

Multi-therapeutic potential of autoantibodies induced by immune complexes trapped on follicular dendritic cells

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Abbreviations: Ab, antibody; Ag, Antigen; CR, complement receptor; CSR, class switch recombination; ECDII, subdomain II of the extracellular domain of rat neu Ag; Endo-S, endoglycosidase-S; FDC, follicular dendritic cells; GC, germinal center; GCR, germinal center reaction; IC, immune complex; LN, lymph nodes; IC₅₀, half maximal inhibitory concentration; SHM, somatic hypermutation; TI, T cell independent.

Induction of autoantibodies (autoAbs) targeting disease drivers/mediators is emerging as a potential immunotherapeutic strategy. Auto-immune complex (IC)-retaining follicular dendritic cells (FDCs) critically regulate pathogenic autoAb production in autoreactive germinal centers (GCs); however, their ability to induce potentially therapeutic autoAbs has not been explored. We hypothesized that deliberate display of clinically targeted antigens (Ags) in the form of ICs on FDC membranes induces target-specific autoreactive GCs and autoAbs that may be exploited therapeutically. To test our hypothesis, three therapeutically relevant Ags: TNF- α , HER2/neu and IgE, were investigated. Our results indicated that TNF- α -, HER2/neu- and IgE-specific autoAbs associated with strong GC reactions were induced by TNF- α -, HER2/neu- and IgE-IC retention on FDCs. Moreover, the induced anti-TNF- α autoAbs neutralized mouse and human TNF- α with half maximal inhibitory concentration (IC₅₀) of 7.1 and 1.6 nM respectively. In addition, we demonstrated that FDC-induced Ab production could be non-specifically inhibited by the IgG-specific Endo-S that accessed the light zones of GCs and interfered with FDC-IC retention. In conclusion, the ability of FDCs to productively present autoAgs raises the potential for a novel immunotherapeutic platform targeting mediators of autoimmune disorders, allergic diseases, and Ab responsive cancers.

Introduction

Follicular dendritic cells (FDCs) reside in the light zones of germinal centers (GCs) where they uniquely retain foreign and self antigens (Ags) in the form of immune complexes (ICs) via Fc and complement receptors for extended periods of time. Engagement of B cell receptors (BCRs) with FDC-retained Ags promotes B cell survival, induction of antibody (Ab) responses, class switch recombination (CSR), somatic hypermutation (SHM), affinity maturation, memory B cells generation, and regulation of serum Ig levels.^{1,2}

We recently showed that FDCs uniquely regulate the form of Ag and the co-signals that B cells encounter in GC reactions (GCRs). In contrast to Ag processing and peptide presentation to T cells, we discovered that FDCs multimerize monomeric Ags by trapping them in ICs via FDC-Fc γ RIIB and then presenting them polyvalently to BCRs with a characteristic periodic spacing

between epitopes of 200–500 Å.^{1–4} This allows simultaneous engagement of multiple BCRs and strong signaling to B cells. In addition to this primary signal, FDCs also provide co-signals including: B cell-activating factor belonging to the TNF family (BAFF), C4b-binding protein (C4bBP), IL-6 and CD21L, and these co-signals can be induced in activated FDCs.^{1,2} Such combination of primary and secondary signals induces tyrosine phosphorylation, B cell activation, proliferation, GC formation and Ag-specific IgM secretion in the absence of T cells and/or T cell factors. Nevertheless, T cells and/or IL-4 and IL-5 promoted IgM production and induced IgG class switching. Furthermore, we documented that a late antigenic signal presented by FDCs to B cells together with FDC-IL-6 were critical for SHM and affinity maturation.^{2,3}

Breach of tolerance is characterized by the display of self-Ag-bearing FDCs in autoreactive GCs producing high affinity

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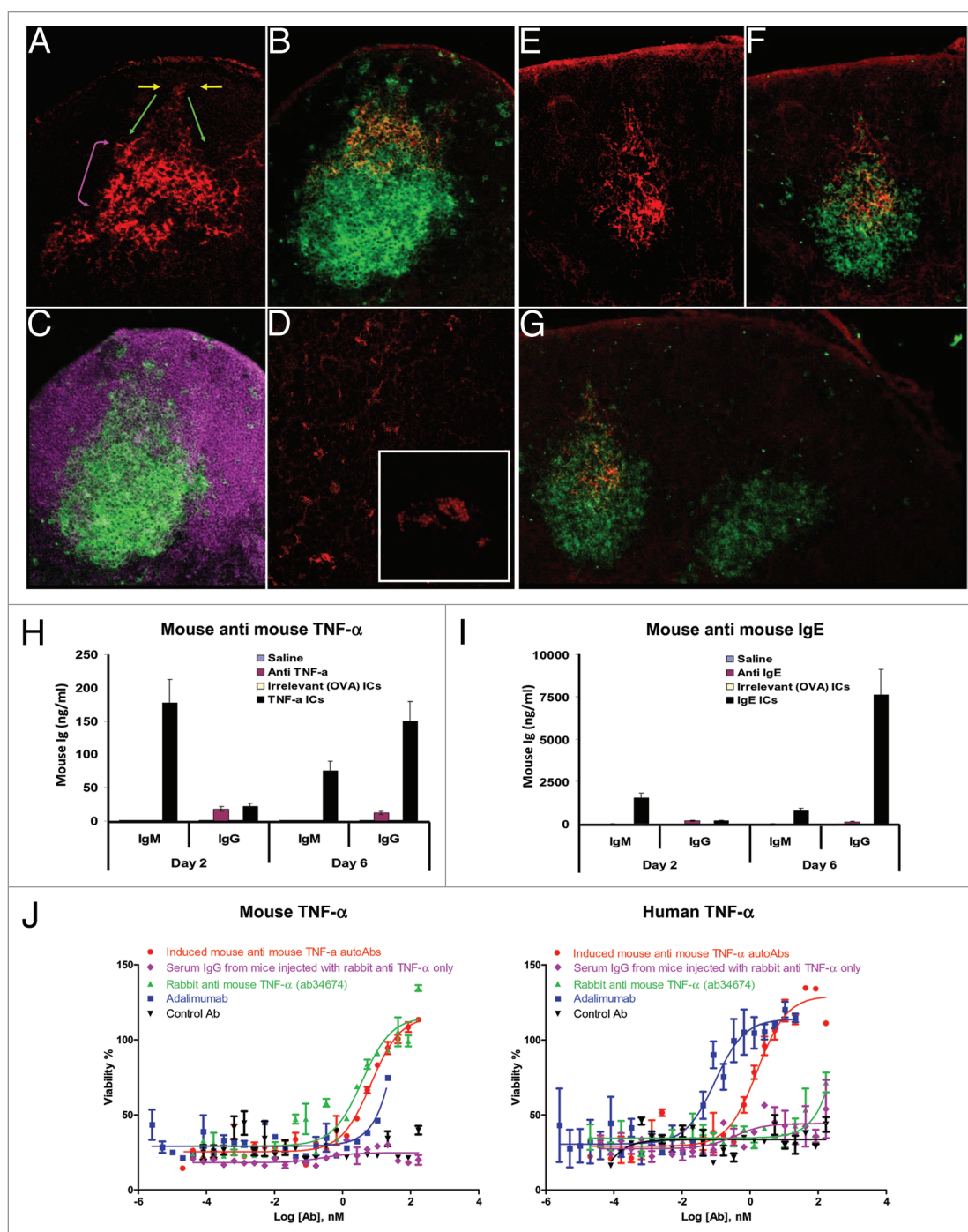


Figure 1. For figure legend see page 2436.

pathogenic autoAbs in secondary and tertiary lymphoid tissues^{5,6} and self-Ag withdrawal inhibits autoAb production.⁵ Currently, breakage of tolerance to endogenous disease mediators is emerging as a potential novel immunotherapeutic strategy;⁷ however, neither the ability of FDCs to induce self-generated autoAbs against therapeutic targets has been investigated nor has its translation into effective therapies been exploited. Thus, we sought to establish: (1) the ability of FDC-trapped self-ICs to induce autoAbs against therapeutic targets, (2) the capacity to inhibit FDC-IC trapping.

