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Soluble antigen arrays disarm antigen-specific B cells to promote lasting immune tolerance in experimental autoimmune encephalomyelitis

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ABSTRACT

Autoreactive lymphocytes that escape central immune tolerance may be silenced via an endogenous peripheral tolerance mechanism known as anergy. Antigen-specific therapies capable of inducing anergy may restore patients with autoimmune diseases to a healthy phenotype while avoiding deleterious side effects associated with global immunosuppression. Inducing anergy in B cells may be a particularly potent intervention, as B cells can contribute to autoimmune diseases through multiple mechanisms and offer the potential for direct antigen-specific targeting through the B cell receptor (BCR). Our previous results suggested autoreactive B cells may be silenced by multivalent 'soluble antigen arrays' (SAgAs), which are polymer conjugates displaying multiple copies of autoantigen with or without a secondary peptide that blocks intracellular cell-adhesion molecule-1 (ICAM-1). Here, key therapeutic molecular properties of SAgAs were identified and linked to the immunological mechanism through comprehensive cellular and *in vivo* analyses. We determined non-hydrolyzable 'cSAgAs' displaying multivalent 'click'-conjugated antigen more potently suppressed experimental autoimmune encephalomyelitis (EAE) compared to hydrolyzable SAgAs capable of releasing conjugated antigen. cSAgAs restored a healthy phenotype in disease-specific antigen presenting cells (APCs) by inducing an anergic response in B cells and a subset of B cells called autoimmune-associated B cells (ABCs) that act as potent APCs in autoimmune disease. Accompanied by a cytokine response skewed towards a Th2/regulatory phenotype, this generated an environment of autoantigenic tolerance. By identifying key therapeutic molecular properties and an immunological mechanism that drives SAgA efficacy, this work guides the design of antigen-specific immunotherapies capable of inducing anergy.

1. Introduction

Many current therapies for autoimmune diseases such as multiple sclerosis (MS) act through nonspecific suppression of the immune response, resulting in global immunosuppression and adverse side effects. Creation of antigen-specific immunotherapies (ASIT) that target and suppress only the offending autoreactive immune cells would address a pressing need for safer and more effective autoimmune therapies. Endogenous mechanisms for maintaining immunological tolerance, such as deletion and regulation of autoreactive B and T lymphocytes, would suggest restoration of immune tolerance is plausible. Therapeutic interventions capable of inducing tolerance, however, remain elusive.

Central immune tolerance is coordinated through silencing by deletion and receptor editing in the bone marrow and thymus, while peripheral tolerance is achieved through a state of antigen unresponsiveness called anergy [1]. Anergy occurs when B or T cells mount an initial response to primary antigenic signal but do not receive sufficient secondary signal to sustain activation [1]. For T cells, successful antigen-specific activation requires receipt of both a primary antigenic signal (via MHCII) and secondary costimulatory signal (from receptors such as CD80 and CD86) presented by an antigen presenting cell (APC) [2–5]. B cells, however, are unique in that they can both be activated in this manner and also play the role of APC [6,7]. In fact, B cells may act as the main APC during induction of many autoimmune diseases because they more efficiently uptake low concentrations of soluble

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antigen via high affinity B cell receptors (BCRs) compared to pinocytosis mechanisms of dendritic cells (DCs) and macrophages [7–10]. B cells also contribute to the induction and pathogenesis of autoimmune diseases such as MS and type 1 diabetes (T1D) through autoantibody production, cytokine secretion, and dysregulated B cell signaling leading to a loss of tolerance [6,11–13].

As both an effector cell and APC with potential for direct antigen-specific targeting, B cells have emerged as an especially promising target for ASIT [1,14]. B cells can be modulated in an antigen-specific manner either indirectly by targeting other APCs or $CD4^+$ T helper cells, or directly by targeting the BCR, a surface receptor with inherent antigen specificity that can bind soluble antigen [1]. Since B cell anergy is the primary endogenous mechanism for silencing autoreactive B cells to maintain immune tolerance, it also presents an attractive strategy for ASIT [15,16]. Anergy can be induced through chronic antigen exposure and/or continuous BCR binding, occupation, and clustering in the absence of secondary costimulatory signal, resulting in reduced calcium flux, downregulation of costimulatory markers CD80 and CD86, and thus impaired capacity for T cell stimulation [17–21]. Importantly, B cell fate can be directed by the form of self-antigen that is encountered, depending on antigen avidity and the degree of antigen receptor crosslinking [22]. For example, oligomeric soluble antigen is reported to induce anergy whereas highly multivalent membrane-bound antigen induces deletion [16,23]. Thus, design and development of an effective B cell-targeted ASIT should take into account appropriate physicochemical properties of delivered antigen in light of known molecular mechanisms for inducing peripheral tolerance.

In previous studies, we investigated multivalent soluble antigen arrays ($SAgA_{PLP:LABL}$) designed to induce tolerance to a specific multiple sclerosis (MS) autoantigen. $SAgA_{PLP:LABL}$ consists of a hyaluronic acid (HA) polymer conjugated with multiple copies of autoantigen ($PLP_{139-151}$) and cell adhesion inhibitor (LABL, specific for intracellular adhesion molecule-1, ICAM-1) peptides. Employing a degradable (hydrolyzable) linker to codeliver PLP and LABL, $SAgA_{PLP:LABL}$ was therapeutic *in vivo* in a murine model of MS (experimental autoimmune encephalomyelitis (EAE)) [24–28] and exhibited antigen-specific binding with B cells, targeted the B cell receptor (BCR), and dampened BCR-mediated signaling *in vitro* [29]. Motivated by results that pointed to sustained BCR engagement as the $SAgA_{PLP:LABL}$ cellular mechanism, we developed a new version of the SAgA molecule using non-hydrolyzable conjugation chemistry. “Click SAgAs” ($cSAgA_{PLP:LABL}$), employing hydrolytically stable covalent conjugation chemistry to link PLP and LABL to HA, achieved higher avidity B cell binding, greater reduction and inhibition of BCR-mediated signaling, and significantly enhanced *in vivo* efficacy compared to hydrolyzable $SAgA_{PLP:LABL}$ [30]. We concluded that non-hydrolyzable conjugation increased the avidity of $cSAgA_{PLP:LABL}$ to drive *in vivo* efficacy through dampened BCR-mediated signaling via a mechanism of sustained action (BCR binding and clustering) on the cell surface.

