ORIGINAL RESEARCH ARTICLES

IMPORTANCE OF TOLL-LIKE RECEPTORS FOR B LYMPHOCYTE SURVIVAL IN PRIMARY SJÖGREN'S SYNDROME

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Abstract

The Sjögren's syndrome is a systemic autoimmune disease characterized by lymphocytic infiltration of the glands responsible for mouth and eyes dryness. A minority of infiltrating B cells is organized as germinal centers while the majority is aggregated into clusters of transitional and marginal zone B cells. The Toll-like receptor 9 (TLR9) recognizes microbial DNA but also, sometimes, the self DNA. It appears to be a key determinant of the survival and differentiation of B lymphocytes. After laser micro-dissection of B cells from salivary glands, analyses by quantitative RT-PCR showed that transitional B cells express high level of TLR9 mRNA unlike B cells from germinal centers. B lymphocytes from healthy donors were sorted by flow cytometry and stimulated in vitro with their TLR9. It induces survival, activation and proliferation associated with phenotypic changes. Transitional B cells exhibited characteristics of the marginal zone, whereas mature B cells expressed follicular germinal center specificities. Finally, IgM and IgG were secreted by both population, but with elevated production of autoantibodies by the transitional B cells. Increased expression of TLR9 by transitional B cells suggests that they may be highly sensitive to differentiate into autoantibody secreting cells through maturation into the marginal zone into the salivary glands. TLR9 might be a target for forthcoming biotherapies.

Le syndrome de Gougerot-Sjögren est une maladie autoimmune systémique caractérisée par une infiltration lymphocytaire des glandes responsable d'une sécheresse buccale et oculaire. Une minorité des lymphocytes B infiltrants est organisée en centres germinatifs tandis que la majorité est regroupée en agrégats de lymphocytes B transitionnels et de la zone marginale. Le Toll-like receptor 9 (TLR9) reconnaît l'ADN microbien mais aussi, parfois, l'ADN du soi. Il apparaît donc comme un élément déterminant de la survie et la différenciation des lymphocytes B. Après micro-dissection laser des lymphocytes B des glandes salivaires, une analyse par RT-PCR quantitative a montré que les lymphocytes B transitionnels expriment fortement l'ARNm de TLR9 contrairement à ceux des centres germinatifs. Des lymphocytes B de donneurs sains ont été triés par cytométrie en flux puis stimulés in vitro par leur TLR9. Il s'ensuit une survie, une activation et une prolifération associées à des modifications phénotypiques. Les lymphocytes B transitionnels présentent des caractéristiques de la zone marginale, tandis que les lymphocytes B matures expriment des spécificités folliculaires des centres germinatifs. Enfin, des IgM et des IgG sont sécrétées par les deux types de population, mais avec une production d'auto-anticorps plus élevée issue de la différenciation des lymphocytes B transitionnels. L'expression accrue de TLR9 par les lymphocytes B transitionnels suggère qu'ils pourraient être particulièrement sensibles à une différenciation en cellules sécrétrices d'auto-anticorps par une maturation vers la zone marginale au sein des glandes salivaires. Le TLR9 pourrait bien devenir la cible des futures biothérapies.

Keywords: Sjögren's syndrome, B lymphocyte, Toll-like receptor 9, differentiation

Introduction

Sjögren's syndrome (SS) is an autoimmune epithelitis (1) hallmarked by a disruption of acinar and ductal structures, the subsequent lymphocytic infiltration of lacrimal and salivary glands (SGs) and their ensuing dryness (2). The role of B cells in the pathophysiology of the disease have attracted interest, since, beyond the production of antibodies (Abs) they accomplish various functions (3-5).