Three Ags currently targeted by monoclonal antibody (mAb) therapy; tumor necrosis factor- α (TNF- α), IgE, and the neu Ag; were tested to assess the ability of FDC-trapped ICs to induce therapeutically desirable autoAbs. ICs of autologous TNF- α or IgE, were injected into normal mice, and the rat neu-Tg FVBN202 mice were challenged with ICs of the subdomain II of the extracellular domain of rat neu antigen (ECDII)^{8,9} that is currently targeted for vaccination in human breast cancer.¹⁰ In each of these three conditions, the ICs were trapped by FDCs with strong induction

Figure 1 (See previous page). AutoAg (-TNF- α in **A–D, H** and -IgE in panels **E–G** and **I**) retention on FDCs induces autoreactive GCs, plasmablasts, and autoAbs. BALB/c mice were passively immunized with 1 mg rabbit anti-mouse-TNF- α or rabbit anti-mouse IgE (kind gift of Professor Daniel Conrad, Virginia Commonwealth University). Twenty four hrs later, one group was challenged s.c. in the back of the neck with a total of 4 μ g/mouse mouse-TNF- α and the other with 100 μ g/mouse mouse-IgE. Control groups challenged with OVA, rabbit anti-OVA, OVA ICs, or saline were included. Two days later mice were bled and 6 d later they were sacrificed and sera and draining LNs were collected. (**A and E**): FDC-reticula in sections of draining LNs were defined by retained auto-ICs labeled with rhodamine conjugated anti-rabbit IgG that binds the rabbit IgG in the FDC-ICs (red) (A: A magenta bracket identifies an FDC-reticulum). This labeling included Ag transport funnels that extended from the sub-capsular sinus into the FDC reticulum in the LN cortex (A: yellow and green arrows). (**B and F**): Autoreactive GCs (FITC-conjugated GL-7⁺ labeled, green) were associated with the IC-bearing FDC reticula (red). Activated B cells (green) engaging FDC-ICs can be seen in orange in both of these overlays in the GC light zones. (**C**): In this overlay, the B220^{hi} mantle of resting B cells (anti-B220 was Cy5.5-conjugated, which yields the pseudo color magenta) surrounds the GL7⁺ green/B220^{dim} GC B cells. (**D**): GC associated plasmablasts were specifically labeled with B lymphocyte-induced maturation protein-1 (Blimp-1). Blimp-1 is a transcription factor and the nuclear labeling is shown at higher magnification in the inset. (**G**): A pre-existing GC, not associated with self-IgE trapping FDC reticulum (right), is shown adjacent to an autoreactive GC associated with IgE-IC-retaining reticulum (left). The lack of a self-IgE-retaining FDC reticulum in the pre-existing GC was confirmed by sectioning through the GC. In addition to the lack of ICs on FDCs, cutting through the whole GC in serial sections did not show indications of an Ag transport site or funnel. These temporary structures can be seen as early as 1 min⁶⁸ and stay for as long as a week as we have previously reported³ and seen in **Figure 3A**. (**H and I**): Measurement of IgM and IgG autoAb responses against TNF- α and IgE. BALB/c mice were challenged with murine TNF- α -ICs (**H**) or murine IgE-ICs (**I**). TNF- α -, and IgE-specific IgM and IgG in the mice sera were measured on 96-well plates coated with 10 μ g/ml of the respective Ag. AutoAbs in the sera against TNF- α and IgE were apparent within 48 h and these autoimmune responses were consistent with FDC-induced T cell independent responses to conventional Ags.³ Note that by day 6, IgG autoAbs were detectable and that the IgM levels had declined. No TNF- α or IgE-specific autoAbs were detectable in controls challenged with OVA ICs or saline. Other controls included injection of the same level of free TNF- α , IgE or irrelevant Ag that induced no anti-TNF- α or -IgE Abs. (**J**): Neutralization of the cytotoxic activity of TNF- α by the induced anti-TNF- α autoAbs. IgG was purified on protein A columns from sera collected at day 6 and concentrated in Centricon centrifugal filters. Mouse fibroblast cell line L-929 (1.5×10^4) in 100 μ l DMEM supplemented with 10% FCS were incubated for 18 h then the medium was removed. Hundred μ l of complete DMEM with 1 μ g/ml Actinomycin D, mouse TNF α (0.1 ng/ml) or human TNF α (0.2 ng/ml), and the antibody of interest, were added to the cell monolayer and incubated 24 h at 37°C in a CO₂ incubator. The starting concentration of the used Abs was 170 nM except for Adalimumab that started at 21.25 nM. An amount of 10 μ l of MTT solution (5 mg/ml in PBS) was added to the wells and incubated for 3 h at 37°C. The medium was then removed and the cells were resuspended in 100 μ l 90% isopropanol 10% DMSO for 15 min, and the optical absorption was measured at 560 nm with a TECAN GENios plate reader. The half maximal Inhibitory Concentration (IC₅₀) was calculated using GraphPad Prism. The induced anti-TNF- α autoAbs neutralized human and murine TNF- α and inhibited their cytotoxic effect on L-929 cells with half maximal Inhibitory Concentrations (IC₅₀) of 7.1 and 1.6 nM respectively. These IC₅₀ values corresponded to 50% neutralizing activity of a rabbit polyclonal anti-mouse TNF- α (IC₅₀ 3.7 nM) (Abcam, ab34674) on mouse TNF- α ; and 5% of Adalimumab (IC₅₀ 0.08 nM) on human TNF- α . Data shown are expressed as mean $\pm$ SEM.

of GCs and autoAb responses. In marked contrast, free Ag that would have unfettered access to BCRs did not load on FDCs and did not induce detectable autoAbs.

FDC-IC de-trapping is critical for inhibition of FDC-dependent autoAb induction when it overshoots the desired therapeutic effect. Endo-S inhibits IgG/FcR interaction and IgG-dependent complement fixation;¹¹ consequently, we reasoned that it could interfere with Ag retention on FDCs and thus inhibit FDC-Ag-induced Ab production. Our results indicated that Endo-S non-specifically inhibits Ag retention on FDCs, as non-selectively it accessed the light zones of GCs, and interfered with the immune response to foreign Ags.

In summary, the unique ability of FDCs to present deliberately loaded autoAg, and induce potentially therapeutic autoAbs provides a novel platform targeting endogenous mediators of chronic diseases where passive administration of mAbs has become integral part of the therapeutic armamentarium. However, the prohibitive cost of mAbs, the development of neutralising Abs (even when fully human) and the inconvenience of frequent injections all contribute to the persistence of a major unmet need that requires the development of alternative approaches. The work we present here represents a stepping-stone toward achieving such a goal.

Results

Deliberate retention of TNF- α and IgE on FDCs induces autoreactive GCs and TNF- α - and IgE-specific autoAbs

FDCs are critically involved in the induction of protective and self reactive Abs in the GCRs.^{1,5} Here, we sought to evaluate the ability of FDCs to induce therapeutically relevant autoAbs. TNF- α and IgE are targeted by at least 6 mAbs¹² and we reasoned that retention of TNF- α or IgE on FDCs would induce self-generated anti-TNF- α or -IgE Abs which potentially eliminates the need for repeated administration of exogenous mAbs.

As hypothesized, the draining lymph nodes (LNs) from TNF- α or IgE IC-challenged mice exhibited GL7⁺ B220^{dim} autoreactive GCs and plasmablasts associated with autoAg-retaining FDC reticula (**Fig. 1A–G**). Mouse IgE and TNF- α -specific IgM autoAbs were detectable by 48 h in the sera of these mice. By day 6, IgG autoAbs were detectable and the IgM levels declined. No autoAb production was detected in the controls (**Fig. 1H and I**).

Minimal cross reactivity with the rabbit Abs was observed in our system possibly due to the rapid clearance of ICs and heterologous Igs by the reticulo-endothelial system. In mice, 80% of rabbit IgG oligomers were located within the liver after 1 h,¹³ less than 3% of rabbit IgG in ICs could be seen in the serum 4 d after IC injection;¹⁴ and around 2% remained in the circulation 3 d after injection of 1 mg native rabbit IgG.¹⁵ No mouse IgG was detectable at day 2 as the recorded levels are not significantly different from the background levels.