Here, we identified SAgA immunological mechanisms using the EAE mouse model to define arising immune tolerance pathways to the PLP antigen used to induce the disease. Splenocyte immune responses were evaluated following *in vivo* and *ex vivo* treatment with click-conjugated (non-hydrolyzable) and hydrolyzable soluble antigen arrays, mixtures of the components, and controls. Targeted immune cell subtypes were identified through flow cytometry binding assays and microfluidic imaging of live cells. Reduced costimulatory signaling was evaluated as a marker of anergy and lasting antigenic tolerance in EAE splenocytes following *ex vivo* and *in vivo* treatment. Cytokines, PLP-specific autoantibody production, and splenic immune cell composition were also evaluated following *in vivo* treatment to determine the shape of the effector response (Th1 vs. Th2, Treg vs. Th17). Through these comprehensive cellular and *in vivo* analyses, we determined that multivalent polymer arrays displaying click-conjugated PLP induced an anergic response in B cells and a subset of B cells that act as potent APCs in autoimmune disease called autoimmune-associated B cells (ABCs), accompanied by a cytokine response skewed towards a Th2/regulatory phenotype. By identifying cornerstone molecular properties and an immunological mechanism that drives SAgA efficacy, this work guides our understanding of the antigen-specific immune response and informs the future design of ASIT.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA) sodium salt (MW 16 kDa) was purchased from Lifecore Biomedical (Chaska, MN). 11-azido-3,6,9-trioxaundecan-1-amine (NH_2-PEG_3-N_3), *N*-hydroxysuccinimide, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), 2-(*N*-morpholino) ethane-sulfonic acid sodium salt (MES), tris(3-hydroxypropyltriazolylmethyl)amine, and sodium ascorbate (NaAsc) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received without further purification. Copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) was purchased from Acros Organics (Geel, Belgium). Alkyne-functionalized peptides bearing an *N*-terminal 4-pentynoic acid (homopropargyl, hp) modification, $hpPLP_{139-151}$ (hp-HSLGKWLGHPDKF-OH) and hpLABL (hp-ITDGEATDSG-OH), were originally synthesized in our laboratory via solid phase peptide synthesis. Larger quantities of both $hpPLP_{139-151}$ and hpLABL peptides were obtained from Biomatik USA, LLC (Wilmington, DE). Unmodified PLP (NH_2-HSLGKWLGHPDKF-OH) peptide was purchased from PolyPeptide Laboratories (San Diego, CA). Incomplete Freund's adjuvant (IFA) and killed *Mycobacterium tuberculosis* strain H37RA were purchased from Difco (Sparks, MD). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). R-phycoerythrin (PE)/Cy7-conjugated anti-mouse CD3, AlexaFluor647-conjugated anti-mouse CD19, Pacific Blue-conjugated anti-mouse CD11c, PerCP-conjugated anti-mouse B220, PE-conjugated anti-mouse CD86, FITC-conjugated anti-mouse CD80, and respective isotype control antibodies were purchased from BioLegend (San Diego, CA). All other chemicals and reagents were analytical grade and used as received.

2.2. Synthesis of click soluble antigen arrays (cSAgAs)

Penn Green-Alk, HA-N_3, and click soluble antigen arrays (cSAgAs) were prepared as previously reported [30]. Briefly, cSAgAs were constructed using a two-step procedure starting from sodium hyaluronate. 3-(ethyliminomethyleneamino)-N,N-dimethylpropan-1-amine (EDC) and N-hydroxysuccinimide (NHS) neat were added to a solution of sodium hyaluronate in MES buffer. After 5 min of stirring, H_2N-PEG_3-N_3 was added and the solution was stirred at room temperature for 24 h before being dialyzed and lyophilized to isolate HA-N_3. The appropriate ligands ($hpPLP_{139-151}$, hpLABL, Penn Green-Alk) were added to a solution of HA-N_3 in deionized water, followed by a premixed solution of tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) and copper (II) sulfate ($CuSO_4{\cdot}5H_2O$) in deionized water. After stirring for 1–2 min, sodium ascorbate (NaAsc) was added and the reaction was allowed to proceed at elevated temperature until the desired conjugation levels were achieved. Following completion of the reaction, the reaction solution was dialyzed and lyophilized. cSAgAs were analyzed qualitatively by FTIR and NMR, and quantitatively by RP-HPLC to determine extent of conjugation (Fig. S1).

2.3. Synthesis of soluble antigen arrays

Soluble antigen arrays ($SAgA_{PLP:LABL}$) were synthesized and characterized as previously reported [24,25]. Aminooxy peptides AoPLP and/or AoLABL were linked to HA using oxime conjugation chemistry. Peptide conjugation was determined through gradient reverse-phase analytical high-performance liquid chromatography (RP-HPLC) following cleavage of peptides in 0.1N HCl.