After exiting the bone marrow, B cells settle in secondary lymphoid organs and progress through sequential stages. They are first designated as transitional B cells identified as $CD21^{+++}CD23^{\pm}IgM^{++}IgD^{++}$ (6). Autoreactive transitional B cells are ineffective in their ability to enter the follicles (FOs). However, possibilities exist that they circumvent this checkpoint. The fate of B cells is thus determined by the affinity of their B cell antigen receptor (BCR) to local Ags, prior to transcription factor (TF) commitment. A weak signal favors expression of Notch-2 receptors (7) and confines the cells to the marginal zone (MZ). In contrast, transitional B cells with strong BCR affinity migrate easily to the FO, where Bcl-6 is up-regulated and germinal centers (GCs) initiated. As a result, the activation-induced cytidine deaminase (AID) gene is transcribed, and promotes somatic mutations and recombinations. The ensuing activating signals provoke maturation of plasma cells (PCs), alongside silencing Bcl-6 and increased Blimp-1 expression (8).

Interestingly, transitional and MZ B cells (9) aggregate in SGs (10). Local excess ligation of Toll-like receptors (TLR) may be central to altered differentiation of B cells (11). Yet, there are no clues to how TLR9 would contribute to SS. In this family of pattern recognition receptors (12), it is an endosomal sensor for unmethylated CpG. Because CpG-responding B cells are particularly common in the MZ B cell population (13), and because CpG drives transitional B cells to the production of Abs (14), TLR9 is central to the pathogenesis of autoimmunity (15) and emerges as a candidate mechanism for promoting the maturation of B cells into SGs. The present work was aimed to explore this hypothesis.

Materials and methods

2.1. Patients and controls

SG biopsy specimens were collected from 40 female patients, ranging in age from 31 to 72 years, and fulfilling the American-European Consensus Group criteria for the diagnosis of SS (16). Clinical details were available, and a particular note of the presence of extraglandular complications was made. None of them suffered from associated lymphoma, nor were they taking steroids or immunosuppressive drugs. Children undergoing routine tonsillectomy supplied the tonsils used as lymphoid organs for comparisons with the SG biopsies. Cord blood B cells were also used for in vitro experimental studies. All patients and donors' parents gave informed consent, and the study was approved by our Ethics Committee.

2.2. Quantitative RT-PCRs

All infiltrates of interest were microdissected using the Veritas system (Arcturus). Total mRNA was extracted with a PicoPure RNA isolation kit and amplified with a TransPlex whole transcriptome amplification kit (Sigma). Transcripts for AID, IRF-4, Bcl-6, Blimp-1, Notch-2, and TLR9 were quantified by RT-PCR using the TaqMan gene expression master mix (Applied Biosystems). The primer sets used have been previously described in detail (10). Relative levels of gene expression were measured by quantitative RT-PCR with SYBR Green master mix reagent in an ABI PRISM 7000 sequence detection system according to the manufacturer's instructions. The number of threshold cycles (Ct) was counted using the $\Delta\Delta$Ct method, with GAPDH transcripts as an internal control.

2.3. Cell culture

Transitional B cells and mature naive B cells from cord blood were identified as $CD24^{high}CD38^{high}$ and $CD24^{+}CD38^{+}$, respectively, and sorted on Epics Altra flow cytometer (Beckman Coulter). They were cultured in RPMI1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 200U/ml penicillin and 100μg/ml streptomycin. For stimulation, B cells were seeded at 5.10^5cells/ml in the presence of 0.25μM CpG-ODN 2006 (Cayla-InvivoGen), or on 5.10^5 NIH-3T3 fibroblasts transfected or not with human CD40L gene and treated with mitomycin C, in the presence of 10μg/ml anti-IgM-coated beads (Irvine Scientific) and 100UI/ml rIL-2 (ImmunoTools).

2.4. Flow cytometry

All mAbs were purchased from Beckman Coulter, unless otherwise specified. We used FITC-conjugated anti-CD21, anti-CD23, anti-IgD (BD Biosciences), and anti-IgM (Dako), PE-conjugated anti-CD24, PE-cyanin 5-conjugated anti-CD38, and PE-cyanin 7-conjugated anti-CD19 for cell surface staining. Intracellular staining for Notch2 and TLR9

were performed after permeabilization of the cells using cytofix/cytoperm permeabilization kit (BD Biosciences) with goat anti-human Notch2 (R&D Systems) revealed using FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) and biotinylated anti-human TLR9 (Imgenex) revealed using PE-cyanin7-conjugated streptavidin, respectively. Cells were examined on FC500 flow cytometer (Beckman Coulter) and the results analyzed with CXP analysis software (Beckman Coulter).