It has been previously reported that induction of autoAbs by self immunization approaches may not be associated with functional activity of the induced Abs.¹⁶ Accordingly, we sought to demonstrate the functional capacity of the induced

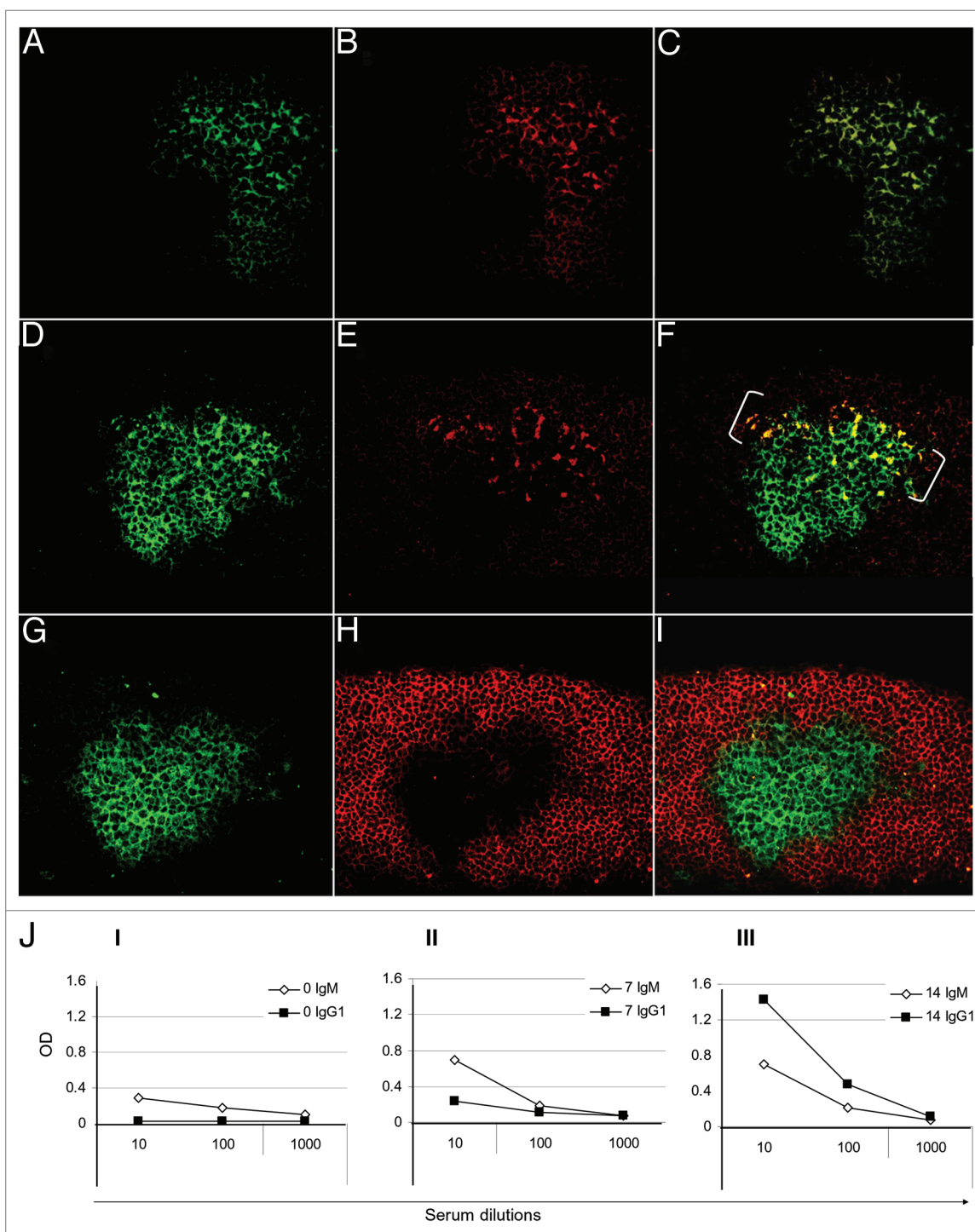


Figure 2. For figure legend see page 2438.

autoAbs in a biological system in order to strengthen the rationale for therapeutic potentials.

Human and mouse TNF- α show approximately 79% homology at the amino acid level, and cross reactivity between the two species has been previously reported.¹⁷ As shown in Figure 1J, the induced anti-TNF- α autoAbs neutralized human and murine TNF- α and inhibited their cytotoxic effect on L-929 cells. In contrast, IgG purified from control mice

injected with rabbit Ab alone displayed no TNF- α neutralization activity, thus, ruling out contribution of the rabbit IgG to TNF- α neutralization and confirming that the TNF- α neutralizing activity displayed by IgG purified from TNF- α IC-injected mice is mediated by the induced mouse anti TNF- α autoAbs.

Despite the documented ability of ICs to bind FDCs in pre-existing GCs¹⁸ and the capacity of the same FDC to trap more than one Ag,¹⁹ IgE ICs were not deposited in all GCs labeled

Figure 2 (See previous page). Retention of the subdomain II of the rat *neu* Ag (ECDII) on FDCs via autologous IgG induces ECDII-specific autoAbs and rIL4 enhances CSR. FVBN202 mice were challenged s.c. with FITC-ECDII complexed with mouse anti-FITC and mouse anti-ECDII Abs (50 µg FITC-ECDII + 25 µl ECDII anti-serum for 1 hr, and then 50 µg mouse anti-FITC Ab for 1 h); then injected on day 7 and 10 with 1 µg rIL-4. Draining LNs were collected and sera were assessed for ECDII-specific Abs. Anti-ECDII sera derived from the ECDII-sensitized FVB mice were used as positive controls for the detection of neu-specific IgM and IgG (data not shown). Cloning and expression of ECDII is detailed in reference 9 (**A–C**): (**A**), represents FITC-ECDII (green) trapped on an FDC reticulum in a draining LN section; (**B**), represents mouse IgG in ICs on an FDC reticulum labeled with rhodamine-conjugated anti-mouse IgG (red), and (**C**) (orange/yellow) represents an overlay of Ag and Ab in the ICs. (**D–F**): (**D**) (green), illustrates activated GC B cells labeled with FITC-conjugated GL7 and FITC-ECDII-labeled FDCs. (**E**) (red), represents rhodamine-conjugated anti-mouse IgG labeling ICs on FDCs; (**F**), represents the co-localization of FITC-conjugated ECDII with rhodamine-IgG giving orange/yellow in the GC light zone located above the GL7⁺ GC B cell rich dark zone (green). The white brackets indicate the extent of the GC light zone. (**G–I**): (**G**) (green) illustrates a GL7⁺ GC. (**H**) (red) illustrates the mantle of IgD⁺ resting B cells labeled with PE-conjugated anti-IgD; (**I**) is the overlay showing the GC surrounded with the resting B cell mantle. (**J**): illustrates measurement of ECDII-specific autoAbs: (**Ji**) illustrates the background levels of anti-ECDII IgM and IgG in sera collected prior to immunization. Within a week of ECDII-IC challenge, FVBN202 mice developed IgM and IgG anti-ECDII responses above background (**Jii**). IL-4 was administered at day 7 and 10 to provide a T cell cytokine to enhance class switching and augment the IgG response at day 14 (**Jiii**).

in **Figure 1G**. Pre-existing GCs can also be colonized by heterologous B cells in a cognate T cell independent fashion;^{20–22} however, it is also true that Ag does not localize in every pre-existing GC and B cells do not home to every vacant follicle in a draining LN. So far, the factors regulating IC deposition, B cell re-entry, and re-utilization of vacant FDC networks in pre-existing GCs²³ have not been fully elucidated.

ECDII subdomain-ICs made with mouse Abs induced ECDII-specific Ig responses

We used rabbit IgG to form ICs in TNF- α , and IgE challenged mice, and the possibility that foreign rabbit IgG served as an adjuvant required for induction of autoAbs could not be ruled out. Therefore, in this experiment (**Fig. 2**) we used ICs constructed with FITC-labeled ECDII complexed with mouse anti-FITC IgG and mouse anti-ECDII raised in wild-type FVB which, in contrast to the FVBN202-Tg mice, are not tolerant to the rat *neu* protooncogen.^{8,9} As predicted, FVBN202-Tg mice challenged with ECDII-ICs, made with murine Abs, developed GCs and ECDII-specific IgM and IgG within a week, when IL-4 was used to enhance IgG secretion (**Fig. 2A–J**).

Although the response to ECDII has not been followed in the absence of IL-4, we reason that CSR occurs without exogenous IL-4. In fact anti-TNF- α and anti-IgE autoAbs switched to IgG by day 6 without cytokine administration (**Fig. 1**) and IgG was detectable in the sera before IL-4 administration (**Fig. 2Jii**). FVBN202 mice are T cell competent and Ig class switching is regulated by T cells and/or T cell factors in TI responses.²⁴ Consequently, we reason that although cognate autoreactive T cell help is lacking, endogenous T cell cytokines can induce class switching in B cells stimulated with Ag-bearing FDCs.

Endo-S non-selectively accesses the light zones of GCs and inhibits Ab production in vivo

Control of FDC-induced autoAb levels is crucial for their potential therapeutic applications. Endo-S inhibits IgG/Fc γ R interaction and the IgG ability to activate complement¹¹; at the same time, FDCs retain ICs via Fc and complement receptors; thus, we reasoned that Endo-S may be able to interfere with IC retention on FDCs. Endo-S has a MW of 106 kDa and its transport in the B cell follicles is regulated by the structural transport pathways in the LN cortex.²⁵ Consequently, we sought to determine first if Endo-S can access the FDC reticulum and localize with the FDC-ICs in vivo. Within one hr of subcutaneous

(s.c.) injection, Endo-S attained high levels in the draining LNs of mice previously (6 d) challenged with ovalbumin (OVA)-ICs in the same place. Endo-S was seen in the IgD resting mantle and co-localized with OVA and LCA-labeled IgG glycans in the FDC networks (**Fig. 3A and B**). Moreover, Endo-S administration 7 d after challenging mice with OVA-ICs dramatically impaired CSR and significantly inhibited OVA-specific IgG levels at day 21 compared with control untreated mice (**Fig. 3C**).

