivery device processing capacity of HUVEC might explain the lack of down-regulation *in vitro*. For this reason, validation of drug delivery systems for selective delivery of siRNA into inflammation-activated endothelial cells should be studied in primary endothelial cells from arterial, micro-vascular and venous origin to provide a solid platform for further application for siRNA delivery *in vivo*.

Research performed in cell systems investigating the inflammatory response might be more accurately interpreted when the local flow status is taken into account [18]. A study by Kurniati et al. demonstrated that the endothelial expression of the receptor tyrosine kinase Tie2 is dependent on flow, with a decrease in flow leading to a decrease in Tie2 expression [19]. One underlying cause of this might be the effects of flow/absence of flow on flow responsive transcription factors like Kruppel-like factor-2 [20]. The absence of flow might affect the expression of other genes as well e.g., upregulation of adhesion molecules upon pro-inflammatory stimulus (TNFα/LPS) or endocytosis/trafficking-related genes. This could have major

implications for interpretation and extrapolation of research on drug delivery into endothelial cells. Ultimately this might result in the selection of 'inappropriate' delivery systems for *in vivo* application. Therefore in the future, during optimization and validation of the SAINT-based siRNA lipoplexes, *in vitro* experiments should be performed under flow conditions to better mimic the *in vivo* situation with regard to shear stress and response to pro-inflammatory stimuli. Initial experiments studying SAINT-O-Somes (liposomal siRNA carrier [21]) association and cargo release under flow conditions demonstrated a significant increase in association and cargo release when compared with static conditions (unpublished data R. Aprianto). Moreover, elevated expression levels of E-selectin were observed, both at mRNA and protein level. The difference between *in vivo* and *in vitro* conditions could affect the molecular mechanisms underlying the internalization, intracellular trafficking and processing of antibody-SAINTPEGargs. We do realize that introduction of flow does not cover all aspects of the complicated translation of the *in vitro* setting to an animal.

Despite evident accumulation in VCAM-1 positive venules, anti-VCAM-1-SAINTPEGarg/PEG2% containing VE-cadherin siRNA did not affect VE-cadherin gene expression *in vivo*. This may be attributed to inadequate siRNA concentrations in the target cells. However, we demonstrated effective *in vitro* gene silencing in low nanomolar range [10], while many other delivery systems designed for *in vivo* application show gene silencing in the micromolar range [22, 23]. Yet, high(er) siRNA concentrations may lead to less specific gene silencing, thereby increasing off-target effects and toxicity related issues [24, 25]. Since, antibody-SAINTPEGargs were effective in silencing VE-cadherin gene expression *in vitro* and co-localized with endothelial cells in VCAM-1 positive lung postcapillary venules *in vivo*, improvement of the siRNA release properties of antibody-SAINTPEGargs should be one of the main emphases in further research. The introduced improvements should however not considerably change carrier characteristics like siRNA encapsulation efficiency, size, zeta-potential and/or antibody conjugation capacity. Based on the knowledge on the intracellular trafficking of SAINT-O-Somes [21], incorporation of acid-sensitive moieties or cell-penetrating peptides could likely improve the siRNA release of PEGylated antibody-SAINTPEGargs *in vivo* [26, 27]. Recently, pH-sensitive lipoplexes demonstrated efficient nucleic acid delivery both *in vitro* and *in vivo* [28]. Clearly there are more factors that could affect drug release like topography of the delivery system and lipid composition [29]. Essential in obtaining more information on these issues *in vivo* is the availability of state-of-the-art techniques that enable quantification of intracellular trafficking and siRNA release of siRNA delivery systems to monitor these processes.

The absence of VE-cadherin down-regulation *in vivo* emphasizes the importance of *in vivo* validation at an early stage in siRNA carrier development, as *in vitro* these antibody-SAINTPEGargs proved to be effective at low nanomolar range in inflammation-activated endothelial cells of different vascular origins. The size of the positively charged anti-VCAM-1-SAINTPEGarg/PEG2% might increase in the circulation through non-specific interactions with blood components, e.g. proteins, which could affect the uptake efficiency. It has been shown that, in blood, nanoparticles are rapidly covered by a selected group of biomolecules (mainly apolipoproteins, e.g. apoE) that form a so-called "corona" [30, 31]. The presence of biomolecules on the nanoparticle surface creates a "molecular signature" affecting the nanoparticle-cell interactions and subsequently altering the route of particle internalization. The route of internalization, then, might affect the eventual fate of the nanoparticle in the body [32]. In addition, it could interfere with the targeting device exposure on the nanoparticle or result in uptake via molecules present on the molecular corona itself. Consequently, it is important to study the developed drug delivery system under physiological conditions, thereby mimicking their (future) biological environment as close as possible. Although surface modifications (such as PEGylation) reduce the binding of additional biomolecules, some association of biomolecules may still occur. In the future, besides chemical grafting, the corona itself could be controlled and used as a novel approach to target (inflammatory) diseases [33], which has been shown for endogenous apoE-based targeting to hepatocytes *in vivo* [34].

