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TLR9 responses of B cells are repressed by intravenous immunoglobulin through the recruitment of phosphatase☆

Jean-François Séité [a,b], Thomas Guerrier [a,b], Divi Cornec [a,b], Christophe Jamin [a,b], Pierre Youinou [a,b,*], Sophie Hillion [a,b]

[a] Université Européenne de Bretagne, France
[b] Brest University Medical School Hospital, Brest, France

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ABSTRACT

One way for intravenous Ig (IVIg) to affect responses of the B cells might be to operate through their TLR7 and TLR9. We confirm the ability of TLR agonists to induce CD25 expression in B cells. For this to occur, sialylated Fc-gamma of IgG included in the IVIg preparation are required. As a result, IVIg suppresses TLR-induced production of the proinflammatory IL-6, but not that of the anti-inflammatory IL-10. That is, IVIg mimics the effects of the MyD88 inhibitor. Finally, as we previously showed that IVIg induces CD22 to recruit the inhibitory SHP-1, we established that this enzyme was also involved in IVIg-induced inhibition of TLR9 signaling. This is the first report to demonstrate such a mechanism underlying the negative impact of IVIg on B lymphocytes.

Intravenous Ig (IVIg), has shown promise in the treatment of various autoimmune diseases [1–3]. This preparation of plasma-derived human IgG, modulates the responses of B lymphocytes, depending on whether these cells are activated or not. Therefore, the ensuing cellular response could vary. There is Ab production when B cells are CD40-stimulated [4], whereas apoptosis develops when they are stimulated through their B cell antigen (Ag) receptor (BCR) [5]. IVIg could interfere with B lymphocyte functions through a multitude of pathways, including the FcγR IIb [6], and through interactions [7] of sialic acid (SA) terminating polysaccharides, with lectin CD22, which is a negative regulator of B cell responses. This possibility is supported by the recent finding [8] that CD22 is involved in the TLR as well as the BCR pathways.

TLRs comprise a family of widely-expressed immune receptors of the innate and the adaptative immune systems [9]. Of the 10 different TLRs identified in humans, TLR3 binds to dsRNA, TLR7 as well as TLR8 bind to ssRNA, while TLR9 binds not only to unmethylated CpG motifs in viral and bacterial DNA, but also to synthetic non-methylated CpG-containing oligodesoxynucleotides. These ligands are collectively referred to as immunostimulatory sequences (ISS). Based on the nature of the triggering signals and aimed at ensuring homeostasis, the immune system through TLRs polarizes towards effective responses to pathogens or favors tolerance to self-components [10–12].

But inappropriate TLR signaling may also contribute to pathophysiology. Aberrant TLR3 ligation participates to the activation of programmed-cell death in salivary epithelial cells from Sjögren's syndrome patients [13]. Furthermore, TLR9 stimulation may exacerbate B cell hyperactivity, and thereby favors the synthesis of anti-dsDNA Ab in SLE [14]. In this respect, several investigators have cast TLR9 with a leading part in the play of B cell-mediated autoimmunity, following activation by self DNA [15]. A defect in MyD88, an adaptor molecule for TLR pathways, abrogates autoAb synthesis in MRL/*lpr* mice [16], whereas deficiencies in TLR7 and TLR9 inhibit the production of anti-dsDNA Abs in NZB/W mice T and attenuate nephropathy in pristine-induced lupus like mice [17]. This leaves open the question of how mammalian DNA, which is not an efficient ligand for TLR9 [18], stimulates B cells through TLR9-mediated signaling. The possibility exists that coengagement of the BCR somehow relaxes the stringency of TLR9 recognition for canonical and/or hypomethylated CpG motifs. Apoptotic chromatin may be also responsible, because, when relapsed in inflammatory context, it is enriched in hypomethylated, and hence stimulatory CpG motifs. In reality, irrespective of their sources, all these CpG-containing sequences could synergize and generate B cell hyperactivity, and

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* Corresponding author. Laboratory of Immunology, Brest University Medical School Hospital, BP824, F-29609 Brest, France. Tel.: +33 298 22 33 84; fax: +33 298 22 38 47.
E-mail address: youinou@univ-brest.fr (P. Youinou).

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ultimately lead to autoimmune states. Thus, inhibitors of TLR9 would be valuable to understand TLR9-mediated responses, and potentially as agents to treat such autoimmune diseases [19].

TLR activation of B cells up-regulates the expression of activation markers, induces proliferation [20], cytokine secretion, terminal differentiation and finally Ig secretion. TLR expression varies from one B cell subset to another [21]. In marked contrast to TLR1 and TLR3, it is recognized that TLR7 and TLR9 are present in all B cell subpopulations. The fine-tuning of the signal mediated through TLRs in human B cells has not been totally elucidated. Several reviews have touched on this topic recently [22,23], and mentioned that two pathways downstream governed by MyD88. On the one hand, NFκB is the major transcription factor in the production of proinflammatory cytokines and the upregulation of inflammatory mediators, such as chemokine receptors. On the other hand, there is an alternative pathway that involves phosphatidyl inositol-kinase, and triggers another phosphatase-dependent cascade to regulate TLR signaling [24]. This pathway also recruits the toll-interacting protein (Tollip) [25], and the IL-1 receptor-associated kinase (IRAK)-M [26], which are both negative regulators, or censors, of the TLRs.

To gain further insight into how IVIg regulates responses of B cells, and successfully improves autoimmune diseases, we have investigated the capacity of IVIg to modulate TLR7- and TLR9-mediated activation of B cells. We present evidence that IVIg down-regulates TLR9-mediated activation in B cells. Most notably, our experiments demonstrate that IVIg reduces CD25 level expression in human B cell subpopulations, inhibits proinflammatory cytokine production, chemokine secretion, and upregulation of their receptors. Finally, we discovered that the molecular mechanism responsible for the downregulation of TLR9-mediated B cell activation by IVIg requires recruitment of SHP-1. This finding motivates further investigations into how IVIg reacts with phosphatases. Whether such strategy could be promoted in the treatment of autoimmune diseases remains to be determined.