Endo-S non-specifically de-traps FDC ICs

Endo-S significantly inhibited FDC-induced Abs and we reasoned that interference with FDC-IC retention mediated this inhibition. Rhodamine-conjugated OVA was loaded on murine FDCs in vivo and in vitro using OVA-specific IgG. FDC-trapped rhodamine-OVA ICs co-localized with FDC-CR1/2 (**Fig. 4A**). Treatment of sections from the draining LNs of rhodamine-OVA challenged mice groups with Endo-S significantly decreased OVA, anti-OVA, and C3 labeling in the CR1/2⁺ FDC reticula (**Fig. 4B**). FDCs trap ICs via Fc and CRs, and Endo-S inhibits IgG/FcR interaction thus detrapping ICs. With shaking, the de-trapped ICs are washed out and significantly weaker signals could be detected from the IC triad; rhodamine-OVA, rabbit anti-OVA and C3. Furthermore, purified murine FDCs loaded with rhodamine OVA-ICs in vitro lost rhodamine labeling upon treatment with Endo-S compared with untreated FDCs (**Fig. 4C**).

Discussion

Data in the present study strongly suggests that deliberate retention of therapeutically targeted Ags on FDCs induces target-specific Abs. When appropriate FDC-ICs were presented in vivo, specific autoAb responses were induced in mice previously unresponsive to self TNF- α , IgE, and ECDII. Moreover, these autoAb responses were associated with autoreactive GCs and plasmablasts in the draining LNs; and the induced anti-TNF- α autoAbs neutralized mouse and human TNF- α and inhibited their cytotoxic effect. In addition, Endo-S inhibited FDC-IC retention; co-localized with FDCs in the light zone of GCs; and non-specifically inhibited FDC-IC-induced Ab production. These results provide a novel FDC-dependent immunotherapeutic platform targeting endogenous mediators of chronic inflammatory, autoimmune, hypersensitivity, and mAb-responsive neoplastic disorders.

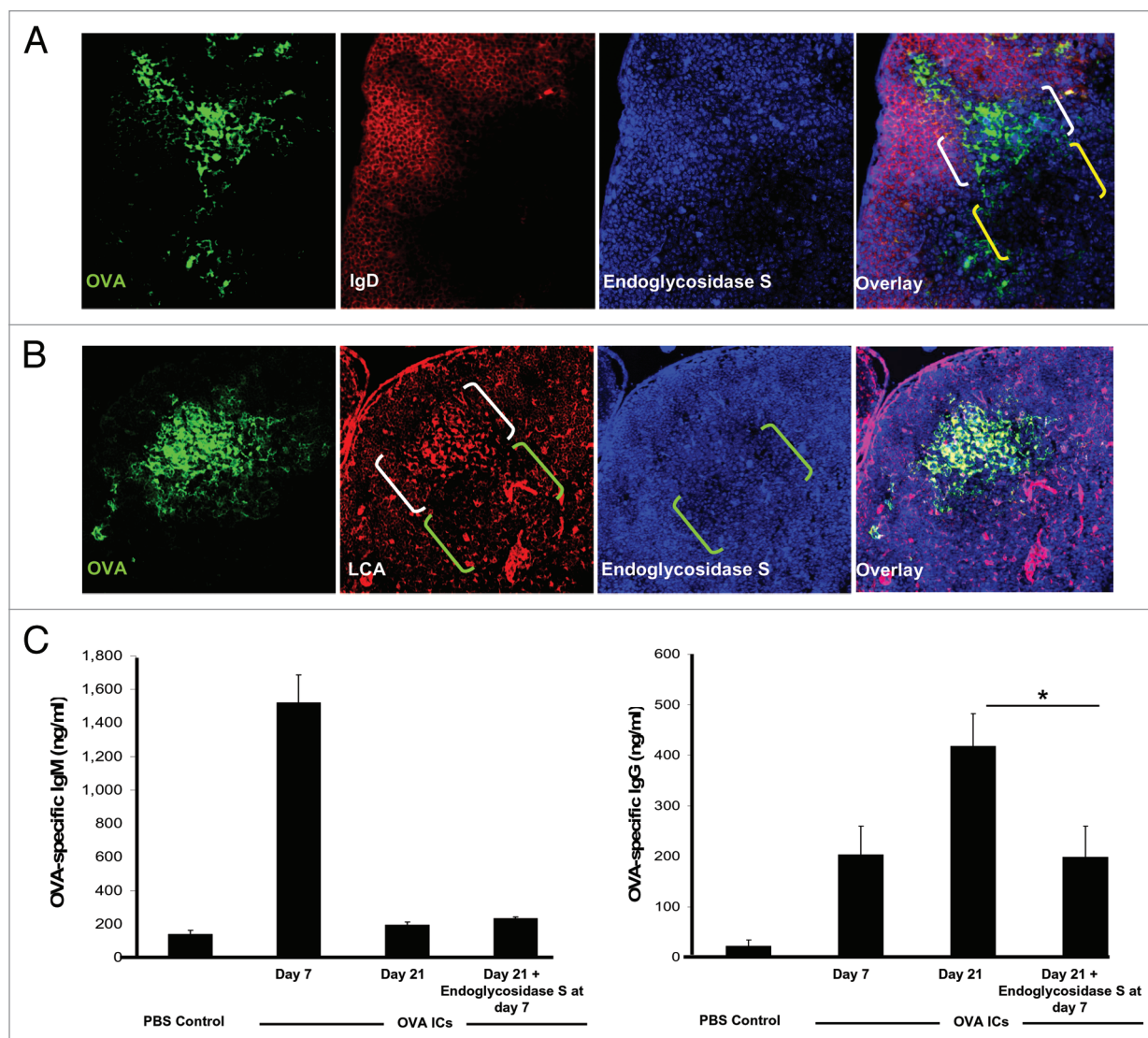


Figure 3. Endo-S targets the GCRs in vivo and significantly inhibits Ag-specific IgG production. The in vivo effect of Endo-S was assessed in a group of OVA-IC challenged mice that were left for 21 d interrupted by 1000 U/mouse Endo-S s.c. injection in the back of the neck on day 7. One hr after injection, a group of mice was sacrificed and Endo-S was labeled in the B cell follicles of the draining LNs, whereas, sera were obtained from the rest of the mice at day 7 and 21 and assessed for OVA-specific IgM and IgG by ELISA. p-values were calculated using unpaired two-tailed Student's t tests, and error bars represent the SEM between samples. Significance was accepted at $\alpha < 0.05$. (A): OVA (green, labeled with FITC-anti-OVA) in the Ag transport funnel is tracking through the resting IgD⁺ B cell mantle (red) from the sub-capsular sinus to the FDC reticulum in the LN cortex. Endo-S (blue, labeled with anti-histidine mAb targeting the histidine tag in the N-terminal of the recombinant enzyme) can be co-localized with the OVA on FDCs in the light zone of the GC [white brackets in the overlay]. Less Endo-S signal is seen deeper (yellow brackets in the overlay, site of the dark zone of the GC) at that time point. (B): OVA (green) on FDCs is co-localizing with Endo-S (blue) and a strong lens culinaris agglutinin (LCA, red) signal (white brackets) labeling the IgG glycans in the FDC-ICs in the GC light zone. The LCA and Endo-S signals are reduced deeper (green brackets, site of the GC dark zone). (C): At day 7; OVA-specific IgM dominated the immune response. Due to CSR, OVA-specific IgG exceeded IgM at day 21. Compared with non-injected mice groups at day 21; Endo-S injected mice at day 7 (day 21 + Endo-S at day 7) showed significantly less OVA-specific IgG (*P value < 0.05).

