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Chitosan as an Immune Modulator: Therapeutic Potential in Cerebral Amyloidopathies

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Chitosan as an Immune Modulator: Therapeutic Potential in Cerebral Amyloidopathies

by

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Each person whose signature appears below certifies that this dissertation in his or her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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FIGURES

**Figures marked by an asterisk were originally published as supplementary material in Cupino, et al. (2018). Unpublished supplementary figures are provided in the Appendix.*

TABLES

ABBREVIATIONS

Aβ	Amyloid-beta peptide
AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BMB	Brain microbleed
C3	Complement protein 3
C5b-9	Terminal end product of complement cascade
CAA	Cerebral amyloid angiopathy
CD59	MAC-inhibitory glycoprotein; Protectin
cDNA	Complementary DNA copied from mature cellular mRNA
CIHS	Complement-intact human serum
CR1	Complement receptor 1
Cs	Chitosan, a linear glucosamine polysaccharide
CsM	Cs microparticles, precipitated by ionic gelation
cvSMC	Cerebrovascular smooth muscle cells
DLS	Diffusion light scattering
DMEM	Dulbecco's modified Eagle cell culture medium
DPBS	Dulbecco's phosphate-buffered saline
EBSS	Earle's balanced salt solution

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GLUT	Glucose transporters (1,4,9 and 10 denotes isoform)
HBS	HEPES buffered saline
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO-1	Heme oxygenase 1
ICH	Intracerebral hemorrhage
LPS	Lipopolysaccharide
LRP-1	Lipoprotein receptor-related protein 1
MAC	Membrane attack complex, see C5b-9
MFI	Mean fluorescence intensity
Mϕ	Macrophages
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of B cells
NT2	Neuronal cell line, shorthand for NTera2
PBS	Phosphate buffered saline
pDNA	Plasmid DNA
RAGE	Receptor for advanced glycosylation end products
RPMI	Roswell Park Memorial Institute cell culture medium
TBS	TRIS buffered saline
TLR	Toll-like receptors
TPP	Tripolyphosphate
TRIS	tris(hydroxymethyl)aminomethane

ABSTRACT OF THE DISSERTATION

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Tanya Larissa Cupino

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
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Dr. Wolff Kirsch, Chairperson

Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA) are neurodegenerative diseases that are pathologically defined by accumulations of amyloid beta (Aβ) in the brain parenchyma and vascular walls, respectively. CAA comorbidity occurs in more than half of advanced AD cases. Aβ can activate complement, an enzymatic cascade that terminates in a cytolytic pore called membrane attack complex (MAC). Protectin (CD59) is a GPI-anchored glycoprotein that physically interrupts formation of MAC. At autopsy, individuals with AD and CAA have high levels of MAC and normal to low levels of CD59 in the tunica media of affected blood vessels. We proposed the introduction of exogenous CD59 gene expression via a plasmid (pDNA) to rescue cerebrovascular smooth muscle cells (cvSMC) from lysis by MAC. Chitosan (Cs) is a linear glucosamine polysaccharide that is well described in a variety of nano- and micro-carrier payload delivery applications and frequently considered biologically inert. In this investigation we examined the stability of chitosan microparticles (CsM) under physiologic conditions and describe the response of primary human cvSMC to CsM and pDNA-loaded CsM (pCsM) as quantified by flow cytometry. We also explored the impact of chitosan polymers, the building blocks of CsM, on CD59 expression in cvSMC. We found that CsM are not stable under standard cell culture conditions while in

cell culture media. CsM effectively delivered plasmid into cvSMC, as evidenced by high CD59 mRNA expression levels 24 h after transfection. However, this effect appeared to be lost in translation and no increase in CD59 surface expression was seen after treatment with pCsM. Surprisingly, Cs alone induced robust and persistent increases CD59 in cvSMC surface expression. These finds challenge assumption that Cs is inert and encourage the use of chitosan-only controls in future transfection experiments.

CHAPTER ONE

AN INTRODUCTION TO AMYLOID, COMPLEMENT AND CHITOSAN

Alzheimer's Disease: Prevalence, Diagnosis and Treatment Options

Alzheimer's Disease (AD) is defined clinically as a progressive neurodegenerative disease that presents with memory loss, moderate cognitive impairment and behavioral disruption (Budson and Solomon 2016). AD is a growing concern in the United States, with 1 in 10 individuals over the age of 65 meeting diagnostic criteria within the last few years (Association 2015). It is expected that the number of people with AD will continue to rise in the coming decades, as a greater proportion of the population reaches the highest risk age categories. In addition to age, risk factors for AD include female gender, apolipoprotein E (ApoE) genotype (Premkumar, Cohen et al. 1996), having a first-degree relative with AD (Bergem, Engedal et al. 1997), cardiovascular disease, and history of traumatic brain injury. The emotional and economic impact of AD is estimated to be in the billions per annum, both in medical costs and lost productivity of patients and their caregivers. Caregivers who may experience significant declines in quality of life (Bosboom, Alfonso et al. 2013). Unfortunately, despite the intense need for a therapeutic intervention, very few options are available to AD patients.

AD is defined pathologically by amyloid-beta plaques and tau tangles in the brain parenchyma of patients affected with clinically identifiable symptoms. Aβ forms complexes with acetylcholinesterase, causing acetylcholine deficiencies (Dinamarca, Sagal et al. 2010). Cholinesterase inhibitors are approved by the FDA to manage AD symptoms (Budson and Solomon 2016). If patients do not tolerate cholinesterase

inhibitors, physicians may proscribe Memantine, a drug that is primarily an NMDA antagonist, but acts on a wide variety of neurotransmitter receptors. Additional psychiatric medications may also be recommended to address behavioral symptoms that are distressing to the patient or caregiver. Despite providing for limited symptom management, these medications do not slow the progression of AD. To develop interventions that arrest the progression of AD requires a willingness to consider additional pathological features that hint at a multifactorial etiology for clinical AD.

Regarding the Amyloid Hypothesis

For the last two decades, *argumentum ad populum* in the field of AD was that Aβ is the primary causal agent in AD (Greenberg and Murphy 1994). This "amyloid hypothesis" states that accumulation of Aβ in the brain parenchyma induces inflammation, which in turn precipitates cognitive decline. However, recent evidence has begun to align with earlier observations that Aβ plaque development and clinical presentation of AD may be caused by the same underling etiology, rather than dementia being a downstream consequence of Aβ (Henderson 1988, Kozlov, Afonin et al. 2017). Evidence for a non-Aβ etiology includes observations that Aβ plaques can be present in the brain without clinical AD memory loss (Lue, Brachova et al. 1996), that Aβ appears to have a neuroprotective effects at physiological levels (Carrillo-Mora, Luna et al. 2014) and that anti-Aβ immunotherapies have failed to yield anything more promising than iatrogenic cerebral vasculopathy (Salloway, Sperling et al. 2009, Braunschweig and Jozsi 2011, Sperling, Salloway et al. 2012, Sakai, Boche et al. 2014). Due in part to the predominance of the amyloid hypothesis, the basic etiology of AD has not yet been

established despite first being described more than a century ago (Hippius and Neundorfer 2003). Though Aβ accumulation may not be the cause of AD, Aβ is harmful when aberrantly deposited, especially in the perivascular plaques seen in advanced AD (Brenowitz, Nelson et al. 2015). Indeed, AD research has often focused on the neuronal impact of Aβ, to the exclusion of the neurovascular unit (Zlokovic 2005). This insistence on a single etiological cause, Aβ in the brain parenchyma, rather than a multi-factor approach that considers blood vessel integrity, may well have negatively impacted the development of efficacious therapies to delay cognitive decline.

Vascular Amyloidosis and Related Complications

Amyloid beta plaques in the tunica media of cerebral and leptomeningeal vessels, a condition known as cerebral amyloid angiopathy (CAA), can be found at autopsy in most AD patients (Jellinger and Attems 2003, Brenowitz, Nelson et al. 2015). With advanced age, amyloid clearance is altered in way that results in perivascular Aβ deposits in the smooth muscle cell layer of the cerebral vessels (cvSMC). These Aβ plaques can directly activate complement. Complement is an enzymatic cascade that terminates in formation of transmembrane pores called membrane attack complexes (MAC). Normally, complement functions as part of the innate antimicrobial defense. Somatic cells are protected from MAC-mediated perforation during infection by a GPI-anchored glycoprotein called CD59 (An, Miwa et al. 2009). CD59 protection against autolysis is lacking for cvSMC in the diseased vessels of individuals with CAA, despite high levels of MAC accumulation (Zabel, Schrag et al. 2013).A more complete summary of Aβ, complement, and MAC as it relates to AD with concurrent CAA, is in CHAPTER TWO.

Challenges in Treating Amyloidopathies

Both AD and CAA share the pathological accumulation of Aβ (Coria, Prelli et al. 1988), and CAA may be associated with advanced AD. Interestingly, the spatial distribution and morphology of the amyloid plaques correlates with the location of the lesion in the vessel wall or brain parenchyma (Nagata, Maruya et al. 2000, Baron, Farid et al. 2014). There is also overlap in the clinical presentation of AD and CAA. Both present with memory loss, though this is the primary symptom of AD and is usually secondary to attention and executive dysfunction in CAA (Budson and Solomon 2016). Vascular fragility should be considered in patients with memory and executive function loss, even though they may only have an AD diagnosis.

Vascular disruption increases the risk of cerebral bleeds with prophylactic aspirin use (Kirsch, McAuley et al. 2009, Biffi, Halpin et al. 2010) and in anti-Aβ immunotherapy (Boche, Zotova et al. 2008). Based on the erroneous assumption of the amyloid hypothesis, in the late 1990s to early 2010s several pharmaceutical companies attempted to develop anti-Aβ immunotherapies to treat AD. Unfortunately, introducing anti-Aβ antibodies in patients with AD induces transient CAA, with evidence of microhemorrhages and other vascular lesions (Boche, Zotova et al. 2008).

Complement is a is probably involved with the increased frequency of neurovascular events. Vascular complement activation and MAC-mediated cvSMC destruction is believed to be a primary cause of vascular fragility in CAA (Itoh, Yamada et al. 1993, Verbeek, Otte-Holler et al. 1998). Anti-Aβ antibodies are known to activate complement in a dose-dependent manner (Crane, Brubaker et al. 2018). Most anti- Aβ clinical trials were discontinued due to lack of positive result, or in a few cases, actual

harm through accelerated cognitive declines. Though profoundly unfortunate, these results are unsurprising given the high rate of CAA in murine models of AD and the frequency of brain bleeds with anti-Aβ therapy in these animals (Wilcock and Colton 2009).

In the general population, elevated levels of brain microbleeds in AD indicate a poor prognosis, with possible feed-forward acceleration of cognitive decline as heme accumulates in the brain (Hanyu, Tanaka et al. 2003, Schrag, McAuley et al. 2010, Brundel, Heringa et al. 2012). Since as many as 9 of 10 patients with AD will develop mild to severe CAA (Jellinger and Attems 2005), there is a clear need for a therapeutic innovation that can protect vascular integrity in the presence of Aβ plaques.

Chitosan as a Gene Therapy Vehicle to Upregulate CD59

Chitin, from which chitosan is derived, is a linear polysaccharide that serves as a structural component in the exoskeletons of arthropods and fungi (Brodaczewska, Donskow-Lysoniewska et al. 2015). Chitin is composed primarily of N-acetylglucosamine residues (Fig S1, n1). Chitosan is derived from chitin by deacetylating at least 40% of the polymer residues to glucosamine (n2). A highly versatile cationic polymer, chitosan (Cs) has been used in a wide variety of applications, from bulk forms that serve as tissue scaffolds (Lin, Chen et al. 2013) and wound dressing (Woo, Choi et al. 2015) to nanovehicles for delivering proteins (Kim, Choi et al. 2013), drugs (Azadi, Hamidi et al. 2013) and nucleic acid constructs (Momenzadeh 2015). Beginning in the late 1990s, chitosan nano- and microparticle (CsM) preparations have been broadly explored as a non-viral alternative in gene therapy (Erbacher 1998). By definition

nanoparticles have a diameter smaller than 100 nm, but a careful reading of the chitosan literature demonstrates a surprising degree of flexibility in nomenclature. In compliance with technical definitions, we have selected the term “microparticle” since our constructs are larger than 100 nm diameter as measured by diffusion light scattering.

Micro- and nanoparticles can be precipitated under mild conditions due to complementary molecular charges between DNA and chitosan. This means that loading Cs with DNA is relatively straightforward: The amine group of chitosan carries a slight positive charge below pH 6.5, which is attracted to the slightly electronegative phosphate backbone of DNA. CsM form spontaneously when DNA is added to dilute Cs preparations. Tripolyphosphate, an ionic cross-linker, was added to reduce CsM size and surface charge for enhanced uptake by cells (Zheng, Tang et al. 2013, Xiao 2017).

CsM are often assumed to be biologically inert in gene therapy applications despite significant evidence to the contrary. Indeed, in the context of AD with CAA chitosan is particularly attractive as a vector due to its bioactivity. For example, Cs prevented Aβ formation in neuron-like cell cultures during exposure to oxidative stress and physically inhibited aggregation of Aβ (Khodagholi, Eftekharzadeh et al. 2010, Dai, Hou et al. 2015). CsM can be targeted directly to Aβ plaques and cross an intact blood-brain barrier (Agyare, Jaruszewski et al. 2014). Moreover, highly deacetylated Cs has innate anti-inflammatory properties that include augmenting Heme oxygenase-1, reducing Cyclooxygenase-2 expression and blocking pro-inflammatory cytokine production by astrocytes exposed to aggregated Aβ (Kim, Sung et al. 2002, Hyung, Ahn et al. 2016). CsM have even been used to deliver DNA that coded for siRNA against Tissue Factor to cultured primary human cvSMC, with successful knockdown of Tissue Factor expression

(Wan 2015). In summary, chitosan has the following desirable qualities for gene therapy applications:

- Readily encapsulates DNA
- Stimulates anti-inflammatory gene expression
- Can be targeted to Aβ plaques
- Directly and indirectly reduces the toxicity of Aβ
- Demonstrates efficacy as a transfection vector in cvSMC

This means Cs has significant potential in CD59-based gene therapy. Prior to completion of the work described herein, there was no published information about the bioactivity of Cs as related to CD59 expression. Further descriptions of the effects of Cs on cvSMC are presented in CHAPTER THREE.

An Oversight in Chitosan Microparticle Size and Stability

In addition to the biological consequences of Cs and cvSMC interactions, a more basic question regarding the physical properties of these particles needed to be is addressed. Multiple authors have described the effect of temperature and pH on size stability in CsM (Lu, Wang et al. 2010, Wang, Qian et al. 2011, Ma, Liu et al. 2012, Abdel-Hafez, Hathout et al. 2014, Jaiswal, Pradhan et al. 2014). However, none have systematically examined the effect of standard mammalian cell culture materials and conditions on particle stability. This was surprising given that 37 °C is a relatively high temperature compared to bench-top experiments carried out at 20-25°C. Additionally, buffered cell culture reagents are tuned to approximate physiologically neutral pH at the 5% CO_2 found in culture incubators. At pH 7.4, this is significantly higher than pH 5-6 at

which most particles are precipitated and characterized. CsM size variability may pose a hurdle to future therapeutic applications. Endocytosis of larger particles requires significantly more energy and is much less efficient than uptake of smaller particles. Thus, a systematic examination of CsM size and stability in a variety of common buffered solutions and standard cell culture conditions was undertaken. Data and analysis of these experiments are presented in CHAPTER THREE.

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CHAPTER TWO

ALZHEIMER'S SILENT PARTNER: CEREBRAL AMYLOID ANGIOPATHY

Cupino, T.L. and M.K. Zabel. (2014)

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Alzheimer's Silent Partner: Cerebral Amyloid Angiopathy

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Abstract

Alzheimer's disease (AD) is the most common form of dementia, which completely lacks a viable, long-term therapeutic intervention. This is partly due to an incomplete understanding of AD etiology and the possible confounding factors associated with its genotypic and phenotypic heterogeneity. Cerebral amyloid angiopathy (CAA) is a common, yet frequently overlooked, pathology associated with AD. CAA manifests with deposition amyloid-beta (Aβ) within the smooth muscle layer of cerebral arteries and arterioles. The role of Aβ in AD and CAA pathophysiology has long been controversial. Although it has demonstrated toxicity at super-physiological levels in vitro, Aβ load does not necessarily correlate with cognitive demise in humans. In this review, we describe the contributions of CAA to AD pathophysiology and important pathological mechanisms that may lead to vascular fragility and hemorrhages. Additionally, we discuss the effect of Aβ on smooth muscle cell phenotype and viability, especially in terms of the complement cascade.

Introduction

While memory loss is the most commonly known symptom of AD, the disease is also marked by steady decline in all aspects of cognitive function; including disruptions of personality, ability to communicate, sensory processing, and basic self-care (McKhann, Knopman et al. 2011). Though AD is the most common form of dementia, almost century after being discovered there are still no viable long-term therapeutic interventions. This may be due in part to an incomplete understanding of AD etiology and possible confounding factors associated with its genotypic and phenotypic

heterogeneity. Alarmingly, demographic projections indicate that as the population ages over the next few decades, a rapid increase in the number of individuals presenting with AD is expected (Alzheimer's 2012). The lack of treatment and growing population at risk for AD demand a reexamination of the premises that have driven research thus far.

Why a new paradigm?

