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Retinoic acid and α-galactosylceramide regulate the expression of costimulatory receptors and transcription factors responsible for B cell activation and differentiation

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ABSTRACT

Mature naïve B cells possess a number of BCR coreceptors and other antigen receptors, including the MHC class I-like molecule CD1d, but little is known of the response of B cells to stimulation by the CD1d ligand, α-galactosylceramide (αGalCer). Previously, we showed that all-trans-retinoic acid (RA) increases the expression of CD1d and the magnitude of CD1d-mediated antibody production *in vivo*. Potential mechanisms could include changes in the expression of costimulatory molecules and transcription factors that regulate plasma cell formation. In the present study, we have used isolated purified B cells and *in vivo* studies to demonstrate that αGalCer and RA initiate a regulated expression of several genes essential for B cell activation and differentiation, such as Pax-5, Blimp-1, IRF-4 and activation-induced cytidine deaminase (Aid). Moreover, whereas αGalCer mainly increased the expression of Pax-5, CD40 and CD86 that are critical for B cell activation, RA predominantly increased $CD138^+$ and Fas^+–PNA^+ B cells, which represent more advanced B cell differentiation. It is also noteworthy that αGalCer enriched a CD19hi subset of B cells, which represent B cells with more differentiated phenotype and higher potential for antibody production. *In vivo*, treatment with αGalCer enriched the CD19hi population, which, after sorting, produced more anti-TT IgG by ELISPOT assay. Together, our data demonstrate that RA and αGalCer can regulate B cell activation and differentiation at multiple levels in a complementary manner, facilitating the progress of B cells towards antibody secreting cells.

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Introduction

Vitamin A and its active metabolite all-trans-retinoic acid (RA) play essential roles in the regulation of innate and adaptive humoral and cell-mediated immunity (Allie et al. 2013; Chen et al. 2008; Engedal et al. 2004; Ertesvag et al. 2009; Iwata et al. 2003; Mora and von Andrian 2009; Morikawa and Nonaka 2005; Ross et al. 2011). Previous studies in tetanus toxoid (TT)-immunization animal models have shown that RA, alone or combined with other immune stimuli, can increase the antigen specific immunoglobulin (Ig) production and promote durable memory responses (Chen et al. 2011; Ma et al. 2005).

B cells, as a type of professional antigen-presenting cells, receive and transmit signals through a variety of receptors including the B cell receptor (BCR), MHC molecules and Toll-like receptors (Chen and Ross 2005; Lang et al. 2001; Minguet et al. 2008). Moreover, several other cell-surface proteins, such as the costimulatory molecules CD40 and CD86, and the BCR co-receptor CD19, also regulate the B cell response to antigens (Baracho et al. 2011; Ertesvag et al. 2009; Sato et al. 1995). Recently, the MHC class I-like molecule CD1d has been shown to be expressed on B cells and capable of presenting glycolipid antigens to modulate B cell function and immune response (Barral et al. 2008; Chen et al. 2011; Devera et al. 2008; Dufour et al. 2008; Lang et al. 2008). It is interesting to note that in mouse spleen, B cells constitute a major cell population expressing CD1d (Chen et al. 2011). Although ample studies have recognized the role of CD1d in NKT cell activation, its role in B cell antigen-presentation and differentiation remains unknown.

CD1d recognizes a natural ligand, α-galactosylceramide (αGalCer), which serves as a prototype of a class of glycolipid antigens that activates iNKT cells when presented to the cell-surface CD1d molecule (Godfrey and Rossjohn 2011; Mallevaey and Selvanantham 2012; Matsuda et al. 2008). Previously, we have reported that RA is a potent regulator of CD1d gene

Abbreviations: αGalCer, α-galactosylceramide; AID, activation induced deaminase; GC, germinal center; IRF, interferon regulatory factor; RA, all-trans-retinoic acid; TT, tetanus toxoid.

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expression in monocytic antigen presenting cells (Chen and Ross 2007); and αGalCer markedly increases B cell proliferation *in vitro*, and synergizes with anti-μ, which, through ligation of the BCR, results in B cell activation (Chen et al. 2011). Moreover, αGalCer *in vivo* increases the antigen-specific antibody production in a TT-immunized mice, and treatment with RA enhances αGalCer-mediated response at both primary and secondary antibody response periods (Chen et al. 2011).

In the present study, we have used a previously defined *in vitro* B cell model as well as *in vivo* studies in intact mice to examine the mechanisms of RA in the regulation of CD1d-mediated B cell activation and differentiation. We hypothesized that one way in which RA may promote αGalCer-mediated B cell differentiation is through the expression of costimulatory molecules, and/or transcription factors that promote B cell activation and differentiation towards plasmacytic cells. We report here that RA and αGalCer are both potent regulators of B cell differentiation. Moreover, the level of CD19 expression helps to define a subset of B cells (CD19hi B cells) that are expanded in the presence of αGalCer and RA; these cells express a higher level of CD1d protein and are enriched in factors needed for B cell differentiation, as identified by CD138 and other markers of germinal center (GC) B cells. Thus, RA and αGalCer promote a B cell differentiation program that includes heightened expression of costimulatory molecules, BCR coreceptor CD19, and critical transcription factors, which are all necessary for improved B-cell antibody production.

