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Exploiting CD22 to Selectively Tolerize Autoantibody Producing B-cells in Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that primarily affects the synovial joints and can lead to bone erosion and cartilage damage. One hallmark of RA is anti-citrullinated protein autoantibodies (ACPA) and memory citrulline-specific B-cells, which have been implicated in RA pathogenesis. While depletion of B-cells with Rituximab improves clinical responses in RA patients, this treatment strategy leaves patients susceptible to infections. Therefore, using Siglec-engaging Tolerance-inducing Antigenic Liposomes (STALs) to selectively target the citrulline-specific B-cells may be beneficial. ACPA production from purified human RA patients' B-cells *in vitro* was achieved through a set stimulation conditions, which includes: BAFF, anti-CD40, IL-21, and LPS. *In vivo* generation of citrulline specific B-cells and ACPA production was accomplished by antigenic liposomes consisting of monophosphoryl lipid A (MPLA) and a cyclic citrullinated peptide (CCP) administered to SJL/J mice. We show that STALs that co-display a high affinity CD22 glycan ligand and synthetic citrullinated antigen (CCP STALs) can prevent ACPA production from RA patients' memory B-cells *in vitro*. These CCP STALs were also effective in inducing tolerance to citrullinated antigens in SJL/J mice. The results demonstrate that tolerization of the B-cells responsible for ACPA can be achieved by exploiting the inhibitory receptor CD22

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Conflict-of-Interest
The authors have no conflict-of-interests to declare

Ethics Approval and Consent Participate
Human Samples: Frozen peripheral blood mononuclear cells (PBMCs) were obtained through AllCells (Alameda, CA) or BioreclamationIVT (Chestertown, MD) as per institutional protocols for human sample acquisition. Janssen Pharmaceutical, LLC Institutional Animal Care and Use Committee approved all experimental procedures involving mice.

Supporting Information Available: This material is available free of charge *via* the Internet

with high affinity glycan ligands. Such a treatment strategy could be beneficial in the treatment of RA.

Keywords

rheumatoid arthritis; anti-citrullinated protein antibodies; liposomes; antigen specific tolerance; auto-antibodies

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease affecting the synovium of the joints, characterized by joint inflammation, bone erosion, loss of bone density, and articular cartilage damage leading to limited mobility[1-3]. Inflammation and disease pathology are mediated by pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) released by infiltrating immune cells within the synovial tissue[4-6]. Current therapies to control inflammation use non-steroidal anti-inflammatory (NSAIDs) or disease modifying anti-rheumatic drugs (DMARDs)[7-9]. However, increased understanding of disease progression has opened doors for novel disease modifying biologic therapies that target anti-inflammatory cytokines (e.g. anti-TNF-α, anti-IL-6), or prevent immune responses within the B- and T-cell compartments[10-13].

Increased knowledge of disease progression has also provided diagnostic tools that identify patients who are at high risk of developing RA. Genetic and serological screens reveal that HLA-DRB1 alleles are highly associated with RA incidence and are strongly associated with the development of anti-citrullinated protein autoantibodies (ACPA)[14]. The association between HLA-DRB1 and ACPA stems from HLA-DRB1 accommodating peptides bearing post-translational modifications of antigens associated with RA, such as citrullination, leading to a break in tolerance and autoantibody formation[15]. Conversion of arginine to citrulline is catalyzed by peptidylarginine deiminases (PAD) enzymes generating the epitope for ACPAs[16]. ACPA are highly correlated with RA disease burden and are predictive of disease onset[17-20].

The discovery of autoantibodies, such as ACPA and rheumatoid factor (RF) and the associated genetic alleles, has changed the way RA is classified. RA patients that develop ACPA or RF are defined as seropositive, while those who do not are seronegative [2]. Seropositive cases represents the majority of patients (~70%)[21], present with more severe bone and joint destruction[22], and respond better to disease modifying biologics such as B-cell depletion (Rituximab)[23] or co-stimulation blockade (CTLA4-Ig)[24]. Disease progression in seropositive patients is also well understood due to longitudinal studies tracking patient pathology[25]. The discovery of ACPA came from a systematic analysis of autoantibodies in RA patients, which defined a common epitope containing a citrullinated motif that occurred in many different proteins like fibrinogen, vimentin, and type II collagen[25-30]. Currently, ACPAs are detected in RA sera using synthetic cyclic citrullinated peptides (CCP) that broadly cover antibodies that recognize the citrullinated epitope[31-34]. CCP-reactive antibodies are specific for RA, not found in healthy individuals, and found only at low levels

in other autoimmune inflammatory conditions such as type 1 diabetes[35] and multiple sclerosis[36].

RA patients undergoing B-cell depletion therapy with Rituximab exhibit improved clinical responses and reduced circulating IgM RF and ACPA, supporting a pathological role for ACPA and strong rationale for targeting ACPA reactive B-cells in RA[37]. Rituximab only targets naïve and memory B-cells, due to the loss of CD20 expression upon differentiation into plasmablasts and plasma cells, yet targeting these populations is sufficient to reduce circulating ACPA and achieve therapeutic efficacy in RA patients, suggesting a plausible approach of RA treatment by depleting antigen-specific memory B-cells. Further support for the role of ACPA comes from preclinical studies evaluating the contribution of ACPA and citrulline-specific B-cells to disease pathology[38-41]. Although the mechanisms that underlie ACPA induction of RA pathology remains under investigation[38, 42-45], there is emerging consensus that citrulline post-translational modifications and ACPA play a significant role in RA disease progression and severity.

As an alternative to depletion of B-cells with Rituximab, more selective targeting of citrulline-specific B-cells could have a similar therapeutic profile for RA without compromising broad B-cell directed immunity[37, 46]. To this end, we have examined the potential of exploiting Siglec-engaging Tolerance-inducing Antigenic Liposomes (STALs) as an approach to deplete the citrulline-specific B-cells. STALs contain both an antigen of interest and a high affinity ligand for the inhibitory B-cell Siglec, CD22. The STALs exploit the inhibitory properties of CD22 by driving clustering of CD22 with the BCR that binds the antigen[47], suppressing B-cell activation, and deleting the antigen-specific B-cells from the B-cell repertoire, leading to antigen-specific tolerance[48-52]. Here, we have used a synthetic cyclic citrullinated peptide, CCP, as the antigen since it is recognized by a broad range of RA pathogenic citrulline-specific B-cells, to generate tolerizing STALs - referred to throughout as CCP STALs - to test the hypothesis that CCP STALs can selectively deplete citrullinated protein-specific B-cells. We show that RA patients have normal expression patterns of CD22 on various B-cell subsets, and that CCP STALs can induce *in vitro* tolerance of citrullinated protein-specific memory B-cells from RA patients via depletion mechanisms. Importantly, these tolerizing effects occur in an antigen-specific manner. Similarly, CCP STALs induce antigen-specific tolerance in SJL/J mice, leading to impaired ACPA responses. Our results demonstrate that selectively silencing the potential pathogenic B-cells in RA patients with high ACPA titers, using STALs, could be beneficial in treating this autoimmune disease.

RESULTS AND DISCUSSION

RA and Healthy Donors Show Similar Profile of B-Cell Subsets and CD22 Expression.