The most widely accepted theory of AD etiology is known as the Amyloid Cascade Hypothesis, which states that overproduction and extracellular aggregation of the Amyloid-beta (Aβ) peptide is the fountainhead of AD. These extracellular Aβ aggregates act to increase neuronal kinase activity and results in phosphorylation of the microtubule associated protein tau (Hardy and Selkoe 2002). Hyperphosphorylation of tau induces formation of intracellular aggregates known as neurofibrillary tangles and alters intracellular transport along the microtubule tracks (Terry, Masliah et al. 1991). This in turn abolishes neuronal communication, resulting in cell death in a spatially conserved pattern and producing deficits in networks that subserve memory and cognition (Palop and Mucke 2010). Aggregation of Aβ and tau are well-established pathophysiological characteristics of AD brain tissue at autopsy. It is also known that in familial forms of AD, mutations in Amyloid Precursor Protein (APP), Presinilin 1 or Presinilin 2 accelerate Aβ accumulation (Waring and Rosenberg 2008). Presinilins function as part of the gamma secretase protein complex, one of three proteolytic enzymes responsible for cleaving APP into Aβ or non-amyloidogenic peptides. Autopsy samples from brain parenchyma of patients with familial AD, which account for less than 5% of all AD cases, present with Aβ and Tau accumulation similar to sporadic AD (Nochlin, van Belle

et al. 1993, Campion, Dumanchin et al. 1999). Additionally, since the APP gene is located on chromosome 21, individuals with Down's syndrome (trisomy 21) invariably develop AD-like dementia (Glenner and Wong 1984). However, it is highly uncertain to what degree familial AD and Down's syndrome recapitulate the initial stages of sporadic AD, which accounts for the vast majority of AD (Alzheimer's 2012). This is the core of the growing debate surrounding the Amyloid Cascade Hypothesis: Is the place of Aβ aggregation at the origin of AD or a downstream effect from an earlier insult (Castello and Soriano 2012)? Additionally, and of considerable concern, multiple recent immunotherapy clinical trials that target and clear Aβ have failed to reverse cognitive loss and in some cases have accelerated it (Doody, Raman et al. 2013, Tayeb, Murray et al. 2013). A full analysis of the arguments involved in the question of Aβ as a cause or effect is beyond the scope of this review; however, we will focus on a commonly overlooked aspect of amyloid pathology in patients with Alzheimer's disease which may contribute to disease onset and severity.

Cerebral amyloid angiopathy: Alzheimer's silent partner?

While the pathological hallmark of AD consists of extracellular Aβ deposits, cerebral amyloid angiopathy manifests with Aβ deposition in the walls of cerebral arteries and arterioles (Vinters 1987). Specifically, Aβ is deposited in the adventitia and media of the involved arterioles. The order in which the vessels of various brain regions are affected tends to follow a general pattern. Most frequently, arteries of the leptomeninges are first to show signs of pathology, followed shortly by penetrating arterioles in the neocortical grey matter (Preston, Steart et al. 2003). Vessels to the

posterior aspects of the brain are especially impacted, most severely in the occipital lobe but also in the parietal region (Tomonaga 1981, Vinters and Gilbert 1983, Pfeifer, White et al. 2002, Attems 2005, Attems, Quass et al. 2007). The frontal cortex has also been reported as a primary site of CAA pathology (Masuda 1985). The next stage of CAA is characterized by Aβ accumulation in vessels of the olfactory cortex, hippocampus, and cerebellum. Finally, the third stage demonstrates involvement of deep grey and white matter (Mann, Pickering-Brown et al. 2001, Thal, Ghebremedhin et al. 2003). Histologically, though Aβ distribution in CAA follows a regionally specific path of cortical arterioles, within each vessel the distribution of Aβ can be patchy and focal, with adjacent histological sections demonstrating heterogeneous degrees of Aβ accumulation.

These Aβ deposits can also be classified into three stages based on severity (Vonsattel, Myers et al. 1991). Initially Aβ accumulates in the adventitia of affected vessels and Aβ begins to intercalate between smooth muscle cells (cvSMC) in the media. Classified as mild CAA, at this stage Aβ is restricted to the tunica media without cvSMC death. Moderate CAA is defined by the replacement of cvSMC by Aβ and thickening of the media without disruption of the blood-brain barrier. As cvSMC are replaced by Aβ, the vessel loses the ability to respond to dynamic changes in blood flow and grows increasingly fragile. Severe CAA denotes extensive Aβ deposition with fragmentation and/or double-barreling of the vessel wall, fibrinoid necrosis, and aneurysm formation(Vonsattel, Myers et al. 1991). This loss of vessel integrity often results in blood extravasation to the perivascular space.

Moderate to severe CAA is a common comorbidity with AD, identified at autopsy in 75-98% of AD patients (Ellis, Olichney et al. 1996, Jellinger and Attems 2003). The

role of Aβ in AD and CAA pathophysiology has long been controversial. Although it may have toxic effects at super-physiological levels, Aβ load does not necessarily correlate with cognitive demise in humans (Lue, Brachova et al. 1996). However, it is known that Aβ deposition in CAA is a major contributor to vascular fragility and hemorrhages of various sizes (Greenberg, O'Donnell et al. 1999). Very small brain bleeds, or microbleeds, were once thought rare and silent. Recently it was found that brain microbleeds (BMB) appear as small, spherical, hypointense foci in magnetic resonance susceptibility weighted imaging (SWI) (Kirsch, McAuley et al. 2009). This has allowed for antemortem clinical analysis of microbleed prevalence. One or more BMBs are found in 16-32% of individuals with AD, but only 3-6% of healthy adults (Roob, Schmidt et al. 1999, Roob, Lechner et al. 2000, Nakata, Shiga et al. 2002, Tsushima, Tanizaki et al. 2002, Hanyu, Tanaka et al. 2003, Jeerakathil, Wolf et al. 2004, Cordonnier, Al-Shahi Salman et al. 2007). Even the smallest bleed can have devastating consequences for the neurons within and surrounding the hemorrhage.

Extensive neuronal death within the core of the hemorrhage results from a variety of insults, including oxidative stress from the breakdown of heme and free iron metabolism through the Fenton reaction (Lara, Kahn et al. 2009, Schrag, Mueller et al. 2011). Additionally, due to the relatively immune privileged state of the brain parenchyma, introduction of novel antigens also elicits a local immune response from microglia, astrocytes, and migrating peripheral immune cells. Together these result in increased production of complement factors, interleukin 1β and nitrous oxide (Brambilla, Couch et al. 2013). Through perivascular propagation of these inflammatory byproducts, nearby blood vessels are also damaged, further weakening them and increasing the risk of

additional bleeding (Rosand, Muzikansky et al. 2005). Indeed, increasing vessel fragility can lead to complete rupture and result in a hemorrhagic stroke. Often health practitioners are unaware a patient has CAA until he or she presents with a hemorrhagic stroke into the brain tissue, a devastating event known as intracerebral hemorrhage.

CAA and the risk of intracerebral hemorrhage

Clinical analysis of these patients describes probable CAA with supporting pathology, probable CAA, or possible CAA. The former is established only by availability of evacuated hematoma or cortical biopsy with demonstrated congophilic Aβ staining in the absence of other diagnostic lesions (Greenberg and Vonsattel 1997). Probable and possible CAA diagnoses are made in patients aged ≥ 55 years presenting with cortical hemorrhage or microbleed upon patient interview and gradient-echo MRI or CT evidence of one or more lobar hemorrhages in the absence of other hemorrhage etiology (Knudsen, Rosand et al. 2001). Based on the limitations of current diagnostic methodologies, it will be important for future exploration to focus on genetic and molecular markers that identify patients harboring vascular Aβ pathology.

Identification of patients with CAA is of paramount consequence due to the association between cerebral microbleeds and frank hemorrhages and the administration of anticoagulant therapy in this population (Vernooij, Haag et al. 2009, Biffi, Halpin et al. 2010). Alarmingly, even regular use of aspirin, a mild anticoagulant, was found to elevate the risk of intracerebral hemorrhage (ICH) in AD (Thoonsen, Richard et al. 2010). Even without the use of anticoagulants, AD patients have increased risk of hemorrhagic stroke compared to their non-demented peers, despite no increase in the risk of ischemic stroke

or stroke overall (Tolppanen, Lavikainen et al. 2013). ICH is the deadliest type of stroke. Accounting for 19% of all strokes, ICH has a survival rate of 48-50% at 30 days and only 38% live to one year post-stroke (Dennis, Burn et al. 1993, Feigin, Lawes et al. 2003, Sacco, Marini et al. 2009). Those that survive often suffer extensive loss of function and diminished quality of life, even compared to peers that have experienced ischemic stroke (Cadilhac, Dewey et al. 2010). ICH commonly results from small vessel hypertension and manifests in the deep nuclei, cerebellum and pons (Massaro, Sacco et al. 1991). Interestingly, ICH in normotensive patients often localizes in a lobar distribution, particularly in the temporal lobe, a region highly susceptible to both CAA and AD (Itoh, Yamada et al. 1993, Mesker, Poels et al. 2011, Sepulcre, Sabuncu et al. 2013). These normotensive patients have been shown to harbor CAA pathology in vessels that rupture (Massaro, Sacco et al. 1991).

A potential tool for identifying patients at risk from ICH is MRI through the SWI signal void caused by iron deposits from microbleeds. Higher prevalence of brain microbleeds correlate with elevated risk of non-traumatic intracerebral hemorrhage (47-56%) compared to microbleeds present with ischemic stroke (18-28%) (Cordonnier, Al-Shahi Salman et al. 2007). Microbleeds are damaging to the brain and diagnostic tools or therapeutic interventions would be best administered prior to vessel rupture. This underscores the need to elucidate the mechanisms of ICH in AD with CAA, especially considering recent pharmacological and genotypic risk factors.

From brain to blood: A switch in Aβ clearance

Microglia play a significant role in Aβ clearance. Activated amoeba-like microglia

phagocytize Aβ and transport it to nearby perivascular space. Normally, Aβ efflux from the perivascular space is mediated by low density Lipoprotein Receptor-related Protein 1 (LRP-1) when interacting with its ligands alpha-2-Macroglobulin and apolipoprotein E (Deane, Wu et al. 2004, Kanekiyo, Liu et al. 2012), while the Receptor for Advanced Glycation End-products (RAGE) mediates its influx from peripheral circulation (Deane, Du Yan et al. 2003). When the balance of exchange is toward the circulation, Aβ is taken up and digested by active macrophages or adheres to red blood cells and to be recycled in the spleen (Coria, Prelli et al. 1988). LRP-1 was decreased on cvSMC isolated from patients clinically and pathologically diagnosed with AD. Depressed LRP-1 follows overexpression of myocardin and serum response factor, which together down-regulate LRP-1 via activation of Sterol Regulatory Element Binding Protein-2 (Chow, Bell et al. 2007, Bell, Deane et al. 2009). Both myocardin and serum response factor increase due to hypoxia. Hypoperfusion of multiple brain regions is linked to both the development and progression of AD (Huang, Wahlund et al. 2002, Johnson, Jahng et al. 2005). Even in young individuals, cerebral ischemia can induce transient amyloidosis due to altered vascular basement membrane composition (Jullienne, Roberts et al. 2014). With age and accumulated insults, cvSMC switch to a non-Aβ clearance phenotypic.

Interestingly, there is a disparity between familial and sporadic AD in the spatial accumulation of Aβ plaques around the cerebral vasculature. Specifically, in sporadic AD there is a spatial association between Aβ plaques in the brain and associated blood vessels near parenchymal plaques. This is not seen in familial AD cases (Armstrong, Cairns et al. 1996). A leading hypothesis for accumulation of Aβ at the vessel wall is an imbalance between Aβ production and clearance; with decreased clearance as the

changing variable in late-onset AD (Fig S2). Together these suggest a local, non-genetic mechanism upstream of vascular Aβ deposition that could play a major role in initiating CAA.

A separate route of Aβ clearance occurs through non-specific bulk flow of brain interstitial fluid. The brain lacks a lymphatic system comparable to the rest of the body, so interstitial fluid must travel by retrograde periarterial transport through the Virchow-Robin Spaces (VRS). The VRS comprise a functional anatomic compartment associated with penetrating cortical arteries, in which the pia mater at the cortical surface forms a network of channels along the abluminal surface of the artery walls in the subarachnoid space (Krahn 1982, Hutchings and Weller 1986, Zhang, Inman et al. 1990). Once solutes, including Aβ, reach the vascular compartment by bulk flow, it is theorized that they are transported up the vessel wall in the VRS via retrograde, pulsatile motion generated through contraction and expansion of the artery (Schley, Carare-Nnadi et al. 2006). Based on tracer studies in mice, solutes are channeled along the basement membrane between smooth muscle cells in the arterial media of cortical arterioles and leptomeningeal arteries (Carare, Bernardes-Silva et al. 2008) down to the Circle of Willis (Szentistvanyi, Patlak et al. 1984). Similarly, the distribution of Aβ intercalated between adjacent cvSMC has been shown in human postmortem tissue (Soontornniyomkij, Choi et al. 2010). However, at the skull-base solutes drain into the cervical lymphatics before entering venous circulation, which may explain the lack of CAA on the large subarachnoid arteries (Cserr and Knopf 1992).

The contribution of perivascular drainage to CAA is not yet fully understood. Bulk flow and perivascular drainage are very slow (Bell, Sagare et al. 2007) and only

responsible for about 10-15% of Aβ clearance, while the majority is transcytosed across the blood brain barrier (Shibata, Yamada et al. 2000). Drainage could thus be a compensatory mechanism when other routes fail, (such as loss of LRP-1) which, when overloaded, results in CAA and backup of Aβ into the parenchyma. Based on the theoretical model of perivascular flow (Schley, Carare-Nnadi et al. 2006), aged arteries effected by arteriosclerosis or inflammation would become rigid, disrupting their contractile function and thus impeding perivascular flow.

The Amyloid Cascade Hypothesis posits that Aβ directly causes the demise of cvSMC. In a transgenic (TgCRND8) mouse model it was observed that Aβ itself causes these changes in cvSMC in cortical arteries (Dorr, Sahota et al. 2012). While this may be true in transgenic animals with Swedish and Indiana APP mutations, wild type Aβ fails to show the same degree of neurotoxic effect on primary human cvSMC *in vitro,* as do Dutch and Flemish mutant forms (Davis and Van Nostrand 1996, Wang, Natte et al. 2000). Specifically, cultured human cvSMC exposed to wild type Aβ show very little change in morphology or viability (Davis and Van Nostrand 1996, Wang, Natte et al. 2000). Simply increasing the load of wildtype Aβ to physiological disease-state levels is not enough to cause cytotoxicity. This begs the question, what entity induces cvSMC toxicity and vascular fragility if not Aβ? We hypothesize elements of the innate immune system may provide an explanation for vascular fragility.

Complement Cascade: A link between Aβ and smooth muscle cell death?

The Aβ toxicity observed in vitro may in large part be due to complement cascade activation. Complement, a potent inflammatory cascade, can cause robust cell death if

allowed to propagate unchecked. This antimicrobial cascade is so named because it functions as a rapid response that complements the slower adaptive immune response. Complement may be activated by any of three pathways: the Classical Pathway, mediated by C1q assembly to C1 at antigen:antibody complexes on the pathogen surface; the Mannose-Binding Lectin Pathway, initiated by direct interaction of mannose-binding lectin with the pathogen surface; or the Alternative Pathway, initiated by spontaneous hydrolysis of C3 at the pathogen surface. All three pathways converge at C3 convertase, an enzyme that cleaves complement component 3 (C3) into C3a and C3b (Fig 1A). C3b itself is utilized to form additional C3 convertase assemblies. C3 convertase amplifies the cascade and C3b accumulates on the cell membrane. Normally, this accumulation of C3b mediates phagocytosis of an infected or pathogenic cell. However, if C3b density continues to increase, C5 convertase will spontaneously assemble and initiate the terminal lytic pathway of the complement cascade. Membrane attack complex is the result. It begins as C5 convertase cleaves C5 into C5b and C5a. C5b binds covalently to the cell membrane and mediates assembly of C6, C7, and C8 (Fig 1B). Together these induce insertion and polymerization of multiple C9 monomers, forming the aptly named membrane attack complex (MAC), a transmembrane pore that punctures and kills the target cell (Fig 1C).

