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Retinoic acid promotes mouse splenic B cell surface IgG expression and maturation stimulated by CD40 and IL-4

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Abstract

Retinoic acid (RA) increases antibody production in vivo but its role in B-cell activation is unclear. In a model of purified mouse splenic B cells stimulated by CD40 coreceptor (as a surrogate of T cell co-stimulation), IL-4, a principal Th-2 cytokine, and ligation of the B-cell antigen receptor, CD40 engagement or IL-4 alone induced B-cell activation indicated by increased IgY1 germline transcripts, cell proliferation, and surface (s)IgG1 expression, while triple stimulation with the combination of anti-CD40/IL-4/anti- μ synergized to heighten B-cell activation. Although RA was growth inhibitory for anti-CD40-activated B cells, RA increased the proportion of B cells that had more differentiated phenotypes, such as expression of higher level of activation-induced deaminase, Blimp-1, CD138/syndecan-1 and sIgG1. Overall, RA can promote B-cell maturation at the population level by increasing the number of sIgG1 and CD138 expressing cells, which may be related to the potentiation of humoral immunity in vivo.

Keywords

retinoic acid; germline transcript; cell division; CD138; Blimp-1; AID

1. Introduction

The two-signal theory of B-cell activation states that both B-cell antigen receptor (BCR) and CD40 engagement, accompanied by cytokines such as IL-4, are required to induce B-cell activation and functional maturation [1-5]. CD40 is a member of the tumor necrosis factor receptor superfamily, and is expressed on variety of cell types including B cells. It mediates the crosstalk between T and B cell through binding to its ligand on T cells [2,6]. IL-4 is a principal regulatory cytokine for B-cell differentiation produced by T helper-2 cells, natural killer cells and NKT cells [7,8]. Stimulation of B cells through CD40 and cytokine IL-4 not only induces a higher level of cell proliferation, but also induces an undefined subset of stimulated B cells to enter a differentiation pathway that leads to the formation of antibody-secreting cells [3,9]. Although signaling through B-cell receptor (BCR), CD40 and IL-4 involve distinguishable pathways and independent effects on B-cell activation, it is the summation of crosstalk between these pathways, and with other signaling networks, that results in a mature B-cell antibody response [10,11].

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All-*trans*-retinoic acid (RA), a natural bioactive metabolite of vitamin A [12], regulates a broad range of biologic processes through binding to specific nuclear retinoid receptors [13], present in many cells types including T and B cells [14,15]. RA is well known for its ability to inhibit cell proliferation and regulate the expression of a large number of genes [13]. Among its many actions, RA plays a crucial role in maintaining an adequate immune response [16]. A dietary deficiency of vitamin A results in dysregulation of both innate and acquired immune responses [17-21], while, conversely, vitamin A and RA supplementation have significantly reduced disease severity and restored the host immune response in animals and humans [22-24], supporting the significance of vitamin A and RA in immune system functions. Moreover, RA can potentiate antibody production by normal animals after vaccination *in vivo* [25-27]; however, the underlying mechanisms are not clear.

Despite the apparently positive effects of RA on antibody production, RA has been shown to be a negative regulator of B cell proliferation in several models, including in B-cells stimulated to proliferate by engagement of the B-cell receptor (BCR) and the BCR-coreceptor CD38 [28,29], and by nonspecific stimulation such as phorbol esters [30]. RA has also been shown to prevent the spontaneous apoptosis of naïve B cells [31]. In previous studies, the presence of a physiological concentration of RA during B cell stimulation through BCR and CD38, a BCR co-receptor [32], reduced the proliferation of B cells while it still induced the differentiation of a population of B cells into sIgG-expressing cells [29]. Another important costimulatory molecule for B-cell activation is CD40, which is activated *in vivo* by the CD40-ligand expressed on activated T cells [2]. This interaction mediates and prolongs the communication between B and helper T cells and provides the secondary signal to activated B cells that promotes their survival and differentiation [2,4,6,7]. Previous reports using several models have shown fairly consistently that CD40 ligation alone can induce B-cell proliferation in human and mouse B cells [4,33], and that CD40 ligation plus IL-4 can promote B-cell isotype switching to IgE and IgG1 [34]. RA has also been reported to increase Ig production by tonsillar B cells stimulated with anti-CD40 in the presence of IL-10 [15]. It is further believed that CD40 ligation along with IL-4 can augment the primary signal mediated by antigen binding to the BCR, allowing the processes of B-cell activation, survival and differentiation to proceed towards a productive humoral immune response [11,35]. However, the ability of RA to regulate CD40 signaling alone and in cooperation with BCR signaling and cytokine stimulation has not yet been reported. In the present study, we have used a model of isolated splenic B cells to examine how RA affects the response of primary B-cells stimulated by T cell signals, such as CD40 ligation or IL4 stimulation, both alone and in combination with BCR engagement. We show that even though RA inhibited B-cell $\gamma 1$ germline transcript ($\gamma 1$ GLT) expression and cell proliferation, which are initial events after stimulation, in response to CD40 ligation, RA did not affect the B-cell proliferation in response to IL-4, suggesting the differential involvement of RA in these different signaling pathways. Despite the early reduction in B-cell proliferation in RA-treated cells, more of the RA-treated cells expressed sIgG1 and CD138 at later times, as well as activation-induced deaminase (AID) and Blimp-1 gene expression. These results suggest that RA may redirect a portion of activated B-cells toward a more differentiated stage, which may account for increased IgG production as has been observed *in vivo*.

