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TLR9 expressed on plasma membrane acts as a negative regulator of human B cell response

Thomas Guerrier [a], Pierre Pochard [a], Ayan Lahiri [a], Pierre Youinou [a,b], Jacques-Olivier Pers [a,b], Christophe Jamin [a,b,*]

[a] EA2216 Immunologie et Pathologie, LabEx IGO, SFR ScInBioS, Université de Brest et Université Européenne de Bretagne, Brest, France
[b] Laboratory of Immunology and Immunotherapy, CHRU Brest, Brest, France

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ABSTRACT

Toll-like receptors (TLRs) are positioned at the interface between innate and adaptive immunity. Unlike others, those such as TLR9, that recognize nucleic acids, are confined to the endosomal compartment and are scarce on the cell surface. Here, we present evidence for TLR9 expression on the plasma membrane of B cells. In contrast to endosomal TLR9, cell surface TLR9 does not bind CpG-B oligodeoxynucleotides. After B cell-receptor (BCR) stimulation, TLR9 was translocated into lipid rafts with the BCR, suggesting that it could serve as a co-receptor for BCR. Nevertheless, stimulation of B cells with anti-TLR9 antibodies did not modify the BCR-induced responses despite up-regulation of tyrosine phosphorylation of proteins. However, CpG-B activation of B cells, acting synergistically with BCR signals, was inhibited by anti-TLR9 stimulation. Induction of CD25 expression and proliferation of B cells were thus down-regulated by the engagement of cell surface TLR9. Overall, our results indicate that TLR9 expressed on the plasma membrane of B cells might be a negative regulator of endosomal TLR9, and could provide a novel control by which activation of autoreactive B cells is restrained.

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1. Introduction

The family of toll-like receptors (TLRs) stands at the junction between innate and adaptive immunity [1]. They are essential in the discrimination between self and non-self. They lead to the development of immune response against a wide variety of pathogens while avoiding abnormal response to endogenous ligands due to the presence of numerous negative regulators [2].

TLRs are differentially expressed by the different subsets of B cells, conferring a large range of functional responses. Thus, transitional and marginal zone B cells are highly sensitive to TLR9 stimulation resulting in activation, proliferation and immunoglobulin production [3]. In these situations, paired BCR and TLR signals up-regulate gene products not induced by BCR or TLR9 alone and can cooperate to facilitate B-cell differentiation [4]. In contrast, follicular B cells are poorly activated due to the presence of regulated events [5]. Identification of these regulatory elements remains a major challenge in view of a control of the TLR9-dependent B cell responses that might be aberrantly activated in autoimmune diseases [6].

Like all TLRs that recognize nucleic acids, TLR9 is confined to the endoplasmic reticulum and to endolysosomes [7]. Activation of TLR9 requires the acidification of endosomal compartments that in turn influences direct binding and interaction with its ligand [8] and leads to its cleavage, a prerequisite of its activation [9,10]. Such intracellular localization and cleavage restrain TLR9 activation to ligands able to reach endolysosomes in sufficient quantities, which is the case for viral and bacterial DNA but normally not the case for self DNA [11]. Thus, a transmembrane TLR9 construct artificially expressed on the cell surface is not functional in its complete form whilst the cleaved mutated form bypasses the requirement of proteolysis and provides sensitivity to mammalian DNA [12]. The intracellular localization and cleavage requirement prevent the recognition of self DNA and preserve tolerance breakdown [11].

However, natural cell surface expression of TLR9 has been reported. Intestinal [13] and gastric [14] epithelial cells have been shown to be able to express TLR9 on their plasma membrane, although the functionality of this TLR9 remains to be clearly established. Moreover, it seems likely that human B lymphocytes can also express cell surface TLR9 [15–17] but its functional role has

* Corresponding author. Laboratory of Immunology, Brest University Medical School Hospital, BP824, F29609 Brest, France. Tel.: +33 2 98 22 33 84.
E-mail address: christophe.jamin@univ-brest.fr (C. Jamin).

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not been determined. In the present study, we wish to evaluate the presence of TLR9 on the plasma membrane of human B cells and identify its function on B cell response.

2. Material and methods

2.1. Isolation of B lymphocytes

Cord blood, peripheral blood and tonsils were collected after informed consents had been obtained. Tissues were minced up and filtered to remove fragments and clumps. Cord blood samples, peripheral blood samples and tonsillar cell suspensions were layered onto Ficoll-Hypaque and centrifuged. Mononuclear cells were incubated with neuraminidase-treated sheep red blood cells and T cells depleted by a second round of centrifugation. All preparations were >95% pure B cells.

2.2. Flow cytometry

All mAb were purchased from Beckman Coulter, unless otherwise indicated. We used phycoerythrin (PE)-conjugated anti-CD24, PE-cyanin5-conjugated anti-CD38 and biotinylated anti-human TLR9 (Imgenex) revealed using PE-cyanin7-conjugated streptavidin. For the activation response, B cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD25.

For the proliferation assay, B cells were preliminary labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) before stimulation and their proliferation evaluated on a FC500 flow cytometer (Beckman Coulter) measuring the decrease in mean fluorescence intensity (MFI) of CFSE.