There is longstanding evidence for complement activation by Aβ *in vivo*. Both classical and alternative complement activation products have been found in senile plaques from AD patients (Eikelenboom, Hack et al. 1989, McGeer, Akiyama et al. 1989). In AD brain tissue, C1q colocalized with Aβ despite a lack of staining for immunoglobulin, the main activator of C1q (Rogers, Schultz et al. 1992). It is important

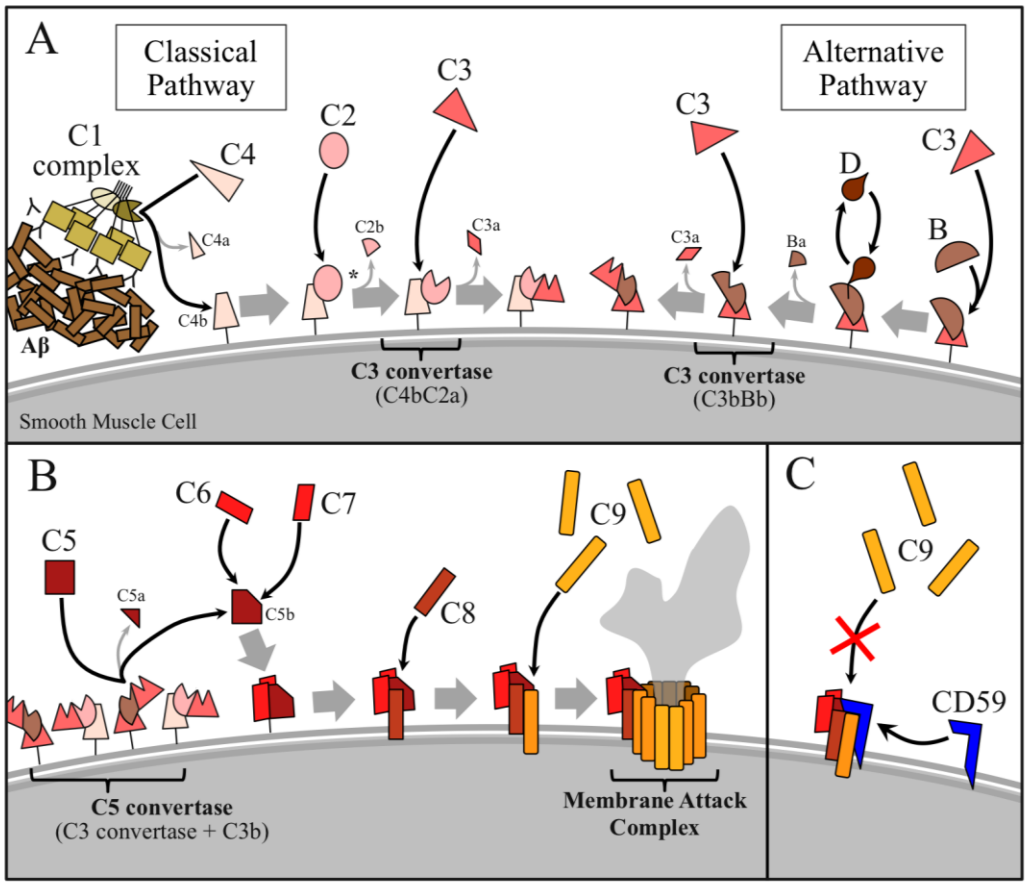

Figure 1. CD59 blocks membrane attack complex formation
Complement activation by Aβ forms membrane attack complex (MAC) unless interrupted by CD59. Complement may be activated via the classical or alternative pathways, which converge at the formation of C3 convertase (A). C3b, produced by C3 convertase, cleaves C5 to initiate formation of MAC (B), unless binding of GPI-anchored MAC-inhibiting protein (CD59) to pre-lytic MAC interrupts polymerization of C9 (C). *Cleavage of C2, like C4, is mediated by the C1 complex. Mannose-binding lectin pathway not shown

to note that anti-Aβ immunotherapy utilizes immunoglobulin that targets Aβ. Assuming that the immunoglobulin is able to activate C1q, this could provide a potential mechanism for the accelerated neurological decline seen in some clinical trials (Racke, Boone et al. 2005). The overlap of Aβ and C1q in AD patients is also found in the leptomeningeal and cortical vessels that stain positive for Aβ (Verbeek, Otte-Holler et al. 1998). Furthermore, C4b, a component of C3 convertase, is also present in Aβ plaques, indicating that C1 is fully assembled and functional. This is supported *in vitro*, where it is observed that Aβ directly activates C1q (Rogers, Schultz et al. 1992). However, early complement activation alone is insufficient to induce cell death. For that, MAC is a prime suspect.

Like complement components from the early cascade, MAC was also found in autopsy samples from AD patients (Lue, Brachova et al. 1996, Webster, Lue et al. 1997, Verbeek, Otte-Holler et al. 1998, Yang, Li et al. 2000). Interestingly, very little MAC was observed in identical brain regions of high pathology controls, which were identified by significant parenchymal Aβ accumulation without concurrent cognitive loss (Lue, Brachova et al. 1996, Tanskanen, Lindsberg et al. 2005). In AD, MAC is found together with plaques in both the brain parenchyma and blood vessels (Lue, Brachova et al. 1996, Verbeek, Otte-Holler et al. 1998). At the cellular level, MAC was found at dystrophic neuritis and colocalized to the periphery of myelin near Aβ plaques (Webster, Lue et al. 1997). *In vitro* evidence reveals that Aβ is a sufficient to directly and fully activate the complement cascade, inducing formation of MAC (Brandt, Pippin et al. 1996, Cadman and Puttfarcken 1997, Webster, Lue et al.). Further analysis shows that the MAC is active, and depresses viability in neuronal cell cultures (NT2) (Brandt, Pippin et al. 1996). Primary human AD brain as well as SH-SY5Y cells (a neuroblastoma line

differentiates into neuron-like cells upon treatment with retinoic acid) exposed to Aβ in culture express mRNA for all complement proteins (C1q, C2, C3, C4, C5, C6, C7, C8, and C9). Furthermore, SH-SY5Y express protein for components C1q, C3 and C9 (Shen, Sullivan et al. 1998). A similar effect is seen in the vasculature.

Postmortem-derived cerebrovascular smooth muscle cells express mRNA for all components of the complement cascade, except C3. However, C3 and C6 mRNA are elevated by introduction of aggregated Aβ (Walker, Dalsing-Hernandez et al. 2008). Under the same conditions, western blot analysis demonstrated expression of C1r, C1s, C3, C4, C6, C7, C8 and C9 by primary cerebrovascular smooth muscle. Upon treatment with Aβ, C3 protein expression is elevated compared to untreated controls. We speculate that these sources of complement components would be most significant prior to the point at which CAA disrupts the endothelium. Once the blood brain barrier is compromised, the cascade components abundant in the circulating plasma will be readily activated by exposed Aβ plaques in the vessel wall.

Work from our lab has demonstrated increased C3b levels in occipital lobe parenchyma of AD patients with CAA compared to AD patients without CAA and pathological controls (Zabel, Schrag et al. 2012). This increase in C3b was accompanied by a differential binding of Aβ to microglial CD11b, was observed predominantly in patients with CAA. We postulated that this mechanism could result in the increased levels of MAC and Aβ seen on cvSMC in CAA. In conjunction with these findings, we also found that the previously described *Complement Receptor 1 (CR1)* gene (rs6656401), which is associated with increased risk of AD (Lambert, Heath et al. 2009) and CAA (Biffi, Shulman et al. 2012), was significantly enriched in patients with CAA

compared to those without CAA (Zabel, Schrag et al. 2012). The *CR1* gene codes for CR1 protein, which is a transmembrane receptor for C3b/C4b, both cleaving complement components and inhibiting their action. CR1 polymorphisms may alter the appropriate protein function and push the complement activation phenotype even further towards MAC deposition. It remains to be determined if complement generation is intended as a protective response; and if so, what factors induce a shift to pathogenesis. Given that smooth muscle cells express complement proteins, microglia are observed to deposit C3b along with Aβ, and Aβ itself is sufficient to activate complement, smooth muscle cells in CAA-afflicted vessels would be particularly vulnerable to complement attack and lysis.

The potent cytolytic effects of MAC necessitate protective countermeasures on somatic cells. Bystander tissue is generally protected from MAC by constitutive expression of various complement inhibitors. Among these is Protectin (CD59), a glycoprotein that blocks polymerization of C9 and thus inhibits formation of MAC. Protectin mRNA and protein is decreased in the hippocampus and frontal cortex of AD compared with age-matched non-demented controls. In SH-SY5Y neuronal cultures, Protectin mRNA is diminished in a dose-dependent manner by exposure to Aβ (Yang, Li et al. 2000). Furthermore, inhibition of Protectin significantly exacerbates the deleterious effects of Aβ alone in neuron cultures (Shen, Sullivan et al. 1998). In blood vessels from patients with AD and CAA, Protectin is not upregulated despite high levels of MAC deposition (Zabel, Schrag et al. 2012). This, together with the observation that Aβ induces expression of complement proteins, invites further investigation into the role of complement and MAC in the consequences to smooth muscle observed with CAA and AD.

Conclusions

As demographic trends point to a rapidly growing population afflicted with AD, the means to stop or even slow disease progression remain elusive. To find a therapeutic intervention, it may soon be necessary to break with old paradigms and pursue new avenues of inquiry. As a common AD comorbidity, CAA may be such an avenue. By achieving a deeper understanding of AD with CAA, and the interplay of complement and Aβ, novel therapeutic targets may be discovered. As an additional point of interest, this could also facilitate removal of such patients from clinical trials using therapies that might exacerbate the CAA phenotype, including anticoagulants (Vernooij, Haag et al. 2009, Biffi, Halpin et al. 2010, Biffi, Plourde et al. 2010) and amyloid-β immunotherapy (Racke, Boone et al. 2005), to 1) reduce the risk of complications, and 2) to remove potential confounding variables that may alter clinical trial outcomes. To ignore CAA, with so high an incidence in AD and so great a potential consequence if left unaddressed, is to ignore a direction of inquiry that is potentially rich in targets for therapeutic intervention.

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CHAPTER THREE

STABILITY AND BIOACTIVITY OF CHITOSAN AS A TRANSFECTION AGENT IN PRIMARY HUMAN CELL CULTURES: A CASE FOR CHITOSAN-ONLY CONTROLS

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Stability and Bioactivity of Chitosan as a Transfection Agent in Primary Human Cell Cultures: A Case for Chitosan-Only Controls

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Abstract

Chitosan polymers (Cs), from which microparticles (CsM) may be precipitated to deliver various intracellular payloads, are generally considered biologically inert. We examined the impact of cell culture conditions on CsM size and the effect of chitosan on CD59 expression in primary human smooth muscle cells. We found that particle concentration and incubation time in biological buffers augmented particle size. Between pH 7.0 and pH 7.5, CsM size increased abruptly. We utilized CsM containing a plasmid with a gene for CD59 (pCsM) to transfect cells. Both CD59 mRNA and the number of CD59-positive cells were increased after pCsM treatment. Unexpectedly, CsM also augmented the number of CD59-positive cells. Cs alone enhanced CD59 expression more potently than either pCsM or CsM. This observation strongly suggests that chitosan is in fact bioactive and that chitosan-only controls should be included to avoid misattributing the activity of the delivery agent with that of the payload.

Introduction

The stability of CsM under cell culture conditions is currently under-reported, with the physical properties of particles measured prior to use with cells but not after incubating in cell culture conditions. Ionic gelation of CsM depends on electrostatic interactions between amine groups of chitosan and hydroxyl moieties in tripolyphosphate molecules. However, many varieties of the cell culture media and salt solutions used in biological applications contain phosphate anion buffers. We explored the relationship of pH and CsM size under conditions typical when culturing human cells in multiple types

of cell culture media. Our hypothesis was that CsM size would be relatively stable under cell culture conditions.

Chitosan polymers are made by deacetylating chitin, a linear N-acetyl-D-glucosamine polysaccharide which functions as a structural component in arthropod exoskeletons and fungi. Chitin is found in a variety of parasitic and opportunistic organisms that infect humans and elicits a strong immune response (Brodaczewska, Donskow-Lysoniewska et al. 2015). The relationship between Cs and the immune system is more complex, with reports of both pro- and anti-inflammatory effects that are directly linked with the physical properties or endotoxin contamination of chitosan (Han, Zhao et al. 2005, Lieder, Gaware et al. 2013, Tu, Xu et al. 2016). Despite these effects, the low toxicity, general biocompatibility, and mild conditions under which chitosan nano- and microparticles are precipitated by ionic gelation make Cs an increasingly popular tool for intracellular payload delivery.

We utilized CsM as an intracellular gene delivery vector with the goal of upregulating CD59 (OMIM 107271, Ensembl 966) via a complementary DNA (cDNA) plasmid encoding for the human gene. CD59 is a GPI-anchored glycoprotein that protects tissues from autolysis during an innate immune response called complement cascade. Complement is activated during infection or inflammation and terminates in a lytic transmembrane pore called membrane attack complex (MAC, Fig 1). CD59 interrupts MAC formation. Vascular Aβ plagues, such as those that occur in advanced Alzheimer's disease, aberrantly stimulate complement in the absence of CD59 upregulation (Zabel, Schrag et al. 2013). This leads to cerebrovascular smooth muscle cells (cvSMC) death, increased vascular fragility and accelerated cognitive decline (Webster, Lue et al. 1997,

Yang, Li et al. 2000). CsM were selected as a non-viral gene therapy vector because they can be targeted to perivascular Aβ plaques and cross the blood brain barrier in animal models (Li 2003, Jaruszewski, Ramakrishnan et al. 2012, Agyare, Jaruszewski et al. 2014, Wan 2015). However, since CD59 is involved with immune function, we included Cs-only controls to rule out potential off-target effects on CD59 expression.

Materials and Methods

Chitosan Polymer Preparation

Chitosan (a gift from Scion BioMedical, Raymond, Washington) derived from Dungeness crab shells (*Cancer magister*, 662 kDa) was dehydrated at 65 °C for 48 h, then dissolved in 4.6 mM glacial acetic acid (0.1% w/v) while stirring on ice for 16 hours. After filtration through a 0.22 μm Stericup from EMD Millipore (Billerica, Massachusetts), the pH was adjusted to 5.55 with 1 M NaOH. Chitosan polymer aliquots were stored at -20 °C. Before use, Cs was thawed overnight at 4 °C. All experiments described herein were from a single preparation of Cs to minimize batch-wise variability. This batch of Cs was 88.3% deacetylated (commercially analyzed by HaloSource, Bothell, WA, chitosan lot number 09-BCCP-0523) and contained <0.250 EU/mL endotoxin (Limulus Amebocyte Lysate assay, Endosafe PTS, Charles River, Charleston, SC).

CD59 Plasmid Cloning and Characterization

TrueClone cDNA plasmids containing human CD59 (Origene, Rockville, Maryland) were expanded in Alpha-Gold competent *Escherichia coli* bacteria (Origene). Transformation of *Escherichia coli* via heat shock was completed according to the

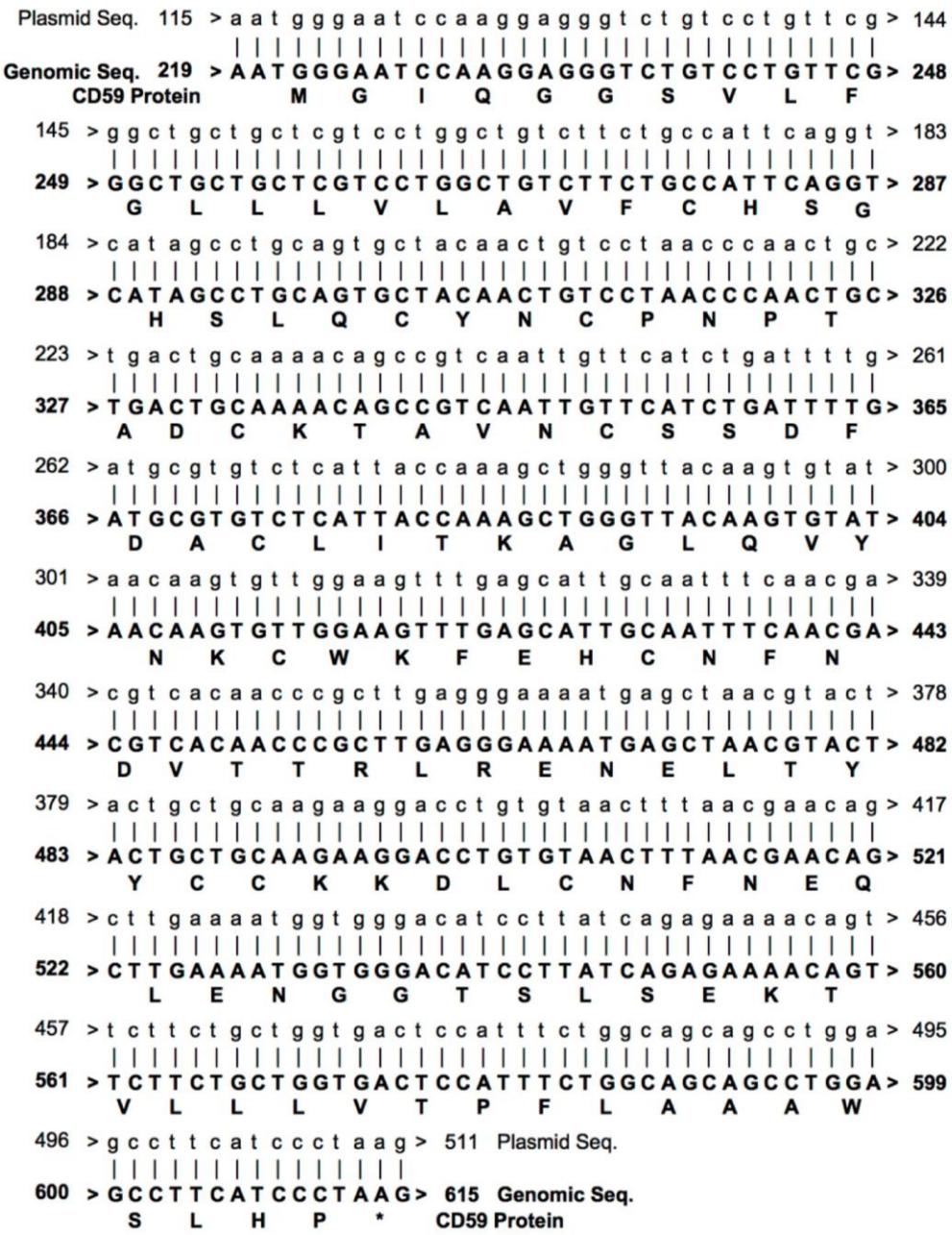

Figure 2. Plasmid, cDNA and protein sequences for the CD59 gene
Sequenced human CD59 from the plasmid clone used herein, compared to the complementary DNA from mRNA that codes for the CD59 protein. *Stop codon.

manufacturer's instructions. Briefly, frozen bacteria were thawed on ice. Plasmids were added and cells incubated for 35 s at 42 °C without shaking. The bacteria were cooled on ice before dilution in S.O.C. medium from Thermo Fisher Scientific (Waltham, Massachusetts). Transformed bacteria were plated on LB agar Ampicillin-100 from Sigma-Aldrich (St. Louis, Missouri) and incubated overnight at 37 °C. Three colonies were expanded separately in LB broth from Invitrogen (Thermo Fisher Scientific) and plasmids extracted via Maxiprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid yield was determined by spectrophotometry (Nanodrop 1000, Thermo Fisher, Delaware) and plasmid DNA (pDNA) was commercially sequenced (Fig 2) by Eton Bioscience (San Diego, California) with forward (VP1.5) and reverse (XL39) primers from Origene.

