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Polyplexes assembled from self-peptides and regulatory nucleic acids blunt toll-like receptor signaling to combat autoimmunity

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ABSTRACT

Autoimmune diseases occur when the immune system incorrectly recognizes self-molecules as foreign; in the case of multiple sclerosis (MS), myelin is attacked. Intriguingly, new studies reveal toll-like receptors (TLRs), pathways usually involved in generating immune responses against pathogens, play a significant role in driving autoimmune disease in both humans and animal models. We reasoned polyplexes formed from myelin self-antigen and regulatory TLR antagonists might limit TLR signaling during differentiation of myelin-specific T cells, inducing tolerance by biasing T cells away from inflammatory phenotypes. Complexes were formed by modifying myelin peptide with cationic amino acids to create peptides able to condense the anionic nucleic-acid based TLR antagonist. These immunological polyplexes eliminate synthetic polymers commonly used to condense polyplexes and do not rely on gene expression; however, the complexes mimic key features of traditional polyplexes such as tunable loading and co-delivery. Using these materials and classic polyplex analysis techniques, we demonstrate condensation of both immune signals, protection from enzymatic degradation, and tunable physicochemical properties. We show polyplexes reduce TLR signaling, and in primary dendritic cell and T cell co-culture, reduce myelin-driven inflammation. During mouse models of MS, these tolerogenic polyplexes improve the progression, severity, and incidence of disease.

1. Introduction

Failure to mount an immune response to an antigen is known as immunological tolerance [1]. When tolerance to self-antigens (e.g., host proteins) is not maintained, inflammation and autoimmune disease can develop. Autoimmune diseases such a multiple sclerosis (MS), type 1 diabetes, lupus, and rheumatoid arthritis, affect over 20 million Americans [2,3]. In MS, the myelin sheath that insulates and protects the axons of neurons is recognized as a foreign antigen [4,5]. Myelin derived antigens are now strongly implicated as the targets of malfunctioning self-reactive cells in MS [6], inflammatory populations that infiltrate the central nervous system (CNS). In the CNS, the attack by these cells drives demyelination of neurons, while secretion of inflammatory cytokines recruits additional immune cells to the site [7].

Cures for autoimmune diseases do not exist and treatment options are limited. Typical therapies rely on regular doses of immunosuppressive drugs or antibodies that are non-specific and can leave patients immunocompromised [8]. Thus, great interest has developed in new treatments that can durably block inflammatory responses against self-antigen without non-specific suppression. One exciting new strategy is co-delivery of an antigen, such as myelin, along with immunomodulatory molecules to redirect responses against self-antigen. Biomaterials offer very attractive features in this context; in addition to co-delivery, they provide routes for efficiently targeting antigen presenting cells (APCs) and for controlled release [9,10]. In particular, synthetic polymers have been used to co-deliver antigen with regulatory signals—small molecules, cytokines, and proteins—to polarize differentiating T cells away from inflammatory cells and toward regulatory T cells (T_{REG}) [11–19]. These populations can control self-reactive effector cells (e.g., T_H1, T_H17) that drive autoimmune

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disease while limiting broad suppression [1]. Expansion and biasing of T cells toward a regulatory response also reduces the absolute number of inflammatory cells and offers the potential for more durable treatments [20,21].

Toll-like receptors (TLRs) are a collection of signaling pathways that recognize pathogen-associated molecular patterns (PAMPs), resulting in secretion of inflammatory cytokines and activation of the immune cells needed to fight infection [22]. Interestingly, a developing body of new literature demonstrates that many TLRs are overexpressed in MS and other autoimmune diseases, as well as in animal models [23–27]. For example, in experimental autoimmune encephalomyelitis (EAE), a pre-clinical mouse model of MS, disease is significantly reduced in TLR9 knock-out mice [23]. This example highlights the importance of TLR9 signaling in driving disease. TLR9 typically activates innate immunity following recognition of a characteristic bacterial DNA sequence called CpG [28]. CpG DNA is a TLR agonist (TLRa) for TLR9, ultimately driving inflammation through the MyD88 pathway that leads to activation of dendritic cells (DCs), macrophages, monocytes, and B cells, along with secretion of inflammatory cytokines [29,30]. For this reason, CpG has been intensively studies as a vaccine adjuvant [29]. In contrast to this common role of CpG as an adjuvant, one group has explored TLR9 antagonists to promote tolerance using GpG, an analog of CpG exhibiting a substitution of guanine for cytosine [31,32]. Like CpG, GpG is unmethylated, single-stranded DNA with a phosphorothioate backbone that can bind TLR9. During cell studies, treatment with GpG suppressed proliferation of inflammatory T_H1 cells. In mice, repeated regular treatment with GpG attenuated EAE—a T_H1-mediated MS model— and further, enhanced induction of tolerance when GpG was mixed just prior to injection with plasmid DNA encoding myelin self-antigens. Thus, we reasoned juxtaposing GpG and self-antigen in polyplex-like nanoparticles might alter the inflammatory signaling associated with recognition of self-antigen, biasing differentiating T cells away from inflammatory phenotypes to help combat autoimmune disease.