A small cohort of RA patients selected for elevated levels of ACPA (≥ 20 units) were documented for sex, age, rheumatoid factor levels (RF), ACPA titers, health assessment questionnaire (HAQ) score, and current medications (Supplemental Figure 1A). While several reports document small differences in the naïve B-cell compartment in RA patients [53-56], no statistically significant differences in B-cell subsets were found in this cohort compared to healthy controls, including the naïve ($CD20^{+}IgD^{+}CD27^{-}$), innate memory

(CD20$^+$IgD$^+$CD27$^+$), and memory (CD20$^+$IgD$^-$CD27$^{int/+}$) and plasma (CD138$^+$) B-cells (Supplemental Figure 1B). CD22 expression on B-cells of RA patients was similar to healthy controls across all B-cell subsets, with strong expression in naïve and memory subsets and very low levels in plasma B-cells as documented previously[57, 58] (Figure 1A **and** Supplemental Figure 1C).

B-cell Antibody Production Correlates with Plasmablast Differentiation *In Vitro.*

To assess ACPA production from RA patient blood samples, an *in vitro* antibody production assay was developed. Various stimulation conditions were evaluated for their effects on B-cell proliferation, differentiation, and antibody production (Supplemental Figure 2A **and** Figure 1B). IgG antibody production correlated with the differentiation of B-cells to IgD$^-$CD27^{hi} cells (R-square=0.7360, Figure 1B), but had no correlation with differentiation to other B-cell subsets (e.g. IgD$^+$CD27^{hi}, IgD$^-$CD27$^-$, and IgD$^+$CD27$^-$) or B-cell proliferation (Supplemental Figure 3). The stimulation condition with anti-human CD40, BAFF, anti-human IgG/IgM, IL-21, and LPS in Stim-3 consistently produced the highest level of total IgG across multiple healthy controls, is comprised of mediators that mimic signals from T-follicular helper cells *in vivo* [59].

To further characterize the IgD$^-$CD27^{hi} B-cells that correlate with IgG antibody production, purified B-cells were incubated under Stim-3 condition and assessed for well-established markers of antibody-producing B-cell lineages and plasmablast/plasma cells. The cells expressed high levels of CD38 and CD95, and low levels of CD22 (Figure 1B **and** Supplemental Figure 2B). CD138 expression was not detected in these B-cell cultures (Supplemental Figure 4) and it is likely that this time point is too early for plasma cell generation and thus CD138 expression[60, 61]. Based on these markers, IgD$^-$CD27^{hi} B-cells can be classified as antibody-secreting cells (ASC), resembling plasmablasts, whose appearance strongly correlates with production of high levels of human IgG antibodies.

Memory B-cells are Responsible for the Generation of Plasmablasts.

Memory B-cells are thought to play an important role in RA disease pathogenesis, being responsible for the local generation of ACPA producing ASCs in the synovium[62, 63]. To assess the role of memory B-cells in the production of IgG and plasmablasts in our *in vitro* assay, B-cells were purified from healthy controls and sorted for naïve (CD20$^+$CD27$^-$IgD$^+$) or memory (CD20$^+$CD27$^+$IgD$^-$) cells (Supplemental Figure 5). As expected, sorted naïve B-cells had no IgG surface expression and were almost entirely surface IgM$^+$, while sorted memory B-cells had a higher percentage of IgG$^+$ cells. These B-cell subsets were cultured under Stim-3 condition without BCR stimulation (anti-human IgG/IgM) for 7 days to evaluate the ability of the two different B-cell types to differentiate into ASC. Plasmablasts were found to be generated only in the cultures of sorted memory B-cells and correlated with production of significant amounts of IgG in culture supernatants (Figure 1C). We conclude that memory B-cells are primarily responsible for the generation of IgG producing plasmablasts in this assay.

Memory RA B-cells are Depleted using Anti-human IgG-STALs.

To selectively target human memory B-cells, we formulated STALs with a synthetic hCD22 ligand, 6'MBP-5F-Neu5Ac (hCD22L) and anti-human IgG Fab fragments to bind to the IgG memory B-cell BCR as a surrogate antigen (αIgG-STALs). Purified B-cells were cultured for 24hr under one of the following conditions: PBS, liposomes decorated with only anti-human IgG (αIgG-liposomes), liposomes decorated with CD22L only (CD22L-liposomes.) or αIgG-STALs presenting both αIgG and CD22L. Cells were then assessed for activation and apoptosis by measuring upregulation of CD69 and PI/Annexin V, respectively. Memory B-cells treated with αIgG-liposomes had greatly increased CD69 expression and negligible increased apoptosis relative to the PBS controls (Figure 2A). In contrast, αIgG-STALs caused no activation of memory B-cells while significantly increasing apoptosis. This effect was dependent on co-presentation of αIgG and CD22 since CD22L-liposomes alone had no effect on either B-cell activation or cell death. In contrast to the impact of αIgG-STALs on IgG-memory B-cells, they had no effect on IgM-naïve B-cells (Supplemental Figure 6). Soluble antigens and autoantibodies may be present in circulation in RA, which could potentially compete with or bind the antigen on STALs to mask its ability to engage the antigen specific B-cells. Therefore, we monitored the effect of STALs in the presence of soluble antigen (purified human IgG) and found that it did not affect the ability of anti-human IgG liposomes to cause B-cell activation or anti-human IgG STALs to inhibit activation and induce apoptosis of memory B-cells (Figure 2B).

αIgG-STALs Prevent Differentiation of Stimulated Memory B-cells to Plasmablasts.

The inflammatory conditions in the RA synovium produces a variety of cytokines that can potentially provide a secondary signal that abrogates the effect of STALs [49, 50]. To assess the impact of such stimulatory conditions on αIgG-STALs, purified B-cells were left unstimulated (PBS) or cultured under Stim-3 conditions for 24hrs, followed by addition of αIgG-Liposomes, αIgG-STALs, or CD22L-Liposomes. Under these conditions, anti-IgG STALs significantly blocked the differentiation of memory cells to plasmablasts, while αIgG-Liposomes or CD22L-Liposomes had no effect on differentiation (Figure 3A, B). The decreased differentiation of plasmablasts also correlated with a near complete abrogation of IgG production relative to the unstimulated control (Figure 3C). Notably, αIgG-STALs had no effect when added 5 days after cells were stimulated (Supplemental Figure 7), consistent with the timeframe in which down regulation of CD22 occurs when B-cells are differentiated into plasmablasts. This data demonstrates that STALs maintain their tolerizing effects on memory B-cells even in the presence of some inflammatory mediators.

CCP STALs Abrogate ACPA Production by RA Patient B-cells *In Vitro.*

We next formulated CCP-STALs to directly assess the impact on ACPA-generating B-cells in RA blood[60, 64-66]. Under the optimized Stim-3 conditions, B-cells from RA patients produced detectable ACPA relative to background levels set by B-cells from healthy controls (Figure 4A). Although the ACPA titers are significantly lower than those in human sera, this likely relates to the low frequency of ACPA producing B-cells. There are, on average, only one ACPA-generating B-cell per 2×10^4 B-cells[64], which translates to ~5 citrulline-specific B-cells per well. Nevertheless, these *in vitro* ACPA responses were consistent and sufficient

to test the effect of CCP STALs on ACPA-producing B-cells. Direct detection of ACPA B-cells was tested exhaustively, however one complication is that the same peptide used to detect ACPA-secreting cells is used for ACPA B-cell STAL targeting and tolerization. Therefore, interpreting results due to competition of binding and detection of ACPA B-cells by ELiSpot or flow cytometry may not be valid.