2.4. Proliferation assay

Freshly isolated B cells were labeled with 5μM CFSE before stimulation. B cell proliferation was evaluated by flow cytometry on FC500 (Beckman Coulter) measuring the decrease in mean fluorescence intensity of CFSE.

2.5. ELISA

Secreted Igs were detected at day 7. Briefly, 96-well plates were coated overnight at 4°C with 10μg/ml purified goat anti-human Ig (Jackson). After washing with PBS-Tween 0.05% and 1h blocking with PBS-BSA 3%, plates were incubated for 1h at 37°C with the supernatants of cultured cells. After washing, plates were incubated for 1h at 37°C with peroxidase-conjugated goat anti-human Ig (Dako) followed by incubation with O-phenylenediamine. The absorbance was measured at 492 nm.

2.6. Immunofluorescence analysis

Anti-nuclear and anti-cytoplasmic antibodies were detected by indirect immunofluorescence (IIF) analysis. Hep-2 cells were permeabilized and fixed in 20% ethanol plus 80% acetone for 5min. Cells were then incubated with the supernatants of cultured cells for 2h at room temperature, washed, and revealed using FITC-conjugated anti-human Ig (Jackson). Slides were mounted in glycerol and examined with an Axioplan fluorescence microscope (Zeiss).

Results

3.1. Detection of TLR9 in the SGs

Based on the suggestion that TLR9 may be required for MZ B-cell development (17) and the finding that some 40% of transitional B lymphocytes are autoreactive (18), we speculated that their differentiation into MZ B cells might be encouraged by engagement of the TLRs. Quantitative RT-PCR of microdissected B-cell structures detected transcripts for TLR9 in the clusters of aggregated MZ B cells, but neither in the GCs, nor in the tonsil GCs (Fig 1). Functional GCs are sites of AID-dependent somatic hypermutation. The microdissected B-cell infiltrated structures were subjected

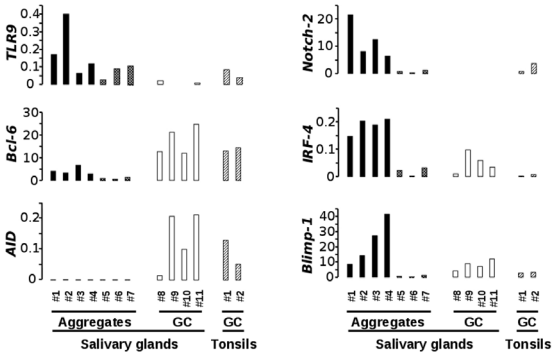

Figure 1 Detection of TLR9, AID, and transcription factors in the salivary glands. Levels of mRNAs were determined by quantitative RT-PCR in tonsillar germinal centers (GC), and in GC and B-cell aggregates from micro-dissected salivary glands.

to quantitative RT-PCR to detect mRNA for AID. The GC-resembling clusters contained AID mRNAs, whilst the GC-nonresembling clusters did not. This dochotomy was substantiated by the differential expression af TFs. Bcl-6 prevents untimely maturation, and IRF-4 enables differentiation of B cells into PCs. Naïve and memory B cells express low levels of Bcl-6, in contrast to FO B cells that up-regulate it throughout the GC passage. Bcl-6, and, to a lesser degree, IRF-4 were mainly expressed in the GCs, supporting their classification. In contrast, the aggregates contained more mRNAs for IRF-4 and Blimp-1 than of Bcl-6. However, only some showed high levels of mRNAs for Blimp-1 and IRF-4, suggesting that PCs were not prevented from emerging in the remaining. In these aggregates, the Notch-2 TF, which is involved in the final stages of MZ B cell maturation, predominated in four infiltrates. Its weak expression implies that, because of the low Bcl-6, as opposed to the high Blimp-1 and IRF-4 expression, these B cells are ready to differentiate into MZ-like B cells and/or into PCs.

