T Cell-Independent Breach of B Cell Tolerance by Self Antigen Retention on Follicular Dendritic Cells

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Abstract

Follicular dendritic cell (FDC) networks supporting germinal centre (GC)-like structures and local auto-antibody production have been recognized in chronically inflamed tissues, including the synovium of rheumatoid arthritis (RA). The mechanisms by which FDCs are involved in auto-antibody production are nevertheless not fully understood. We hypothesized that auto-immune complex (IC) retention on FDCs can breach B cell tolerance and induce auto-antibody production; and we sought to investigate the mechanisms and modulation of FDC-induced auto-antibodies. To test our hypothesis, IC retention on RA synovial FDCs was examined using scanning confocal microscopy. In vitro GC reactions differentially reconstituted with T cells, complement, and ICs were set up to assess auto-antibody induction by IC-loaded FDCs. Breach of B cell tolerance with IC-retaining FDCs was investigated in sHEL-Tg mice, and modulation of FDC-IC retention and auto-antibody production by the IgG-specific Endoglycosidase-S was verified. Our results showed that fibrinogen retention can be revealed on FDCs in RA synovial follicular structures. In vitro, fibrinogen-IC-loaded FDCs induced T cell-independent (TI) fibrinogen-specific IgM, whereas non-specific T cell help was required for IgG production. Furthermore, retention of HEL-ICs on FDCs broke B cell tolerance and induced HEL-specific auto-antibodies in sHEL-Tg mice. Finally, Endoglycosidase-S interfered with FDC-IC retention and significantly inhibited FDC-induced antibody production. In conclusion, the ability of FDCs to productively present auto-antigens provides a mechanism that could help explain their pathogenic role in autoimmune disorders. Interference with FDC-IC retention using Endoglycosidase-S inhibits FDC-induced auto-antibody production, and can be potentially used to ameliorate auto-antibody-mediated autoimmune diseases.


Introduction

ENGAGEMENT of B cell receptors (BCRs) with follicular dendritic cell (FDC)-retained antigens (Ags) is critical for 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].

We recently showed that FDCs uniquely regulate the form of Ag and the co-signals that B cells encounter in germinal centre (GC) reactions (GCRs) [2]. In contrast to Ag processing and peptide presentation to T cells, we discovered that FDCs multimerize monomeric Ags by trapping them in immune complexes (ICs) via FDC-Fc-gamma Receptor IIB (–FcγRIIB) and then presenting them polyvalently to BCRs with a characteristic periodic spacing between epitopes of 200-500 Å. This allows simultaneous engagement of multiple BCRs and strong signalling 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 by the ICs themselves [3]. Toll-like receptor (TLR) ligands [4], and collagen type-1 [5]. 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

Abbreviations:

CSR : Class switch recombination.
Endo-S : Endoglycosidase-S.
FDC : Follicular dendritic cells.
GC : Germinal centre.
GCR : Germinal centre reaction.
IC : Immune complex.
HEL : Hen egg lysozyme.
RA : Rheumatoid arthritis.
SHM : Somatic hypermutation.
TD : T cell-dependent.
TI : T cell-independent.
IL-4 and IL-5 promoted IgM production and induced IgG class switching [6]. 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 [7,8].

Breach of tolerance in autoimmune diseases is characterized by the display of IC-bearing FDCs in autoreactive GCs producing high affinity pathogenic auto-Abs in secondary and tertiary lymphoid tissues [9,10]. It has been demonstrated that the FDC positive follicular units in the rheumatoid arthritis (RA) synovium and the salivary glands of Sjögren's syndrome express the molecular machinery to support local auto-Ab production independently of new B cell influx from the systemic circulation [11]. However, the mechanisms by which FDCs are involved in local auto-Ab production in autoimmune diseases have not been fully elucidated. The difficulty in assessing FDC functions, and the lack of FDC-IC retention modulators have all contributed to the slow progress in these areas. Thus, in this study, we sought to establish: (a) the ability of FDC-trapped self-ICs to induce auto-Abs, (b) the mechanisms underpinning this induction, and (c) the capacity to modulate these auto-Ab responses.

Fibrinogen plays a critical role in several inflammatory autoimmune diseases [12], including RA, and we used it in the current study to investigate the ability of FDC-ICs to induce auto-Ab production and the capacity of Endoglycosidase-S (Endo-S) to modulate its retention on FDCs. In RA, the auto-Ab response to native fibrinogen spreads to citrullinated fibrinogen and precedes the onset of arthritis [13]; and fibrinogen-ICs can be demonstrated in the sera and inflamed synovia [14,15]. Endo-S has a specific activity on the conserved N-linked glycan located in the heavy chain of IgG [16] compromising IgG recognition by FcRs [17], and the IgG ability to activate complement [18].

Here we show that auto-IC retention on FDCs induces IgM auto-Abs in a TI fashion, and that non-cognate T cells and complement promote IgG auto-Ab production. Moreover, interference with FDC-auto-IC trapping significantly inhibits auto-Ab responses. This ability of FDCs to productively present auto-Ags provides a mechanism that could help explain their pathogenic role in autoimmune diseases, and its inhibition by Endo-S can be potentially used to interfere with the local Ag-driven auto-Ab production in chronically inflamed tissues.