Accordingly, RA patient B-cells were left unstimulated or stimulated, as described above, with the addition of liposomes decorated with CCP peptides (CCP-Lip) or CCP-STALs thirty minutes later. As shown in Figure 4B, CCP-STALs significantly reduced ACPA production to levels in the unstimulated control. The depletion was consistent across multiple donors and repeated data (Figure 4C). In contrast, CCP-Lip increased the production of ACPA, as expected due to crosslinking of the BCR with the citrulline-reactive B-cells. It is also notable that while CCP-STALs prevented ACPA production from RA patient samples, they did not affect total IgG production under the same conditions from the same wells as tested for ACPA (Figure 4D). This is consistent with the previous findings of affecting only the specific antigen attached to the liposome [50] and this shows tolerization of B-cells that recognize CCP, while other memory B-cells are unaffected.

CCP-STALs Induce B-cell Tolerance to ACPA Production *In Vivo.*

SJL/J mice are known to generate antibody responses against citrullinated antigens[67]. Therefore, we tested the ability of CCP-Lip to generate ACPA-like responses in SJL/J mice. Since CCP-Lip is considered a T-independent type II antigen, we added a TLR4 ligand, MPLA, to CCP-Lip as an adjuvant. Indeed, CCP-Lip with MPLA generated a robust ACPA antibody response relative to CCP-Lip without MPLA (Supplemental Figure 8). For the experiment, SJL/J mice initially treated with PBS, CCP-STALs, or CCP-Lip containing MPLA. Two weeks after the initial injection, all groups of mice were challenged with CCP-Lip containing MPLA, and ACPA titers were measured the following 2 weeks. Mice treated initially with CCP-STALs had significantly reduced total ACPA titers at all time points compared to the group that was treated immunized with two doses of CCP-Lip, and significantly reduced titers at day 21 and day 28 compared to mice that received CCP-Lip only at day 14 (Figure 5). These data demonstrate that liposomes decorated with a cyclic citrullinated peptide and a high affinity ligand for CD22 can tolerize CCP-specific B-cells in ACPA production *in vivo*.

Discussion.

B-cells have gained attention as therapeutic targets in RA, largely due to positive clinical and radiological responses seen in patients receiving Rituximab for B-cell depletion therapy. Rituximab targets CD20 on naïve and memory B-cells, causing depletion of these CD20-expressing cells primarily through complement-dependent cytotoxicity[68]. Other monoclonal antibodies targeting B-cells, such as CD19 and CD22 conjugated to toxins or radionuclides are currently being developed and tested[37]. Prolonged B-cell depletion, however, limits the use of these therapies due to loss of protective humoral immunity and increased risk for infection[37]. In principle, a more selective therapy that targets B-cells directly involved in the

pathology of RA would be more tolerated and maintains a functional humoral immunity for protection from infections.

B-cells that recognize citrullinated proteins are of interest for RA given evidence for ACPA levels correlated with disease onset and pathogenesis, and the potential of these B-cells to secrete cytokines and present antigen to T-cells, playing a pathogenic role beyond antibody production. Memory B-cells are the major cytokine producers and T-cell activators in RA patients, and are enriched in the synovial fluid, synovial tissues, and intra-tissue like lymphoid structures in the joints[69-72]. Memory B-cells specific for citrullinated antigens in the peripheral blood express both IgA and IgG isotypes[64], and clinical responses correlate with the number of these cells in the blood[73-75]. Transplantation of lymphoid tissues from joints in RA joints into SCID mice results in production of class-switched ACPA[63, 76]. Together, these data suggest that memory B-cells specific for citrullinated antigens are key progenitors of the ASCs that secrete ACPA, making them a logical population for targeted B-cell therapy.

Cyclic citrullinated peptides were originally developed to mimic epitopes on citrullinated proteins recognized by ACPA and could be used diagnostically to detect ACPA in RA patient sera. We took advantage of CCP to therapeutically target ACPA B-cells using CCP-STALs. We showed here that treating B-cells from RA patients *in vitro* with CCP-liposomes induced ACPA formation, while CCP-STALs suppressed formation of ACPA. Furthermore, mice treated with CCP-STALs had an impaired ability to mount an antibody response upon a subsequent challenge with CCP antigenic liposomes. The results suggest the potential for use of CCP-STALs for inducing tolerance and reducing ACPA formation. Although CCP does not recognize 100% of ACPAs, second and third generation CCPs have been shown to cover > 95% of ACPAs[33], providing an opportunity to create CCP-STALs that recognize nearly all ACPAs.

STALs met the expectation for antigen-specific targeting of B-cells since CCP-STALs suppressed ACPA, but did not impact induction of non-ACPA (total) antibody[50]. Similarly, αIgG-STALs targeted memory B-cells (IgG^+) but not naïve B-cells (IgM^+). Moreover, we found that the αIgG-STALs inhibitory effects on memory B-cells was not abrogated when exogenous IgG $F(ab')_2$ was added. This could be due to the fact that the soluble antigen used is divalent while the large number of IgG F(ab) molecules decorating the liposomes surface is highly multimeric, increasing avidity to the surface IgG BCRs on the B-cell. ACPA in patient serum, amounting to 10-200 μg/ml[77], could also potentially interfere with CCP-STALs and would be important to address in the context of an ongoing disease where such antibodies would be present.

In summary, we have shown that ACPA producing B-cells from RA patients can be selectively suppressed *in vitro* using CCP-STALs, as illustrated in Figure 6. Depletion of antigen-specific memory B-cells which develop into ACPA-producing ASCs, may have multiple benefits in RA patients that present with high ACPA titers, including: depleting the local and systemic pool of ACPA-producing B-cells, reducing ACPA titers and their ability to antagonize the immune system through forming immune complexes, removal of the citrulline-specific B-cells that can capture and present citrullinated antigens to T-cells, and

reducing inflammatory cytokine secretion. Since STALs do not directly deplete long-lived plasma cells, even though they may indirectly affect this population through depleting the memory B-cells, a combination therapy that depletes both the long-lived plasma cells and the antigen-specific naïve and memory B-cells may ultimately be the most efficacious while keeping protective humoral immunity. Such a targeted immunotherapy provides an attractive alternative to current treatments for RA.

Conclusions.

Memory B-cells are responsible for plasmablast generation *in vitro*. Targeting memory B-cells using STALs is able to prevent the activation of memory B-cells and ultimately resulting in reduction of antibodies. Targeting ACPA memory B-cells using STALs decorated with a cyclic citrullinated peptide was able to reduce ACPA production *in vitro*. SJL/J are able to produce ACPA after injection with an antigenic liposome. STALs can significantly reduce ACPA production *in vivo*. These results demonstrate that STALs could be an effect therapy for the depletion of ACPA B-cells.