Microparticle Precipitation

Chitosan microparticles (CsM) were precipitated from chitosan polymers (Cs) with and without plasmid DNA (pDNA) containing human CD59 in weakly acidic Vehicle (pH 5.6, Fig 3) by ionic gelation as previously described (Csaba 2009) with minor modification. Three mL of sodium tripolyphosphate (TPP, Sigma Aldrich) in nanopure water (0.07% w/v) was added dropwise to 7 mL of thawed Cs while stirring at 5000 RPM. For CsM containing plasmid DNA (pCsM), 10 μg of pDNA encoding human CD59 on a cytomegalovirus promoter (Origene, pCSMV6-XL5 vector) was added to 7 mL of Cs. After stirring for 2 min, TPP in nanopure water was added as described above. After mixing for 10 min, each microparticle solution was centrifuged (Beckman, Brea, California) at 2500 RCF for 10 min to remove large aggregates. Microparticles in the

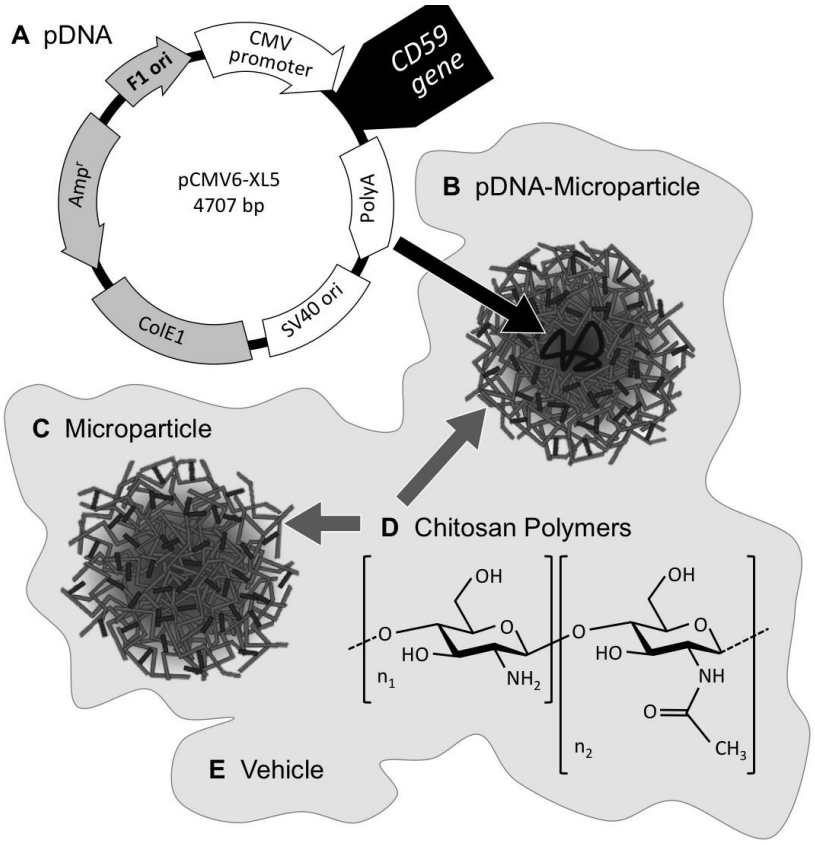

Figure 3. Illustration of the various treatment groups
Plasmid DNA containing human CD59 on a high-yield promoter (A, pDNA) was incorporated into chitosan microparticles (B, pCsM). Both pCsM and microparticles without pDNA (C, CsM) were precipitated from chitosan polymers (D, Cs). All chitosan treatments were prepared and delivered in mildly acidic Vehicle (E, pH 5.6).

retained supernatant were left undisturbed for 16 h at room temperature prior to experimental use.

Microparticle Yield

Microparticle yield was quantified by the equation (CsM Wt ÷ Cs Wt) × 100% where CsM Wt was the dry weight of post-centrifugation CsM and Cs Wt was the dry weight of the Cs used to make the CsM. To acquire dry chitosan weight, aqueous samples were placed in clean, pre-weighed 1 mL tubes and dried for 48 h at 35 °C. Once dried, the tubes were sealed and allowed to cool before weighing.

Microparticle Characterization

CsM in solution were mixed with selected cell culture media at a concentration of 1-part CsM solution to 1, 2, 5 or 10 parts serum-free Medium 231 (Gibco, Invitrogen), high glucose DMEM (GlutaMAX, Gibco), low glucose DMEM (Gibco) or RPMI 1640 (Gibco). These concentrations roughly correspond to 50%, 30%, 20% and 10% CsM solution in culture media. Samples were pipetted into disposable semi-micro cuvettes (Sigma-Aldrich) and either immediately sealed with plastic paraffin film (Parafilm, Neenah, WI) or incubated under cell culture conditions (5% CO_2 at 37 °C) for 2 h and then sealed with Parafilm. Size was measured by dynamic light scattering (DLS, Nicomp 380 ZLS, Particle Sizing Systems, Port Richey, Florida) after allowing 10 min for temperature equilibration. Once equilibrated, size measurements were recorded every 60 seconds. After five consecutive minutes of stable size readings were achieved, the final value was recorded as the particle size. Control mixtures were sealed immediately after pipetting, and size measured without incubation. Following sizing, the pH of each sample

was measured (ThermoOrion, Thermo Fisher Scientific). All data are shown as Mean±SEM, $n \geq 5$.

Smooth Muscle Cell Culture

Primary human cvSMC from ScienCell (Catalog #1100, Carlsbad, California) were cultured under standard conditions (5% CO_2 at 37 °C) in Medium 231 containing 5% Smooth Muscle Growth Supplement (Gibco), 0.1 mg/mL Primocin (Invivogen, San Diego, California), and 100 U/mL each penicillin and streptomycin (Life Technologies, Thermo Fisher Scientific) in BioCoat Collagen I culture flasks (Corning, New York). Cells were passaged using 0.05% Trypsin EDTA (Gibco) after washing with sterile Dulbecco's Phosphate Buffered Saline (Cellgro, Mediatech, Corning). For flow cytometry and RT-qPCR experiments, cvSMC were plated in BioCoat Collagen I 96-well plates (Corning) at $1x10^4$ cells/well. After two days in growth-supplemented media, the cells were transitioned to Smooth Muscle Differentiation medium (Gibco). Cell morphology was evaluated visually (Fig S3) prior to treatment.

Treatment of Smooth Muscle Cells with Microparticles

After differentiating for 24 h, cells were incubated for 2 h under standard culture conditions with 50%, 30%, 20% or 10% CsM in serum-free Medium 231 (% v/v). All treatments except pDNA were delivered in Vehicle. The plasmid quantity used in the pDNA-only treatment was equivalent to the amount of pDNA delivered by pCsM, adjusted for CsM percent yield. After treatment, cvSMC were allowed to recover for 0, 8, 24, 48 or 72 h with daily fresh media every 24 h prior to staining and analysis. Dead cell controls for flow cytometry were treated with 70% EtOH for 1 h prior to staining. Untreated live cell controls were incubated in serum-free Medium 231 for 2 h without

exogenous treatment. Lipid-based transfection methods were attempted but uniformly resulted in overwhelming loss of cell viability, rendering this method of transfection impractical in differentiated primary cvSMC.

Flow Cytometry

Cells were washed with warm PBS (Lonza, Allendale, New Jersey) and incubated in Far Red LIVE/DEAD stain (Invitrogen) according to the manufacturer's instructions with the minor modification of 1 μL/mL dye concentration. Cells were then gently washed with 0.05% Triton-100X in PBS (PBST) and fixed in 4% paraformaldehyde. Cells were blocked in PBST with 1% bovine serum albumen (PBSTB). Mouse monoclonal anti-CD59 [MEM-43] antibodies (Sawada, Ohashi et al. 1989) directly conjugated to fluorescein isothiocynate (FITC) from Abcam (ab18237, Cambridge, Massachusetts) were diluted to 0.5 μL antibody per 100 μL in PBSTB and incubated for 1 h in the dark at room temperature. FITC-labeled anti-CD59 antibodies were mixed with an equal concentration of unlabeled Mouse monoclonal anti-CD59 [MEM-43/5] (Abcam, ab9183) as the specificity control. After washing to remove excess antibodies, the cells were trypsinized (Gibco) and analyzed using a 7-color MACSQuant analyzer (MiltenyiBiotec, Auburn, California). Calibration with standardized beads (Miltenyi) was performed prior to each run. Flow cytometry data were processed with Flowjo data analysis software (TreeStar, Ashland, Oregon) available through the Flow Cytometry Education and Training Core Facility in the Center for Health Disparities and Molecular Medicine at Loma Linda University. The gating strategy included only intact cells (Fig 4). All data shown as Mean±SEM, $n \geq 5$.

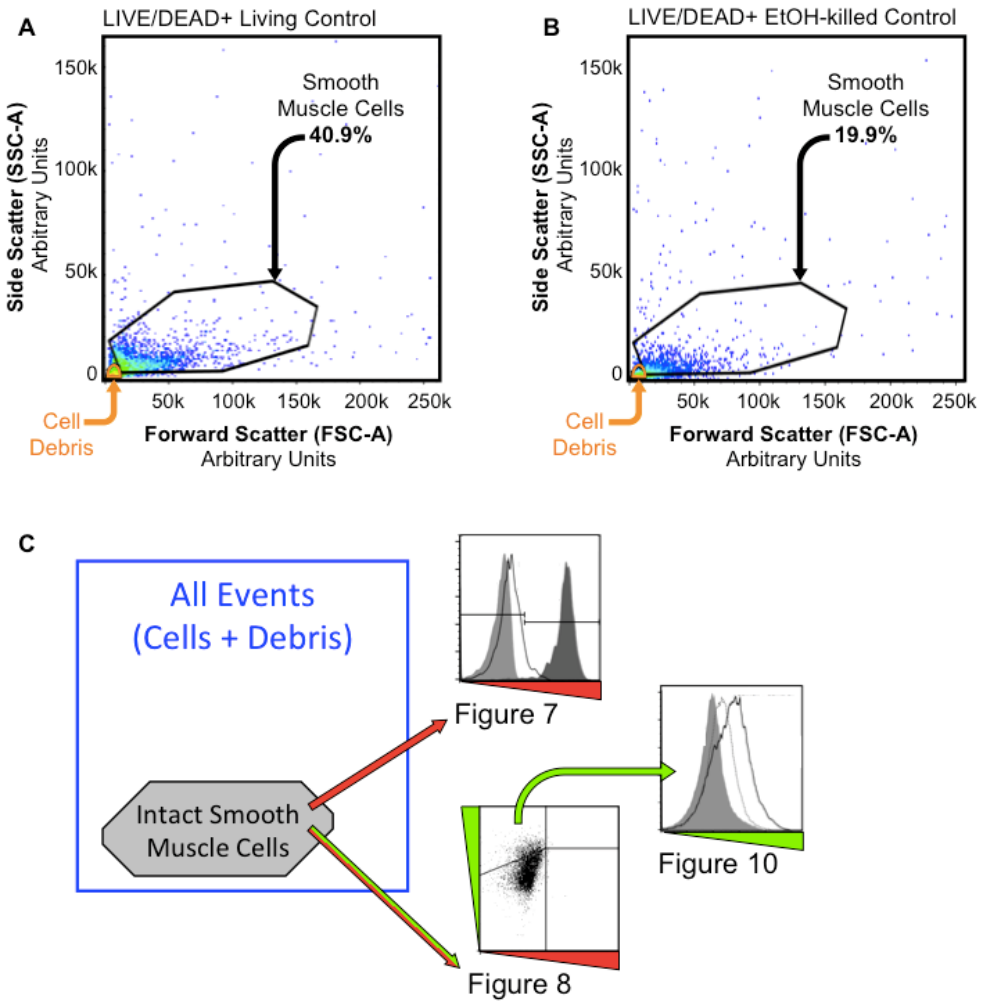

Figure 4. Flow cytometry gating stratagem for smooth muscle cells
Every particle passing through the flow cytometry detector triggers an event marked on a density map. The Smooth Muscle Cells gate (SMCg, black) was drawn to include intact cells, both living and dying. The total events in SMCg is a large proportion of all events for living cells (A) than EtOH-killed cells (B), because dead cvSMC rapidly degenerate into cellular debris fragments (orange). The SMCg contains 100% of the cvSMC in each measured sample, and from it the percent live cells (Red, LIVE/DEAD stain), percent CD59 and CD59 expression (Green, CD59-FITC antibodies) can be determined (C).

RNA extraction and cDNA synthesis

RNA extraction and on-column DNA digestion were performed using the Quick-RNA MicroPrep kit (Zymo Research, Irvine, California) without modification as described by the manufacturers. RNA purity and concentration were determined by spectrophotometry (Nanodrop 1000). The extracted RNA was subsequently converted to cDNA via reverse transcription using the iScript Advanced cDNA Synthesis kit (BioRad, Hercules, California) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction

All RT qPCR experiments, including primer efficiency, used SsoAdvanced Universal SYBR Green Supermix (BioRad). Reactions were performed on the BioRad CFX96 ThermoCycler with the following primers: CD59 Forward 5′-GAT TTT GAT GCG TGT CTC ATT ACC-3′, CD59 Reverse 5′-CAC CAT TTT CAA GCT GTT CG-3′, PKG Forward 5′-CAG TTT GGA GCT CCT GGA AG-3′, PKG Reverse 5′-GCA TCT CTT GGC CAC TAG C-3′. Reactions were performed in triplicate on two independent experiments and analyzed using the CFX Manager 3.0 software (BioRad). CD59 expression was normalized to the housekeeping gene PGK1 and calculated relative to an untreated control using the $2^{-\Delta\Delta CT}$ method. Data shown as Mean ± SEM, n = 2 in triplicate.

Statistical Analysis

One-way between-subjects ANOVA was performed to detect treatment differences in percent live and CD59-positive cells for dilutions and time-points of interest using StatPlus 6.0 (AnalystSoft Inc, Alexandria, Virginia). Three-way between-subjects ANOVA was performed with treatment, dilution, and time as fixed factors, followed by

Tukey post-hoc tests for possible pairwise differences, using SAS version 9.3 (SAS Institute Inc, Cary, North Carolina). Untreated controls (n = 52) were excluded from three-way ANOVA to preserve the factorial design, as the untreated cells had only a single dilution level. Viability of cells exposed to 20%, 30% and 50% treatments were compared to concurrent data for 10% treatment (Fig 5). Student's paired t-Test with two-tailed distribution was used to determine significance between each treatment group and untreated controls for RT-qPCR data (T-Test, Microsoft Excel, Redmond, Washington). Significance was considered at $p \leq 0.05$.

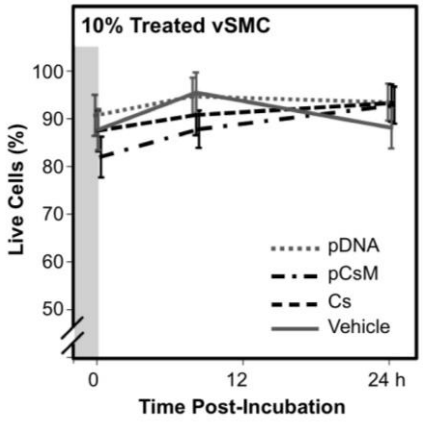

Figure 5. Smooth muscle cell viability is preserved during low-dose treatment

There is no difference in viability at any time between treatment with 10% pCsM, CsM or Cs in cell culture media. These data were used as the baseline against which 20%, 30% and 50% treatments were analyzed for figures 7,8 and 9.

Results and Discussion

Particle Size Stability

In Vehicle alone (100% CsM) the size and pH (288±16 nm and 5.7±0.1 respectively) were unchanged by incubation under cell culture conditions (37 °C, 5% CO_2) as determined by diffusion light scattering. Microparticle yield was 65±6%

Cell Culture Conditions and Microparticle Size

We observed that CsM size is highly variable after incubation under cell culture conditions in buffered culture media. Specifically, CsM diameter increased dramatically from 372±11 nm to 707±304 nm during incubation (10% CsM in Medium 231), with notably higher variability in final particle size (Fig 6A-B). At 20% and 30% CsM in Medium 231, pH and mean particle diameter were essentially unchanged by incubation under cell culture conditions. CsM size was destabilized by incubation in DMEM and RPMI culture media under cell culture conditions (Table 1). Higher pH correlated with larger particle size, and incubation under culture conditions increased size variability.