Material and Methods

Human and mouse FDCs were isolated from palatine tonsils and C57BL/6 lymph nodes (LNs) respectively after enzymatic digestion and preparation of single cell suspensions. FDCs do not express CD45, and they were pre-enriched by depleting the CD45+ cells from the washed single cell suspensions using anti-human and anti-mouse CD45 MicroBeads (Miltenyi Biotech, 130-045-801, 130-052-301). For human FDCs, the labelling pattern of the mouse anti-human follicular dendritic cell mAb (clone CNA.42, IgM) (Dako, M7157) was first verified on tonsillar sections and used for labelling human FDCs in the CD45+ fraction (2µg/ml per 107 cells for 1 hr on ice) followed by positive selection using anti-mouse IgM MicroBeads (Miltenyi Biotech, 130-047-302). Eighty five per cent of the purified cells were positive for the human FDC-
specific mouse anti-CD21 long isoform mAb as assessed by flow cytometry. Murine FDCs were isolated as we have previously described [19]. The characteristic dendritic morphology of purified FDCs was visualized by differential interference contrast (Nomarski) microscopy.

**Lymphocytes:**

Human CD 19+ B cells, CD3+ T cells, and mouse B220+ B cells were immuno-magnetically selected from human tonsils and mouse spleens using mouse anti-human CD19 MicroBeads (Miltenyi Biotech, 130-050-301), mouse anti-human CD3 MicroBeads (Miltenyi Biotech, 130-050-101), and B220 MicroBeads (Miltenyi Biotech, 130-049-501) respectively. Purified FDCs, B cells, and T cells were used in setting up in vitro GCRs.

**Setting up in vitro GC reactions:**

In vitro human autoreactive GCRs were differentially set up with 10^5 B cells, 2x10^4 T cells, 2x10^4 FDCs, 5 ng human fibrinogen (Merck Chemicals UK, 341578) and 10 ng anti-fibrinogen from the purified ACPA+ IgG. Fibrinogen ICs were incubated with human complement (Sigma, S 176-1 ML) (at 1:12 final dilution) for 2 hr to generate complement fragments. The IC-complement mixture was then added to the FDCs for entrapment, and then the FDCs were added to the cultures. Ten units of Endo-S were added to inhibit IC trapping on FDCs in certain cultures, and the cultures were maintained for 6 days at 37°C in a CO2 incubator then assessed for fibrinogen-specific IgM and IgG auto-Ab production by ELISA. Cell clusters were visualized by light microscopy and pictures were taken at day 2 and 5.

T cell-deficient murine in vitro GCRs were also set up to assess cytokine-induced CSR as previously detailed [6] and 10 ng/ml recombinant mouse IL4 (R&D, 404-ML-010) and IL5 (R&D, 405-ML-005) were added and the culture supernatant fluids were collected at day 7 and assessed for mouse IgG and IgM by ELISA.

**Mice and immunization:**

C57BL/6 & C57BL/6-Tg (ML5sHEL) 5Ccg/J (sHEL-Tg) mice were purchased from the Jackson Laboratory. Mice were housed in accordance with the Institutional Animal Care and Use Committee guidelines at Virginia Commonwealth University and Queen Mary University of London; and the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (PIL 70/23296).

Groups of sHEL-Tg mice were passively immunized with 1 mg rabbit anti-HEL intra-peritoneal injection (i.p.) followed 24 hr later with 100 µg HEL subcutaneous (s.c.) (unconjugated or rhodamine-conjugated) in the loose skin of the back of the neck. Six days later the mice were sacrificed and sera and draining LNs were collected. Control groups challenged with HEL alone or anti-HEL alone were included.

**Immunohistochemistry and confocal microscopy:**

Ten µm thickness frozen sections were cut from the synovial tissue samples, human tonsils, and mouse LNs, fixed in absolute acetone, air-dried and blocked with serum-free protein block (Dako, X0909). The sections were labelled for FDCs, B cells, T cells, complement, Endoglycosidase-S, glycans, and an array of FDC and B cell Ags expressed in the GCs [A list of Abs used in the study is shown in Table (1)]. Sections were mounted with anti-fade mounting medium, Vectashield (Vector Laboratories), cover-slipped, and examined with a Leica TCS-SP2 AOBs confocal laser scanning microscope. Four lasers were used: Diode laser (405nm); Argon (488nm); HeNe (543nm); HeNe (633nm) [far red emission is shown as pseudo-colors]. Parameters were adjusted to scan at 1024x1024 pixel density and 8-bit pixel depth. Emissions were recorded in two or three separate channels, and digital images were captured and processed with Leica Confocal, LCS Lite software, and Image-J for quantitative assessment of immunohistochemistry.

**Flow cytometry:**

Heat-inactivated human AB serum (Sigma, H4522-20ML) was used to block non-specific binding to the human FDC FcRs. The cells were labelled with the human FDC-specific mouse anti-CD21 long isoform mAb (Dendritics, DXO120) or isotype control followed by PE-conjugated rat anti-mouse IgG1 (Biolegend, 406608). Purified human FDCs loaded with rhodamine-conjugated fibrinogen ICs were treated with 10 U Endo-S for 1 hr at 37°C with shaking, and the IC retention was compared with unloaded FDCs. Data were collected and analyzed using BD FACS Calibur in the Flow Cytometry Core Facility of William Harvey Research Institute.

**ELISA:**

Fibrinogen-specific IgG and IgM in the culture supernatant fluids were measured on 96-well plates coated with human fibrinogen (10 µg/ml) and blocked with 5% BSA. HEL-specific IgM and IgG
in the mice sera were similarly assessed on plates coated with HEL [4,6].

**In situ effect of Endo-S on FDC-IC retention in tonsillar sections:**

Ten µm tonsillar sections were treated with 20 U Endo-S at 37°C for 1 hr on a shaker, then labelled with anti-human C3, or anti-human IgG together with the FDC-marker CNA.42, and compared with untreated sections using confocal microscopy.