FDCs present Ags to B cells in a highly immunogenic form and membrane-bound Ags seem to be the predominant form of Ag that mediates B cell activation in vivo.²⁶ In contrast to low valency self-Ags such as small soluble proteins; periodically displayed multivalent Ags such as multimerized FDC-bound Ags; extensively cross-link BCRs, maintain downstream signaling, and induce B cell activation and Ab production.²⁷ Moreover, the immunogenicity of FDC-ICs is protected by the high levels of FDC-FcγRIIB that minimizes serious inhibition of B cell

activation upon BCR/FcγRIIB cross linking.^{3,28} Not only do FDCs present Ags in ICs in a multivalent pattern, but also they provide complement-mediated B cell co-stimulation which decreases the B cell activation threshold and can overcome B cell anergy.²⁹

More than 20 mAbs are currently approved for human therapy; however, the high manufacturing cost, generation of anti-mAb antibodies, and the inconvenience of frequent injections are major challenges in mAb therapy. We reason that

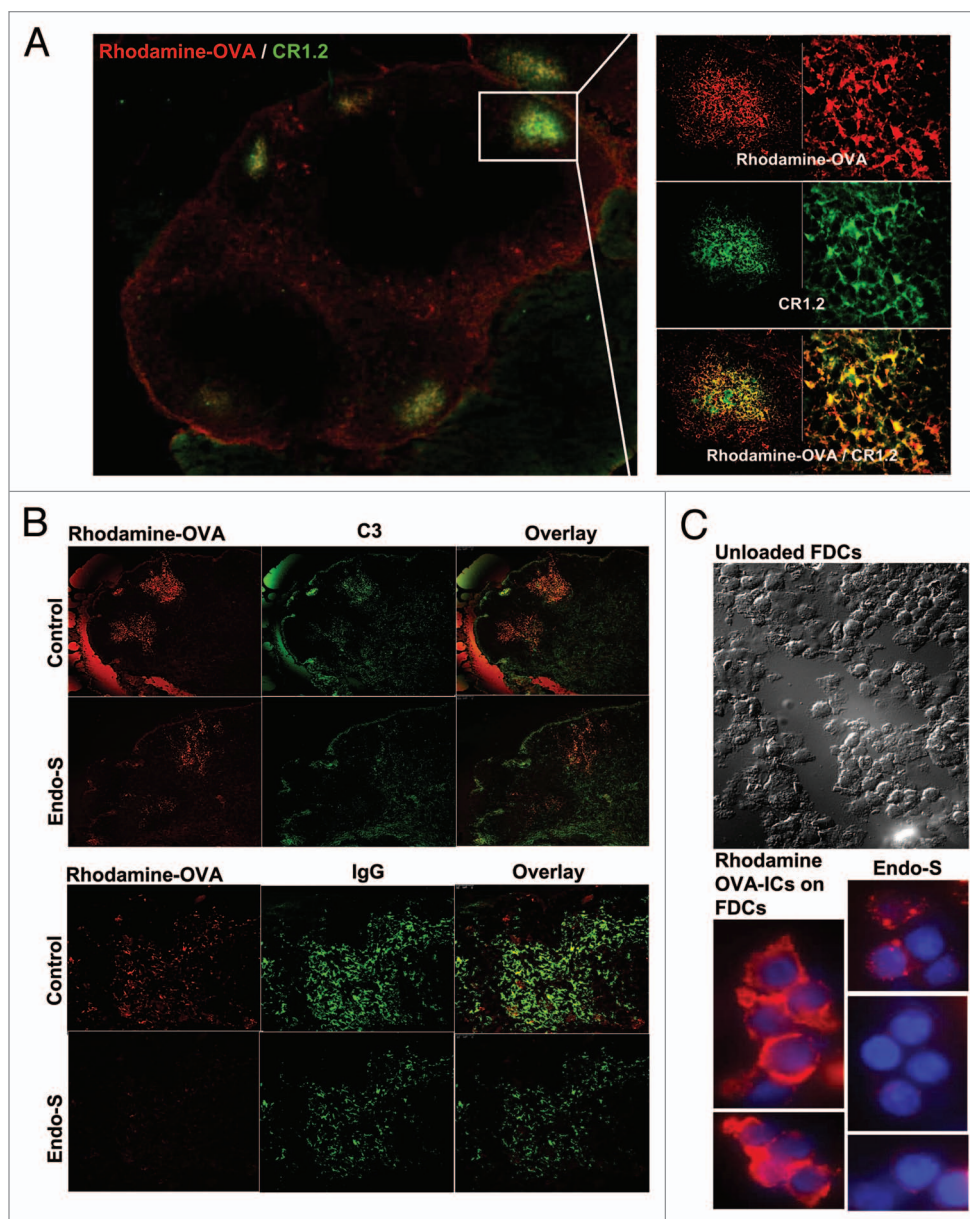


Figure 4. Endo-S unloads FDC-trapped ICs. OVA was conjugated to rhodamine, then rhodamine-conjugated OVA was loaded on murine FDCs in vivo and in vitro using OVA-specific IgG. For in situ assessment, 10 μ m sections from the draining LNs were prepared 7 d after OVA-IC challenge and treated with 20 U Endo-S at 37°C for 1 h on a shaker, then rhodamine-OVA, rabbit-anti-OVA, and mouse C3 were compared with untreated adjacent sections using confocal microscopy. For the effect of Endo-S on purified FDCs, murine FDCs loaded with rhodamine-OVA ICs were treated with 10 U Endo-S for 1 h at 37°C with shaking, and examined by confocal microscope. **(A):** Rhodamine-OVA was trapped in multiple FDC reticula in the draining LNs in the presence of rabbit anti-OVA IgG. The orange color indicates co-localization of rhodamine-OVA (red) with FDC-CR1/2 (green). A single FDC reticulum is shown at high magnification where the reticular nature of FDCs can be seen. **(B):** Endo-S-treated LN sections show less rhodamine-OVA (red), rabbit anti-OVA (green, labeled with goat anti-rabbit IgG FITC), and mouse C3 (green, labeled with FITC-conjugated goat anti-mouse C3) than untreated controls. **(C):** Murine FDCs were isolated as we have previously described,⁴ and the characteristic dendritic morphology and clustering nature of purified unloaded FDCs was visualized by differential interference contrast (DIC, Nomarski) optics. Compared with untreated rhodamine-OVA-IC-loaded FDC controls, Endo-S-treated FDCs labels less for rhodamine-OVA (red). DAPI stains the FDC nuclei (blue).

deliberate induction of autoAbs to proteins currently targeted by mAb therapy has the potential to overcome these limitations. Our reasoning takes advantage from the partial tolerance in the B cell repertoire manifested in several diseases by the development of autoAbs including cytokine-specific autoAbs.^{30,31} FDCs uniquely multimerize Ags,^{1,2,4} and presentation of self/neo proteins in

repetitive arrays induces autoAbs to neo-Ags in transgenic mice,³² as well as to cytokines in humans⁷ and immune-tolerant animal models.³³

Single domain proteins and peptides are normally poorly immunogenic but, in our study, we were impressed by the ability of FDC-loaded ECDII (145 aa) to induce Abs in unresponsive

mice. Tailored induction of autoAbs to selected antigenic sub-domains may be critical to avoid unwanted responses. For example, the Cε3 fragment of IgE can be targeted instead of the whole IgE molecule. Cε3 can specifically bind the α chain of FcεRI, and blocking this binding can inhibit IgE-mediated release of inflammatory mediators. The Cε3 fragment is masked upon binding of IgE to FcεRI, thus Cε3-specific Abs are unable to bind cell-bound IgE and would be non-anaphylactogenic.³⁴ In addition, T cell defects, and inhibition of certain anti-tumor immune responses⁸ are major challenges in cancer immunotherapy. In the FVBN202 mouse mammary carcinoma (MMC) model, the T cell response induces loss of the target neu Ag in an IFN-γ-dependent fashion leading to emergence of MMC variants refractory to HER-2/neu-targeted immunotherapy and predisposes to tumor relapse.⁸ Induction of Abs by FDC-ICs is TI,¹⁻³ therefore it bypasses the requirements of cognate T cell help and uniquely maintains the sensitivity of cancers to targeted immunotherapy thus minimizing recurrence.⁸

FDCs retain Ags via Ag-specific Abs and complement for years,³⁵ and therapeutic unloading of FDC-retained Ags has not been successful before. Endo-S specifically acts on the conserved N-linked glycan of IgG thus compromising IgG recognition by FcRs and the IgG ability to activate complement. Consequently, Endo-S interferes with the effector functions of autoAbs and ameliorates several autoimmune conditions.¹¹ Ag retention on FDCs is critically involved in Ig CSR, SHM, and provides a constant Ag depot for memory B cell re-stimulation and effective long-term serological memory.^{1,6,36-42} Although the contribution of long-lived memory and plasma cells is important, continuous antigenic stimulation by persisting antigen is critical for maintenance of Ig levels.⁴³ In fact B cell memory is short-lived in the absence of Ag, and interference with FDC-reticula attenuates autoreactive GC formation, reduces autoAb titers and memory B lymphocyte generation.^{5,6,36,37,44,45} Detrapping of FDC-Ag has not been previously studied, and results presented here provide evidence that Endo-S effectively unloads FDC-trapped Ags. The impact of Endo-S on CSR was evident within the 3 week assessment period (Fig. 3) and IgG levels were significantly inhibited in Endo-S-treated mice. Although the long-term effect of Endo-S has not been determined, we reason that Endo-S-mediated withdrawal of FDC Ags will inhibit recall responses, Ab affinity maturation, Ag-driven memory B cell activation and plasma cell differentiation leading to a sustained reduction of autoAb production. Endo-S may also deglycosylate IgG-BCR which may affect BCR signaling, and consequently FDC-B cell interaction in the GC and this is currently under investigation.