The silencing efficacy of siRNA containing antibody-SAINTPEGargs could also be improved by incorporation of newly engineered lipids/polymers or improved derivatives of the SAINT-C18 lipid. Moreover, incorporation of a ionizable amino lipid such as heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate [35] could improve the siRNA release characteristics of antibody-SAINTPEGargs. In environments where the pH is below the pKa of the ionizable lipid (pH $\leq$7.0), the amino group is protonated and interacts with the negatively charged siRNA, thereby promoting the self-assembly of the siRNA encapsulating nanoparticle. Subsequently, in the acidic environment of endosomes (pH<pKa; e.g., pH 4.0), the amino group of the ionizable lipid becomes positively charged and associates with the anionic endosomal lipids [36]. This interaction enables the destabilization of the endosomal membranes and facilitates the release of siRNA into the cytoplasm. Lipid nanoparticles containing these ionizable amino lipids are today the leading delivery technology for siRNA with 5 siRNA formulations currently in various stages of clinical

development [37]. Consequently, synthesis and screening of a large number of SAINT-based ionizable lipids with various types of linkers connecting the (newly developed) amino group and the acyl chains of SAINT might improve the silencing capacity of antibody-SAINTPEGarg both *in vitro* and *in vivo*.

Additionally, once the most effective formulation has been validated in primary inflammation-activated endothelial cells of different origin, and proven to be effective in silencing disease-associated target genes, a combination of multiple siRNA molecules recognizing different target mRNAs involved in different signaling pathways could enhance the therapeutic potential of siRNA-based therapies. This is from a pharmacological point of view advantageous compared to targeting a single inflammatory protein since the inflammatory process is complex and heterogeneous in response to disease [4, 38]. In recent years, the importance of posttranscriptional regulation of inflammation by microRNAs (miRNAs) has furthermore become increasingly apparent. Targeting miRNAs could have advantages for treatment of complex inflammatory diseases since miRNA recognition of the target mRNA is imperfect, resulting in binding many target RNAs, leading to the silencing of multiple target genes [39, 40].

The major limitation of siRNA use in the clinic is the development of safe and effective systemic delivery systems. Although there are significant advancements in siRNA design and chemical modifications that increase both stability and reduce immunogenicity, there is still a need for further improvement towards efficient cellular uptake, target site accumulation and intracellular release. These improvements of selective and efficient delivery systems will be essential for siRNA-based formulations to obtain the potential to become therapeutics.

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Chapter 7

Nederlandse samenvatting

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Short interfering RNA's (siRNA's) zijn dubbelstrengs nucleïnezuur moleculen opgebouwd uit een klein aantal basen; ze hebben de bijzondere eigenschap dat ze in de cel met complementaire strengen messenger RNA (mRNA) kunnen complexeren, die als gevolg daarvan worden afgebroken en daardoor hun genetische code-boodschap (message) niet kunnen afleveren aan de eiwitsynthese-machinerie van de cel. Dit leidt er vervolgens toe dat het door het mRNA gecodeerde eiwit niet gevormd kan worden. Dit mechanisme opent dus de mogelijkheid de vorming van een ongewenst eiwit, bijvoorbeeld een eiwit dat betrokken is bij het ontstaan van een ziekte, te verhinderen en daarmee het ontstaan of verergeren van die ziekte tegen te gaan. siRNA zou op deze manier als een medicijn gebruikt kunnen worden. De moeilijkheid hierbij is echter dat het siRNA in de bloedbaan gemakkelijk wordt afgebroken en dat het molecuul als zodanig niet een cel kan binnendringen. Om aan deze problemen het hoofd te bieden kan het RNA verpakt worden in een dragersysteem dat wel in staat is het binnenste van een cel te bereiken en dat voortijdige afbraak in de bloedbaan verhindert. Het dragersysteem dat wij hebben ontwikkeld bestaat uit een aantal specifieke vetachtige moleculen (lipiden) dat met een aantal siRNA moleculen een deeltje vormt met een diameter van circa 100-200 nm (0,0001-0,0002 mm). Hoewel erg klein, kan dit deeltje na injectie in de bloedbaan niet gericht de daarachter liggende cellen, zoals ontstekingscellen of tumorcellen, bereiken. Om ons dragersysteem toch relatief eenvoudig *in vivo* toe te kunnen passen hebben wij als doelcel de bloedvatendotheelcel gekozen. Deze staat in direct contact met het bloed en is dus meteen toegankelijk voor het siRNA-drager complex.