**Conditional activation of Endo-S and Western blotting:**

Endo-S (Genovis, A0-IZ1-050) was biotinylated using EZ-Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce, 21935). Biotinylated Endo-S was inactivated by DNP-thermolysin for 45min at 37°C then DNP-thermolysin was immuno-precipitated before addition of mouse anti-biotin IgG mAb (Jackson Immuno Research, 200-002-211) or ChromPure Mouse IgG, whole molecule (Jackson Immuno Research, 015-000-003). Reactions were set up at 1 Unit Endo-S per 1 µg IgG for 30 mins at 37°C in phosphate buffered saline (PBS). In other experiments the functional recovery of thermolysin-inactivated Endo-S was attempted using sera from mice immunized with Endo-S. IgG glycosylation status was assessed by SDS/PAGE and lectin blot analysis using HRP-conjugated Lens Culinaris Agglutinin (LCA) (US biological, L1665-25).

**Statistical analysis:**

\( p \)-values were calculated using unpaired two-tailed Student’s \( t \)-tests. Error bars represent the mean ± SD between samples. Significance was accepted at alpha <0.05.

**Results**

**Fibrinogen trapping on RA-FDCs:**

FDCs characteristically trap unprocessed Ags on their membranes for extended periods of time. Lacking Ag-specific receptors, FDCs rely on Fc and complement (C) receptors to engage Ags in the form of Ag/Ig/C (immune) complexes (ICs). FDCs in primary B cell follicles minimally trap ICs, whereas, in GC-containing secondary B follicles FDCs maximally retain Ags in the form of ICs and critically participate in B cell activation, SHM, and affinity maturation. It has been previously reported that primary B cell follicles are absent in inflamed RA synovia [20], emphasizing the role of Ag recognition in the generation of inflammation-associated lymphoid organogenesis. Here, we sought to determine if RA-FDCs trap ICs; and whether native fibrinogen is incorporated in the ICs retained on FDCs in the follicular structures of RA synovia. RA synovial tissue samples were labelled for FDCs, B cells, hIgG, C3, and fibrinogen and examined by scanning confocal microscopy. Single fluorescence channel recording and multi-colour overlays revealed that FDCs and B cells co-localize in the GC-like structures of the RA synovia (Fig 1 A-C), where FDCs retained hIgG (Fig. 1-A), C3 fragments (Fig. 1-B), and fibrinogen (Fig. 1-C). In 3 RA synovial samples with follicular organization, 25% of the GC-like structures were positive for fibrinogen retention on FDCs. In addition, within the same GC-like structure, some FDCs retained fibrinogen, and others were negative (Fig. 1-C, CNA.42/fibrinogen overlay).

**Auto-Ag-bearing FDCs alone can induce IgM auto-Abs while IgG auto-Ab production requires auto-Ag-bearing FDCs and T cells:**

FDCs are critically involved in the induction of GCRs, whereas T cell help is required for CSR and SHM. We have recently shown that FDCs in athymic nude mice and in T cell-deficient in vitro GCRs support GC development and IgM Ab production against OVA. Blockade of FDC-FcγRIIB and neutralization of the FDC-associated CD40 Ligand (FDC-C4bBP) or FDC-BAFF inhibited the ability of FDC-ICs to induce these T cell–independent (TI) IgM responses.

In the current study we sought to evaluate the ability of human FDCs loaded with fibrinogen ICs to induce fibrinogen-specific auto-Abs in in vitro GCRs. using purified human FDCs (Fig. 2-I). Purified human FDCs were characteristically in clusters with extensive dendrites as seen by the Nomarski optics just after isolation (Fig. 2-I A), and more than 85% were positive for the FDC-specific CD21 long isoform mAb [21] (Fig. 2-I B). In the in vitro GCRs, we observed that the FDCs, B cells, and T cells clustered as early as 48 hr after co-culture, and the clusters increased in size with time (Fig. 2-I C). Assessment of fibrinogen-specific Ab production at day 6 (Fig. 2-II) indicated that fibrinogen-specific IgM was induced in B cell cultures stimulated with fibrinogen-IC-loaded FDCs in the absence of T cells, which was not increased significantly by the addition of complement to these cultures (Fig. 2-II A). These data indicate that cross linking of BCRs by ICs on FDCs is sufficient to induce IgM in the presence of other FDC-derived co-signals but not to induce CSR (IgG production) in the absence of T cells or T cell
factors. In addition, in the presence of fibrinogen ICs and complement, anti-fibrinogen IgG was not measurable in B cell cultures deficient of FDCs and T cells (Fig. 2-II B), FDCs alone (Fig. 2-II C) or T cells alone (Fig. 2-II D).

Notably, fibrinogen-IC-loaded FDCs induced fibrinogen-specific IgG in the presence of T cells and the response was significantly enhanced by complement (Fig. 2-II E). These data indicate that T cells are critical for CSR of autoreactive B cells, and that complement augments, but is not an absolute requirement for IgG auto-Ab production by autoreactive B cells stimulated with FDC-auto-ICs. As demonstrated later (Fig. 3-III A and B), IL-4 and IL-5 can induce CSR and IgG production in the absence of cognate T cell help in in vitro GCRs.

**Neo-self-Ag trapping on FDCs induces autoreactive GCs and auto-Abs in sHEL-Tg mice in vivo; and recombinant IL-4 and IL-5 promote CSR in the absence of T cells in vitro:**

To confirm the ability of FDC-ICs to breach B cell tolerance and induce autoreactive GCRs and auto-Abs in vivo, we used the well-verified soluble hen egg lysozyme (sHEL) transgenic (Tg) mouse model, as it is known that B cells in sHEL-Tg mice are profoundly anergic to T cell dependent (TD) and TI BCR-mediated stimulation with sHEL in vivo and in vitro. We reasoned that presentation of HEL to B cells in the form of ICs would induce HEL-specific B cell activation in an FDC-dependent fashion. Immunohistochemistry of the draining LNs of HEL-IC-challenged sHEL-Tg mice revealed HEL trapping on FDCs in the presence of rabbit anti-HEL and GL-7 + GCs were induced in association with the IC-retaining FDCs (Fig. 3-I A-N). As predicted, sHEL-Tg mice produced HEL-specific IgM and IgG in response to HEL-ICs but not to sHEL (Fig. 3-II A,B).