2.3. Cultures of B lymphocytes

B cells were cultured in RPMI1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin at $2 \cdot 10^5$ cells/well in 96-well culture plates. They were stimulated with 0.25 μM CpG-B 2006 (Cayla-InvivoGen), or 10 μg/ml anti-IgM-coated beads (BioRad) in the presence of 100 U/ml recombinant IL-2 (ImmunoTools) or 10 μg/ml anti-TLR9 Abs (clone 26C593.2, Imgenex, or clone eB72-1665, eBioscience) cross-linked on 10 μg/ml anti-mouse IgG or anti-rat IgG (Jackson ImmunoResearch Laboratories) coated plates.

2.4. Immunofluorescence analysis

B cells were stained with mouse anti-human TLR9 (Imgenenex) revealed with FITC- or tetramethylrhodamine-5,6-isothiocyanate (TRITC)-conjugated donkey anti-mouse IgG (Jackson). They were co-stained with either rabbit anti-human IgM (Dako) revealed with TRITC-conjugated donkey anti-rabbit IgG (Jackson) or TRITC-conjugated cholera toxin B (CTB, Sigma), or with FITC-conjugated CpG-B (Invivogen). Cells were then fixed in 4% paraformaldehyde, cytospined and analyzed with a TCS-NT confocal imaging system (Leica). Control mouse IgG with either FITC-conjugated or TRITC-conjugated donkey anti-mouse, and control rabbit IgG with TRITC-conjugated donkey anti-rabbit did not reveal background fluorescence.

2.5. Isolation of lipid rafts

Based on their insolubility in non-ionic detergent and their low density leading to their separation on a discontinuous sucrose gradient, lipid rafts were isolated from B cell plasma membranes. To this end, tonsillar B cells were first stimulated or not with 10 μg/ml rabbit anti-IgM cross-linked with sheep anti-rabbit IgG (Sigma) for 10 min at 37 °C. After washing at 4 °C in TNE buffer (25 mM Tris–HCl pH7.5, 140 mM NaCl and 1 mM EDTA), cells were incubated for 30 min in 1% Triton X-100 in TNE buffer containing anti-proteases cocktail (Sigma). One ml of supernatant was mixed with 1 ml 85% sucrose, covered with 3 ml of 35% sucrose and 1.5 ml 5% sucrose, and centrifuged for 17 h at 180,000 × *g* at 4 °C. Eleven fractions were collected from the bottom upwards, the latest corresponding to the lipid rafts, and analyzed by Western blot.

2.6. Western blot assay

Cell surface expressed proteins were purified using the Cell Surface Isolation Kit (Pierce) according to the manufacturer's instructions. Samples were separated by SDS-PAGE electrophoresis and proteins transferred on polyvinylidene difluoride (PVDF) membranes. After 1 h of saturation with 5% milk in 0.1% Tween 20 buffer, PVDF membranes were incubated in the presence of either rat anti-TLR9 (Imgenex), rabbit anti-CD20 (Interchim), mouse anti-β-actin (Abcam), rabbit anti-EEA1 (Abcam), horseradish peroxidase (HRP)-conjugated anti-IgM heavy chain (Dako), or biotinylated CTB (Sigma). After washes, HRP-conjugated anti-rat, anti-rabbit or anti-mouse immunoglobulins (all from Jackson), or HRP-conjugated streptavidin (Amersham) were added, revealed using the ECL Advance kit (GE Healthcare) and membranes analyzed with Quantity One Software (BioRad).

For the activation assay, stimulated cells were lysed and proteins separated as specified above. Detection of phosphorylated tyrosine and phospho ERK was performed using mouse anti-phosphotyrosine (Abcam) and mouse anti-phospho ERK (BD Biosciences), revealed with HRP-conjugated anti-mouse immunoglobulins as above.

2.7. Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using chi-squared test for comparisons of percentages. Significance was assessed at $P < 0.05$.

3. Results

3.1. TLR9 is expressed on B cell plasma membrane

Expression of cell surface TLR9 on non-permeabilized B lymphocytes was determined by flow cytometry (Fig. 1A) and assessed as MFI. Mature B cells and transitional B cells isolated from cord blood expressed low level of TLR9 with a MFI of 1.8 ± 0.1 and 2.2 ± 0.1, respectively. TLR9 expression was elevated on peripheral blood B lymphocytes (MFI of 16.1 ± 1.1) and intermediate on tonsillar B cells (MFI of 6.3 ± 0.7).

To become active, endosomal TLR9 must be cleaved. A soluble fragment is generated in the endosomal lumen which can bind to the transmembrane cleaved form. We looked for a cleavage form of plasma membrane TLR9. Proteins from the surface of B cells were biotinylated, and plasma membranes lysed. Biotinylated proteins were purified on NeutrAvidin column and separated by SDS-PAGE. Western blot analysis using anti-TLR9 mAb revealed a 130 kDa band corresponding to the entire form of TLR9 and a supplementary 60 kDa band corresponding to the cleaved fragment (Fig. 1B). Western blots were repeated on the whole cell lysates without biotinylation. Densitometric analyses led to determine the ratio of cleaved form (60 kDa) of TLR9/entire form (130 kDa) of TLR9 (Fig. 1C). It was interesting to note a higher ratio with the biotinylated cell surface proteins indicating that most of TLR9 receptors on the B cell surface are cleaved. Overall, our results suggest

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