2. Materials and methods

2.1. Reagents

All-*trans*-retinoic acid (Sigma, St. Louis, MO) was prepared as a concentrated stock in ethanol and diluted with medium just before application. Antibodies used for B cell stimulation were anti-CD40 (BD Biosciences, San Jose, CA), and anti-mouse IgM F(ab')₂ fragment (Cat. #115-006-075, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). IL-4 was from R&D Systems (Minneapolis, MN). Carboxyfluorescein diacetate, succinimidyl ester (CFSE)

was from Molecular Probes. Anti-mouse IgG1-PE antibody was from Molecular probe and anti-mouse CD19-PE-Cy7 antibody was from BD biosciences.

2.2. B cell isolation and culture

Naïve splenic B cells were isolated from adult (>8 wk) C57BL/6 male or female mice (Charles River Laboratories, Willmington MA) by negative selection using antibodies against non-B cells coupled with density beads (StemCell Technologies, Vancouver, Canada). The purity of the recovered B cells (CD19⁺/B220⁺/CD38⁺) was above 95%. Isolated B cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5×10^{-5} M β -mercaptoethanol, 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA), at 37°C in a 5% CO₂/95% air incubator. For each experiment, RA and stimuli were added to cells concomitantly, and cells were harvested as indicated in each figure.

2.3. B cell proliferation assay

Splenic B cells were added to 96-well flat-bottomed plates (Corning, 2×10^5 cells/well) and treated with and without stimuli (described in figure legends), without and with addition of RA. RA was added at a concentration of 20 nM in all experiments, which is similar to the physiological concentration in plasma [36]. Cells were cultured for 72 h. [³H]-Thymidine (0.2 μ Ci/well, Amersham Biosciences Corp, Piscataway, NJ) was added to cells for the last 6 h of culture, after which the cells were harvested to determine the incorporation of [³H]-thymidine by liquid scintillation spectrometry [29].

2.4. RNA isolation and reverse transcription-PCR

Total RNA was isolated from cultured B cells using RNeasy mini kit (Qiagen). 0.5 μ g of total RNA was subjected to reverse transcription. One-tenth of the RT reaction was subjected to ³³P-labeled PCR [37], and the data was confirmed by quantitative real-time PCR with SYBR Green labeling (BioRad, Hercules, CA). Primers used in the study were Ig γ 1 germline transcript [38], AID [29] and Blimp-1. The primer sequences for Blimp-1 were: forward, 5-TGGACATGGAGGACGCGGATATG, reverse, 5-GGTGGCAGGGATAGGCTTAATAG. GAPDH was also amplified at the same time for internal control [37].

2.5. Flow cytometry analysis

All staining was carried out using 0.1 μ g of each antibody for 1×10^5 cells at 4° for one hour. Anti-CD19-PEcy7 and anti-IgG1-PE was used to detect the surface IgG1 B cells. Cells were washed and resuspended in PBS. Propidium iodide was added to cells 5 min before flow analysis to ensure the viability of cells. Isotype-matched control antibodies were used to set up the gates. Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

For tracking cell division, purified B cells were stained with CFSE and then subjected to treatment and cultured for up to 5 days. The cell division was assessed at time of harvesting [29].