CSR is critically regulated by T cells and/or T cell factors in TD and TI responses [22]. In HEL-Tg mice, tolerance within the T-cell compartment was substantiated in the original description of the model [23] which excludes the possibility of cognate B cell-T cell interaction in the context of immune responses to HEL. We reasoned that although cognate autoreactive T cell help may be lacking, T cell cytokines may be able to induce CSR in B cells once stimulated with FDC Ags that efficiently crosslink the BCRs. Indeed, the addition of rIL-4 and rIL-5 in the absence of T cells enhanced IgM and class switching to IgG in B cells stimulated by cross-linking the BCRs with anti-IgD-IC-bearing FDCs in vitro within 48 hr (Fig. 3-III A,B).

**Interference with auto-Ag retention on human FDCs inhibits FDC-induced auto-Ab production in vitro:**

Inhibition of FDC-mediated auto-Ab production is critical for interference with the auto-Ab-mediated pathology in autoimmune diseases. Auto-IC retention on FDCs induced autoreactive GCRs and auto-Ab production in vitro (Fig. 2) and in vivo (Fig. 3), thus, we hypothesized that interference with FDC-IC trapping would inhibit FDC-induced Ab production. As expected, addition of Endo-S significantly inhibited fibrinogen-specific IgG production in FDC-T cell-enriched cultures with or without complement (Fig. 4-I A,B). We reasoned that this inhibition of auto-Ab production in the in vitro GCRs by Endo-S is directly related to loss of B cell stimulation by the Ags retained in FDC-ICs. To test our reasoning, purified human FDCs loaded with rhodamine-conjugated fibrinogen were treated with Endo-S, washed and compared with untreated cells by immuno-fluorescence and flow cytometry. Endo-S-treated FDCs showed significantly less rhodamine signal by immuno-fluorescence with a clear left-shift quantifiable by flow cytometry compared to untreated cells indicating loss of rhodamine-conjugated fibrinogen from the surface of FDCs (Fig. 4-II).

**Endo-S de-traps pre-bound FDC-ICs in secondary lymphoid tissues:**

IC retention on FDCs is universally mediated by Fc and complement receptors in secondary and tertiary lymphoid tissues. Similar to the follicular structures in the RA synovia (Fig. 1-A,B), CNA-42-labeld FDCs in the tonsillar sections co-localized with hIgG and complement labelling (Fig. 5-I). Exceptionally the FDC network in the T cell-rich narrow band of the outer zone (white arrows) was devoid of IgG retention which confirms earlier studies that FDCs are not involved in Ag presentation to T cells.

Endo-S interferes with IC retention on FDCs by inhibiting IgG and complement engagement with Fcγ and complement receptors respectively. Consequently, we sought to determine if Endo-S can de-trap ICs retained on FDCs in tonsillar sections. As demonstrated in Fig. (5-II,III), Endo-S-treated tonsillar sections showed less human IgG and C3 labelling in association with the FDC marker CNA.42 compared to control untreated sections.
Partial recovery of Endo-S activity against targeted Abs in vitro:

Endo-S interfered with IC retention on purified FDCs in vitro (Fig. 4) and non-specifically de- trapped FDC-retained ICs in tonsillar sections in situ (Fig. 5). The non-specific activity of Endo-S may interfere with normal immune responses thus minimizing its potential application in autoimmune disorders. We reasoned that if Endo-S can target selected groups of Abs; this would improve its potential in vivo utility. Full-length Endo-S is required for its activity [24], and we sought to test our reasoning that the split fragments of tagged Endo-S can be reassembled by anti-tag Abs, but not other Abs, thus partly recovering its function against the anti-tag IgG. As shown in Fig. (7), antibiotin IgG was partially deglycosylated in conditions treated with split biotin-tagged Endo-S whereas non-specific IgG was spared.

Table (1): List of antibodies used in the study.

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CD20\(^+\) B cell aggregates associated with the FDC marker CNA.42 (red) in 3 different RA GC-like structures are shown in A (green), B, and C (blue). FDCs trap Ags in the form of Ag/Ab/C\(^-\) complexes (ICs) and the co-localization of this triad with the FDC marker in RA synovia is shown in A-C. (A) hIgG (blue), CD20 (green), and CNA.42 (red) are imaged in separate channels and a 3-color overlay is shown next to them. Co-localization of hIgG with FDC-CNA.42 (magenta overlay) is shown as inset at the lower left corner of the 3-color overlay. hIgG also co-localizes with several CD20 \(^+\) cells indicating that these cells are IgG\(^+\) B cells. (B) C3 (green), CD20 (blue), and CNA.42 (red) are shown in 3 separate channels with a 3-color overlay in an adjacent frame. The orange overlay of C3 and CNA.42 is show in the inset at the lower left corner of the 3-color overlay. (B) Fibrinogen (green), CD20 (blue), and CNA.42 (red) are shown in 3 separate channels with a 3-color overlay in an adjacent frame. Fibrinogen retention on RA-FDCs (orange overlay) is shown at higher magnification below the 3-color panel. Images are representative of 3 different synovial samples with follicular structures. Scale bar 100 \(\mu\)m.
(I) Purified human FDCs were used to set up in vitro GCRs. (A) Due to reticular formation in vivo, purified human FDCs, as seen by the Nomarski optics, tend to remain in clusters, and the dendritic morphology is seen in multiple cells (scale bar = 10 µm). (B) Flow cytometric analysis of purified human FDCs. Compared to the isotype control (4%), >85% of the purified FDCs are positive to the human FDC-specific CD21 long isoform mAb (91%). (C) In FDC-T cell-enriched cultures, the size of B cell clusters increases with time as they proliferate in the in vitro GCRs due to interaction with FDC-ICs and provision of T cell help.