METHODS

B-cell Purification and Staining.

Frozen peripheral blood mononuclear cells (PBMCs) were obtained through AllCells (Alameda, CA) or BioreclamationIVT (Chestertown, MD) as per institutional protocols for human sample acquisition. Cells were thawed in accordance with the manufacture's protocol. B-cells were purified by negative enrichment (B-cell isolation kit II, Miltenyi), resulting in 91-98% pure $CD20^+$ B-cells. B-cells were suspended in FACS Buffer (0.2% BSA, BD Bioscience) containing Human Fc Block (BD Bioscience) on ice for 10 minutes. Primary antibodies were added at a concentration directed by the manufacturer (Biolegend) and left for 1hr on ice. The following antibodies directly toward the human antigens were used (clone; fluorophore): CD20 (SJ25C1; APC-Cy7), CD27 (CM-T271; Brilliant Violet 510, APC), IgD (IA6-2; FITC), IgM (MHM-88; Pacific Blue), CD38 (HB-7; PE-Cy7), CD138 (DL-101; APC), IgG (M1310G05; Percp/Cy5.5), CD22 (HIB22; PE), CD95 (DX2; Brilliant Violet 421), and CD69 (FN50; FITC)). B-cells were centrifuged (300 RCF, 5 min), washed 3x with FACS buffer, and were stored on ice until analyzed by flow cytometry. For Annexin V staining, the cells were stained in accordance with the manufacturer's protocol (BD Biosciences). Flow cytometry data was obtained on a CantoII flow cytometer (BD Biosciences) with up to ten fluorochromes and analyzed using FlowJo software (TreeStar).

B-cell Proliferation and IgG Antibody Production.

Purified B-cells were cultured with DMEM containing: 10% FBS, 2% Penicillin and Streptomycin, 1% L-glutamine, MEM Non-Essential Amino Acids (NEAA), and sodium pyruvate (ThermoFisher), described below as standard culturing media, in a 96-well U-bottom plate at 2×10^4 cells per/well in a volume of 200 μl. Combinations of additional factors were added for 7 days, including: $F(ab')_2$ anti-human IgG/IgM (Jackson ImmunoResearch, 100 ng/mL), B-cell activating factor (BAFF; eBioscience, 100 ng/mL), anti-human CD40 (eBioscience, 100 ng/mL), IL-4 (eBioscience, 10 ng/mL), IL-21

(eBioscience, 50ng/mL), LPS (Invivogen, 1 ng/mL), and CD180 (eBioscience, 100 ng/mL). After 7 days, plates were spun down (300 RCF, 5 min) and the supernatant analyzed for total human IgG antibody (Ready-Set-Go total human IgG ELISA Kit, Invitrogen). B-cell proliferation was measured using CellTiter-Glo (Promega).

B-Cell Sorting and *in vitro* Stimulation.

B-cells were sorted on a FacsAria (BD Biosciences) cell sorter for memory ($CD20^+CD27^+IgD^-$) or naïve B-cells ($CD20^+CD27^-IgD^+$) using a 70μm nozzle. After-sorting, purity of each B-cell subset population was determined and cells were cultured in standard culturing media in a 96-well U-bottom plate at 1×10^5 cells per/well in 200 μL. All wells were stimulated with the following cocktail for 7 days: BAFF (100 ng/mL), anti-human CD40 (100 ng/mL), IL-21 (50 ng/mL), and LPS (1ng/mL), referred to below as the standard stimulation conditions or Stim-3 below. After 7 days, the plate was spun down (300 RCF, 5min) and the supernatant was harvested and total human IgG determined by ELISA. The remaining cells were also stained for specific B-cell markers to look at subsets.

In Vitro B-Cell Activation and Evaluation of Cell Death.

B-cells from healthy controls were cultured in standard culturing media in a 96-well U-bottom plate at 1×10^5 cells per/well in 200 μl. Following plating of B-cells, one of the following treatments was added into the well for 24 hours: PBS, anti-human IgG Liposomes (αIgG Lip), STALs decorated with anti-human IgG and hCD22L (αIgG STALs), or liposomes decorated with hCD22L alone (CD22L Lip). The final concentration of liposomes used in these assays was 40 μM, based on total lipids. Following 24hrs, the plate was spun down (300 RCF, 5min) and stained as described above for the B-cell markers labeled in each figure. All results were normalized to the unstimulated control (PBS) and fold change was determined for both activation ($CD69^+$) and cell death (PI^+ and Annexin V^+). For the addition of soluble protein, B-cells were cultured in identical conditions, as stated above, with soluble human IgG (5 ng/mL, Sigma) added into the cultures of all the wells prior to the addition of liposomes.

In Vitro B-Cell Activation and Analysis of Plasmablast Generation.

B-cells were cultured in standard culturing media in a 96-well U-bottom plate at 1×10^5 cells per/well in 200 μL. Cells were cultured with standard stimulation conditions starting on day 0 for 7 days. The following treatments were initiated on day 1: (a) PBS, (b) αIgG Lip, (c) αIgG STALs, or (d) CD22L Lip. On day 7, the plates were spun down at (300 RCF, 5min) and supernatants were harvested from each well for the quantitation of total human IgG. B-cells were stained for plasmablast-like differentiation markers indicated in each of the figures.

In Vitro RA and Healthy Donors B-Cell Activation and Measurement of ACPA Production.

B-cells from healthy donors or RA patients were cultured in standard culturing media in a 96-well U-bottom plate at 1×10^5 cells per/well in 200 μl. Cells were cultured with standard stimulation conditions starting on day 0 for 7 days. On day 7, the plates were spun down (300 RCF, 5 min) and supernatants were collected and tested for anti-citrullinated protein

antibodies (ACPA titer) in accordance with the protocol from Inova Diagnostics CCP3 IgG kit. polyethyleneglycol-distearoyl phosphoethanolamine (PEG-DSPE, Avanti Polar Lipids) was conjugated to a peptide known to bind ACPA producing B-cells, CFFCP (HSTKRGHAKSRPV-Cit-GHQ-(CHQEST-Cit-GRSRGRC)-GRSGS-OH)[78], herein called CCP. DSPE-PEG-CCP was synthesized by an outside vendor (Peptide International) to 99% purity and was decorated onto the liposomes as described below. B-cells from RA patients were cultured in standard culturing media in a 96-well U-bottom plate at 1×10^5 cells per/well in 200 μl. The B-cells were either left unstimulated or stimulated with standard stimulation conditions for 7 days with liposomes decorated with CCP (CCP Liposome), or liposomes decorated with CCP and hCD22L (CCP STALs). Plates were spun down (300 RCF, 5min) and ACPA production was measured from the supernatants. All results were normalized to the stimulated condition and assessed for fold changed differences in ACPA titers. From the same supernatant, total human IgG production (Invitrogen) was assessed to determine the effect of CCP liposomes or CCP STALs on overall IgG production.

Immunization of Mice with CCP Liposomes or CCP STALs.