Endotheelcellen bekleden de binnenkant van alle bloedvaten en spelen een belangrijke rol bij ziekteprocessen, zoals de vorming van bloedvaten in tumoren en de rekrutering van ontstekingscellen, of witte bloedcellen, bij ontstekingsziekten. Endotheelcellen brengen in ziekteprocessen allerlei eiwitten tot expressie zoals adhesie ("plak")-moleculen op de celmembraan, die in gezonde endotheelcellen niet of in veel mindere mate voorkomen. Door ons dragersysteem uit te rusten met een moleculaire structuur die een dergelijk adhesiemolecuul herkent kunnen we het systeem specifiek naar de betreffende cellen toesturen. Als herkenningsmolecuul kunnen antilichamen tegen de adhesiemoleculen worden gebruikt. Het siRNA-drager complex bindt zo gericht aan endotheelcellen in door chronische ontstekingen of tumoren aangedaan weefsel en zal daar door middel van het ingebouwde specifieke siRNA een ziekte-geassocieerd eiwit kunnen uitschakelen.

Het in dit proefschrift beschreven onderzoek richt zich op de ontwikkeling van een op lipiden gebaseerd siRNA dragersysteem dat geschikt is voor efficiënte en endotheel-specifieke afgifte van siRNA *in vivo*. Het door ons ontwikkelde siRNA dragersysteem ("lipoplex") is gebaseerd op het synthetische, positief geladen amfifiele lipide SAINT-C18 en voorzien van endotheel-specifieke antilichamen gericht tegen E-selectine en vasculair cel adhesiemolecuul-1 (VCAM-1). Deze eiwitten komen vooral tot (over)expressie op ontstoken endotheelcellen. We stelden eerst vast dat deze lipoplexen *in vitro* in staat waren siRNAs af te leveren in geactiveerde primaire, uit humane navelstreng, geïsoleerde endotheelcellen geactiveerd met tumor necrose factor-alfa (TNFα). Daarna werd in een diermodel vastgesteld of, en in hoeverre, deze op SAINT-C18 gebaseerde siRNA-bevattende lipoplexen in staat zijn *in vivo* genexpressie te onderdrukken.

Microvasculaire endotheelcellen spelen een actieve rol als regulator in de patho(physio)logie van acute of chronische ontstekingen. We verwachtten daarom dat endotheel-specifieke afgifte van gen-specifiek siRNA een goede strategie zou kunnen zijn voor de behandeling van (chronische) ontstekingen door het onderdrukken van de expressie van ontstekings-geassocieerde genen. In **hoofdstuk 2** wordt een overzicht gegeven van recente ontwikkelingen in het ontwerpen van op lipiden en polymeren gebaseerde carriers voor siRNA voor de behandeling van (chronische) ontstekingen door middel van specifiek ingrijpen op het vaatendotheel. In dit verband wordt het concept "endotheliale heterogeniteit" beschreven; dit houdt in dat de eigenschappen van het vaatendotheel in verschillende organen en/of onderdelen van organen niet identiek zijn. Dit geldt *a fortiori* voor endotheel in door ziekte aangedane organen en heeft voor de hand liggende consequenties voor het ontwerpen van endotheel-getargete therapeutische interventies. Voorts wordt uitvoerig aandacht besteed aan belemmeringen die een obstakel vormen voor de klinische uitvoerbaarheid van op siRNA gebaseerd farmacologisch ingrijpen op (chronische) ontstekingsprocessen. Verder worden nieuwe weefsel technologieën besproken die de ontwikkeling van cel- of weefselspecifiek getargete siRNA dragersystemen kunnen bevorderen. Voorbeelden daarvan zijn: 1. "precies gesneden weefselslices", een techniek waarbij een klein stukje weefsel met daarin alle celtypes van het desbetreffende weefsel wordt uitgesneden en die aldus een experimentele brug vormt tussen een *in vitro* celkweek model en de *in vivo* situatie en 2. laser microdissectie, een techniek die het mogelijk maakt om lokale farmacologische effecten in het vaatendotheel te analyseren in specifieke vasculaire segmenten.