3.2. Proliferative response of transitional B cells to TLR9 stimulation

In order to evaluate the role of TLR9 stimulation in the survival and differentiation of B cells, transitional $CD24^{high}CD38^{high}$ B cells and mature $CD24^{+}$ $CD38^{+}$ B cells were sorted from cord blood and stimulated with type B CpG-ODN. Transitional B cells proliferated in response to TLR9 stimulation. Frequencies of

proliferating cells were closed to those observed with mature naive B cells (Fig. 2). Three division cycles were at least induced after 4 days of stimulation with CpG-ODN, while the response was lower following activation with

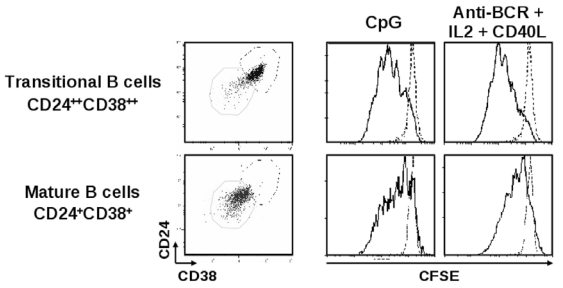

Figure 2 The proliferative response of transitional B cells. Transitional CD24high CD38high B cells and mature CD24+CD38+ naive B cells were sorted by flow cytometry for in vitro stimulation. They were stained with CFSE and stimulated for 4 days with 0.25μM CpG-ODN 2006 or with 10 μg/ml anti-IgM and 100UI/ml rIL-2 on CD40L-transfected fibroblasts. The proliferative response was evaluated on flow cytometer by the dilution of CFSE expression. Representative experiments are shown where dotted histograms correspond to CFSE staining at the onset of the culture.

combined CD40L, anti-IgM Abs and IL-2. These preliminary experiments indicate that transitional and mature naive B cells proliferated similarly in response to TLR9 stimulation.

3.3. Particular maturation of transitional B cells in response to TLR9 stimulation

After two days of stimulation with CpG-ODN, the maturation status of transitional B cells was established. There was a downregulation of the density of CD24 and CD38 molecules leading to the emergence of a $CD24^{-}CD38^{+}$ subpopulation. These cells were IgM^{high} IgD^{low} $CD21^{high}$ $CD23^{low}$ and Notch 2^{high}. Mature naive B cells did not differentiate similarly. Thus, the emerging $CD24^{-}CD38^{+}$ B cells were IgM^{low} IgD^{high} $CD21^{low}$ $CD23^{low}$ and $Notch2^{low}$ (Fig. 3). These results strongly suggest that MZ-like B cells can arise specifically from transitional B cells in response to TLR9 stimulation.

3.4. Terminal differentiation of transitional B cells following TLR9 stimulation

The antibody production of transitional B cells stimulated with CpG-ODN was then assessed. Following a 7-day period, stimulated B cells differentiated into antibody producing cells with elevated amount of IgM and low levels of IgG secreted in the supernatants (Fig. 4). Mature naive B cells were sensitive to TLR9 activation as well, but leading to the secretion of IgM and very few IgG. In contrast, transitional and mature naive B cells stimulated with

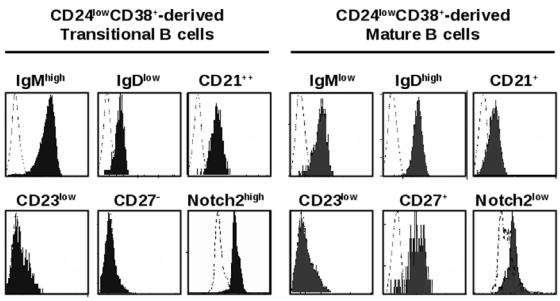

Figure 3 The specific maturation of transitional B cells. Transitional CD24high CD38high B-cells and mature CD24+ CD38+ naive B cells were sorted form cord blood and stimulated with 0.25μM CpG-ODN 2006 or with 10μg/ml anti-IgM and 100UI/ml rIL-2 on CD40L-transfected fibroblasts. After two days, the phenotype of the emerging CD24-CD38+ population was determined. Representative experiments are shown where dotted histograms correspond to isotype controls.