Janssen Pharmaceutical, LLC Institutional Animal Care and Use Committee approved all experimental procedures involving mice. Female SJL/J mice were obtained from Jackson laboratory at 6 weeks of age. Whole blood (50 μl) was collected via tail vein bleeds to obtain the serum after centrifugation (17,000 RCF, 1 min). Serum was aliquoted and stored at −20°C. PBS or liposomes were delivered via the lateral tail vein (I.V.) in a volume of 100 μl/mouse. Briefly, mice were injected on day 0 with PBS, CCP liposomes with 5% (mol %) MPLA (Invivogen) as an adjuvant (100 μM of liposomes were given, based on total lipid), or CCP STALs (100 μM of liposomes were given, based on total lipid). All groups were challenged on day 15 with CCP liposomes with 5% (mol %) MPLA (250 μM of liposomes were given, based on total lipid). Mice were bled weekly for serum and sacrificed two weeks after CCP liposome challenge.

Measurement of ACPA Titers from *in vivo* Animal Models.

Neutravidin high-binding plates were coated (O/N, 4°C) with a biotinylated CCP peptide (Peptide International) (Biotin-HSTKRGHAKSRPV-Cit-GHQ-(CHQEST-Cit-GRSRGRC)-GRSGS-OH) at 100 μl/well of 10 μg/ml stock solution in PBS. The following day, plates were washed 6x with PBS-T (0.1% Tween 20) and blocked (2 hour, RT) with PBS with 0.1% Tween 20 and 1% BSA, and mouse sera were applied at 1:1000 dilution. Plates were incubated (2 hour, RT) with samples (100 μl/well), washed 6x with PBS-T, and incubated (1 hour, RT) with the appropriate HRP-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology). Following 6 washes in PBS-T, plates were developed (15 minutes, RT) in 75μl/well of TMB substrate (ThermoFisher) and quenched with 75μl/well of 2N H_2SO_4. Absorbance was measured at 450 nm, and the numerical titer was based on the standard from the Inova Diagnostics CCP3 kit.

Generation of High Affinity Murine and Human CD22 Ligands.

The synthesis of the high affinity mCD22L, described as ^{BPA}Neu5Gca2-6Galb1-4GlcNAc-lipid, and high affinity hCD22L, described as ^{MPB}Neu5Aca2-6Galb1-4GlcNAc-lipid, were prepared as described previously[48, 49].

Liposomes and Protein-lipid Conjugation.

A detailed protocol for conjugation of proteins to PEG-DSPE and liposomal preparation can be found in detail elsewhere[50, 79, 80]. Preparation of CCP-PEG-DSPE was commercially prepared (Peptide International; Louisville, KY). Briefly, all liposomes were composed of distearoyl phosphatidylcholine (DSPC, Avanti Polar Lipids), cholesterol (SigmaAldrich), PEG-DSPE, pegylated lipids in a 60:35:5 molar ratio and were extruded through an 800 nm, 200 nm, and finally 100 nm filter a minimum of 20 times each, followed by running the liposomes on a CL-4B column to remove any unconjugated protein or ligand. Liposomal compositions used in these studies consisted of 0.03% anti-human IgG F_{ab} fragment or 0.1% CCP as the antigens, with or without 1.5% CD22L (mouse- or human-specific ligands), all of which were linked to PEG-DSPE. Care was taken to ensure that the total mol% of PEG was 5% in all liposomes.

Statistics.

GraphPad Prism was used to determine statistical significance; test used for statistical significance is indicated in each figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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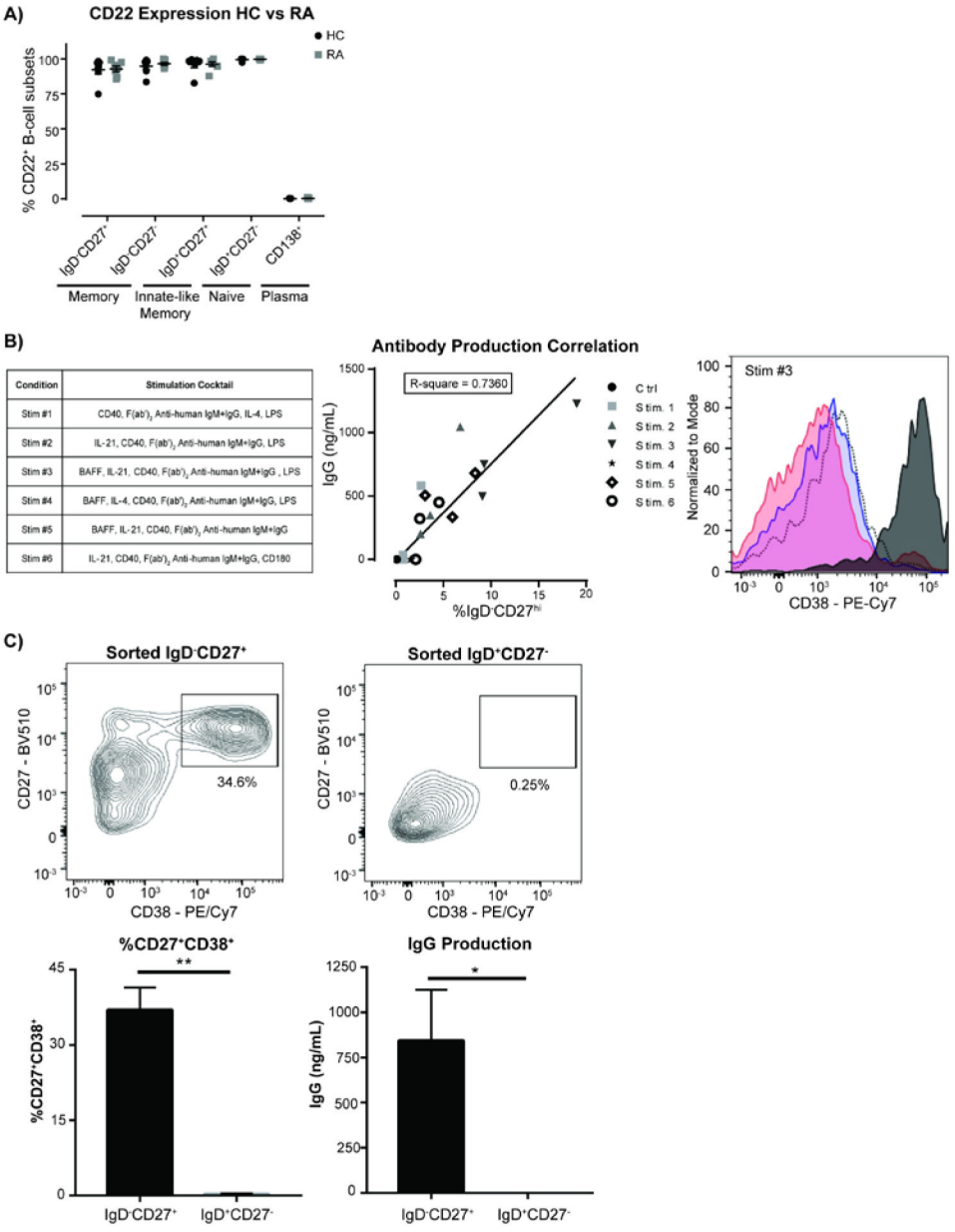

Condition	Stimulation Cocktail
Stim #1	CD40, F(ab')2 Anti-human IgM+IgG, IL-4, LPS
Stim #2	IL-21, CD40, F(ab')2 Anti-human IgM+IgG, LPS
Stim #3	BAFF, IL-21, CD40, F(ab')2 Anti-human IgM+IgG , LPS
Stim #4	BAFF, IL-4, CD40, F(ab')2 Anti-human IgM+IgG, LPS
Stim #5	BAFF, IL-21, CD40, F(ab')2 Anti-human IgM+IgG
Stim #6	IL-21, CD40, F(ab')2 Anti-human IgM+IgG, CD180

Figure 1.