While our results indicate that Endo-S activity is not Ag specific and can debilitate protective immunity, they provide novel evidence that previously retained FDC-ICs can be de-trapped without structural disruption of FDC reticula. Subunit expression of Endo-S and tagging may provide selective targeting of specific Ab/Ag complexes. For example, molecular engineering of TNF-α-peptide- or Cε3-baited Endo-S subunits that dimerize and restore function only in the presences of anti-TNF-α or anti-IgE would provide selective activity against FDC-TNF-α-ICs or TNF-Cε3-ICs respectively.

FDCs trap a diverse array of Ab- and complement-opsonized molecules,² and induction of potentially therapeutic autoAbs by FDC-ICs would be unrestricted. Moreover, our results with the ECDII subdomain indicate that therapeutic targets can be loaded on FDCs in the presence of autologous IgG. Accordingly, Ags can be tagged so that they bind specific Abs that naturally exist in the host thus sidestepping the need to inject foreign Abs. For example, the average levels of anti-phosphorylcholine (PC) IgG in normal human subjects is 35 μg/ml,⁴⁶ and injection of PC-tagged Cε3, for example, would form PC-Cε3/anti-PC ICs that instantly loads on FDCs and induces anti Cε3 autoAb response. Success with anti-PC would provide us with an approach to easily load Ags of all kinds on FDCs in humans without introduction of foreign Igs. Furthermore, the hapten-conjugated self Ag may recruit non-cognate T cell help which could enhance CSR and affinity maturation. As previously shown,⁴⁷ Ag presenting cells will take up the hapten-conjugated protein and present the foreign hapten epitope sequences to T helper cells, which will become activated and provide help to B cells presenting the same hapten epitopes to the activated T cells. Due to immune tolerance, only T helper cells that recognize hapten peptides are expected to become activated whereas autoreactive T-cell responses will be limited or absent.⁴⁷

Despite the evidence that the induced autoAbs are functionally active, the longevity of the response has not been followed over extended periods of time. Nevertheless, our studies on athymic nude mice challenged with OVA-ICs indicated that OVA-specific IgM was present in the sera within 48 h and these IgM levels were maintained for a minimum of 2 mo.³ In fact there is a substantial evidence that TI B cell memory exists,^{48,49} and in the presence of non-cognate T cell help, and the provision of FDC-derived B cell co-stimulatory signals including C4bBP that engages B cell CD40, we think that TI memory will be generated and autoAb levels maintained thus minimizing the need for repeated vaccine administrations. Although the affinity of the induced Abs has not been directly measured, the anti-TNF-α autoAbs displayed 50% neutralizing activity of a comparable rabbit polyclonal anti-mouse TNF-α within 6 d. Due to induction of GCRs, we reason that the autoAb affinity will progressively increase with time, and this will improve the functional capacity of the induced Abs leading to effective disease control. Studies investigating maintenance and affinity maturation of FDC-IC-induced Ig secretion are currently in progress.

Given the excellent activity of the induced autoAbs in vitro, we plan to assess the in vivo efficacy and Endo-S-mediated regulation of the induced anti-TNF-α, -IgE, and -HER2/neu autoAbs in disease models of arthritis, asthma, and mammary cell carcinoma respectively. Due to its multi-mechanistic effect on Abs, FDC-ICs, and possibly IgG-BCR, Endo-S is a promising GCR regulator; however, its non-selective nature needs to be rectified before it can be exploited therapeutically.

Several approaches of auto-immunization have been developed over the last ten years making use of the incomplete tolerance in the B cell repertoire. Therapeutic vaccines comprising virus-like particles (VLPs) covalently conjugated with disease-associated self-molecules to induce neutralizing autoAb responses have been successfully tested in animals and humans without serious

adverse events.⁵⁰ Although multiple injections were required to maintain high Ab levels, the efficacy of the induced Abs by VLPs were higher compared with mAbs used to control similar disease conditions.^{31,51-53} Furthermore, the first clinical trials performed with the second generation of these vaccines have demonstrated that the induced autoAb responses are reversible with limited autoreactive T-cell activation.^{54,55} Owing to the natural difference in individuals' immune responses, patient-to-patient variability and poor responders were associated with the use of some VLP-based vaccines.⁵⁶ Induction of active immunotherapy depends on several factors in the host's immune constitution, and bypassing the MHC-TCR requirement in our FDC-IC-based approach may minimize such factors contributing to variability and poor responses.

Induction of target-specific Abs by auto-immunization does not necessarily imply functionality of the induced autoAbs. For example, HER2-specific Abs were induced by vaccinating breast cancer patients with a HER2 protein vaccine; however it could not induce receptor internalization and degradation.¹⁶ Moreover, anti-TNF- α autoAbs were induced against denatured but not native TNF- α which compromised their therapeutic utility.⁴⁷

Auto-immunization by self IC retention on FDCs uniquely integrates several strengths that boost its translational potential and future practical exploitation. First, the TI nature of the response provides safety against autoreactive T cell activation. Even with hapten-tagged targets, T cell responses will be directed to the foreign tag peptides while autoreactive T-cell responses will be limited or absent thus avoiding possible harmful auto-inflammatory responses.^{32,54} Being TI, this approach can benefit patients who; in addition to their primary target-mediated disease; suffer from concomitant T cell dysfunction, disorders affecting Ag presenting cells or limited MHC-II repertoires that may be unable to load certain peptides. In addition, the TI nature of the response makes it rapid and target-specific IgM can be induced within 48 h. Remarkably, sustained protection was observed in mice just 24 h after IC challenge that compared with responses 8 d after Ag alone.^{57,58} Besides its therapeutic potential, induction of TI autoAb secretion by self Ag retention on FDCs may help explain the presence of certain autoAbs in diseases where T cell tolerance is intact, or the MHC repertoire is non-permissive.

Second, the ability of Endo-S to inhibit Ag retention on FDCs offers a novel GC regulator. In addition to its ability to directly interfere with the effector functions of autoAbs,¹¹ this study provides the first evidence that Endo-S significantly reduces Ag retention on FDCs, which would inhibit B cell activation and plasma cell differentiation by the FDC-Ag depot.^{6,36-42} This would downregulate long-term Ab production, thus providing a termination mechanism for deliberately induced autoAbs against targets of clinical importance.

Third, the unique ability of FDCs to trap an extensive array of molecules and microbial fragments provides a

universal immunotherapeutic platform that can possibly target unlimited number of candidate self Ags. Added to the long-term nature of retention, preservation of the native structure of target Ags⁴ promotes induction of polyclonal autoAbs that bind several antigenic epitopes, and may experience cycles of affinity maturation in the GCR leading to enhanced autoAb efficacy. We reason that the polyclonal nature of the induced autoAbs conferred higher cross-reactivity between human and mouse TNF- α beyond that known for mAbs targeting TNF- α . Through binding to a range of epitopes, polyclonal Abs can sterically hinder the Ab-coated TNF- α from binding to its target in multiple species; and recent studies suggested that polyclonal Abs are faster and more efficient than mAbs in clearing TNF- α from patients sera.⁵⁹ In fact, the use of polyclonal Abs is currently expanding as a third generation Ab therapeutics to address the inability of mAbs to target mutation-prone Ags and molecules that require binding at multiple sites.⁶⁰⁻⁶⁴ The inherent high specificity of mAbs for a single epitope is a likely limiting factor, while, polyclonal Abs exhibit high tolerance toward Ag modification.⁶⁵ This is particularly important in anti-cancer therapy where mAbs lose their effectiveness against mutated cancer cells; whereas, polyclonal Abs would provide multi-targeted approach that minimizes the emergence of tumor cell escape variants.^{66,67}