In **hoofdstuk 3** wordt het endotheel-specifieke siRNA dragersysteem

beschreven dat wij voor het bovenbeschreven doel hebben ontworpen. Dit systeem (lipoplex) bestaat uit het kationische amfifiele lipide 1-methyl-4-(cis-9-dioleyl) methyl-pyridiniumchloride (SAINT-C18) en het neutrale helper lipide 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) waaraan anti-E-selectine en/of anti-VCAM-1 antilichamen zijn gekoppeld (SAINTarg). Wij lieten zien dat koppeling van deze antilichamen aan het SAINT-C18 lipide *in vitro* leidde tot een verhoogde opname in endotheelcellen van het verpakte siRNA, en tot specifieke gen onderdrukking door siRNA. Teneinde deze *in vitro* efficiënte en specifieke SAINTargs geschikt te maken voor *in vivo* toepassing modificeerden wij ze met polyethyleen glycol (PEG) waardoor ze langer in het bloed blijven circuleren en dus een grotere kans hebben selectief aan het bloedvatendotheel te binden. Deze gePEGyleerde SAINTargs bleken echter slechts in geringe mate in staat om siRNA specifiek af te leveren in de doelcel, waarschijnlijk omdat de PEG ketens effectieve interactie van het antilichaam met de endotheelcel in de weg staan. Door het antilichaam te koppelen aan het distale uiteinde van het PEG molecuul (resulterend in SAINTPEGargs) werd deze sterische hindering voorkomen en kon het siRNA specifiek en efficient afgeleverd worden in de endotheelcel. Met dit systeem toonden we aan dat zowel anti-E-selectine als anti-VCAM-1-SAINTPEGargs, met daarin een siRNA dat specifiek is voor het endotheliale VE-cadherine gen, *in vitro* het betreffende mRNA en daarmee de expressie van het gen kunnen down-reguleren in zowel arteriële, microvasculaire als veneuze endotheelcellen. Met het oog op de *in vivo* toepassing van deze getargete siRNA dragersystemen was het van belang dat vastgesteld werd dat SAINTPEGargs stabiel zijn in de tijd en het ingesloten siRNA beschermen tegen afbraak door serum RNases.

De in hoofdstuk 3 beschreven resultaten bevestigen de conceptuele bruikbaarheid voor de functionele targeting van siRNA bevattende anti-E-selectine en anti-VCAM-1 SAINTPEGargs naar ontstoken endotheel *in vitro* en rechtvaardigden daarmee verder *in vivo* onderzoek in met TNFα behandelde muizen als model voor ontstekings-gerelateerde endotheel activatie. In **hoofdstuk 4** laten we in dit muizenmodel eerst zien dat intraveneus toegediende gePEGyleerde SAINTPEGargs een gunstiger farmacokinetisch profiel vertonen dan ongePEGyleerde SAINTPEGargs, zonder daarbij de lever- en nierfunctie(s) van de muizen te beïnvloeden. De anti-VCAM-1 -SAINTPEGargs bleken in verhoogde mate te worden opgenomen in ontstoken organen van TNFα behandelde muizen, met name in lever en long. Met behulp van confocale laser scanning microscopie hebben we aangetoond dat in de long anti-VCAM-1-SAINTPEGargs selectief afgeleverd worden aan endotheelcellen

die het adhesie eiwit VCAM-1 tot expressie brengen. Ondanks de hoge mate waarin anti-VCAM-1-SAINTPEGargs, met daarin een VE-cadherine-specifiek siRNA, in long endotheelcellen worden opgenomen, resulteerde dit niet in down-regulatie van het endotheel-specifieke VE-cadherine gen. Met behulp van laser dissectie microscopie hebben we getracht een eventueel vaatbed-specifiek farmacologische effect, dat mogelijk gemaskeerd werd in hele-long homogenaten aan te tonen, maar ook in dit geval werd geen down-regulatie waargenomen. Het ontbreken van aantoonbare down-regulatie van VE-cadherine genexpressie in hele-long en laser microdissectie RNA homogenaten impliceert dat *in vivo* geen internalisatie optreedt en/of dat het intracellulaire transport van anti-VCAM-1-SAINTPEGargs en/of het vrijmaken van siRNA uit het dragersysteem in aan inflammatie onderhevige endotheelcellen niet effectief is.