CD22 expression on B-cell subsets in rheumatoid arthritis patients and human memory B-cells are the major source of antibody secreting cells in an *in vitro* differentiation assay. a) CD22 expression in different B-cell subsets from HC and RA blood was shown. (N=7 individual donors for RA and 7 matched HC donors, unpaired t-test). b) Total IgG antibody production from *in vitro* B cell differentiation under different stimulation conditions as listed in the table was determined in culture supernatants, with each symbol representing a different healthy control sample. (N=3 independent experiments from 3 individual healthy control donors, linear regression). c) Generation of plasmablasts and production of IgG from naïve or memory B-cells were tested in the B cell *in vitro* differentiation assay. Sorted IgD $^{+}$CD27^{-} naïve B cells and IgD^{-}CD27^{+} memory B cells from healthy donor blood were

stimulated (Stim #3) and expression of B cell markers in cells after 7-day cultures were assessed by flow cytometry. Supernatants from day 7 stimulated B cell cultures were collected for measurement of total IgG production by ELISA (N=3 independent experiments with 3 individual healthy donors, unpaired t-test).

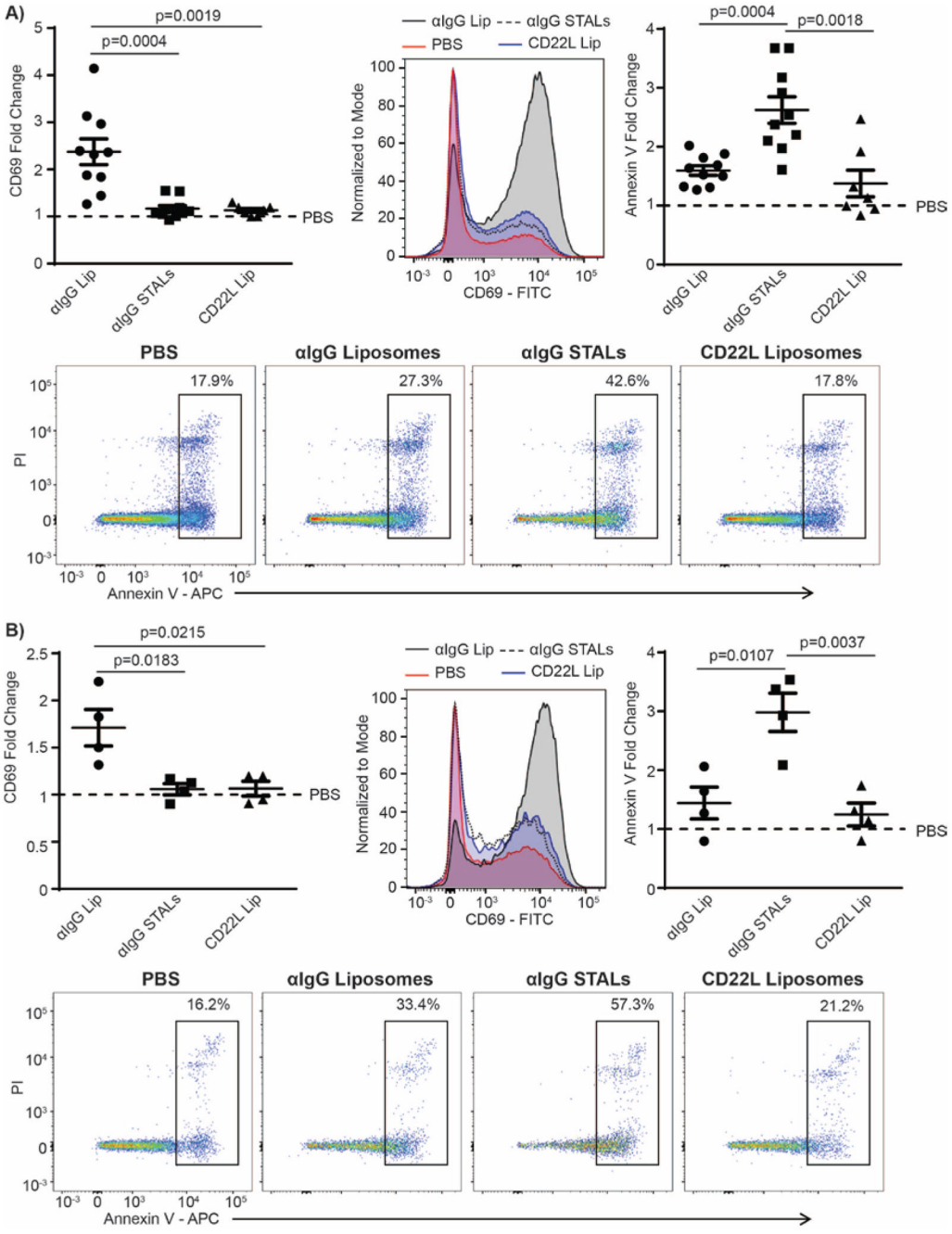

Figure 2.

IgG^{+} expressing B-cells are depleted by anti-human IgG STALs even in the presence of soluble IgG antibody. a) Human memory B-cells were isolated from healthy control PBMCs and incubated under one of the following conditions: unstimulated (PBS), anti-human IgG-liposomes (αIgG Lip), anti-human IgG+hCD22L (αIgG STALs), or CD22L liposomes alone (CD22L Lip) for 24 hrs. After 24hrs, cells were stained for markers of activation (CD69) and cell death (Annexin V/PI) (N=10 for PBS, αIgG, and αIgG STAL, N=7 for CD22L Lip, each independent experiments, pooled data, one-way ANOVA with multiple comparisons). c) B-cells were cultured under the same conditions as above, except that

soluble IgG (5 ng/mL) was added to the wells prior to the addition of PBS or the liposomes (N=4 for PBS, αIgG, αIgG STALS, and CD22L Lip, each an independent experiment, pooled data, one-way ANOVA with multiple comparisons). Representative FACS analysis data on activation marker CD69 and cell death markers annexin V and PI uptake from one healthy donor are shown.