In conclusion, this is the first potential FDC-IC-based immunotherapy where deliberate autoAg retention induces autoreactive GCRs and functionally active autoAbs against endogenous drivers/mediators of chronic inflammatory, autoimmune and hypersensitivity diseases as well as mAb-responsive cancers. A high affinity self-generated Ag-specific polyclonal response that is maintained for long periods may be more efficacious than the currently available therapeutic mAbs. FDC-retained self-Ags that induce autoreactive GCs may provide these features and induction of this response would only require injection of an appropriate IC. Though further work will be clearly required to translate our results into patients' treatments, the reliance on natural components of the immune system, the ease of formulation, and the cost-effective nature of this auto-immunization approach will enhance its translational potential as a novel multi-dimensional immunotherapeutic platform.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- El Shikh ME, Pitzalis C. Follicular dendritic cells in health and disease. *Front Immunol* 2012; 3:292; PMID:23049531; <http://dx.doi.org/10.3389/fimmu.2012.00292>
- El Shikh ME, El Sayed RM, Sukumar S, Szakal AK, Tew JG. Activation of B cells by antigens on follicular dendritic cells. *Trends Immunol* 2010; 31:205-11; PMID:20418164; <http://dx.doi.org/10.1016/j.it.2010.03.002>
- El Shikh ME, El Sayed RM, Szakal AK, Tew JG. T-independent antibody responses to T-dependent antigens: a novel follicular dendritic cell-dependent activity. *J Immunol* 2009; 182:3482-91; PMID:19265126; <http://dx.doi.org/10.4049/jimmunol.0802317>
- Sukumar S, El Shikh ME, Tew JG, Szakal AK. Ultrastructural study of highly enriched follicular dendritic cells reveals their morphology and the periodicity of immune complex binding. *Cell Tissue Res* 2008; 332:89-99; PMID:18236080; <http://dx.doi.org/10.1007/s00441-007-0566-4>
- Manzo A, Bombardieri M, Humby F, Pitzalis C. Secondary and ectopic lymphoid tissue responses in rheumatoid arthritis: from inflammation to autoimmunity and tissue damage/remodeling. *Immunol Rev* 2010; 233:267-85; PMID:20193005; <http://dx.doi.org/10.1111/j.0105-2896.2009.00861.x>
- Gray D. Immunological memory: a function of antigen persistence. *Trends Microbiol* 1993; 1:39-41, discussion 41-2; PMID:8044459; [http://dx.doi.org/10.1016/0966-842X\(93\)90026-N](http://dx.doi.org/10.1016/0966-842X(93)90026-N)
- Kopf M, Bachmann MF, Marsland BJ. Averting inflammation by targeting the cytokine environment. *Nat Rev Drug Discov* 2010; 9:703-18; PMID:20811382; <http://dx.doi.org/10.1038/nrd2805>
- Kmiecik M, Knutson KL, Dumur CI, Manjili MH. HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses. *Eur J Immunol* 2007; 37:675-85; PMID:17304628; <http://dx.doi.org/10.1002/eji.200636639>
- Morales JK, Kmiecik M, Graham L, Feldmesser M, Bear HD, Manjili MH. Adoptive transfer of HER2/neu-specific T cells expanded with alternating gamma chain cytokines mediate tumor regression when combined with the depletion of myeloid-derived suppressor cells. *Cancer Immunol Immunother* 2009; 58:941-53; PMID:18979098; <http://dx.doi.org/10.1007/s00262-008-0609-z>
- Baxevas CN, Voutsas IF, Gritzapis AD, Perez SA, Papamichail M. HER-2/neu as a target for cancer vaccines. *Immunotherapy* 2010; 2:213-26; PMID:20635929; <http://dx.doi.org/10.2217/imt.09.89>
- Allhorn M, Collin M. Sugar-free antibodies--the bacterial solution to autoimmunity? *Ann N Y Acad Sci* 2009; 1173:664-9; PMID:19758213; <http://dx.doi.org/10.1111/j.1749-6632.2009.04739.x>
- Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol* 2010; 10:301-16; PMID:20414204; <http://dx.doi.org/10.1038/nri2761>
- Finbloom DS, Plotz PH. Studies of reticuloendothelial function in the mouse with model immune complexes. I. Serum clearance and tissue uptake in normal C3H mice. *J Immunol* 1979; 123:1594-9; PMID:479594
- Bockow B, Mannik M. Clearance and tissue uptake of immune complexes in complement-depleted and control mice. *Immunology* 1981; 42:497-504; PMID:7016741
- McLean D, Cooke MJ, Wang Y, Green D, Fraser PE, George-Hyslop PS, et al. Anti-amyloid-beta-mediated positron emission tomography imaging in Alzheimer's disease mouse brains. *PLoS One* 2012; 7:e51958; PMID:23284831; <http://dx.doi.org/10.1371/journal.pone.0051958>
- Ren XR, Wei J, Lei G, Wang J, Lu J, Xia W, et al. Polyclonal HER2-specific antibodies induced by vaccination mediate receptor internalization and degradation in tumor cells. *Breast Cancer Res* 2012; 14:R89; PMID:22676470; <http://dx.doi.org/10.1186/bcr3204>
- Marmenout A, Fransen L, Tavernier J, Van der Heyden J, Tizard R, Kawashima E, et al. Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor. *Eur J Biochem* 1985; 152:515-22; PMID:3932069; <http://dx.doi.org/10.1111/j.1432-1033.1985.tb09226.x>
- Horie K, Chen D, Hoshi H. Development of immune complex trapping: experimental study of lymphoid follicles and germinal centers newly induced by exogenous stimulants in mouse popliteal lymph nodes. *Histol Histopathol* 1999; 14:11-21; PMID:9987645
- Radoux D, Heinen E, Kinet-Denoël C, Tihange E, Simar L. Precise localization of antigens on follicular dendritic cells. *Cell Tissue Res* 1984; 235:267-74; PMID:6705031; <http://dx.doi.org/10.1007/BF00217850>
- Schwickert TA, Lindquist RL, Shakhov G, Livshits G, Skokos D, Kosco-Vilbois MH, et al. In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* 2007; 446:83-7; PMID:17268470; <http://dx.doi.org/10.1038/nature05573>
- Schwickert TA, Alabyev B, Manser T, Nussenzweig MC. Germinal center reutilization by newly activated B cells. *J Exp Med* 2009; 206:2907-14; PMID:19934021; <http://dx.doi.org/10.1084/jem.20091225>
- Lycke NY, Bemark M. The role of Peyer's patches in synchronizing gut IgA responses. *Front Immunol* 2012; 3:329; PMID:23181060; <http://dx.doi.org/10.3389/fimmu.2012.00329>
- Or-Guil M, Wittenbrink N, Weiser AA, Schuchhardt J. Recirculation of germinal center B cells: a multilevel selection strategy for antibody maturation. *Immunol Rev* 2007; 216:130-41; PMID:17367339
- Vos Q, Lees A, Wu ZQ, Snapper CM, Mond JJ. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol Rev* 2000; 176:154-70; PMID:11043775; <http://dx.doi.org/10.1034/j.1600-065X.2000.00607.x>
- Gonzalez SF, Degen SE, Pitcher LA, Woodruff M, Heesters BA, Carroll MC. Trafficking of B cell antigen in lymph nodes. *Annu Rev Immunol* 2011; 29:215-33; PMID:21219172; <http://dx.doi.org/10.1146/annurev-immunol-031210-101255>
- Harwood NE, Batista FD. Early events in B cell activation. *Annu Rev Immunol* 2010; 28:185-210; PMID:20192804; <http://dx.doi.org/10.1146/annurev-immunol-030409-101216>
- Hinton HJ, Jegerlehner A, Bachmann MF. Pattern recognition by B cells: the role of antigen repetitiveness versus Toll-like receptors. *Curr Top Microbiol Immunol* 2008; 319:1-15; PMID:18080412; http://dx.doi.org/10.1007/978-3-540-73900-5_1
- El Shikh ME, El Sayed R, Szakal AK, Tew JG. Follicular dendritic cell (FDC)-FcgammaRIIB engagement via immune complexes induces the activated FDC phenotype associated with secondary follicle development. *Eur J Immunol* 2006; 36:2715-24; PMID:17013985; <http://dx.doi.org/10.1002/eji.200636122>
- Lyubchenko T, Dal Porto JM, Holers VM, Cambier JC. Cutting edge: Complement (C3d)-linked antigens break B cell anergy. *J Immunol* 2007; 179:2695-9; PMID:17709481
- Browne SK, Holland SM. Immunodeficiency secondary to anticytokine autoantibodies. *Curr Opin Allergy Clin Immunol* 2010; 10:534-41; PMID:20966748; <http://dx.doi.org/10.1097/ACI.0b013e3283402b41>
- Spohn G, Keller I, Beck M, Grest P, Jennings GT, Bachmann MF. Active immunization with IL-1 displayed on virus-like particles protects from autoimmune arthritis. *Eur J Immunol* 2008; 38:877-87; PMID:18253928; <http://dx.doi.org/10.1002/eji.200737989>
- Link A, Bachmann MF. Immunodrugs: breaking B- but not T-cell tolerance with therapeutic anticytokine vaccines. *Immunotherapy* 2010; 2:561-74; PMID:20636009; <http://dx.doi.org/10.2217/imt.10.30>
- Sauerborn M, Brinks V, Jiskoot W, Schellekens H. Immunological mechanism underlying the immune response to recombinant human protein therapeutics. *Trends Pharmacol Sci* 2010; 31:53-9; PMID:19963283; <http://dx.doi.org/10.1016/j.tips.2009.11.001>
- Kopp MV. Omalizumab: Anti-IgE therapy in allergy. *Curr Allergy Asthma Rep* 2011; 11:101-6; PMID:21243454; <http://dx.doi.org/10.1007/s11882-010-0173-4>
- Mandel TE, Phipps RP, Abbot AP, Tew JG. Long-term antigen retention by dendritic cells in the popliteal lymph node of immunized mice. *Immunology* 1981; 43:353-62; PMID:7251058
- Barrington RA, Pozdnyakova O, Zafari MR, Benjamin CD, Carroll MC. B lymphocyte memory: role of stromal cell complement and FcgammaRIIB receptors. *J Exp Med* 2002; 196:1189-99; PMID:12417629; <http://dx.doi.org/10.1084/jem.20021110>
- Gray D, Skarvall H. B-cell memory is short-lived in the absence of antigen. *Nature* 1988; 336:70-3; PMID:3263573; <http://dx.doi.org/10.1038/336070a0>
- Klaus GG, Humphrey JH, Kunkl A, Dongworth DW. The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. *Immunol Rev* 1980; 53:3-28; PMID:7009406; <http://dx.doi.org/10.1111/j.1600-065X.1980.tb01038.x>
- MacLennan IC, Gray D. Antigen-driven selection of virgin and memory B cells. *Immunol Rev* 1986; 91:61-85; PMID:3089914; <http://dx.doi.org/10.1111/j.1600-065X.1986.tb01484.x>
- Tew JG, Phipps RP, Mandel TE. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol Rev* 1980; 53:175-201; PMID:7009404; <http://dx.doi.org/10.1111/j.1600-065X.1980.tb01044.x>
- Wrarmert J, Ahmed R. Maintenance of serological memory. *Biol Chem* 2008; 389:537-9; PMID:18953719; <http://dx.doi.org/10.1515/BC.2008.066>
- Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* 2010; 10:787-96; PMID:20948547; <http://dx.doi.org/10.1038/nri2868>
- Lanzavecchia A, Bernasconi N, Traggiai E, Ruprecht CR, Corti D, Sallusto F. Understanding and making use of human memory B cells. *Immunol Rev* 2006; 211:303-9; PMID:16824137; <http://dx.doi.org/10.1111/j.0105-2896.2006.00403.x>
- Vinuesa CG, Sanz I, Cook MC. Dysregulation of germinal centres in autoimmune disease. *Nat Rev Immunol* 2009; 9:845-57; PMID:19935804; <http://dx.doi.org/10.1038/nri2637>
- Anolik JH, Ravikumar R, Barnard J, Owen T, Almudevar A, Milner EC, et al. Cutting edge: anti-tumor necrosis factor therapy in rheumatoid arthritis inhibits memory B lymphocytes via effects on lymphoid germinal centers and follicular dendritic cell networks. *J Immunol* 2008; 180:688-92; PMID:18178805