One simple class of biomaterials particularly well-suited for the co-delivery strategy above is polyplexes- nano-structured complexes that spontaneously assemble due to electrostatic condensation when nucleic acid is mixed with a cationic polymer [33–35]. Polyplexes condense cargo to a high density that is easily internalized by cells and offer protection from enzymatic degradation. These and other advantages have been exploited in a variety of applications ranging from transfection to vaccination. For the former, great effort has been invested to develop cationic polymers that are non-toxic and offer features (e.g., proton-sponge capacity) that overcome specific barriers to DNA and RNA delivery such as endosomal escape. In the vaccine area—both prophylactic and therapeutic—the particulate nature of polyplexes promotes uptake by APCs, and further, polyplexes allow tuning of vaccine specificity by simply replacing a particular DNA plasmid or RNA molecule with different sequences. Despite these useful properties, only a handful of reports, all in the past 3–4 years, have explored polyplexes to modulate immune function or promote immunological tolerance. In one example, receptors involved in the development of diabetes were targeted in mice by condensing plasmid encoding a soluble ligand for this receptor [36]. In a second example, mice were treated during diabetes with polyplexes formed using chitosan to condense plasmids encoding interleukin 4 (IL-4) and interleukin 10 (IL-10), regulatory cytokines that suppress inflammatory cytokines [37]. The Mellor group has studied a system using poly(-ethylenimine) (PEI) to condense plasmid DNA that is free of PAMPs, limiting activation of common inflammatory pathways and cytokines that are also often active during autoimmune diseases [38–40]. Although none of these approaches incorporate self-antigen, we hypothesized polyplexes including such components might allow induction of antigen-specific tolerance. Further, a number of reports demonstrate that commonly used biomaterials, such as chitosan, PLGA, and polystyrene, exhibit intrinsic immunogenicity that drives inflammation [10,41–45]. This characteristic could be detrimental when developing therapies for autoimmune disease, as intrinsic inflammation from a delivery vehicle could exacerbate disease. Thus, therapeutics that eliminate carrier components but still offer features of biomaterials such as co-delivery and cargo protection, could offer simpler and lower-risk treatments.

To realize the possibilities discussed above, we designed polyplex-like structures assembled from GpG and a myelin peptide (myelin oligodendrocyte glycoprotein, MOG) modified with cationic arginine residues. This strategy is unique in four ways. First, these nanoparticles consist entirely of immune signals, and are free of all carriers or supports. The polyplexes thus mimic attractive features of traditional biomaterials, while eliminating the intrinsic immunogenicity described above. This is highly relevant for autoimmune and inflammatory conditions where such traits might worsen disease. Second, we are targeting suppression of TLR signaling to promote tolerance, a new idea arising in immunology research that is untapped in the biomaterials field. This is particularly important for autoimmune therapies owing to the intriguing recent studies revealing TLR signaling is overactive in human autoimmune diseases such as multiple sclerosis, diabetes, lupus, and rheumatoid arthritis. Third, because the polyplexes are built entirely from immune signals, they juxtapose both the self-antigen and the regulatory signal at very high densities. This feature provides highly efficient loading (i.e., 100%) relative to polymers or matrices used to load and deliver cargo, as well as the co-delivery needed to polarize T cells away from inflammatory phenotypes. Last, these immune polyplexes are novel because they include self-antigen for specificity, but do not rely on expression of any plasmid component; the nucleic acid cargo is an antagonistic TLR ligand that directly interacts with these receptors. Using this platform, below we show that MOG-GpG polyplexes are readily internalized by APCs, decrease TLR9 signaling, and are non-toxic. Treatment of DCs reduces activation and inflammatory cytokines, biasing myelin-specific T cell function away from inflammatory phenotypes during co-culture studies. In a mouse model of MS (EAE), polyplex treatment significantly reduces both the severity and incidence of disease.

2. Materials and methods

2.1. Materials

GpG DNA (5'-TGA CTG TGA AGG TTA GAG ATG A-3'), CpG DNA (5'-TCC ATG ACG TTC CTG ACG TT-3'), and a control oligonucleotide (5'-TCC TGA GCT TGA AGT-3') were purchased from IDT (Coralville, IA). MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Genscript (Piscataway, NJ) with a FITC tag on the N-terminus and either one or two arginine (R) residues on the C-terminus ($MOGR_1$, $MOGR_2$). TE buffer and ethidium bromide were purchased from Amresco (Solon, OH). DNase I kits with 10X reaction buffer were purchased from New England Biolabs (Ipswich, MA). RPMI-1640 media and Molecular Biology Grade Water were purchased from Lonza (Allendale, NJ) and fetal bovine serum (FBS) was supplied by Corning (Tewksbury, MA). β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). 20X PBS, HEPES, and non-essential amino acids were purchased from VWR (Radnor, PA). L-glutamine, penicillin-streptomycin, and DAPI were purchased from Thermo Fisher Scientific (Grand Island, NY). Spleen Dissociation Medium and CD4 negative selection kits were from