Hoofdstuk 5 beschrijft pogingen om de effectiviteit van de siRNA complexen te verhogen. Als eerste testten we een op SAINT-gebaseerd siRNA dragersysteem waarin Calf Thymus-DNA (CT-DNA; nucleïnezuur) werd ingebouwd met als doel een versnelde of meer complete (voor activiteit noodzakelijke) verwijdering van het siRNA uit het dragersysteem te bewerkstelligen. Een dergelijke verhoogde vrijmaking zou de gen onderdrukkende activiteit van het siRNA mogelijk kunnen verhogen zonder toxiciteitsverschijnselen te veroorzaken. Dit dragersysteem werd gemaakt door siRNA met CT-DNA te mengen, en vervolgens te complexeren met het SAINT-C18-DOPE lipidenmengsel. CT-DNA blijkt weliswaar een positief effect te hebben op de deeltjes grootte (kleiner) van de antilichaam-gekoppelde SAINTPEGargs, maar verhoogde de gen down-regulatie niet. Als een tweede modificatie van het siRNA dragersysteem werd het systeem uitgerust met zowel anti-E-selectine als anti-VCAM-1 antilichamen om de gen onderdrukkende capaciteit te verhogen. De gedachte hierachter was dat als het siRNA dragersysteem is uitgerust met antilichamen gericht tegen meerdere ontstekingsepitopen op endotheelcellen, de endotheel-binding-specificiteit en -affiniteit zal toenemen en aldus de opname van het dragersysteem en daarmee de gen onderdrukkende effectiviteit van het siRNA zou verhogen. Ook in dit geval werd echter geen verhoogde siRNA afgifte aan geactiveerde endotheelcellen waargenomen.

Samenvattend laat dit proefschrift zien dat gePEGyleerde op SAINT-gebaseerde siRNA dragersystemen geschikt zijn voor functionele *in vitro* aflevering van siRNA in "ontstoken" (=geactiveerde) primaire arteriële, microvasculaire en veneuze endotheelcellen. Onderzoek in met TNFα behandelde muizen toonde aan dat, ondanks de hoge opname van gePEGyleerde anti-VCAM-1-SAINTPEGargs met

daarin VE-cadherine-specifiek siRNA in longweefsel, dit niet resulteert in down-regulatie van het endotheliale VE-cadherine gen. Vervolgonderzoek zal zich moeten richten op de verbetering van de efficiëntie van *in vivo* siRNA vrijmaking uit met antilichaam gekoppelde SAINTPEGargs om aldus een sterkere down-regulatie van ziekte-geassocieerde genen in endotheelcellen te realiseren.

Appendices

Dankwoord

Eindelijk, het proefschrift is klaar! Bij het schrijven van het dankwoord begint het langzaam door te dringen dat het toch echt gelukt is. Ik kijk met veel plezier terug op een geweldige en leerzame onderzoeksperiode. Onderzoek doen doe je nooit alleen! Daarmee breekt dan ook het moment aan om iedereen met wie ik de afgelopen jaren heb mogen werken en die (in welke vorm dan ook) heeft bijgedragen aan de totstandkoming van dit boekje te bedanken.

Mijn promotor prof. dr. G. Molema, beste Ingrid, ik ben zeer dankbaar dat jij mijn promotor bent geweest. Op wetenschappelijk vlak heb ik veel van jou mogen leren. Ik heb jou leren kennen als iemand met een onuitputtelijke passie voor wetenschap (volgens onze e-mail correspondentie slaap je hooguit tussen 01.00 en 07.00 uur), een oprechte compassie voor mensen, een positieve houding en een kritische blik. Je leidde me op tot zelfstandig onderzoeker, maar wanneer ik dreigde te ontsporen zette jij me weer op de rails!

Mijn co-promotor dr. J.A.A.M. Kamps, beste Jan, ik wil jou bedanken voor jouw eindeloze geduld en tijd die jij hebt genomen om mijn vele vragen, over met name "delivery-related issues" te beantwoorden. Ik wil mijn waardering uitspreken voor de moeite die je hebt genomen om mij (altijd) snelle, duidelijke en uitgebreide antwoorden te geven. Het staat al 3 jaar in de planning, maar het wordt nu toch ECHT tijd dat wij gaan toeren in één van mijn Benzen ☺.