1. Materials and methods

1.1. Preparation of B cells and culture

MNCs were prepared from tonsils or peripheral blood by Ficoll-Hypaque centrifugation. T-cells were rosetted, and the resultant B cell-enriched suspensions were recovered. Their purity was confirmed by FACS analysis, using fluorescein isothiocyanate (FITC)-conjugated anti-CD19 and PE-conjugated anti-CD5 mAbs, both from Beckman-Coulter (Villepinte, France). The B cell-enriched preparations contained 98% B lymphocytes. All cell cultures were carried out at 37 °C in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 200 U/ml penicillin and 1 μg/ml streptomycin. Supernatants were collected and stored at −80 °C for cytokine production analysis. This research was approved by the Institutional Review Board at the Brest University Medical School Hospital and informed consent of the patients' parents was obtained according to the Declaration of Helsinki.

TLR-mediated activation was achieved using 0.5 μM CpG against tcg-tcg-ttt-tgt-cgt-ttt-gtc-gtt (InvivoGen, San Diego, CA) for TLR9, 1 μg/ml resiquimod (InvivoGen, Toulouse, France), referred to as CL097 thereafter, for TLR7 and TLR8, 100 ng/ml LPS for TLR4, 10 μg/ml polyinosinic:polycytidylic acid (poly (I:C)) or 100 ng/ml of the macrophage-activating lipopeptide − 2 kDa for TLR3. All TLR ligands were added to the medium 15 min before the addition of IVIg, in order to avoid association with IVIg prior to their internalization into B cells.

Phosphatase inhibition activity was achieved by treating the cells with 25 μM of pervanadate (Sigma–Aldrich, St Louis, MO) during 3 h, and specific SHP-1/-2 inhibition carried out using 200 μM 8-hydroxy-7 (6 sulfonaphtalen-2-yl) diazenyl-quinoline 5 sulfonic acid disodium salt (NSC-87877) (Sigma–Aldrich) during 30 min at 37 °C.

MyD88 was inhibited by treating cells with 0.25 mM MyD88 homodimerization inhibitory peptide (DRQIKIWFQNRRMKWKK RDVLPGT) (Imgenex, San Diego, CA) for 6 h at 37 °C. Inhibition specificity was checked with a control peptide (DRQIKIWFQ NRRMKWKK) under the same conditions.

1.2. IVIg preparations

IVIg preparations were purchased from the Laboratoire du Fractionnement Biologique (Les Ulis, France), and dissolved in serum-free RPMI-1640 medium to a concentration of 100 mg/ml. SA-IgG was purified from the bulk of IVIg preparations by lectin-affinity chromatography using a *Sambucus nigra* agglutinin (SNA) agarose column (Vector Laboratories, Burlingame, CA). Enrichment of the eluate was confirmed by Western blotting (WB) with biotinylated SNA at 4 μg/ml and horseradish peroxidase (HRP)-conjugated streptavidin.

For preparing Fab and Fc fragment, IVIg preparations were digested with 2 mg/ml papain in 0.04 M cystein–PBS for 6 h at 37 °C. The resulting Fab and Fc parts were separated from the undigested IVIg using a Sepharose S300 column, and the Fc fragments further separated from the Fab fragments on a protein G column. All purification steps were carried out using a fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden). Purity of the fragments was assessed by WB using Abs specific either for Fab or for Fc. Heat-aggregated IVIg were obtained by heating monomeric IVIg for 30 min at 63 °C. In some experiments, Herceptin® (Genentech, South San Francisco, CA) was used at 20 mg/ml as a control for high concentrations of Ig.

1.3. Dosage of cytokines

IL-6, IL-10, IFN-γ were quantified in supernatants using ELISAs according to BD Biosciences' instructions (San Diego, CA). The detection limits of IL-6, IL-10, IFN-γ using this protocol were 3 pg/ml, 2 pg/ml and 0.08 IU/ml, respectively.

1.4. RNA extraction and quantitative RT-PCR

Total mRNA was extracted using the RNAble method (Eurobio, Paris, France), and cDNA synthesized by reverse transcription in 20 μl with Superscript II RNase H-RT (Invitrogen, Cergy-Pontoise, France). Quantitative RT-PCRA was conducted in 20 μl mixtures containing 50 ng template cDNA, 1× Sybr Green PCR Master mix (Applied Biosystems, Foster City, CA), and 500 nM of each primer.

The used primers were GACTTGCCTGGTGAAAA-TCATCACTG plus GGGTCAGGGGTGGTTATTGCATC for IL-6, GGCTACCACATCCAA GGAA-GGCAG plus CCAATTACAGGGCCTCGAAAGAG-TC for 18S, TGGGCCGACT-GAACATCA plus CGGG-TCATGCCGTAATTCTT for Tollip, and CTCGGAA-TTTCTCTGCCAAG plus GTGG-GAGGATCTTCA GCAAA for IRAK-M.

Of note, IL-10 and IFN-γ mRNA expression were studied in a Taqman assay. All assays included a negative control which consisted of the reaction mixture with no template, and the mixture with 18S rRNA primers as an internal control. Comparison of cycle thresholds was completed with the $2^{-\Delta ct}$ method using 18S as an internal control.

1.5. FACS analyses

B cell activation was measured by FACS, using FITC-conjugated mouse anti-CD25 mAb (Dako, Glostrup, Denmark), PE-conjugated