(II) In vitro GCRs were differentially set up with B cells, T cells, FDCs, fibrinogen, anti-fibrinogen, and complement. (A) Fibrinogen-specific IgM was induced in B cell cultures stimulated with fibrinogen-IC-loaded FDCs in the absence of T cells. Addition of exogenous complement to these cultures insignificantly increased IgM production. Addition of ICs and complement to B cells in the absence of FDCs and T cells (B), FDCs (C), or T cells (D) induced neither IgM nor IgG. (E) Fibrinogen-IC-loaded FDCs induced fibrinogen-specific IgG in the presence of T cells and the response was significantly enhanced by exogenous complement. Data are represented as mean ±SD and are representative of three experiments of this type. Results are presented after subtraction of the fibrinogen-specific Ig levels used to form ICs, and significance is expressed as * $p<0.05$, and **$p<0.01$. 

Fig. (2): Auto-IC retention on FDCs induces auto-Ab production in vitro.
Fig. (3): In vivo auto-IC trapping on FDCs breaches B cell tolerance in the sHEL-Tg mouse model, and Ig class switching can be induced in purified B cells using IL-4 and IL-5 in the absence of T cell help.

(I) Challenge of sHEL-Tg mice with HEL-ICs induced GCs associated with HEL-retaining FDC-reticula. sHEL-Tg mice were passively immunized with rabbit anti-HEL and then challenged with HEL (unlabeled in panels A-G, and rhodamine-conjugated in panels H-N). (A) HEL-ICs trapped in two adjacent FDC-reticula labelled blue with AMCA-conjugated goat anti-rabbit IgG that binds rabbit IgG in HEL-ICs made of HEL and rabbit anti-HEL. (B & C): GL7+ GC B cells (panel B, green) are surrounded by a mantle of resting B cells (panel C) labelled with PE-conjugated anti-IgD (red). (D-G): Overlays illustrating co-localization of the FDCs and the mantle B cells (panel D), the FDCs and the GC B cells (panel E), the GC and the mantle B cells (panel F), and the GCs including the FDCs surrounded by the mantle B cells (panel G) (scale bar = 100 µm). (H-J): Rhodamine-conjugated HEL (red in panel H) trapped on FDCs with rabbit anti-HEL (blue in panel I). The ICs appear purple in the overlay in panel J. (K-N): FITC-conjugated GL-7 Ab labelled GC B cells (green in panel K) interfaced with FDC-ICs labelled with rhodamine-conjugated HEL (red, in panel L). The AMCA labelled rabbit anti-HEL appears blue in panel M and the rhodamine-labelled HEL with the blue Ab appears purple in the ICs that engage the activated B cell membranes (green) in the overlay in panel N (scale bar = 10 µm).

(II) Induction of auto-reactive GCRs correlated with secretion of HEL-specific IgM and IgG on day 6 after HEL-IC challenge. The IgM data in (A) and IgG data in (B) illustrate that sHEL-Tg mice did not produce IgM or IgG in response to challenge with HEL alone. In marked contrast, passive immunization with anti-HEL alone induced HEL-specific IgG which we attribute to the formation of HEL-ICs that loaded on FDCs and broke B cell tolerance to HEL. However, the response was significantly higher when HEL-ICs were deliberately introduced in the sHEL-Tg mice. The HEL-specific IgM, which is optimal within 48 hr, had switched to IgG by day 6 despite the lack of HEL-specific cognate T cell help in these mice, and IgM can be seen only in mice that were deliberately challenged with HEL-ICs at day 6. Results are representative of at least three separate experiments, 3 mice per group were used, and data are represented as mean ±SD.

(III) In the absence of cognate T cell help, IL-4 and IL-5 induce CSR in vitro. rIL-4 & IL-5 enhanced the IgM response and promoted IgG class switching in purified B cells stimulated with IC-bearing FDCs in vitro. One hundred thousand B cells were stimulated in vitro with 100 ng/ml anti-IgD in the form of ICs on FDCs. (A) In the absence of IL-4 and IL-5, B cells produced IgM but not IgG. (B) Addition of 10 ng/ml IL-4 and IL-5 doubled IgM levels and induced IgG production within 48 hr. Data are represented as mean ±SD and are representative of three experiments of this type.
Fig. (4): Endo-S inhibits human auto-Ab production in vitro and interferes with IC retention on human FDCs.

(I) Fibrinogen-specific IgG was significantly inhibited by Endo-S in FDC-T cell enriched cultures. (A) Fibrinogen-ICs induced fibrinogen-specific IgG in vitro in the presence of both FDCs and T cells, and the IgG levels were complement-enhanced. (B) Addition of Endo-S significantly inhibited IgG production in the presence or absence of complement. Data are represented as mean ± SD and are representative of three experiments. Significance is expressed as * \( p < 0.01 \).