Prof. dr. G.L. Scherphof, beste Gerrit, dank dat jij mij in het eerste jaar (tijdelijk) hebt willen begeleiden toen Ingrid en Jan beiden afwezig waren. Ook bedankt voor het lezen ('de puntjes op de i zetten'!) van mijn Nederlandse samenvatting. Ik laat je weten wanneer we gaan toeren!

I would like to thank the members of the reading committee, prof. dr. V.R. Muzykantov, prof. dr. J.F.J. Engbersen, and prof. dr. K. Poelstra, for evaluating (and approving) my thesis.

Peter en Titia, mijn paranimfen, ik vind het super dat jullie op deze dag naast mij staan. Peter, bedankt voor al die leuke dingen die we gedeeld hebben - werkgerelateerd en daarbuiten. Het maken van promotiefilmpjes was altijd dolle pret. We moeten snel weer een biertje pakken! Titia, dank voor de wetenschappelijke discussies, praktische suggesties en voor de gezelligheid op de AiO-kamer. Wat hebben we een lol gehad in Oberjoch! Helaas was de afloop iets minder.

A

Henriëtte, mijn 'lab-moeder', het was een voorrecht om met jou te mogen werken! Bedankt voor de gezellige momenten, en niet te vergeten al het werk dat je hebt verzet (o.a. het vullen van honderden telpotjes; figuur 1, 2 en 3 van hoofdstuk 4 zijn hier het resultaat van). Ook bedankt voor het oppassen op Lieve (samen met Gerrit). Ik hoop dat wel elkaar blijven zien! Henk, zonder jou geen endotheelcellen (en dus geen experimenten) of zelfgemaakte bramenjam. Je bent een man uit duizenden! Rianne, bedankt voor je praktische ondersteuning/adviezen, maar vooral voor jouw hulp met de lay-out. Het is een super boekje geworden! Piotr, my friend, we share great memories which I will never forget. Thanks for all your help and have fun in the States ☺. Roel (ook een trotse papa ☺), ik zal onze goede gesprekken missen - hopelijk blijven we contact houden. Gesiena, nog even en dan is jouw boekje ook af. Dank voor de leuke tijd die we hebben (gehad) zowel op het lab als daarbuiten! Francis, wat was het soms gezellig op de AiO-kamer, niet? Jill, geweldige humor, en een kritische onderzoekster. Mirjan, dankjewel voor je suggesties wanneer ik een kleuring moest doen. Peter H., bedankt voor je altijd kritische blik op nieuwe resultaten. Matijs, dank voor de leuke gesprekken over wetenschap. My former office mates: Elise, Gopi and Neng. It was great working with you guys! Wish you all the best and I hope we will stay in touch. Betty, bedankt voor de (korte) leuke tijd. Nog even en dan ben je Klinisch Chemicus. Also a word of thanks to all (other) former EBVDT-members and students.

Hierbij wil ik ook de studenten bedanken die ik heb mogen begeleiden. Mariëlle, Marijn en Pranov, dank voor jullie inzet en gezelligheid! Veel succes met jullie verdere carrières.

Verder wil ik graag alle (oud)collega's van het MB-U lab, MB-Z lab, het UMIC-centrum, de flow-cytometrie en het secretariaat bedanken. Als ik alle namen ga noemen ben ik bang dat ik mensen zal vergeten. Ik wil daarom *iedereen* bedanken voor de gezellige sfeer op het lab en een ieders bereidheid om vragen te beantwoorden en hulp te bieden waar nodig. Mede dankzij jullie heb ik een bijzondere tijd gehad die voorbij is gevlogen!

Ook alle mensen van de Centrale Dienst Proefdieren, en Arjen Petersen in het bijzonder (wat zijn die handen van jou toch handig), heel erg bedankt voor jullie bijdrage aan dit proefschrift. Niet te vergeten de muizen, zonder jullie geen hoofdstuk 4!

A

We are causing animals to be born,
causing them to live through a variety of unusual experiences,
and causing them to die.
This is a form of power that cannot be taken lightly.
Today is an opportunity to acknowledge the animals' role in what we do.
To acknowledge that without them,
our research and teaching would be fundamentally altered.
To thank the animals seems logically inappropriate because their contribution was taken, not given.
Yet, I am grateful for, and even dependent upon, their role.

From 'The Gathering to Reflect on the Use of Animals in Research and Teaching' at the University of Guelph (Ontario, Canada) in 1993 by O'Neill, Taylor and Davis

Ook de mensen van het Radionuclidenlaboratorium van de afdeling Laboratoriumgeneeskunde wil ik bedanken. Specifiek bij naam wil ik noemen Frank, Jan, Janna en Sicco. Dankjulliewel voor het altijd willen beantwoorden van mijn (vele) vragen.