Phosphate, pH and Particle Size

When grouped by pH, CsM size distributions were smaller (373±97 nm) below pH 7.0 and larger (1773±458 nm) above pH 7.5 (Fig 6C). CsM demonstrated high size variability and an abrupt shift in particle size distribution between pH 7.0 and 7.5 CsM. This may be driven by changes in the intermolecular charge states of phosphate moieties. Tripolyphosphate, the cross-linker used to precipitate CsM, is an unlikely culprit since it is stably deprotonated between pH 5.3 and 8.5 (Lim and Seib 1993). However, most cell culture media and physiologic salt solutions contain phosphate anions, even if primarily buffered by carbonate (Table 2). We speculate that CsM size instability after introduction

to culture media is driven by exogenous phosphate anions. The abrupt CsM size shift and bimodal size distributions within individual measurements occur near pH 7.2 (Fig 6D-E), corresponding with the pH at which the second hydroxyl on phosphate is deprotonated (Fig 6F-G, pKa2 7.198) (Lide). Our observation that particle size measurements stabilized more rapidly below pH 7.2 (5.0±0.4 min, n = 12) than above pH 7.2 (9.1±0.8 min, n = 13) in culture media, supports the hypothesis that CsM hydrodynamic radii are in flux after pH increases above the pKa2 of phosphate anions. We speculate that increased charge density from deprotonated phosphate anions in culture media above pH 7.2 may stimulate particle aggregation.

Though it is possible to exclude inorganic phosphate with Good's buffers (Table 2) for in vitro transfection, the utility of these for in vivo models is limited at best. At pH7.4 and 0.87-1.45 mM phosphate (Fischbach and Dunning 2009), blood serum may also destabilize CsM size. Particle uptake by living cells is both size and energy dependent. Microparticles with a diameter of 200-500 nm are readily endocytosed in caveolin-mediated transfection (Rejman, Oberle et al. 2004, Peng, Tseng et al. 2011). Unsurprisingly, transfection efficacy decreases as CsM size grows. Larger particles are taken up by macropinocytosis, a process so energy intensive that enriched culture media is required for successful transfection (Rejman, Oberle et al. 2004, Nimesh, Thibault et al. 2010, Hoemann, Guzman-Morales et al. 2013). Enhanced stability of CsM size under physiologic conditions would keep particles small, reducing uptake stress in cells during gene delivery applications.

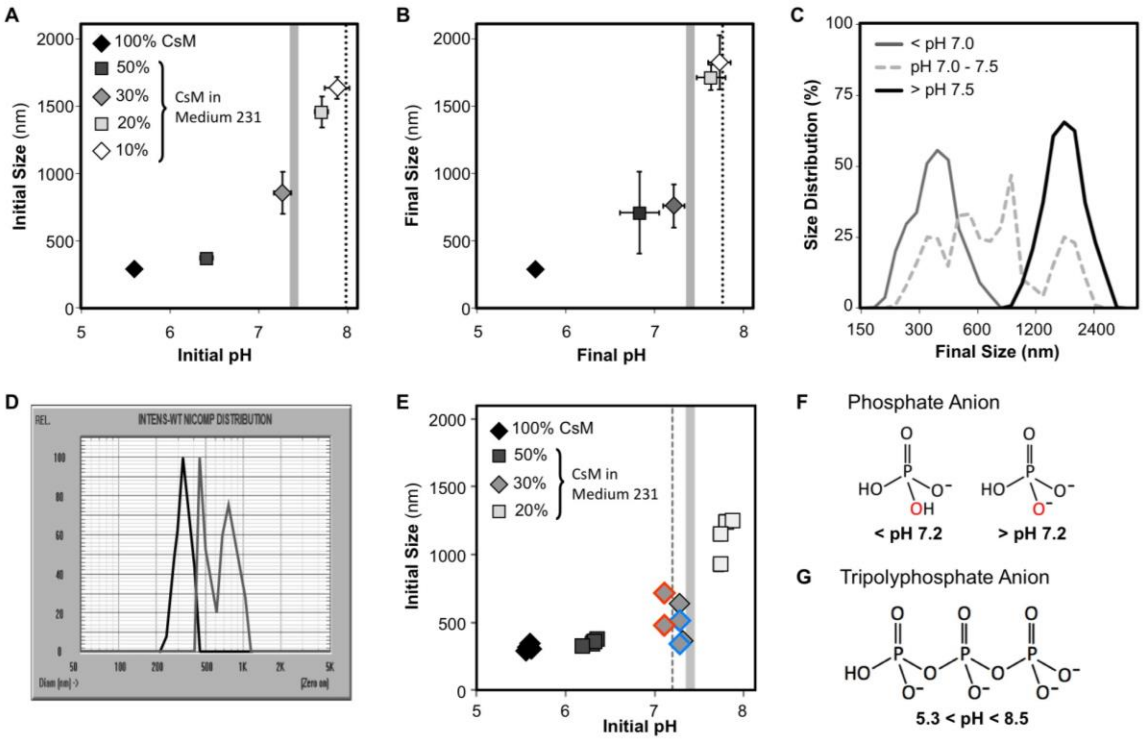

Figure 6. Relationship of pH and particle size is a function of phosphate charge
Decreasing CsM concentrations in Medium 231 drives pH-dependent swelling of particle size (A). Dotted line in (A) and (B) illustrates pH of Medium 231 alone. Incubation under standard human cell culture conditions (5% CO_2, 37°C) increased particle variability approaching physiologic pH (B, vertical grey bar). A discrete shift in CsM size distribution occurred between pH 7.0 and pH 7.5 (C). Monomodal (black) and bimodal (grey) CsM size populations were observed in separate 30% CsM preparations prior to incubation (D, representative screen capture). Bimodal pairs (red and blue) occur near pH 7.2 (E, dashed line), which is the pKa_2 for phosphate anions (F). Tripolyphosphate is stable throughout the observed pH range (G).

Table 1. CsM size is destabilized by incubation in DMEM and RPMI culture media under standard cell culturing conditions.

CsM size is destabilized by incubation in DMEM and RPMI culture media under standard cell culturing conditions.

% CsM in Culture Medium	Size ± SEM (nm)			pH ± SEM		
		DMEM[a]		RPMI	DMEM	
		High Glucose[b]	Low Glucose[c]	1640[d]	High Glucose	Low Glucose
100%[e]	253 ± 19	301 ± 25	300 ± 25	5.58 ± 0.03	5.67 ± 0.03	5.66 ± 0.04
50%	1590 ± 345	1642 ± 170	380 ± 19	7.11 ± 0.15	7.42 ± 0.16	6.73 ± 0.08
30%	1851 ± 187	2298 ± 104	1828 ± 342	7.48 ± 0.10	7.65 ± 0.05	7.32 ± 0.07
20%	1913 ± 254	1716 ± 148	2094 ± 164	7.75 ± 0.12	7.96 ± 0.11	7.72 ± 0.07
10%	1596 ± 85	2021 ± 153	2019 ± 185	7.89 ± 0.08	8.11 ± 0.08	7.71 ± 0.06
0%	–	–	–	8.04 ± 0.20	7.96 ± 0.10	7.80 ± 0.08

[a] DMEM, Dulbecco's Modified Eagle Medium.
[b] High glucose, 25 mM dextrose.
[c] Low Glucose, 5.6 mM dextrose.
[d] RPMI, Roswell Park Memorial Institute Medium.
[e] Size and pH of particles incubated in Vehicle without cell culture media.

Table 2. Summary of phosphate content in common culture media and buffered salt solutions.

Summary of phosphate content in common culture media and buffered salt solutions.

	Culture Media			Buffered Salt Solutions				
Organic & Inorganic Buffers	Medium 231[a,i]	DMEM[b] (mM)	RPMI[c] (mM)	DPBS[d] (mM)	EBSS[e] (mM)	HBSS[f] (mM)	HBS[g] (mM)	TBS[h] (mM)
Sodium Bicarbonate (NaHCO3)	Yes	44.0	18.4	–	26.2	4.2	–	–
Potassium Phosphate Monobasic (KH2PO4)	Yes	–	–	1.5	1.0	0.44	–	–
Sodium Phosphate Dibasic (Na2HPO4)	Yes	–	5.6	8.1	–	0.34	–	–
Sodium Phosphate Monobasic (NaH2PO4)	–	0.9	–	–	–	–	–	–
HEPES[j]	Yes	–	–	–	–	–	25.0	–
TRIS[j]	–	–	–	–	–	–	–	20.0

Most common culture media contains phosphate, even if primarily buffered by carbonate. Of the buffered salt solutions, only Good's buffers are typically phosphate-free. (Concentrations shown are averages; exact values may vary slightly by vendor.)

[a] Medium 231, vSMC-specific culture medium.
[b] DMEM, Dulbecco's Modified Eagle Medium.
[c] RPMI, Roswell Park Memorial Institute Medium.
[d] DPBS, Dulbecco's Phosphate-Buffered Saline.
[e] EBSS, Earle's Balanced Salt Solution.
[f] HBSS, Hank's Balanced Salt Solution.
[g] HBS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Buffered Saline.
[h] TBS, tris(hydroxymethyl)aminomethane (TRIS)-Buffered Saline.
[i] Components listed by the vendor but exact concentrations are proprietary.
[j] Zwitterionic molecules commonly known as Good's buffers.

Cell Viability and pH

Cerebrovascular smooth muscle cell viability was determined based on a gating strategy that utilized FarRed LIVE/DEAD-stained living and dead controls as well as an unstained living control (Fig 7A). The viability of untreated living control cells was 92±2%.

CsM uptake is enhanced by mildly acidic pH in serum-supplemented culture media (Sato, Ishii et al. 2001, Lavertu 2006, Nimesh, Thibault et al. 2010). However, prolonged exposure to extracellular acidosis has deleterious consequences, even in immortalized cell lines (Nimesh, Thibault et al. 2010, Nahaei 2013). We observed that primary human cvSMC are especially sensitive to low pH *in vitro* and suffer substantial viability loss after incubation below pH 7 (Fig 7E, 50% Vehicle treatment). Exposure to acidic conditions *in vivo* induce cvSMC to undergo functional changes that result in vasodilation (Dabertrand, Nelson et al. 2012), altered gene transcription (Christou, Bailey et al. 2005) and pH-dependent cell death (Brenninkmeijer, Kuehl et al. 2011). The potential for enhanced uptake of CsM in acidic culture media must be balanced by cell viability considerations and by the translational potential of the model system.

Within blood vessels, the aptly named acid-sensing ion channel 1 (ASIC1) plays a significant role in the cvSMC response to acidosis (Lin, Jin et al. 2014). ASIC1 stimulates upregulation of a transcription factor called nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via AKT signaling (Chen, Liu et al. 2016). In turn, NF-κB regulates the expression of multiple genes related to stress and inflammation (Muanprasat, Wongkrasant et al. 2015). One such gene is heme oxygenase (HO-1). The HO-1 gene has a binding site for NF-κB and is upregulated in cvSMC exposed to

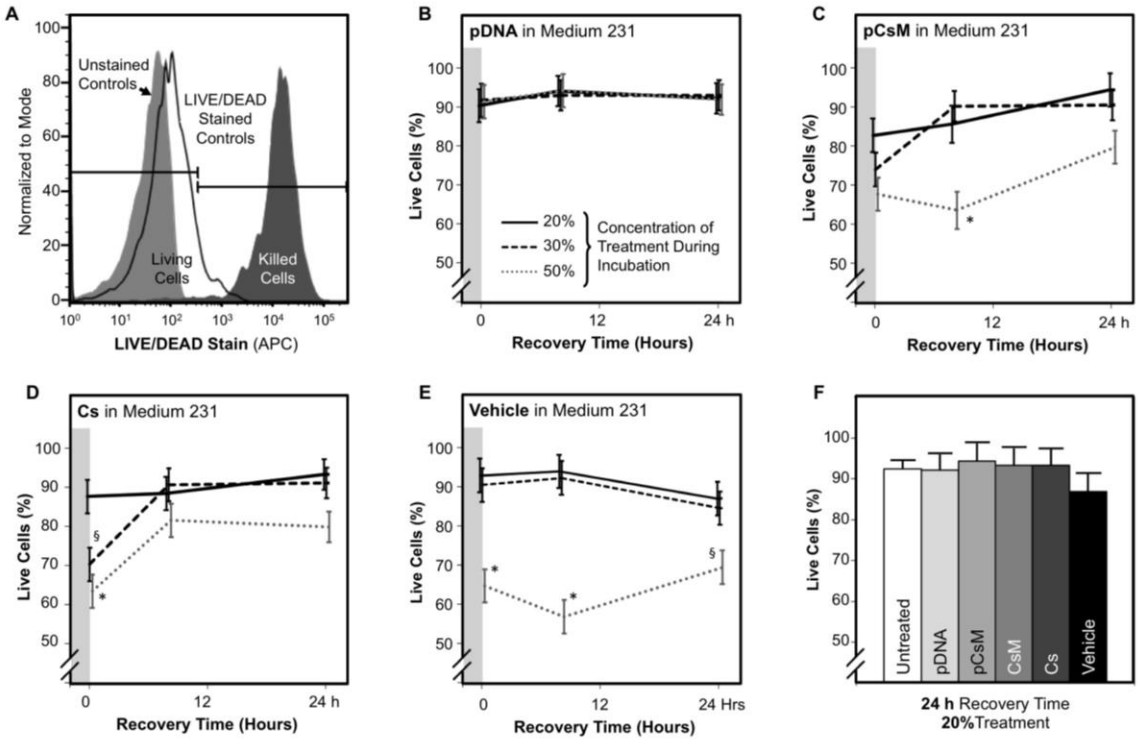

Figure 7. High-dose vehicle damages smooth muscle cells in the absence of chitosan

Flow cytometry gates for cell viability were based on living and EtOH-killed controls (A). (B) Incubation for 2 h (grey bar) with pDNA had no impact on cvSMC viability (B). Fifty percent pCsM (C) and Cs (D) stabilized cvSMC viability by 24 h post-incubation, whereas cell exposed to Vehicle in the absence of chitosan (E) did not recover. Cell viability was unchanged after 24 h for all 20% treatment groups (F). (*$p \leq 0.01$, $^{\S}p \leq 0.05$)

extracellular acidosis (Lavrovsky, Schwartzman et al. 1994). Chitosan augments the normal HO-1 response to noxious stimuli (Khodagholi, Eftekharzadeh et al. 2010). Whether by HO-1 or another mechanism, Cs appears to stabilize cvSMC viability after exposure to acidotic stress (Fig 7D-E). However, Cells treated with 50% Vehicle, in the absence of Cs, continued to suffer the same rate of cell death long after the noxious stimuli of low pH was removed. Due to concerns regarding the impact of low pH on cvSMC viability, further analysis excluded 50% treatment groups.

Cell Viability and Chitosan

Incubation with thirty percent Cs also transiently depressed cvSMC viability (Fig 7D). Specifically, 30% Cs treatment resulted in transiently decreased viability (to 70±5%) immediately after treatment. This appears to be directly related to chitosan exposure; and was due neither to pH (7.2±0.3) nor the presence of Vehicle (Fig 7E). However, viability quickly stabilized after 30% Cs treatment. It is interesting to note that a restorative effect was seen in 50% Cs treatment, despite the deleteriously high concentration of Vehicle. Given the transient deleterious Cs effects we observed, further analysis excluded 30% treatment concentrations. Twenty percent Cs and Vehicle had no impact on cvSMC viability (Fig 7F), so this concentration was selected for further analysis.

Chitosan's time- and concentration-dependent properties (sometimes protective, sometimes deleterious) may arise from physiologic responses to the polymer's physical properties. The anti-inflammatory influence of chitosan is well established and includes protecting neuron-like cells during exposure to oxidants (Khodagholi, Eftekharzadeh et al. 2010), mitigating pro-inflammatory astrocyte responses to Aβ (Kim, Sung et al. 2002)

and attenuating nitric oxide release in stimulated microglial (Wei, Ma et al. 2012).

Conversely, chitosan is also known to induce pro-inflammatory responses and has even been successfully used as vaccine adjuvant (Pattani, Patravale et al. 2009, Carroll, Jin et al. 2016). Instigation of pro- or anti-inflammatory responses appears to be dependent on both the molecular weight and degree of deacetylation in a given Cs (Da Silva, Chalouni et al. 2009). High molecular weight (230 kDa) chitosan requires more time for transcytosis and results in a greater loss in viability than lighter constructs (3.8 kDa) (Chae, Jang et al. 2005). Additionally, Cs that is less than 85% deacetylated remains pro-inflammatory, similar to the effect of the chitin from which it is derived (Shibata, Foster et al. 1997). Highly deacetylated Cs (≥95% glucosamine residues) has anti-inflammatory properties (Han, Zhao et al. 2005, Tu, Xu et al. 2016). At 88% deacetylated, the Cs used herein is between these limits. We suggest that the ratio of deacetylation, and potential skew toward pro- or anti-inflammatory off target effects, be taken into consideration when developing CsM-based translational gene therapy.