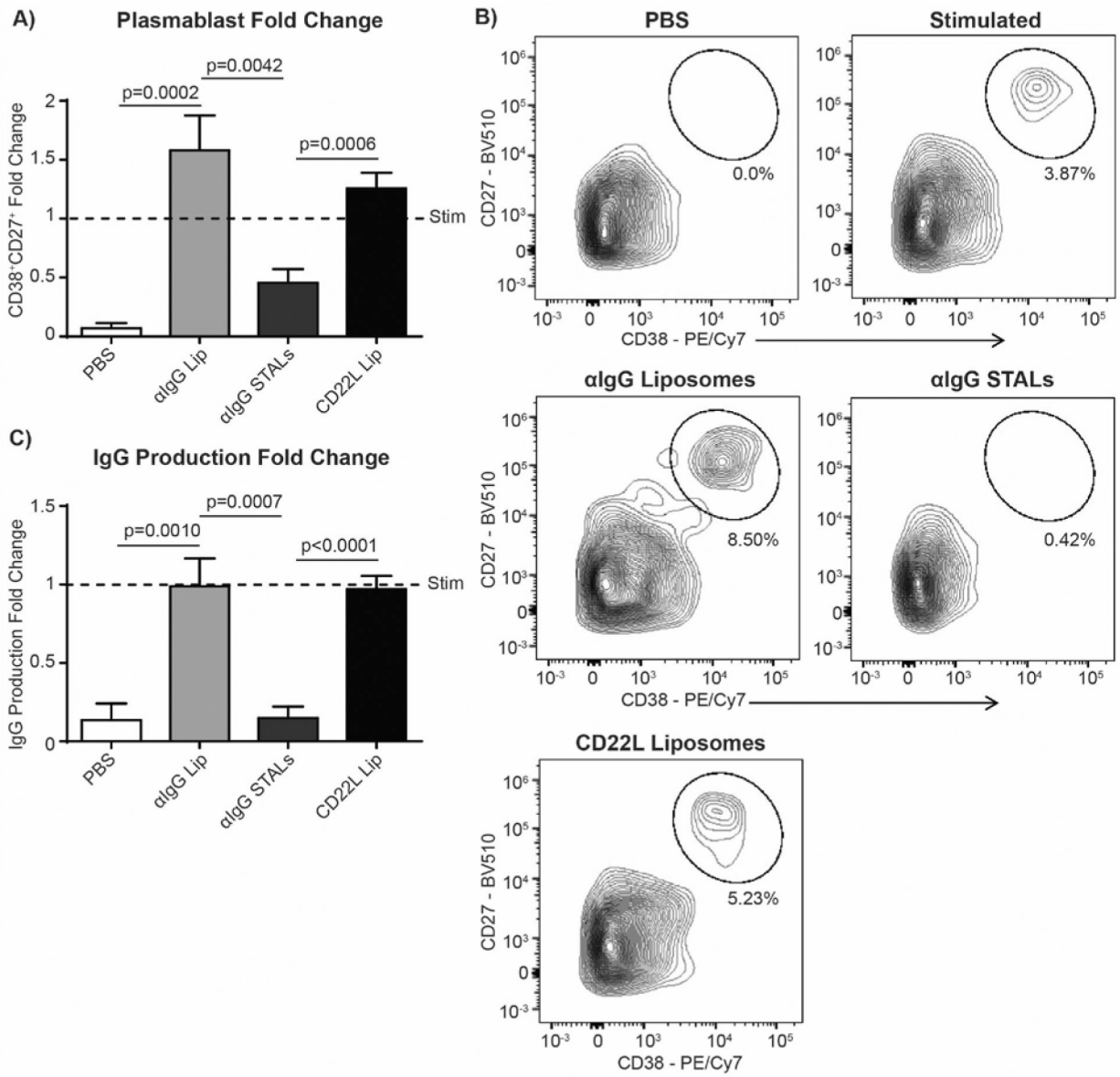

Figure 3.

Generation of plasmablasts is inhibited using anti-human IgG STALs. a) Human B-cells were isolated from healthy control PBMCs and plated at 1×10^5 stimulated under Stim #3 conditions for 24hr or PBS, followed by 7 days under one of the following conditions: unstimulated (PBS), anti-human IgG-liposomes (αIgG Lip), anti-human IgG+hCD22L (αIgG STALs), or CD22L liposomes alone (CD22L Lip) for the entirety of the study. After 7 days, each well was harvested for flow cytometry analysis of B-cell subsets (N=8 each an independent experiment, pooled data, one-way ANOVA with multiple comparisons). B) Representative plots are shown in b) and c) supernatant from each experiment was assessed for total IgG (N=8 each an independent experiment, pooled data, one-way ANOVA with multiple comparisons).

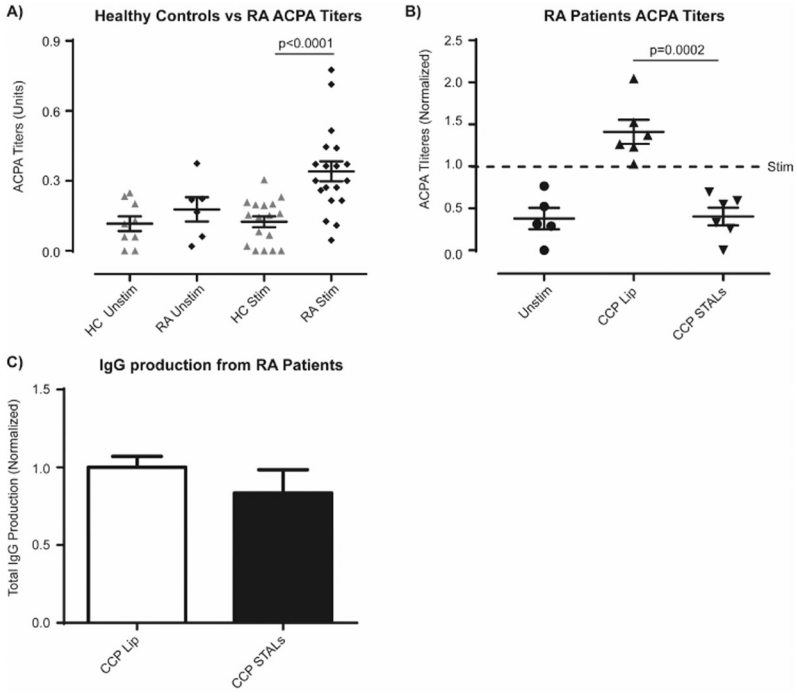

Figure 4.

CCP STALs prevent ACPA production from rheumatoid arthritis patients *in vitro.* a) Human B-cells were isolated from healthy control (N=4 each an independent experiment, pooled data, one-way ANOVA with multiple comparisons) or rheumatoid arthritis (N=4 each an independent experiment, pooled data, one-way ANOVA with multiple comparison) patients' PBMCs. RA patients were pre-screened for high titers of ACPA. B-cells were plated and either left unstimulated (PBS), or stimulated under standard stimulating conditions for 7 days, and ACPA titers in individual wells are shown. b) B-cells were isolated from RA patient PBMCs and left unstimulated or stimulated in accordance with the above conditions with CCP decorated-liposomes (CCP Lip) or CCP+hCD22L decorated liposomes (CCP STALs) for the entirety of the study. Supernatants from the wells were assessed for ACPA titers and normalized titers of individual patient are shown (N=6 each an independent experiment with an individual RA patient sample, unstim. N=5 (one patient did not have enough B-cells for all conditions), Stim. N=6, CCP Lip N=6, CCP STALs N=6, pooled data, one-way ANOVA with multiple comparisons). c) Total IgG titers in supernatants was measured (N=4 for CCP Lip and CCP STALs, each an independent experiment with an individual RA patient sample, pooled data, one-way ANOVA with multiple comparisons).