(II) Effect of Endo-S on purified human FDC-IC retention. ICs of rhodamine-conjugated fibrinogen and rabbit anti-human fibrinogen were loaded on purified human FDCs at 37°C overnight. The cells were washed, treated with Endo-S and compared with untreated cells using fluorescence microscopy and flow cytometry. By immunofluorescence, the rhodamine signal (red) from the Endo-S treated FDC group was lower indicating less fibrinogen retention on FDCs. DAPI (blue) labels the nuclei of FDCs. This was confirmed by flow cytometry where the mean fluorescence intensity of the Endo-S-treated FDCs shifted to left compared to the untreated group (scale bar = 10 µm).
(I) Human FDCs intensely label with anti-human IgG and C3. Labelling of tonsillar sections with anti-human IgG (blue) maximally co-localizes with the FDC-specific marker CNA.42 (red). The T cell-rich band in the outer zone (indicated by white arrows) labels with the FDC marker but is devoid of IgG labelling. In addition, complement fragments are retained on FDCs in the B cell follicles as indicated by colocalization of anti-human C3 labelling (green) with the FDC marker CNA.42 (red) although complement stains areas beyond the FDC network (scale bar = 100 µm).

(II, III) Endo-S unloads FDC-retained human IgG and C3 in tonsillar sections. Adjacent tonsillar sections were labelled with anti-human IgG and C3 (red) that co-localized with the FDC marker CNA.42 (green). As shown, Endo-S treated sections display less IgG and C3 labelling in the CNA.42-stained (green) FDC reticulum (scale bar = 100 µm). Analysis of the area fraction % across multiple sections indicated that human FDC-retained IgG and C3 was significantly decreased in Endo-S-treated FDCs compared to control sections. Data, mean ±SD, are representative of three experiments of this type.
(I) Recovery of split Endo-S activity was attempted using sera from mice challenged with Endo-S. As shown in the Lens Culinaris Lectin blot next to the MW marker (lower panel), this attempt failed as indicated by the lack of deglycosylation activity in the inactive Endo-S alone (lane 2 - human IgG / lane 6 - mouse IgG), the anti-Endo-S sera alone (lane 3 human IgG / lane 7 mouse IgG), and the inactive Endo-S in the presence of anti-Endo-S sera (lane 1 human IgG / lane 5 mouse IgG). Only active Endo-S deglycosylated human IgG (lane 4) and mouse IgG (lane 8). Protein blot stained with Coomassie blue is shown in the upper panel.

(II, III) Selective partial recovery of tagged split Endo-S on anti-tag Abs. Endo-S was tagged with biotin (biotin-EndoS) then split with DNP-thermolysin (inactive biotin-EndoS). DNP-thermolysin was immunoprecipitated prior addition of non-specific mouse IgG (II) or mouse anti-biotin IgG (III). Protein blot stained with Ponceau red is shown in the upper panel.

(II) Control non-specific IgG, anti-biotin IgG, Endo-S and biotin-Endo-S were run next to the MW marker in the first 4 lanes respectively. Endo-S (lane 5) and biotin-Endo-S (lane 6) deglycosylated the non-specific mouse IgG, whereas inactive Endo-S (lane 7) and inactive biotin-Endo-S (lane 8) lacked deglycosylation activity as indicated by strong glycan signal.

(III) The same controls were run in lanes 1-4, Endo-S deglycosylated the biotin-specific mouse IgG (lane 5), and in comparison to the inactive biotin-Endo-S (lane 7), both biotin-Endo-S (lane 6) and inactive biotin-Endo-S in the presence of mouse anti-biotin IgG (lane 8) exerted IgG deglycosylation effect.
During chronic infections, inflammations, and tissue damage TLR-Ligands (TLR-Ls) engaging TLRs on B cells favour autoreactive B cell activation and the secretion of potentially cross-reactive Abs. Binding of these cross-reactive Abs with self Ags (fibrinogen) released due to extensive tissue destruction forms auto-ICs that can be trapped on FDCs via Fc and complement receptors. Consequently: (1) FDC activation will be induced by ligation of FDC-Fc γRIIB (3) with self-ICs and FDC-TLRs (4) with TLR-Ls, which in turn enhances expression of FDC-Fc γRIIB, ICAM, VCAM, BAFF, & IL-6. (2) In a TI fashion, activated FDCs induce autoreactive B cell activation via (a) Extensive BCR cross-linking with multimerized self-Ags, (b) lowering the B cell activation threshold by inhibition of B-cell-Fc γRIIB/BCR cross-linking and promotion of BCR/B cell-CD21 co-ligation via FDC- CD21 ligand (CD21L), (c) provision of FDC-derived B cell survival and differentiation co-signals, and (d) Stable FDC-B cell synapse formation for delivery of FDC-co-stimulatory signals via ICAM-1 and VCAM-1 interaction with LFA-1 and VLA-4. (3) Engagement of B cell-CD40 with FDC-C4bBP together with IL-6 & BAFF produced by activated FDCs induce auto-Ab class switching & affinity maturation either directly or via non-cognate T cell-derived IL-4, IL-5, and IFN-γ-dependent pathway.

Self Ag-specific auto-Abs are secreted by the activated autoreactive B cells, bind self Ags forming auto-ICs that cause more tissue damage and more ICs are loaded on the FDCs. By interfering with IC retention on FDCs, Endo-S can inhibit auto-Ab secretion and abort the vicious circle of tissue damage and IC formation.
Discussion

Data in the present study strongly support the notion that auto-IC retention on FDCs critically regulates auto-Ab production. In a TI fashion, fibrinogen-specific IgM auto-Abs were induced in vitro by FDC-trapped fibrinogen-ICs, whereas, non-cognate T cell help and complement promoted fibrinogen-specific IgG anti-Ab secretion. In addition, when appropriate FDC-ICs were presented in vivo, specific auto-Ab responses were induced in sHEL-Tg mice previously unresponsive to HEL. Moreover, these auto-Ab responses were associated with auto-reactive GCs in the draining LNs. Endo-S interfered with FDC-IC retention in vitro and in-situ; and inhibited FDC-IC-induced auto-Ab production. These results provide novel support for the hypothesis that auto-IC retention on FDCs induces TI auto-Ab responses, and that interference with FDC-IC retention inhibits Ab production.