Mijn collega's van de afdeling Farmaceutische Gen Modulatie, bedankt voor jullie interesse en de altijd opbeurende gesprekken. Dankzij jullie ga ik elke dag met plezier naar mijn werk.

Tot slot wil ik mijn vrienden en familie bedanken. Ondanks dat het voor sommigen misschien een raadsel is geweest waar ik mee bezig ben, bedankt voor jullie steun, aanmoedigingen en interesse!

Enkele mensen wil ik graag expliciet bedanken. Mijn 'grote' broers, Gerwin en Arjan, betere broers had ik mij niet kunnen wensen! Mijn schoonzussen, Nicole en Marijke, jullie zijn schatten van vrouwen. Lieve pap en mam, dit boekje is voor jullie. Dank dat jullie zo trots op mij zijn, de onvoorwaardelijke steun, en gewoon om er te zijn wanneer ik jullie nodig had! Zonder jullie was dit proefschrift er nooit gekomen! We zijn niet al te vaak als familie bij elkaar, maar ik geniet van de momenten dat we als familie bij elkaar zijn.

Ook de familie van Anouk: Wouter, Elly, Rick en Pamela wil ik graag bedanken. Wij kennen elkaar nu bijna 15 jaar, bedankt voor alle leuke momenten en bemoedigingen de afgelopen periode.

Lieve Anouk, mijn steun en toeverlaat! Wat zou ik zonder jou moeten?! We begonnen dit avontuur met zijn tweetjes, en eindigen het met zijn drietjes. Jij en Lieve zijn de grootste schatten in mijn leven. Ik hou van jullie!

Augustus 2014,

Niek

List of publications

Articles

Leus NG, Morselt HW, Zwiers PJ, Kowalski PS, Ruiters MH, Molema G, Kamps JA. VCAM-1 specific PEGylated SAINT-based lipoplexes deliver siRNA to activated endothelium in vivo but do not attenuate target gene expression. *Int J Pharm. 2014 Jul 20;469(1):121-31*.

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'Lipid-based siRNA carriers for anti-inflammatory vascular interventions'. (oral presentation)

Cardio Vascular Conference (2012), Noordwijkerhout, NL.
'Specific delivery of small regulatory RNAs to diseased endothelium using a novel lipid-based carrier'. (oral and poster presentation)

Gordon Research Conference on 'Drug carriers in Medicine & Biology' (2012), Waterville Valley, NH, USA.
'Lipid-based siRNA carriers for vascular interventions'. (poster presentation)

21st Mountain/Sea Liposome Workshop (2012), Oberjoch, Germany.
'Targeting small regulatory RNAs to diseased endothelium using a SAINT-based delivery device'. (oral presentation)

5th International Liposome Society conference (2011), London, UK.
'Specific delivery of small regulatory RNAs to diseased endothelium using a novel lipid-based carrier'. (poster presentation)

Cardio Vascular Conference (2011), Noordwijkerhout, NL.
'Targeting small RNAs to diseased endothelium using a novel SAINT-based delivery device'. (oral and poster presentation)

3rd international symposium on 'Cellular Delivery of Therapeutic Macromolecules' (2010), Cardiff, UK.
'SAINTargs as novel targeted delivery system for siRNA'. (poster presentation)

4th Ameland Liposome Workshop (2009), Hollum, NL.
'SAINTargs as novel targeted delivery system for siRNA - a new class of therapeutic drugs'. (oral presentation)

Funding

Jan Kornelis de Cock Stichting (2012). 'PEGargs; een innovatief drug delivery systeem ter bestrijding van kanker of chronische ontstekingen'.

Jan Kornelis de Cock Stichting (2010). 'SAINTargs: een drug delivery device voor *in vivo* siRNA afgifte.