Cell sorting was performed using a Coulter Elite flow cytometer. To separate cells based on physical properties of forward angle light scatter and side scatter, the gates were set as later illustrated (see figure 3B). Cells in the A and B gates were collected separately for gene expression study. To remove the pre-existing CD138 positive cells, spleen cells were stained with an anti-CD138-PE antibody. CD138-negative cells were collected to perform the experiment described in figure 5.

2.6. Statistics

Data are reported as the mean $\pm$ SEM. Groups were compared by Student's *t*-test with two-tailed distributions for two groups of samples with equal variance. *P* values of less than 0.05 were considered significant.

3. Results

3.1. RA consistently inhibits $\gamma 1$ germline transcript expression during B cell activation induced by CD40 and IL-4

CD40 engagement is an important step that provides the secondary signal for B cell activation [2-4,7], and IL-4 plays essential roles in the stage specific activation/maturation of B cell [39]. We first tested how a physiological concentration of RA (20 nM) would affect the CD40 and IL-4-mediated B-cell activation by examining the expression of $\gamma 1$ GLT, one of the first characteristics of B-cell activation, in isolated mouse splenic B cells. Cross-linking of CD40 by an agonistic antibody rapidly increased $\gamma 1$ GLT expression (detected at 1.5 h after stimulation, Figure 1A) and induced a steady increase in the level of $\gamma 1$ GLT during the 48-h experiment, whereas B cells without stimulation had a barely detectable level of $\gamma 1$ GLT expression (shown in Fig. 1B, Con). Similarly, IL-4 was also a strong inducer of $\gamma 1$ GLT (Fig. 1B). CD40 ligation was additive with BCR ligation while it synergized with IL-4 to induce a much higher level of $\gamma 1$ GLT expression (Fig. 1B). Addition of RA significantly inhibited the increase of $\gamma 1$ GLT level in each of these conditions. These data support the involvement of a physiological level of RA in the regulation of $\gamma 1$ GLT expression induced by multiple cell surface receptor-initiated pathways, such as anti-CD40, anti- μ , IL-4/IL-4 receptor.

3.2. RA differentially affects B-cell proliferation under the influence of CD40 and IL-4

Next we tested how a physiological concentration of RA (20 nM) would affect the CD40 and IL-4 stimulated B-cell activity by monitoring the proliferation of mouse splenic B cells. Cross-linking of CD40 and BCR stimulation both significantly induced B-cell proliferation (Figure 2), while the combination was strongly synergistic. RA significantly reduced B-cell proliferation induced by each of these stimuli alone and in combination. IL-4 alone was a significant mitogen for B cells in terms of [3 H]-thymidine incorporation (Fig. 2), and IL-4 also synergized with CD40 and added to BCR ligation, resulting in a higher level of thymidine incorporation. However, RA did not inhibit IL-4-induced B-cell proliferation, and no longer inhibited the proliferation induced by CD40 ligation when IL-4 was added. These results indicate that the IL-4 signaling pathway that controls B-cell proliferation can be regulated separately from the pathways activated by CD40 and BCR.

B cells were also subjected to CFSE labeling to monitor cell division within the 5-day experimental period [40]. A consistent pattern was observed comparing CFSE dilution with B-cell proliferation results (data not shown), which confirmed the differential effect of RA on CD40 and IL-4 induced B-cell growth.

3.3. RA positively regulates B-cell differentiation after combined stimulation

Although CD40 and IL-4 each exert distinctive effects on B-cell activation and differentiation, they are insufficient for complete B-cell differentiation [41]. Therefore we combined these factors with anti- μ , the primary signal for B cells, to study their combined effects on B-cell differentiation (referred to as triple stimulation). Triple stimulation resulted in a marked increase of sIgG1 expression in the whole population, which included an increase in the percentage of sIgG1-positive cells and in the mean fluorescence intensity per cell. While RA had no effect alone, it significantly augmented the level of sIgG1 induced by triple stimulation (Figure 3A).

B cells were then analyzed for the distribution of subpopulations. After triple stimulation, the B-cell population as a whole became more heterogeneous, as evidenced by a spread in size and granularity (side and forward scatter, respectively) when compared to the unstimulated control cells (data not shown). We categorized the B cells into three groups (A, B and C) based on their characteristics on the forward and side scatters (Fig. 3B). As gate C contains less than 3% of cells, only gates A and B were further analyzed and shown in Tables 1 and 2. After triple stimulation the percentage of B cells in the B gate increased from 7.1 to 18.9%, while after triple stimulation in the presence of RA, 23.7% of cells were in the B gate. Triple stimulation also significantly increased the percentage of cells in the A gate, from an average of 39.9 to 61.6%, while RA reduced this group of cells (Table 1).