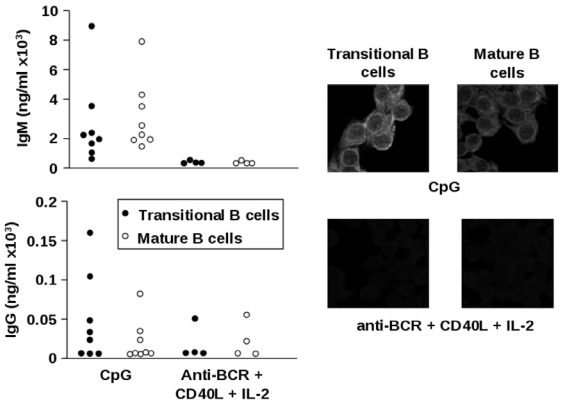

Figure 4 The terminal differentiation of transtional B cells. Transitional CD24high CD38high B cells and mature CD24+ CD38+ naive B cells were sorted and stimulated for 7 days with 0.25μM CpG-ODN 2006 or with 10μg/ml anti-IgM and 100UI/ml rIL-2 on CD40L-transfected fibroblasts. The production of IgM and IgG were determined by ELISA, and the polyreactivity evaluated by immunofluorescence staining of Hep2 cells. Representative experiments are shown.

CD40L, anti-IgM and IL-2 produced lower levels of IgM and of IgG. However, antibody specificity determined by immunofluorescence staining of Hep2 cells was strikingly different between transitional and mature naive B cells. A higher frequency of autoantibodies was produced by CpG-stimulated transitional B cells compared with mature naive B cells. Interestingly, autoantibodies were hardly detectable when transitional B cells or mature B cells were stimulated with CD40L, anti-IgM and IL-2 stimulation.

Discussion

Our data suggests that TLR ligands are invol-

ved in the positive selection of self-reactive B cells, within the SGs of patients suffering SS. TLR9 favors the differentiation of transitional B cells. During the autoimmune epithelitis proponents, the disease may be triggered by coxsackie virus (19) or retrovirus (20) infection of epithelial cells. As a consequence, microbial agents may contribute to the engagement of TLR9 into B cells, and thereby to their transformation into MZ B cells. TLR9 stimulation can thus activate transitional B cells to differentiate into antibody- secreting cells through the development of MZ-like B cells. Interestingly, mature B cells can also respond to TLR9 stimulation but through the development of follicular-like B cells. It appears that peripheral B cells at the immature transitional stage are especially sensitive to differentiate into MZ B cells when TLR9 signaling is engaged.

This raises also the possibility that aberrant stimulation of TLR9 in patients could favor the development of MZ B cells qualified to participate to the pathogenesis (21). This hypothesis is supported by the fact that the majority of infiltrated B cells is aggregated and comprised of transitional-like and MZ-like B cells. These populations express high level of IRF-4 and Notch-2 mRNA but not Bcl-6. IRF-4 is a transcriptional factor which negatively regulates the Bcl-6-dependent germinal center response (22), whereas Notch-2 is indispensable for the development of transitional B cells into MZ B cells (23). The presence of TLR9 on aggregated B cells might thus be an important component for the differentiation of transitional B cells into autoantibody secreting MZ B cells.

Finally, we demonstrated that TLR9 activation of transitional and mature cord blood B cells induces survival signals, up-regulates expression of activation markers and triggers a proliferative response followed by terminal differentiation associated with switch commitment for the secretion of IgM and IgG. One may conclude that, not only do the TLRs deliver a tonic signal to keep B lymphocytes alive, but they also confer those autoreactive B cells with a MZ-like phenotype. Such TLR9 might then become interesting target for treatment of primary SS. The question arises as to whether blockade of TLR9 would ameliorate the clinical features of these diseases.

Acknowledgements

This work was supported by the Institut Français de Recherche Odontologique. Thomas Guerrier was supported by a grant from the Fondation pour la Recherche Médicale. Thanks are due to Geneviève Michel and Simone Forest for their help with the typing of the manuscript.

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