Chitosan as CD59 Gene Therapy Adjuvant

Initial Effects on CD59 Expression

In addition to modulating cell viability, chitosan transiently reduced the average amount of CD59 expressed per cells. Mean fluorescent intensity (MFI) was utilized as a proxy for relative change in CD59 expression after validation with appropriate specificity controls (Fig 8A). Vehicle initially reduced the amount of CD59 expressed per cell (Fig 8B). Chitosan, especially Cs, exacerbated this Vehicle-mediated suppression of CD59. At 10% chitosan, only Cs depressed CD59 expression (Fig 9). It is important to note that the CD59 measured herein was confined to the outer cell membrane since flow cytometry

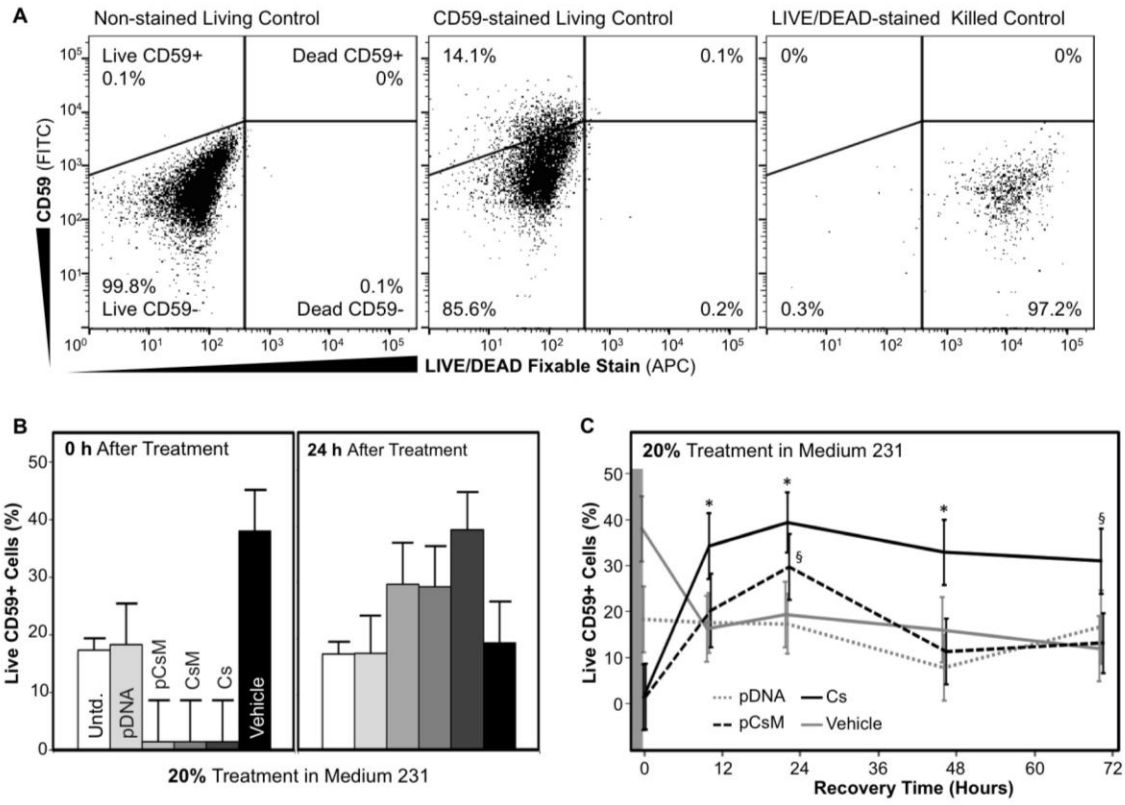

Figure 8. CD59+ cell numbers initially decrease after chitosan, but later resurge
Flow cytometry gates to determine Live CD59-positive cvSMC were based on living and EtOH-killed controls (A). Chitosan (pCsM, CsM, Cs) initially decreased the number of CD59-positive cells (B, $p \leq 0.001$), an effect that was rapidly, potently and durably reversed, especially in the case of Cs (C, $^{*}p \leq 0.01$, $^{\S}p \leq 0.05$).

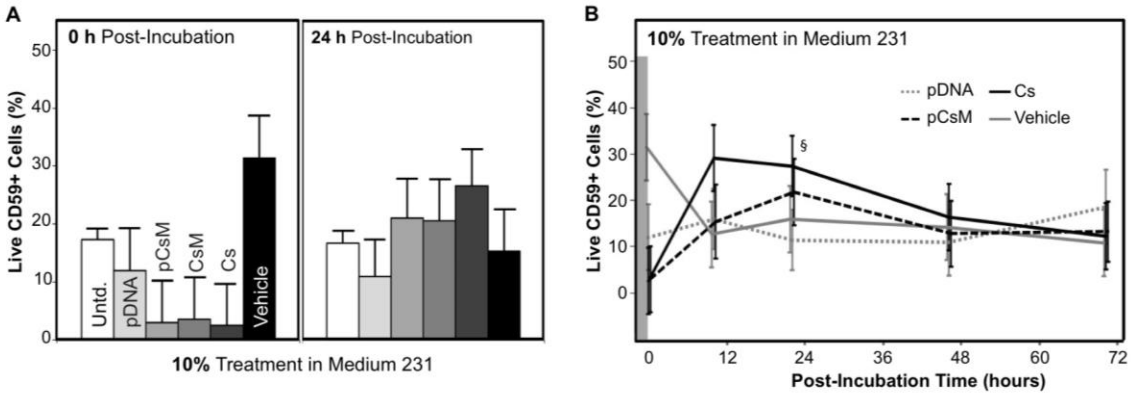

Figure 9. Low-dose chitosan decreases CD59+ cell numbers without later resurgence Chitosan (pCsM, CsM, Cs) initially decreased the number of CD59-positive cells, (A, $p \leq 0.001$), an effect that was reversed rapidly but not robustly (B, $^{\S}p \leq 0.05$).

was conducted on fixed, non-permeabilized cvSMC, despite the presence of CD59 in vesicles within the cells (Fig S4).Thus, the transient depression of CD59 may be related to alterations in the normal cycling of CD59 between the cell surface and Golgi apparatus (Nichols, Kenworthy et al. 2001) or to membrane turnover as CsM are taken up through endocytosis. Chitosan similarly decreased the proportion of CD59-positive cells, to such an extent that CD59-postive cvSMC were almost undetectable immediately following incubation with 20% chitosan particles and polymer (Fig 10B). Vehicle alone increased the percent of cvSMC that were CD59-positive. Chitosan similarly decreased the proportion of CD59-postive cells, to such an extent that CD59-positive cvSMC were almost undetectable immediately following incubation with 20% chitosan particles and polymer (Fig 10B). The impact of Vehicle on CD59 may be due to deployment of an intracellular pool of CD59 that is sequestered until cells are exposed to noxious stimuli (Gordon, Papazaharoudakis et al. 1994). The effect of chitosan on CD59 is so strong that it undercuts the Vehicle-driven increase in CD59 on the surface of cvSMC.

Chitosan appears to initially push cvSMC into a CD59-negative phenotype with much lower levels of CD59 expression overall. Low-dose Cs (10%) demonstrated a similar impact on CD59 (Fig 11). Chitosan's effect on CD59 may be due to transcriptional changes as well as altered trafficking. Genomic human CD59 contains multiple NF-κB binding sites, and this transcription factor is sufficient to stimulate CD59 expression (Du, Teng et al. 2014). Though chitin is known to augment NF-κB expression (Da Silva, Chalouni et al. 2009), chitosan can temporarily reduce NF-κB translocation into the nucleus (Wei, Ma et al. 2012, Tu, Xu et al. 2016). It may be that chitosan is temporarily suppressing NF-κB-driven transcription of CD59.

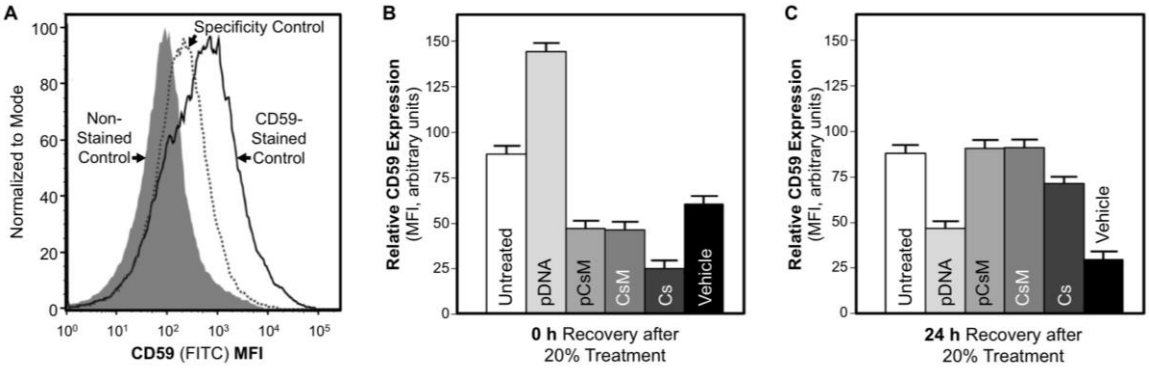

Figure 10. Complex CD59 expression response to plasmid, chitosan and vehicle
Mean fluorescent intensity from flow cytometry was used to approximate average CD59 expression cvSMC (A). CD59 is transiently depressed by pCsM, CsM and Cs (B, $p \leq 0.001$) but recovers to baseline by 24 h despite the presence of Vehicle (C). In the absence of chitosan, Vehicle continues to depress CD59 expression at 24 h post-incubation.

Delayed Responses to Chitosan

Despite the initial suppression of CD59, Cs provoked robust (39±7% vs. 17±2% in untreated controls, Fig 10B) and durable increase in CD59-postive cvSMC that peaked 24 h after incubation (Fig 10C). This effect was present but less pronounced after 10% Cs treatment (Fig 11B), suggesting a dose-dependent response to chitosan. Despite the initial increase, Vehicle did not have a lasting impact on the number of CD59-positve cvSMC, which returned to baseline (18±7%) by 8 h post-incubation (Fig 10C). In the absence of noxious stimuli from Vehicle, CD59 would be reprocessed into storage vesicles within the cell. However, it is unlikely that the persistent increase in CD59-positive cells was driven by a transcriptionally independent mechanism.

In contrast, the average CD59 expression per cell was at baseline 24 h after chitosan exposure. Superficially it seemed that chitosan had no effect on surface expression; however, CsM and Cs were actually counterbalancing the deleterious impact of Vehicle. Vehicle-mediated depression of CD59 was due to altered CD59 mRNA transcription (Fig 12). These results suggest that chitosan robustly influences a large number of cells to express CD59 at normal-positive levels, in part by ameliorating the decrease in CD59 mRNA caused by Vehicle.

Transfection Potential

CD59 mRNA was increased 24 h after treatment with naked and CsM-encapsulated pDNA (Fig 12). Plasmid DNA (pDNA) strongly, yet briefly, enhanced expression of membrane bound CD59 (Fig 10B) in relatively few cvSMC (Fig 8B). Treatment with 10% pDNA elevated CD59 to a lesser degree (Fig 11). Unless plasmid DNA is translocated into the nucleus, or otherwise protected from degradation, pDNA-driven

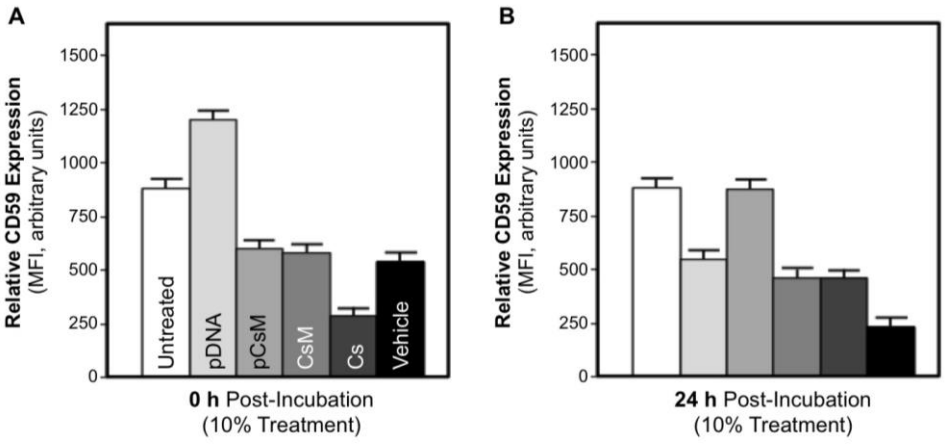

Figure 11. CD59 expression responds similarly to low-dose treatments
CD59 is transiently depressed by pCsM, CsM and Cs (A, $p \leq 0.001$) but recovers by 24 h in the case of pCsM (B) despite the presence of Vehicle, which drives a further decline in CD59 expression.

changes in gene expression have a relatively short half-life of 24 h, (James and Giorgio 2000). The proportion of CD59-positve cvSMC, however, was not altered by pDNA exposure. This indicates that though relatively few additional cvSMC were turned CD59-positive, surface expression in cells that took up naked plasmid was enriched to the point that mean expression was skewed upwards. Twenty-four hours after incubation with pDNA, CD59 expression was depressed relative to untreated controls (Fig 10B) though CD59 mRNA remained elevated (Fig 12).

The decoupling of CD59 mRNA and CD59 surface expression levels could occur at any point. Between transcription and surface expression of mature CD59 a variety of regulatory mechanisms act on translation, GPI-anchoring, glycosylation, intracellular trafficking, and surface stability of the protein (Tashima, Taguchi et al. 2006, Bonnon, Wendeler et al. 2010, Ghosh, Vaidya et al. 2014). Indeed, CD59 may be expressed as either GPI-anchored glycoprotein or secreted in a soluble form (Wheeler, Rudd et al. 2002). Excessive accumulation of GPI-anchored CD59 may push post-translational modifications that favor the soluble form of CD59. Bare plasmid is sufficient to drive transient increases in CD59 surface expression at the expense of CD59 at later time points. Plasmid that is protected by CsM dramatically enhanced CD59 mRNA long after the initial exposure (Fig 12). However, CD59 surface expression was not increased by pCsM (Fig 11C). These results highlight the importance of measuring both mRNA and the desired endpoint of gene expression, with and without plasmid, to determine if chitosan-mediated transfection yields functionally relevant protein as a result of the gene construct or bioactivity of the polymer itself.

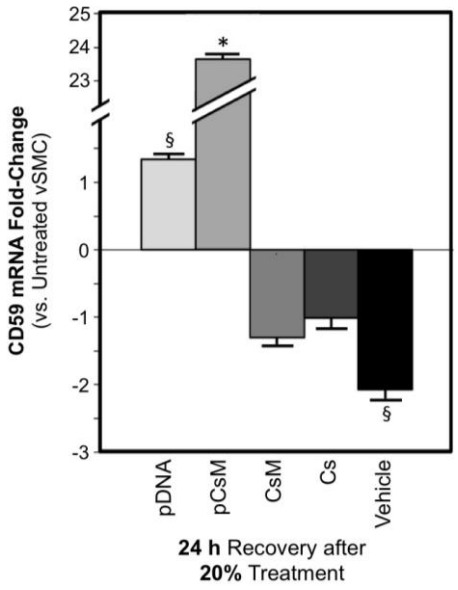

Figure 12. Chitosan effectively delivers plasmid to enhance CD59 mRNA expression

CD59 mRNA was elevated after treatment with pDNA ($p<0.05$) and pCsM ($p<0.01$) but down regulated by treatment with Vehicle ($p<0.05$). Chitosan-only treatments had negligible effects on CD59 mRNA expression.

Acetylation, Inflammation and Translocation

Since chitin is a structural element utilized by several pathogenic organisms, it is unsurprising that acetylated glucosamine residues are recognized by toll-like receptors (TLRs). TLRs play a pivotal role in the innate immune system, sensing potentially dangerous molecular motifs and priming immune cells to respond. TLR4 recognizes the acetylated glucosamine residues of chitin (Zhang, Liu, Peng, Han, & Yang, 2014). Both the NF-κB and Activator Protein 1 (AP-1) transcription factors are downstream of TLR4 signaling and both are activated in an inflammatory chitosan response (Roy et al., 2016; Zheng et al., 2016). Interestingly, TLR4 also recognizes endotoxin (Paramo, Tomasio, Irvine, Bryant, & Bond, 2015). Endotoxin contamination is a critical concern for implantable and injectable chitosan preparations. At sufficiently high levels, endotoxin in chitosan preparations can skew results toward false-positive inflammatory outcomes (Lieder et al., 2013). The extremely low level of endotoxin of the chitosan used in this investigation (< 0.25 EU/ml) is below threshold set by the FDA for injectable sterile water. The importance of including both the degree of deacetylation and endotoxin burden cannot be overstated when reporting the effects of chitosan-based constructs on inflammatory markers and outcomes.

Somewhat less clear is the etiology of chitosan's anti-inflammatory properties. Chitosan appears to suppress inflammation through phosphorylation of AMP-activated protein kinase (AMPK) (Kunanusornchai, Witoonpanich et al. 2016). AMPK is best known for driving translocation of glucose transporter 4 (GLUT4) to the surface of adipose and striated muscle cells during exercise (Kurth-Kraczek, Hirshman et al. 1999). In cvSMC glucose transporters 1, 9, 10 and 12 predominate, with high levels of GLUT1

associated with the proliferative or contractile phenotype (Pyla, Poulose et al. 2013). High levels of GLUT1 are implicated in blood-brain barrier dysfunction in the presence of vascular Aβ plaques (Merlini, Meyer et al. 2011). The specific triggers of AMPK activation, role of AMPK on modulating the various GLUT isoforms in cvSMC and affinity of GLUT-9, -10 and -12 for glucosamine are yet to be elucidated. However, it is highly likely that AMPK is involved in the cvSMC response to Cs because, in addition to driving GLUT translocation, AMPK increases functional surface expression of CD59 (Iwasaki, Miwa et al. 2013). AMPK activation also drives nuclear accumulation of Nrf2, an antioxidant transcription factor that increases, among other genes, the expression of HO-1 (Hyung, Ahn et al. 2016, Joo, Kim et al. 2016). The role of AMPK as a mediator in the anti-inflammatory properties of chitosan may indeed be a very promising line of inquiry.