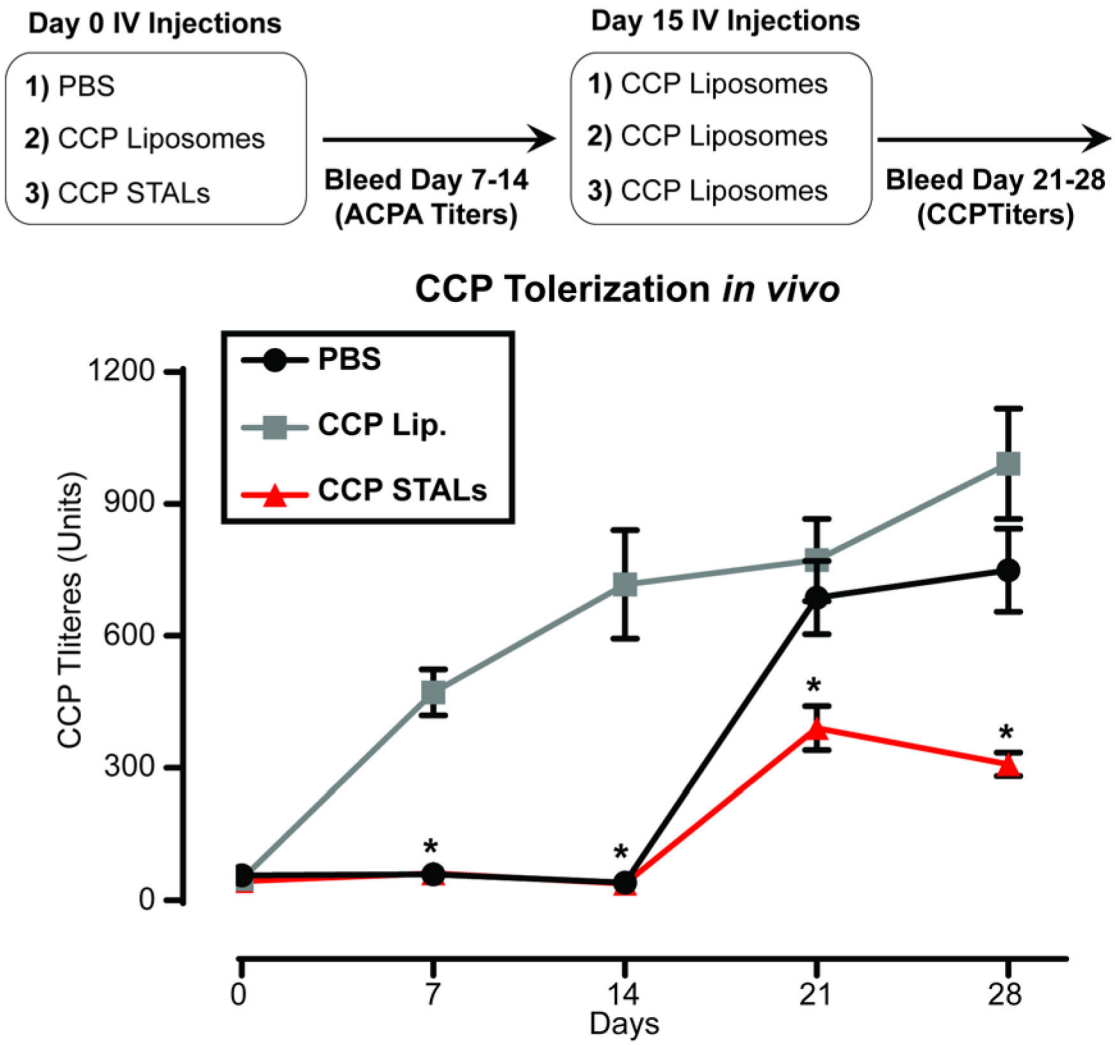

Figure 5.

ACPA production in SJL/J mice are tolerized upon STAL treatment *in vivo.* a) SJL/J mice were treated with indicated conditions on Day 0, followed by immunization on Day 14 with CCP liposomes containing MPLA. ACPA titers were measured once a week for 4 weeks (N=16 mice from 2 independent experiments, pooled data). Significance was measured between CCP Lip and CCP STALs at all time points (*p<0.0001, unpaired t-test) or between CCP STALs and PBS on days 21 and 28 (*p=<0.001, unpaired t-test).

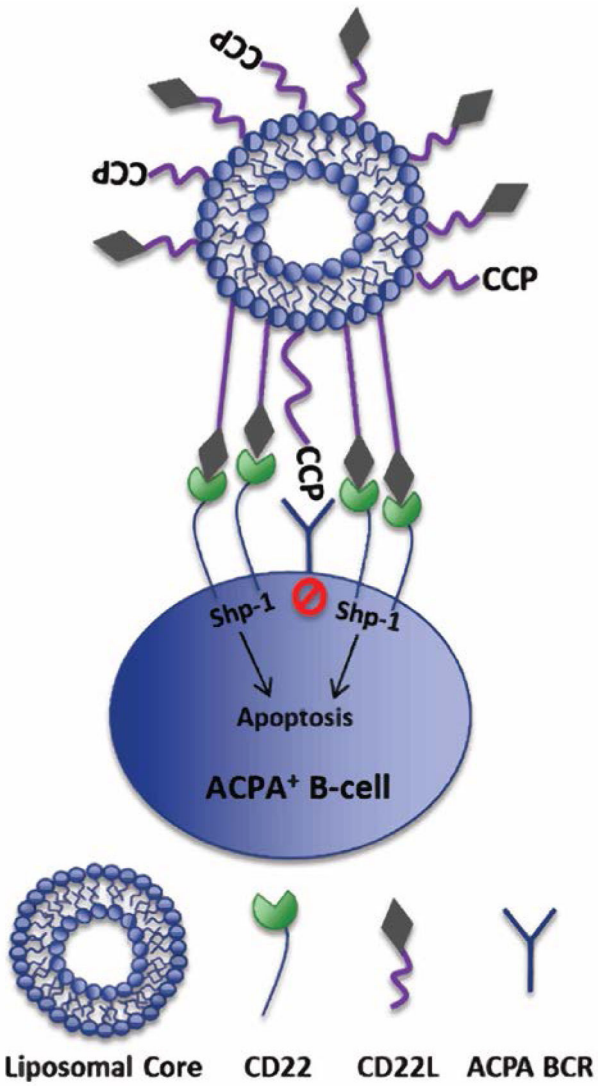

Figure 6.

Proposed mechanism of targeting citrulline-specific B-cells using a CCP-STAL. CCP on STAL targets citrulline protein-specific B cells by binding to citrulline-specific BCR. Human CD22 synthetic ligand (CD22L) on STAL binds CD22 on B cells, causing clustering of CD22 to BCR and SHP-1 recruitment in BCR signaling pathway. This clustering and SHP-1 recruitment causes antigen-specific B cell tolerization possibly through an apoptotic mechanism.