Auto-Ags are crucial for induction and maintenance of autoimmunity; and auto-Ag withdrawal inhibits disease progression [9]. We reason that several mechanisms are involved in the ability of FDC-retained auto-Ags to breach B cell tolerance and induce auto-Ab production.

First, 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 [25]. The high density, periodic geometry, and complement-enhanced immunogenicity of FDC-trapped ICs are extremely efficient in B cell stimulation that they can induce recall responses in immune milieu with high titres of Ag-specific Abs [1]. 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 signalling, and induce B cell activation and Ab production [26]. Moreover, the immunogenicity of FDC-ICs is protected by the high levels of FDC-FcRIIB that minimizes serious inhibition of B cell activation upon BCR/FcRIIB cross-linking [3,6]. In fact, the expression of FcRIIB is significantly reduced on memory B cells and plasmablasts in certain autoimmune disorders and this reduction is associated with high levels of auto-Ab secretion [27].

Second, not only do FDCs present Ags in ICs in a multivalent pattern, but also they provide complement-mediated B cell co-stimulation [28]. Complement receptor (CR)-mediated complementation of BCR signals can overcome B cell anergy [29], and activation of complement is involved in the pathogenesis of systemic autoimmune diseases via the classical and the alternative pathways. The lack of significant increase in IgM responses upon addition of complement in the current study was noticed before [6], and it seems that BCR/CR cross-linking is maximally operating in switched B cells as indicated by enhanced IgG production in complement-containing cultures.

Third, in addition to complement, FDCs provide other co-stimulatory signals that play critical roles in triggering B cell activation, and these accessory molecules are subject to regulation. Engagement of FDC-FcRIIB by ICs, FDC-TLR ligation, and collagen type-1 stimulation induce FDC activation and differentially upregulate the FDC accessory molecules ICAM-1, VCAM-1, FcRIIB, C4bBP, BAFF, IL-10, IL-17, and IL-6. Infections at the onset of many autoimmune diseases may directly activate FDCs by ligating TLRs, and thus enhance FDC-B cell interactions. In addition, FDCs are the major source of IL-6 in GCs [7] and IL-6, directly or via IL-17, promotes autoimmunity [30]. Moreover, FDC-BAFF is critical for FDC-dependent B cell activation [6], and BAFF is directly involved in rescue, activation, and follicular homing of autoreactive B cells [31].

Overall, the context of self-Ag presentation to B cells by FDCs, is highly efficient as illustrated by our in vitro and in vivo data. For example, the induction of anti-HEL Abs in sHEL-Tg mice where serum HEL levels are over 17 ng/ml is remarkable. In these mice, there is ample HEL to bind all BCRs and tolerize the rare HEL specific B cells. Nevertheless, injection of HEL-ICs induced potent anti-HEL responses in these tolerant mice; while HEL, which would not be trapped by FDCs, did not.

B cell tolerance is frequently incomplete, and peripheral blood B cells can be induced in vitro to produce ACPA using CD40L polyclonal stimulation and non-cognate anti-CD3-activated T cell help [32]. We reason that, in the course of autoimmune disorders, the initial Ab needed to trap self-Ags on FDCs may be similarly induced by molecular mimics or by polyclonal B cell activation, which may occur as a consequence of infections that herald autoimmunity [33]. However, once ICs form, they would be trapped by FDCs and the autoreactive B cells would be stimulated in a TI manner. In fact, circulating ICs containing citrullinated fibrinogen are present in ACPA+ RA patients [34] and earlier studies indicated that the FDC-positive follicular structures in RA synovia are surrounded by ACPA-positive plasma cells [31].
Antibodies targeting citrullinated fibrinogen are lacking in a significant number of RA patients [35], and our results showed that native fibrinogen is retained on some FDCs in the RA synovial follicular structures. We reason that the auto-Ab repertoire may initially target native self proteins then extend to their post-translationally modified forms. In fact, synovial protein microarray analyses demonstrated strong autoreactive B cell responses to epitopes derived from predominantly native fibrinogen and spreading to citrullinated fibrinogen in 20% of RA patients [13]. Moreover, in collagen induced arthritis, the autoimmune response initially targets the native inducing autoantigens and a limited set of citrullinated epitopes in the pre-disease and acute disease phases; whereas, the chronic phase of the diseases is dominated by auto-Abs targeting citrullinated epitopes with minimal specificity to their native counterparts [36].

The FDC-induced auto-IgM responses are unlikely to involve cognate T cell help. However, the subsequent CSR and affinity maturation likely stem from the combined activity of T cell factors and FDCs that provide late antigenic signals and IL-6 needed for optimal SHM and affinity maturation [7,8]. This interpretation is consistent with studies indicating that cognate T cell help may be dispensable for initial autoreactive B cell activation, although T cells can regulate ongoing autoimmune responses [37]. Moreover, important T cell factors involved in B cell co-stimulation in GCs may be derived in a non-cognate fashion. First, and most likely, the BAFF, IL-6, and -IL-15 that FDCs produce can induce T cell IL-4, IL-5, IFN-γ, and TNF-α, and these cytokines can promote B cell activation and CSR as indicated in our results. Second, efficient BCR cross-linking by repetitive Ags on FDCs may prime B cells adequately for bystander T cell-mediated CSR and enhanced IgG responses [26]. Finally, B cell activation by FDC-Ags upregulate B cell CD83/CD86 that may stimulate some T cell help [38].