Conclusions

Chitosan microparticles are neither stable nor inert in culture with primary human cells. CsM size under cell culture conditions appears to be heavily influenced by the phosphate anions used to buffer pH in culture media. Despite their large size, 20% pCsM successfully and stably transfected vascular smooth muscle cells as evidenced by enhanced CD59 mRNA, which remained elevated 24 h after treatment. However, this did not translate to increased expression of mature surface protein. Instead, chitosan polymers in the absence of pDNA facilitate a dramatic time- and dose-dependent increase in the number of CD59-positive cells. These observations emphasize the need for careful study design and cautious interpretation of results when chitosan-based transfection

strategies are used with unlabeled gene constructs, especially in the absence of chitosan-only controls. Though the specific mechanisms by which chitosan mediate enhanced CD59 expression are beyond the scope of this report, the variety of potential targets invites further investigation. We conclude that, though elucidation of the specific mechanisms by which chitosan modulates CD59 expression are beyond the scope of this report, the direct bioactivity of chitosan should be further explored since it might be usefully harnessed as an adjuvant therapy in chitosan-based applications.

Acknowledgements

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CHAPTER FOUR

CONCLUDING REMARKS ON CHITOSAN, SMOOTH MUSCLE AND COMPLEMENT

Chitosan as a non-viral transfection vector

The purpose of this dissertation was three-fold: First, we explored the stability of chitosan microparticles (CsM) under standard cell culture conditions. Next, we measured the direct impact of chitosan polymers on CD59 expression in cultured cvSMC. Finally, we assessed the efficacy of CsM containing plasmid DNA (pCsM) as a transfection vector in human cerebrovascular smooth muscle cells (cvSMC).

Enhancing Microparticle Stability

The stability of CsM depends heavily on pH and the composition of the media in which they are incubated. Inorganic phosphate is a major buffer component in most cell culture media and is present in appreciable quantities in blood serum. Positively charged chitosan readily captures anionic phosphate even if precipitated with a cross-linker. This poses challenges in cell culture, where pH varies over incubation times. Changes in pH can induce abrupt shifts in particle size (Fig 6).

One way to reduce the attraction between CsM and phosphate is to coat the outside of the particles with an appropriate negatively charged polymer. This prevents any unbound amino groups from capturing free anions. Pectin is a hetropolysaccharide that is rich in negatively charged galacturonic acid. When introduced to pre-formed CsM and pCsM, pectin reduces the latent CsM surface charge, as measured zeta potential (Fig S5).

Sufficiently high levels of pectin relative to chitosan can even reduce the zeta-potential to almost null. Unfortunately, high concentrations of pectin appear to drive aggregation of CsM (Fig S6), an undesirable side effect in the context of gene therapy applications, where smaller is better.

Additional work is needed to determine the pectin concentration and pectin:chitosan ratio that is optimal for withstanding incubation under cell culture conditions. Pectin may also be useful in coating CsM prior to use *in vitro* to reduce adsorption of CsM to the negatively charged glycocalyx of blood cells and vessels walls. Insulating CsM against off-target binding would keep them suspended in serum for a longer time. Thus, it may be possible to reduce the dose of CsM needed for a therapeutic effect.

Considerations in CD59 Expression

Endotoxin contamination is a concern when working with Cs given its highly electropositive charge, which readily binds negative lipopolysaccharides (LPS) in solution. Indeed, this property is utilized in Cs-based purification methods (Li, Shao et al. 2003). The chitosan used herein was dissolved at < pH 2.5 for more than 48 hours and only endotoxin-free deionized water was used in preparation of Cs and CsM. All chitosan preparations were kept at pH 5.6 until used under sterile cell culture conditions, which are adequate to produce and maintain low levels of endotoxin (Ribeiro, Xu et al. 2010). Direct testing of our Cs revealed extremely low levels of endotoxin (< 0.25 EU/ml) and supported the assertion that acidic conditions decontaminate chitosan.

There is a direct relationship between endotoxin and CD59 surface expression in human cells (Schieren, Janssen et al. 1992). However, the effects of LPS were short-lived and resolve by 5 h after treatment with endotoxin, well before our first measured time

point (12 h, Fig 8). In our experimental setup, endotoxin-driven changes in CD59 would only be detectible as enhanced expression at 0 h, immediately following two hours of incubation with chitosan. However, at this initial time point CD59 expression is dramatically decreased by Cs, CsM and pCsM. This speaks to the direct effects of Cs or pH on cvSMC rather than the effect of endotoxin contamination and provides further evidence that these preparations are essentially endotoxin-free.

Avoiding Pitfalls in Transfection

Given our findings, Cs appears to alter surface expression of certain proteins. From this we infer that any gene-therapy application of CsM requires appropriate controls to rule out off-target effects. To use Cs for unlabeled gene delivery without proper controls invites misinterpretation. Additionally, incongruences between mRNA expression and CD59 localization encourage experimental design that samples multiple points along the path from gene. Testing the culture media and membrane-free cell lysates would shed light on the fate of CD59 expressed from CsM-delivered plasmids.

Chitosan itself may have potential as an adjuvant therapy, but only if the interactions of the construct are well characterized. This is even truer for *in vivo* applications, where multiple cell types will be exposed to the CsM. Based on the current literature, the physical properties of Cs can influence the balance of pro- and anti-inflammatory gene expression (Kim, Sung et al. 2002, Hyung, Ahn et al. 2016) (Kim, Sung et al. 2002, Chen, Wang et al. 2008, Tu, Xu et al. 2016). A construct may even be tunable to an application by adjusting the degree of deacetylation and molecular weight of the polymers used in the CsM. While the ability to tailor CsM increases the potential

for a specific outcome, it also raises the probability that careless analysis could lead to erroneous conclusions. Appropriate controls are essential in testing CsM.

Cultured cerebrovascular smooth muscle as a model system

The use of cerebrovascular smooth muscle as a model system for Cs-based gene delivery is not without precedent in the literature (Xia 2013, Wan 2015, Zhou 2016). However, special care must be taken when interpreting results from cultured smooth muscle cells rather than organ or whole animal systems. It has long been known that culturing vascular smooth muscle cells (vSMC) can disrupt contractile morphology and cytoskeletal profiles (Absher, Woodcock-Mitchell et al. 1989). We attempted to verify the physiologically relevant contractile phenotype by light microscopy (spindle-shaped cells, Fig S3) and immunohistochemistry (appropriate smooth muscle alpha-actin stress fibers, Fig S4). Though imperfect, cultured primary human vascular smooth muscle cells (vSMC) are one of the first steps needed to determine chitosan's safety and efficacy as a gene therapy vector for treating vascular diseases.

Chitosan and Smooth Muscle Cells

The first description of Cs' effect on vSMC was published more than two decades ago (Inui, Tsujikubo et al. 1995). Inui, et al. reported that Cs alone did not cause vSMC proliferation but did increase total protein tyrosine phosphorylation Since that time, is has been established that Cs may in fact retard vSMC proliferation (Chupa, Foster et al. 2000), an effect that appears to be mediated by suppression of NFATc1 (Du, Kou et al. 2016). Though multiple tyrosine kinases are phosphorylated in response to chitosan (Inui,

Tsujikubo et al. 1995), identification of the pathways activated in vSMC by chitosan exposure is far from complete and welcomes further investigation.

Release of DNA from CsM requires particle uptake. This process is associated with adsorption of large pCsM complexes on the surface of the cells (Ishii, Okahata et al. 2001). Though the mechanism is not clearly understood, DNA release from particles and transport to the nucleus occurs in response to acidification in lysosomes (Thibault, Astolfi et al. 2011). Transfection efficacy depends at least in part on cell type (Douglas, Piccirillo et al. 2008). CsM appear to cause distress to human cerebral vSMC (Fig 7, Fig S7), so reducing the metabolic demands of uptake would be beneficial. Decreasing particle size is a most effective way to ease the metabolic demands of endocytosis (Rejman, Oberle et al. 2004). Particle size can be can be decreased by altering exogenous pH (Nimesh, Thibault et al. 2010) or carefully selecting chitosan with a low molecular weight and degree of deacetylation. However, there is a goldilocks zone: very short Cs polymers do not effectively carry DNA and long, highly deacetylated Cs does not release plasmids despite long exposures to lysosomal conditions (Thibault, Nimesh et al. 2010). Adding a cross-linker, such as tripolyphosphate, can also increase transfection efficiency (Zheng, Tang et al. 2013) Adding chitinase, the enzyme that degrades chitosan, to CsM formulations makes DNA release more efficient in a variety of cell lines (Zuo, Sun et al. 2008). Finding the optimal physical properties for high efficiency transfection in cvSMC is an open field for continued study.

Insulting with Complement

Another step toward a therapeutic intervention in CAA is to develop an *in vitro* model of the established pathophysiology. Toward this end, we activated complement-

intact human serum (CIHS) in the culture media with either high- or low-doses of aggregated amyloid beta (Aβ, Fig S8). As previously described (Bradt, Kolb et al. 1998), complement was activated in a dose-dependent manner by Aβ and resulted in formation of membrane attack complex. When CIHS was introduced into plates containing cvSMC, then immediately activated with Aβ, the cells showed signs of membrane disruption in the presence of both concentrations, though high-dose Aβ triggered a more potent response. Exhaustive complement activation by cobra venom factor resulted in complete destruction of all cells. Future work will look at the capacity of Cs-stimulated CD59 to protect cells from disruption by Aβ-induced complement.

Conclusions

In 1906 Dr. Alois Alzheimer presented a case report in Tübingen, Germany. He described the condition of a woman he had observed while visiting the United States. In so doing gave the first account of a disease that would later bear his name (Hippius and Neundorfer 2003). His case report was published in 1909, the same year that vascular Aβ plaques were found by Dr. Gustav Oppenheim in 6 of 14 patients with Alzheimer's disease (AD) at autopsy (Biffi and Greenberg 2011). Despite the early discovery of a relationship between AD and cerebral amyloid angiopathy (CAA), by the end of the twentieth century, research was heavily focused on Aβ in the brain parenchyma, often to the exclusion of the neurovascular unit. The role of complement in AD with CAA has been far from mainstream. This is despite significant evidence that complement is involved in the pathophysiology of cognitive decline in amyloidopathies. However, the AD field is not the only one to forget early lessons.

More than half a century ago the bioactivity of Cs was described in rats (Hillyard, Doczi et al. 1964). Three decades later, Cs was first used to encapsulate DNA fragments (Alexakis, Boadi et al. 1995) and within two years, Cs began to be developed as a transfection vector (Venkatesh and Smith 1997). Perhaps because of the early reliance on qualitative measurements (Roy 1999, Park 2000, Kumar, Behera et al. 2002) and fluorescent gene expression (Cui 2001, Mao 2001), the CsM field developed a blind spot regarding the bioactivity of Cs. Even now, contemporary publications may fail to include chitosan-only controls, and in so doing invite the possibility of misinterpreting transfection data.

This dissertation challenges the dominate hypotheses held in two distinct fields with the objective of achieving an interdisciplinary therapeutic: A chitosan-based gene transfection vector that may potentially be used to upregulate CD59. CD59, the critical glycoprotein that is essential to controlling bystander lysis in the presence of Aβ-activated complement. In some distant future we may learn if Cs is, or is not, the most effective approach to combat vascular fragility in AD with CAA. Along the way we hope that in some small part our contribution will challenge you to question your assumptions and consider carefully your negative controls.

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APPENDIX

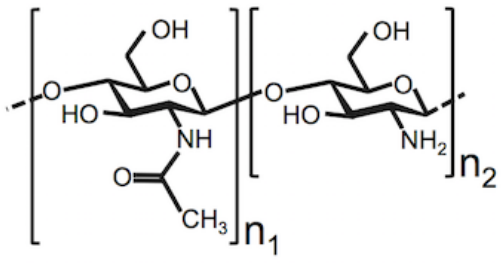

Figure S1. Illustration of a chitosan polymer structure
Chitosan polymer consists of at most 40% N-acetyl-D-glucosamine (n1) residues randomly interspersed among D-glucosamine (n2) residues. The percent deacetylation is calculated using the equation ($n_2 \div n_1$) x 100%.

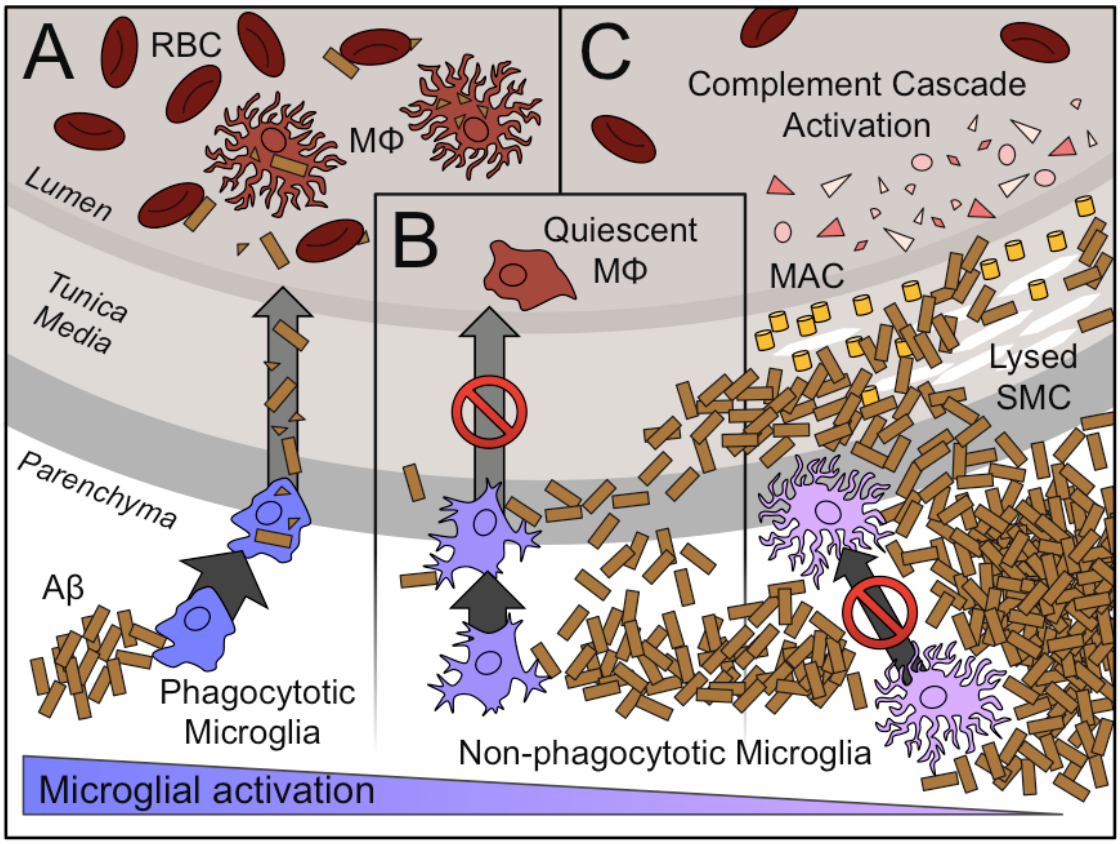

Figure S2. Aβ accumulates due to cvSMC and microglial dysfunction
In young, healthy individuals Aβ is transported to the perivascular space by active microglia and shuttled across the tunica media by cvSMC expressing LRP-1 where it is taken up by macrophages (Mϕ) or adsorbs to red blood cells (RBC) for later degradation and disposal (A). Disruption of LRP-1 mediated transcytosis due to hypoxia or other stressors results in accumulation of Aβ along cerebral vessels (B). Aged microglia become less effective at removing Aβ, and eventually fail to be activated by accumulating Aβ. Meanwhile, accumulating vascular Aβ stimulates complement cascade resulting in MAC formation and cvSMC lysis (C).

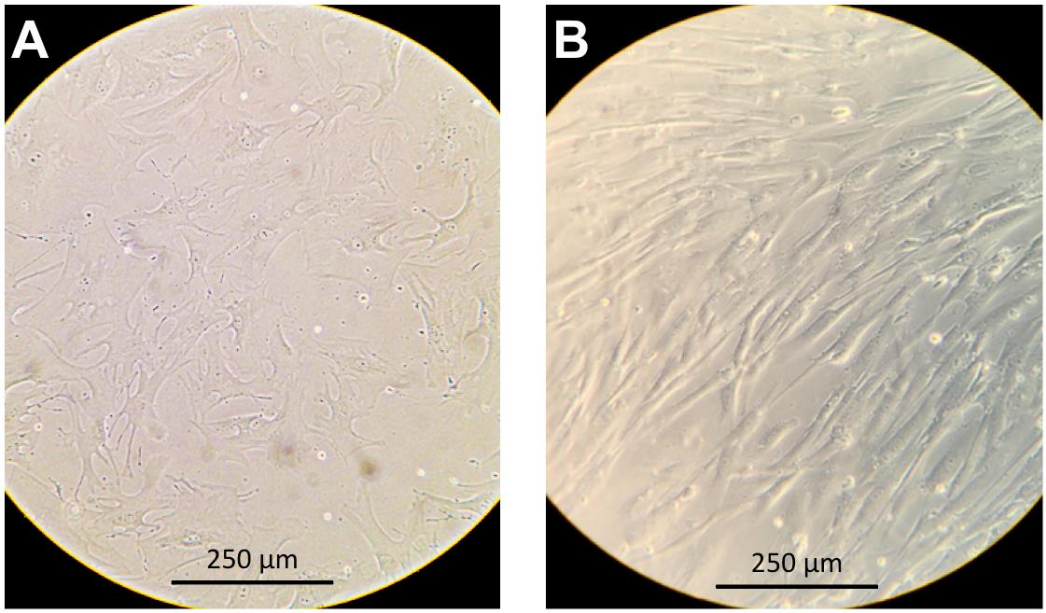

Figure S3. Representative image of cultured primary human cvSMC
Images were taken at 400x magnification before (A) and after (B) switching from growth media (5% FBS) to differentiation media (1% FBS).