A

Curriculum Vitae

Niek Leus werd geboren op 13 maart 1982 te Almelo. In 1998 behaalde hij zijn MAVO diploma aan het St. Canisius College te Almelo. In datzelfde jaar begon hij met de opleiding Laboratorium & Procestechniek (MLO; specialisatie Klinische Chemie en Hematogie) aan het ROC Twente te Hengelo. In 2002 rondde hij deze opleiding af met "veel genoegen". Na het behalen van het MLO diploma startte hij met de bachelor opleiding Medische Biochemie aan de Saxion Hogeschool te Enschede die hij in 2005 (versneld) afrondde op het afstudeeronderzoek 'Hypermethylatie van genen als diagnostische marker bij cervixneoplasie' uitgevoerd onder leiding van Dr. Bea Wisman bij de afdeling Gynaecologische Oncologie in het Universitair Medisch Centrum Groningen (UMCG). In 2005 begon hij aan het schakelprogramma voor de studie Moleculaire Biologie & Biotechnologie aan de Rijksuniversiteit Groningen. Begin 2006 rondde hij dit programma af, waarna hij werd toegelaten tot de Master opleiding Moleculaire Biologie & Biotechnologie met als specialisatie Moleculaire Biologie. Tijdens zijn Masteropleiding werkte hij aan twee onderzoeksprojecten van elk 8 maanden. Bij de afdeling Moleculaire Microbiologie (Rijksuniversiteit Groningen) werkte hij aan de 'Assembly of the pilus of the type IV secretion system in *Neisseria gonorrhoeae*' onder begeleiding van Dr. Chris van der Does. Voor zijn tweede onderzoeksproject werkte hij in het laboratorium van Prof. dr. Janet Stavnezer, Department of Molecular Genetics and Microbiology (University of Massachusetts Medical School, Worcester, MA, USA) onder begeleiding van Dr. Jeroen Guikema aan het project 'DNA breaks in immunoglobulin class switching and chromosomal translocations'. Hij behaalde zijn Master titel in de Moleculaire Biologie in 2008, waarna hij, na een aanstelling van 6 maanden bij de afdeling Hematologie (UMCG), begon met zijn promotieonderzoek bij de afdeling Medische Biologie in het UMCG onder begeleiding van Prof. dr. Ingrid (Grietje) Molema en Dr. Jan Kamps. Hier werkte hij aan de ontwikkeling van een op lipiden gebaseerd siRNA dragersysteem voor specifieke *in vivo* afgifte van siRNA aan endotheelcellen met als ultiem doel de expressie van bepaalde ziekte-geassocieerde genen te onderdrukken. De resultaten van dit onderzoek worden beschreven in dit proefschrift. Sinds december 2013 is Niek werkzaam als 'postdoctoral fellow' in de onderzoeksgroep van Dr. Frank Dekker bij de afdeling Farmaceutische Gen Modulatie van de Rijksuniversiteit Groningen.

Niek Leus was born on March 13, 1982, in Almelo, the Netherlands. After graduating from high school in 1998, he started studying Clinical Chemistry and Hematology at the "regional teaching center" ("ROC") Twente. After obtaining his diploma in 2002, he continued his education at the Saxion University of Applied Sciences in Enschede where he obtained a bachelor degree in Medical Biochemistry. His graduation research project ('Hypermethylation of genes as a diagnostic marker of cervix neoplasia') was performed at the Department of Gynecologic Oncology (University Medical Center Groningen; UMCG) under supervision of Dr. Bea Wisman. In 2005, after obtaining his Bachelor degree, he entered a training program to obtain admission to the Master Molecular Biology & Biotechnology at the University of Groningen. Early 2006 he successfully completed this program and enrolled the Master program Molecular Biology & Biotechnology with the specialization Molecular Biology. During his Master training he worked on two research projects of eight months each: 1. 'Assembly of the pilus of the type IV secretion system in *Neisseria gonorrhoeae'* under supervision of Dr. Chris van der Does (Department of Molecular Microbiology, University of Groningen) and 2. 'DNA breaks in immunoglobulin class switching and chromosomal translocations' in the laboratory of Prof. dr. Janet Stavnezer at the Department of Molecular Genetics and Microbiology of the University of Massachusetts Medical School (Worcester, MA, USA) under supervision of Dr. Jeroen Guikema. He obtained his Master degree in 2008, and after a 6-months stay at the Department of Hematology (UMCG), he started his PhD project at the Department of Medical Biology (UMCG) under supervision of Prof. dr. Ingrid (Grietje) Molema en Dr. Jan Kamps. His research focused on the development of an efficient endothelium-specific lipid-based siRNA delivery system with the ultimate aim to apply this *in vivo* for the effective silencing of disease-associated genes involved in the pathophysiology of inflammatory diseases and cancer. The results of these studies are presented in this dissertation. Since December 2013, Niek is employed as a postdoctoral fellow in the research group of Dr. Frank Dekker at the Department of Pharmaceutical Gene Modulation (University of Groningen).