46. Purkall D, Tew JG, Schenkein HA. Opsonization of *Actinobacillus actinomycetemcomitans* by immunoglobulin G antibody reactive with phosphorylcholine. *Infect Immun* 2002; 70:6485-8; PMID:12379734; <http://dx.doi.org/10.1128/IAI.70.11.6485-6488.2002>
47. Waterston AM, Gumbrell L, Bratt T, Waller S, Gustav-Aspland J, L'hermenier C, et al. Phase I study of TNF α AutoVacIne in patients with metastatic cancer. *Cancer Immunol Immunother* 2005; 54:848-57; PMID:15754205; <http://dx.doi.org/10.1007/s00262-005-0661-x>
48. Defrance T, Taillardet M, Genestier L. T cell-independent B cell memory. *Curr Opin Immunol* 2011; 23:330-6; PMID:21482090; <http://dx.doi.org/10.1016/j.coi.2011.03.004>
49. Good-Jacobson KL, Tarlinton DM. Multiple routes to B-cell memory. *Int Immunol* 2012; 24:403-8; PMID:22451529; <http://dx.doi.org/10.1093/intimm/dxs050>
50. Jennings GT, Bachmann MF. Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. *Annu Rev Pharmacol Toxicol* 2009; 49:303-26; PMID:18851703; <http://dx.doi.org/10.1146/annurev-pharmtox-061008-103129>
51. Tissot AC, Spohn G, Jennings GT, Shamshiev A, Kurrer MO, Windak R, et al. A VLP-based vaccine against interleukin-1 α protects mice from atherosclerosis. *Eur J Immunol* 2013; 43:716-22; PMID:23254454; <http://dx.doi.org/10.1002/eji.201242687>
52. Guler R, Parihar SP, Spohn G, Johansen P, Brombacher F, Bachmann MF. Blocking IL-1 α but not IL-1 β increases susceptibility to chronic *Mycobacterium tuberculosis* infection in mice. *Vaccine* 2011; 29:1339-46; PMID:21093494; <http://dx.doi.org/10.1016/j.vaccine.2010.10.045>
53. Bachmann MF, Jennings GT. Therapeutic vaccines for chronic diseases: successes and technical challenges. *Philos Trans R Soc Lond B Biol Sci* 2011; 366:2815-22; PMID:21893545; <http://dx.doi.org/10.1098/rstb.2011.0103>
54. Röhn TA, Bachmann MF. Vaccines against non-communicable diseases. *Curr Opin Immunol* 2010; 22:391-6; PMID:20338740; <http://dx.doi.org/10.1016/j.coi.2010.02.009>
55. Spohn G, Guler R, Johansen P, Keller I, Jacobs M, Beck M, et al. A virus-like particle-based vaccine selectively targeting soluble TNF- α protects from arthritis without inducing reactivation of latent tuberculosis. *J Immunol* 2007; 178:7450-7; PMID:17513796
56. Bachmann MF, Whitehead P. Active immunotherapy for chronic diseases. *Vaccine* 2013; 31:1777-84; PMID:23415932; <http://dx.doi.org/10.1016/j.vaccine.2013.02.001>
57. Brady LJ. Antibody-mediated immunomodulation: a strategy to improve host responses against microbial antigens. *Infect Immun* 2005; 73:671-8; PMID:15664904; <http://dx.doi.org/10.1128/IAI.73.2.671-678.2005>
58. Houston WE, Kremer RJ, Crabbs CL, Spertzel RO. Inactivated Venezuelan equine encephalomyelitis virus vaccine complexed with specific antibody: enhanced primary immune response and altered pattern of antibody class elicited. *J Infect Dis* 1977; 135:600-10; PMID:404363; <http://dx.doi.org/10.1093/infdis/135.4.600>
59. Rasmussen SK, Rasmussen LK, Weilguny D, Tolstrup AB. Manufacture of recombinant polyclonal antibodies. *Biotechnol Lett* 2007; 29:845-52; PMID:17310321; <http://dx.doi.org/10.1007/s10529-007-9331-8>
60. Waltz E. Polyclonal antibodies step out of the shadows. *Nat Biotechnol* 2006; 24:1181; PMID:17033641; <http://dx.doi.org/10.1038/nbt1006-1181>
61. Newcombe C, Newcombe AR. Antibody production: polyclonal-derived biotherapeutics. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 848:2-7; PMID:16893686; <http://dx.doi.org/10.1016/j.jchromb.2006.07.004>
62. Rasmussen SK, Næsted H, Müller C, Tolstrup AB, Frandsen TP. Recombinant antibody mixtures: production strategies and cost considerations. *Arch Biochem Biophys* 2012; 526:139-45; PMID:22820097; <http://dx.doi.org/10.1016/j.abb.2012.07.001>
63. Logtenberg T. Antibody cocktails: next-generation biopharmaceuticals with improved potency. *Trends Biotechnol* 2007; 25:390-4; PMID:17681390; <http://dx.doi.org/10.1016/j.tibtech.2007.07.005>
64. Berry JD, Gaudet RG. Antibodies in infectious diseases: polyclonals, monoclonals and niche biotechnology. *N Biotechnol* 2011; 28:489-501; PMID:21473942; <http://dx.doi.org/10.1016/j.nbt.2011.03.018>
65. Frandsen TP, Næsted H, Rasmussen SK, Hauptig P, Wiberg FC, Rasmussen LK, et al. Consistent manufacturing and quality control of a highly complex recombinant polyclonal antibody product for human therapeutic use. *Biotechnol Bioeng* 2011; 108:2171-81; PMID:21495017; <http://dx.doi.org/10.1002/bit.23166>
66. Haurum JS. Recombinant polyclonal antibodies: the next generation of antibody therapeutics? *Drug Discov Today* 2006; 11:655-60; PMID:16793535; <http://dx.doi.org/10.1016/j.drudis.2006.05.009>
67. Sharon J, Liebman MA, Williams BR. Recombinant polyclonal antibodies for cancer therapy. *J Cell Biochem* 2005; 96:305-13; PMID:16052504; <http://dx.doi.org/10.1002/jcb.20536>
68. Szakal AK, Holmes KL, Tew JG. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells, including cells with dendritic morphology. *J Immunol* 1983; 131:1714-27; PMID:6619542