Materials and methods

Animals, preparation of splenocytes, B cell isolation and cell culture

Animal protocols were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Adult female BALB/c mice (approximately 8 weeks old, from Charles River Laboratories, Wilmington, MA) were used for animal experiment, and to obtain splenic B cells for *in vitro* and *ex vivo* study as described previously (Chen et al. 2011).

Briefly, mouse spleens were minced and a single cell suspension was incubated with an antibody cocktail that removes all other cells and retains B cells from the spleen, a negative selection process that enriches B cell without affecting B cell activity (Stemcell Technology, Vancouver, Canada). The purity of isolated B cells was approximately 95% affirmed by flow cytometry analysis based on CD19 staining. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 5×10^{-5} M β-mercaptoethanol from Invitrogen (Grand Island, NY).

For the *in vivo* animal experiments, 8-week-old female mice were immunized with tetanus toxoid (TT, 10 μg/mouse) subcutaneously in the lower flank, with or without αGalCer (2 μg/mouse). Some groups of mice were assigned to treatment with oral doses of RA (37.5 μg/mouse in 10 μl of canola oil) for 5 days. After 4 weeks, the mice received a second dose, 10 μg of TT, and the spleens were collected 7 days later for analysis. Half of the spleen tissue from each animal was used to isolate mononuclear cells for flow cytometry analysis or cell sorting, the other half of the spleen was embedded with OCT compound and stored at −80 °C for immunofluorescence staining.

Antibodies and reagents

Antibodies used for flow cytometry analysis were: CD19-PEcy7, CD19-APC (clone 1D3), Fas-PE and CD138-PE (BD Biosciences, San Jose, CA), CD1d-FITC and IgD-Alexa 647 (eBiosciences, San Diego, CA), IgG1-Alexa 488 (Invitrogen, Grand Island, NY), and CD19-FITC (clone MB19-1 from BioLegend, San Diego, CA). Peanut agglutinin (PNA)-fluorescein was from Vector laboratories Inc. (Burlingame, CA). αGalCer (Alexis Biochemicals, San Diego, CA), and βGalCer (Sigma–Aldrich, St. Louis, MO), were both used at the concentration of 100 ng/ml. The anti-μ antibody was utilized in B cell proliferation (1 μg/ml; cat#115-006-075 from Jackson Laboratory, Bar Harbor, ME), *Escherichia coli* LPS (100 ng/ml) served as a pan-B cell stimulator (055:B5, from Sigma–Aldrich).

Flow cytometry analysis and sorting

For each assay, 10^5 cells were incubated with 0.1 μg of fluorescent-labeled antibody for one hour at room temperature. Cell proliferation activity was measured by CSFE labeling as described previously (Chen and Ross 2005). Cell viability was tested by trypan blue, and propidium iodide was used to identify and gate live cells for flow cytometry analysis. Non-stained and isotype-control antibody-stained cells were used to determine the gates for analysis with the Accuri C6 software.

To sort B cells based on their CD19 expression, B cells were stained with anti-CD19-PEcy7 antibody and gated into CD19hi and CD19lo subgroups. Approximately 10^6 cells, phenotype hi or lo, were collected using BD Cytopeia Influx sorter for further analysis. In order to validate CD19hi/lo populations, two different anti-CD19 antibodies raised by different antigenic epitopes (clone ID3 from BD Biosciences, and MB19-1 from BioLegend) were used for detection of CD19, and yielded similar results.

Quantitative real time-PCR (qPCR)

B cell RNA was extracted using Qiagen mini kit and subjected to qPCR (Bio-Rad). The relative expression level was determined after normalizing to the expression of the housekeeping genes HPRT and tubulin-1. The PCR condition and the primer sequences for Pax-5, Aid (or *Aicda*), Blimp-1 and HPRT have been previously reported (Chen and Ross 2005). IRF-4 primers were, forward: 5′-TGATCGACCAGATCGACAGC, reverse: 5′-GTTATGAACCTGCTGGGCTGG; tubulin-1 primers were, forward: 5′-ATGGAGCCCTGAATGTTGAC, reverse: 5′-CTCAAAGCAAGCATTGGTGA.

Immunostaining

Spleen tissues were embedded in OCT compound and cryosections were prepared (8 μm). The sections were then fixed with ice-cold acetone for 10 min and subjected to staining with fluorescent conjugated antibodies to IgM and Ki-67 in PBS with 1% BSA for 2 h at room temperature. Isotype control serum was used as negative control. The section was analyzed by digital microscopy using CellSens image software from Olympus.

ELISPOT assay

Spleen mononuclear cells were stained with CD19-PEcy7 antibody and subjected to flow sorting to collect CD19hi and CD19lo B cells. About 10^6 cells were used to perform the ELISPOT assay as described previously (Chen et al. 2011; Ma et al. 2005).

Statistical methods

Means, SE, and *P* values were determined using Prism software (GraphPad Software, Inc.). *P* values were calculated by *t*-test or ANOVA followed by Tukey's post hoc analysis unless specified in the legend. $P < 0.05$ was considered significant.

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