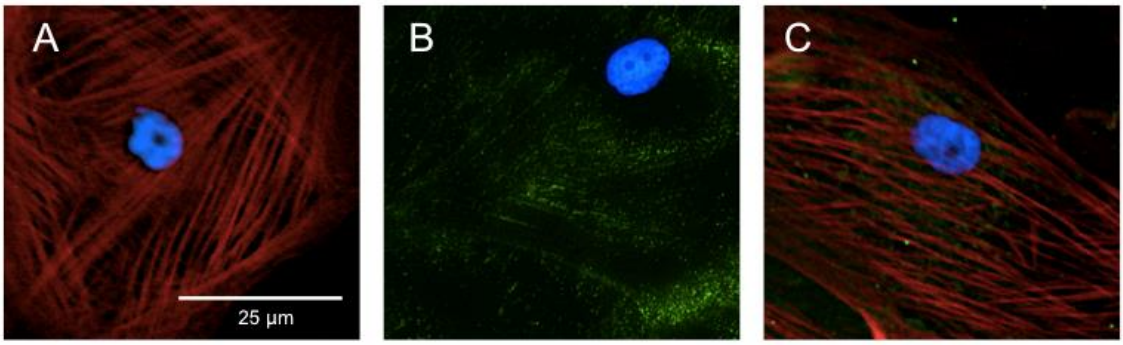

Figure S4. CD59 and smooth muscle actin expression in resting cvSMC
Smooth muscle α-actin (red) showing relative normal stress fiber arrangements in cvSMC cultured on a collagen matrix (A). Punctate arrangement of CD59 (B, green) in resting cvSMC appears to follow cytoskeletal elements but is independent of smooth muscle α-actin (C). Nuclei (blue) were counterstained with DAPI.

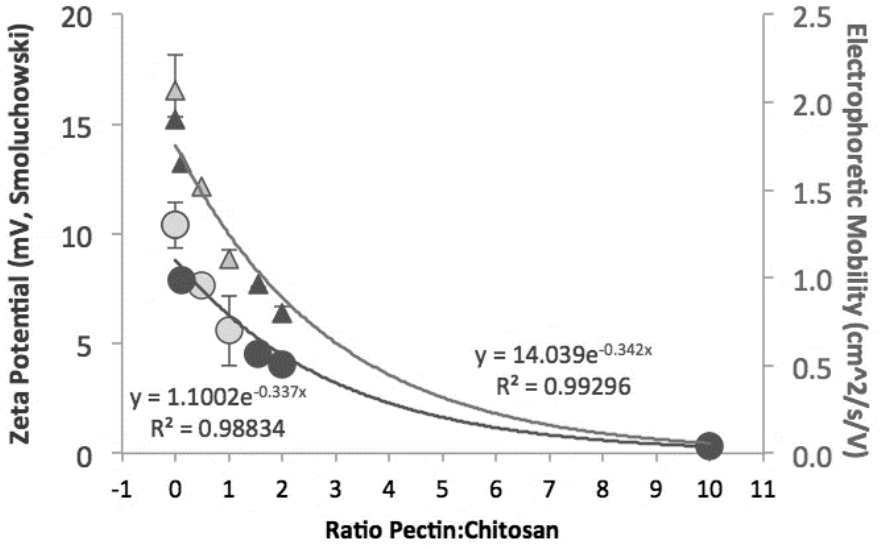

Figure S5. Pectin decreases zeta potential, electrophoretic mobility and size
Increasing the pectin to chitosan ratio diminished zeta-potential (left axis) exponentially in CsM. This relationship was conserved across both CsM (light gray triangles) and pCsM (dark gray triangles). Similarly, responsiveness to the presence of an electric field, measured as electrophoretic mobility (right axis) decreased as pectin increased for both CsM (dark gray circles) and pCsM (light gray circles). CsM were measured with a Nicomp DLS, pCsM with a Wyatt DLS.

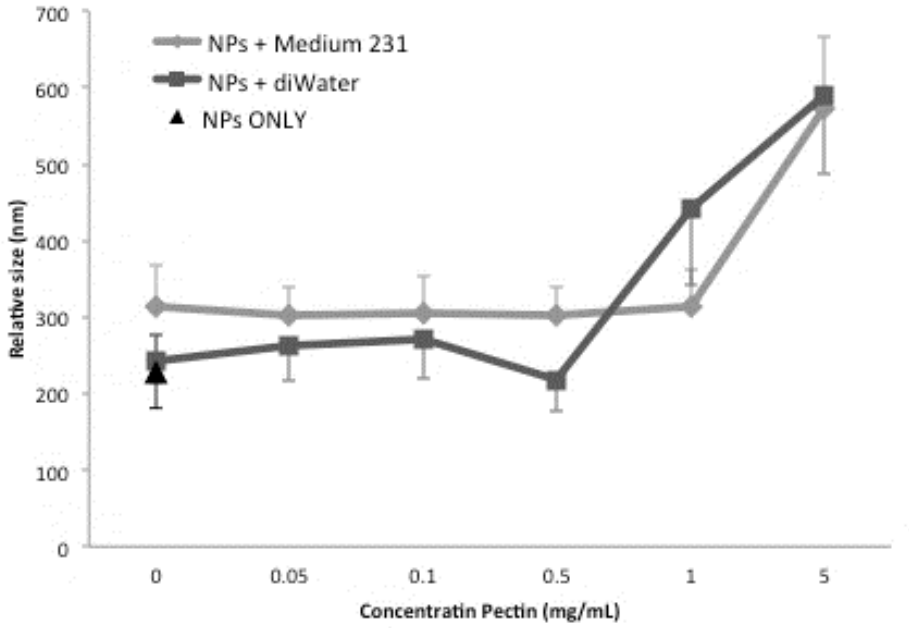

Figure S6. CsM size is a function of pectin concentration
For a given concentration of CsM (designated NPs above), increasing pectin increased particle size in both Medium 231 (gray diamonds) and Water (dark gray squares) immediately after mixing.

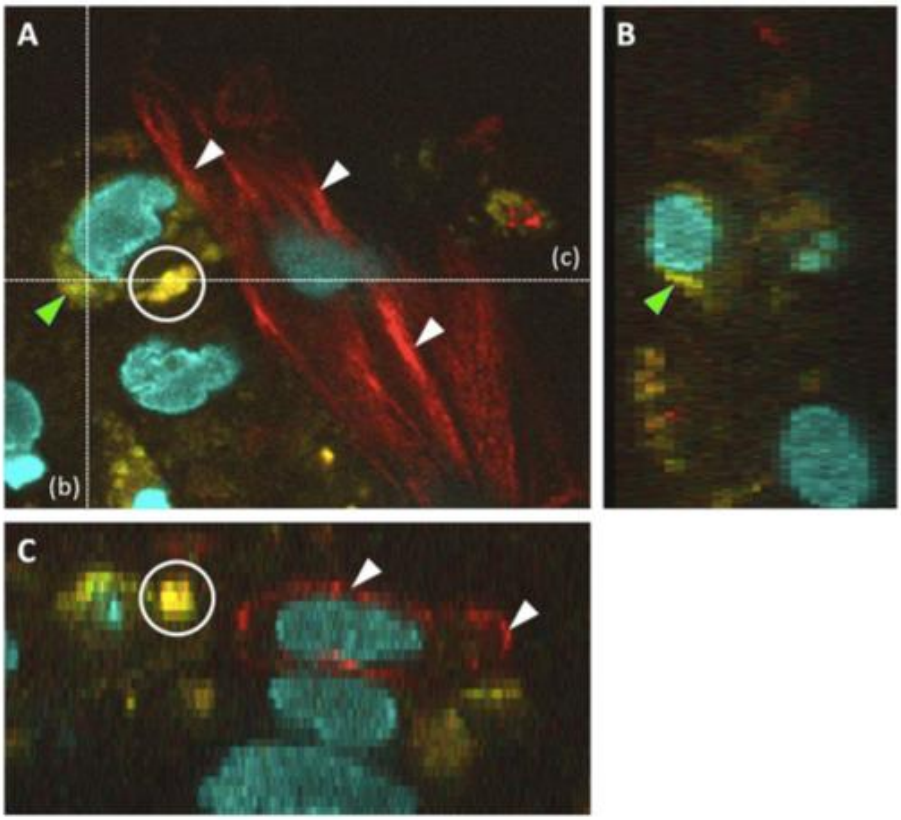

Figure S7. Chitosan particles internally co-localize to actin in cvSMC
Chitosan microparticles labeled with 6-hydroxycoumerin (green) appear to co-localized with actin (red) in cultured cvSMC. In a cell with minimal CsM uptake, actin is organized into normal stress fivers under the plasma membrane (white arrow heads). However, cytoskeletal disruption is readily apparent in a cell with a high burden of CsM (green arrowhead). Disorderly clumps of cytoskeleton and chitosan (white circles) are not conducive to survival. Panel B displays a vertical slice through line (b), and panel C displays a horizontal slice along line (C). Nuclei (cyan) were counterstained with DAPI.

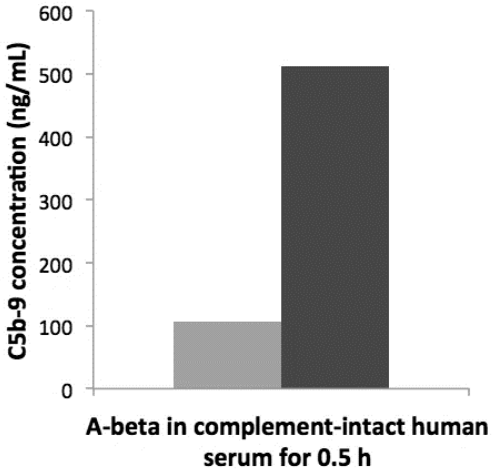

Figure S8. Membrane attack complex forms in response to Aβ
Both low-dose (0.5 μM, gray) and high-dose (5 μM, dark gray) Aβ (labeled "A-beta" above) stimulated formation of membrane attack complex (C5b-9) in the presence of complement-intact human serum. Values normalized to DMEM-only control; cobra venom factor induced 2825 ng/ml C5b-9.

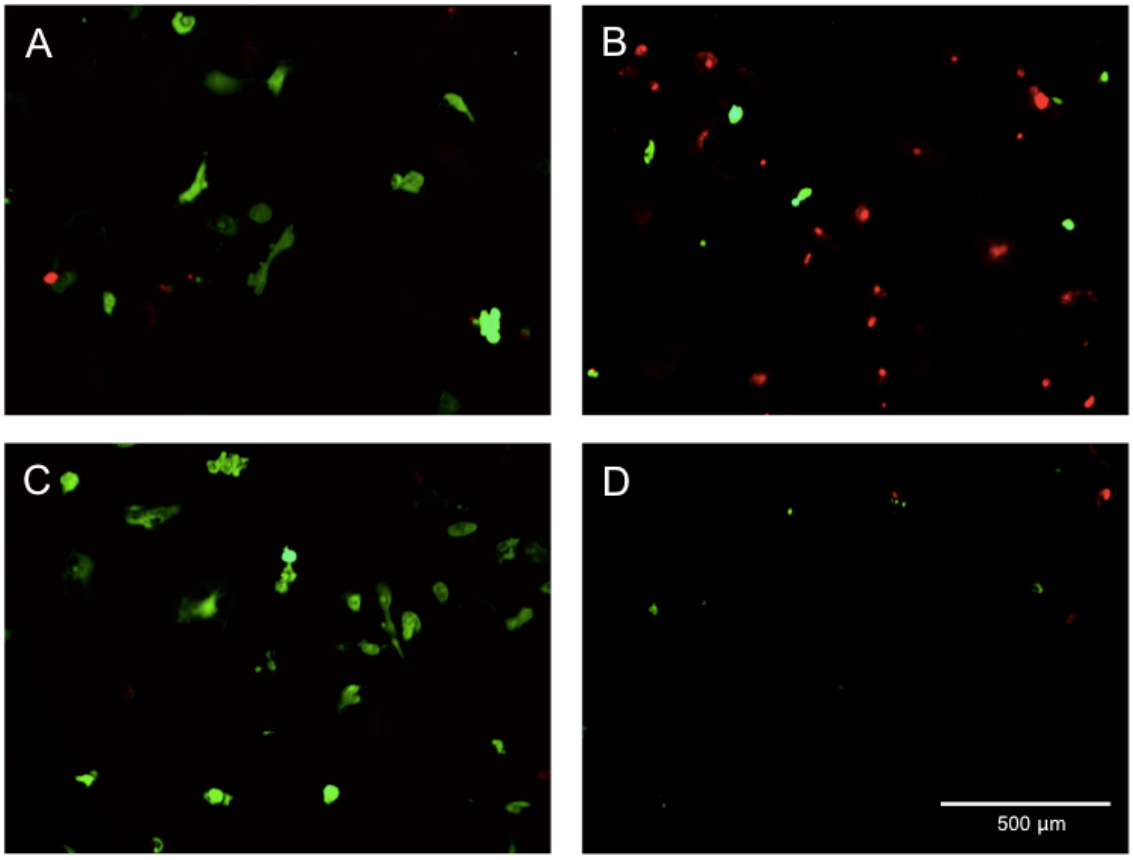

Figure S9. Complement activation by Aβ kills cvSMC *in vitro*
Primary human cvSMC incubated with complement-intact human serum (10% v/v in DMEM) in the presence of (A) 0.5 µM Aβ have more live cells (green) than dead (red) in comparison to (B) 5 µM Aβ. Most cells remained alive after exposure to (C) DMEM without human serum. Only scattered cell fragments remain after complete complement activation with (D) Cobra venom factor.

Supplementary Materials and Methods

Immunohistochemistry for Figure S4

Untreated cells were washed briefly with warm PBS then fixed with 3% PFA. After fixation, cvSMC were rinsed with PBS containing 0.05% Triton-X (PBST) before permeabilization with 5% Triton-X in PBS. Cells were again rinsed with PBST before incubation with IMAGE IT FX Signal Enhancer (Invitrogen). This was followed by blocking for 2 h with 0.01 g/mL bovine serum albumin in PBST. Cells were then incubated with a 1:1000 dilution of mouse monoclonal anti-CD59 [MEM-43/5] antibody from Abcam (ab9183) or a 1:500 dilution of mouse monoclonal anti-alpha smooth muscle actin [1A4] from Abcam (ab7817). MEM-43/5 primary antibodies were counterstained with 1:1000 Alexa Fluor 594 conjugated donkey anti-mouse IgG (A-21203, ThermoFisher). 1A4 antibodies were counterstained with 1:1000 Alexa Fluor 488 donkey anti-mouse (A-21202, ThermoFisher). Cover slips were mounted on slides using Antifade Mounting Medium with DAPI (Vectashield, Burlingame, California). An EVOS FL Cell Imaging System imaging was used to capture images within 24 h of mounting cover slips on slides.

Pectin-coated CsM for Figures S5 and S6

Pectin was dissolved (1mg/ml) in either deionized H_2O or Medium 231 and various volumes (5 ml, 2.5 ml, 1 ml, 0.5 ml, 0.25 ml and 0.1 ml) were individually aliquoted into Falcon tubes. CsM were precipitated as described above (Cupino 2018) Undiluted CsM were characterized, then CsM volume was adjusted with Vehicle so that 1 ml of CsM

solution contained 1 mg of chitosan. CsM was then introduced (1 ml per aliquot), inverted vigorously and characterized immediately by DLS (Nicomp or Wyatt).

Fluorescent CsM and Confocal Imaging for Figure S7

Primary human cvSMC (a gift from Dr. Harry Vinters) were cultured under standard conditions (5% CO_2, 37 °C) in DMEM with 10% fetal bovine serum (FBS, Gibco). Cells were plated in multiwell culture slides and after 24, 6-hydroxycoumerin-labeled CsM (a gift from Dr. Samuel Hudson) were introduced. After two hours, cells were rinsed, fixed, permeabilized with 10% (v/v) Triton-X and incubated with IMAGE IT FX Signal Enhancer (Invitrogen). Alpha-actin was visualized by labeling with [1A4] clone (abcam) followed by an AlexaFluor 647-congugated anti-mouse secondary (ThermoFisher). The multiwell attachment was removed prior to DAPI counterstaining (Vectashield) and confocal imaging (Nikon). *This data was generated in collaboration with Dr. Matthew Zabel.*

Complement Activation by Aβ and ELISA for Figure S8

The total membrane attack complex to which cvSMC were exposed in the above experiment was quantified by MicroVue C5b-9 Plus Enzyme Immunoassay (Quidel) according to the manufacturer's instructions. Briefly, 100 µl of each control, specimen and standards were pipetted into individual wells of the provided plate. SC5b-9 conjugate was added, and the recommended incubation times and temperatures were followed. Stop solution was introduced before reading at 450 nm on a plate reader (Tecan, Magellan software package).

SMC and Live/Dead Fluorescent Microscopy for Figure S9

Amyloid-β (H-1194, Bachem) or Aβ-scramble (H-2972) were incubated for 5 days at 37 °C to induce aggregation. Primary human cvSMC (a gift from Dr. Harry Vinters) were cultured as described above, before plating on collagen-coated coverslips in 6-well plates. After 24 h in standard DMEM with FBS, the media was changed to DMEM supplemented with 10% complement-intact human serum. Aggregated Aβ was added immediately for a final concentration of either 0.5 μM or 5 μM. Cobra venom factor, a known activator of complement was as a positive control. Negative controls included complement-intact human serum stimulated by cobra venom factor and heat-inactivated human serum 5 μM Aβ-scramble. After incubating at room temperature for half an hour, cells were rinsed with PBS and stained with 2 μM Calcein AM and 8 μM Ethidium homodimer-1 per the LiveDead assay instructions (ThermoFisher).

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