In contrast to the MHC-restricted nature of peptide presentation by DCs to T cells, presentation of ICs by FDCs to B cells crosses MHC and species barriers [39]; which may explain development of certain autoimmune disorders independent of specific MHC phenotypes. Citrulline immunity has strong MHC association [40], and more than 20 genes have been found to be implicated with ACPA-positive disease [41]; however, one third of RA patients are ACPA negative [42] and auto-Ab production in these patients may be induced by breach of tolerance in the FDC/B cell axis rather than the DC/T cell axis. In fact, it was suggested that break-age of T cell tolerance is not necessary to generate activated autoreactive B cell or disease-associated auto-Abs [43]. However, once autoreactive B cells are activated by FDC-auto-ICs, B cell-dependent T cell activation may follow. B cells are efficient APCs, and the BCR-mediated selective uptake and presentation of Ags by activated Ag-specific B cells is superior to Ag uptake and presentation by DCs and macrophages. B cells capture ICs from the FDC membranes [38] which can then be processed and presented as MHC-peptides to induce both T cell activation and T cell help later in the course of the disease [44].

FDCs retain Ags via Ag-specific Abs and complement, and IC-loaded FDCs provide constant Ag depot for memory B cell re-stimulation [45], however, unloading FDC-retained Ags has not been attempted before. Endo-S inhibits IgG-Fc γR and IgG-complement interactions thus interfering with the effector functions of auto-Abs and ameliorates autoimmune conditions [46]. Our results provide novel evidence that Endo-S, in addition to its ability to interfere with the direct auto-Ab effector functions, significantly reduces Ag retention on human FDCs, which would inhibit Ag-driven B cell activation and plasma cell differentiation leading to maintained inhibition of auto-Ab production. In addition, subunit expression of Endo-S and tagging with clinically relevant baits (e.g. cyclic citrullinated peptides in RA) may provide selective targeting of specific Abs and Ag-Ab complexes. We are currently investigating the multi-dimensional effect of Endo-S in the GCR.

In conclusion, we have demonstrated that FDC-retained auto-Ags induce T1 auto-Ab production, which can be modulated by Endo-S. This partly contributes to the pathophysiological role of FDCs in autoimmune disorders. The TI nature of the mechanism offers an explanation for auto-Ab secretion in diseases where T cell tolerance is intact, or the limited MHC repertoire is non-permissive. Endo-S-mediated inhibition of auto-Ag retention on FDCs would reduce pathogenic auto-Ab production; however, the non-selective nature of Endo-S needs to be rectified before it can be exploited therapeutically. Though further work will be clearly required to translate these observations into patients’ treatments, this study provides unique insights into the mechanisms of FDC-dependent, TI autoreactive B-cell activation and auto-Ab production as well as the rationale underlying the attempt to exploit inhibition of FDC-auto-IC retention therapeutically.
References


الملخص العربي

تُلاحظ شبكات من الخلايا التغصنية الجراوية الداعمة للتفاعلات الإنشائية وإنتاج المستضدات الذاتية في العديد من الأنسجة التي تعتني النهاية مزمنا بما في ذلك الأنسجة الليمفية المنجلية للمفاصل المنجلية في مرض الروماتويد، على أن تالي ارتباط الخلايا التغصنية الجراوية بإنتاج المستضدات الذاتية لم يتم تفهمه بالكامل حتى الآن.

وقد افترضنا أن بقاء المعقدات المنجلية الذاتية على الخلايا التغصنية الجراوية يؤدي إلى إبطال تحلل الخلايا الليمفية الذائبة لأسباب الجسم مما يتسبب في إنتاج أجسام مضادة ذاتية، وعليه فقد استنفنا في هذا البحث اختبار آليات وتنظيم إنتاج الأجسام مضادة ذاتية السبب بالخلايا التغصنية الجراوية. ولإختبار فرضيتنا فحصنا بقاء الفيرونينجي على الخلايا التغصنية الجراوية في الأنسجة الليمفية المنجلية للمفاصل المنجلية في مرض الروماتويد باستخدام الميكتوسكوب البصائ الماسح. كما أعدنا تفاعلات إنشائية في الأنسجة المنجلية. نجهل أن تفاعلات من الخلايا الليمفية المنجلية والبروتينات المنجلية. يشكل اختبارنا إبطال تحلل الخلايا الليمفية ذاتية لسعة الجسم في فنون منحة ممثة للبروتين بضائ للدجاج. كما اختبرنا قدرة إنزيم الإندوجينزدوزيتر على تنظيم بقاء المعقدات المنجلية على الخلايا التغصنية الجراوية وإنتاج الأجسام مضادة ذاتية. وقد أثبت النتائج بقاء الفيرونينجيين على الخلايا التغصنية الجراوية في الأنسجة الليمفية المنجلية للمفاصل المنجلية في مرض الروماتويد، وأن تحميل الفيرونينجيين على الخلايا التغصنية الجراوية كمقدرات منجلية في تفاعلات إنشائية داخل الأنسجة المنجلية يؤدي إلى إنتاج أجسام مضادة ذاتية من النوع M دون الاستمام على الخلايا الذائبة في حين تتحت التفاعلات الإنشائية. بالإضافة إلى ذلك، نحن نعلم أن تحميل الفيرونينجيين على الخلايا التغصنية الجراوية كمقدرات منجلية في كسر تحلل الخلايا الذائبة إنتاج أجسام مضادة للبروتين. وبهذا نستطيع أن إزالة إنزيم الإندوجينزدوزيتر تقليل بقاء المعقدات المنجلية على الخلايا التغصنية الجراوية وكذلك تبطي إنتاج الأجسام مضادة ذاتية. وإنما فإن هذه الخلايا التغصنية الجراوية على عرض المستضدات الذائبة يفسر أحكام هذه الخلية في الأمراض المنجلية، كما أن قدرة إنزيم الإندوجينزدوزيتر على تنظيم هذا الخروض يمكن استخدامه كطريق لعلاج تلك الأمراض المنجلية.