We then determined the differentiation-related characteristics of the A and B gate cells in terms of their sIgG expression and CFSE dilution in stimulated B cells both with and without RA. After triple stimulation, a much higher percentage of cells in the B gate (composed of larger sized cells) were sIgG-positive cells (Table 2). Moreover, fewer of these cells had undergone CFSE dilution. B cells that were smaller and resided within the A gate had a lower percentage of sIgG1 expressing cells and a higher percentage of CFSE diluted cells. The presence of RA significantly decreased the % of cells with CFSE dilution in both gates while RA increased the sIgG1 expression in A gate cells ($p < 0.05$). B gate cells already had a very high percentage of cells express sIgG1 and no further change was observed. These data demonstrated that the combined triple stimulation through BCR/CD40/IL-4 significantly altered B cell subpopulations, while RA consistently promoted the development of a greater number of larger cells located in the B gate that expressed a higher level of sIgG1 and had undergone fewer cell divisions.

3.4. RA regulates gene expression in B cell populations

To study the expression of activation-associated genes in these two groups of B cells, we determined the gene expression of AID, an enzyme required for class switch recombination, and Blimp-1, a differentiation-specific transcription factor, in these two groups of B cells in addition to $\gamma 1$ GLT. We reasoned that cells in the B gate, if representing a more differentiated population, would express these factors at a higher level. B cells activated by triple stimulation without or with RA were sorted on day 5 and two fractions of cells (A and B gates, as shown in Fig. 3B) were collected. Total RNA was extracted from these cells, and the $\gamma 1$ GLT, AID and Blimp-1 gene expression levels were determined. As shown in Figure 4A, B and C, $\gamma 1$ GLT, AID and Blimp-1 showed a similar pattern to one another. Whereas $\gamma 1$ GLT, AID and Blimp-1 mRNA were readily detectable in B-cells in the B gate, they were very low or virtually absent in the B-cells within the A gate. RA did not alter Blimp-1 expression level, but it increased the level of AID mRNA and decreased the $\gamma 1$ GLT level. Thus, each of these genes associated with B-cell differentiation — $\gamma 1$ GLT, AID and Blimp-1 [29] — was enriched or exclusively located within the B-gate B cells, the group that was expanded by RA (see Table 1). These data further suggested that the B gate contained cells that had reached more differentiated stages along the pathway to the formation of plasmacytic cells. To further test this, we evaluated whether RA promotes triple-stimulated B cells to differentiate into cells resembling early plasma cells by measuring the cell-surface expression of CD138 (syndecan-1), a marker of plasmacyte differentiation. As shown in Figure 5A, on day 5 of culture, CD138-positive cells were increased by triple stimulation with anti-CD40/anti- μ /IL-4 and further up-regulated by RA, consistent with the expression pattern of sIgG1. Moreover, >90% of the CD138-positive cells were located in the B gate (Figure 5B), further demonstrating that the B gate contained the more differentiated B-cells displaying this marker of the plasmacytic phenotype.

4. Discussion

Relatively little is known regarding the interactions of RA with CD40 and IL-4 in immune cell activation. In previous studies, RA was shown to induce CD40 protein expression on promyelocytic leukemic cell lines, HL-60 and NB4, during their differentiation [42,43], suggesting a regulatory role of RA in the activation phase of the humoral response through CD40 signaling [44]. RA also was shown to inhibit IgE production by human peripheral blood mononuclear cells stimulated by cross-linking of CD40 and exposure to IL-4 *in vitro*, but RA failed to inhibit the IgE response of ovalbumin-sensitized mice *in vivo* [45-47]. In the present study, we have examined the effects of RA on T cell derived signals mediated through CD40 and IL-4 in murine B-cells during the activation and differentiation processes leading to sIgG expression.

It is known that B cells undergo differentiation and isotype switching soon after stimulation. Germline transcript expression initiates the process of immunoglobulin (Ig) class switch recombination and therefore is a required step for Ig expression [48]. In our present studies, we first showed that RA markedly reduces stimulation-induced $\gamma 1$ GLT expression, regardless of the stimuli we used (Fig. 1). Although germline Ig gene transcription is understood to be an essential prerequisite step in the process of immunoglobulin gene class switch recombination [48], it is not clear whether quantitative differences in the level of GLT and its persistent expression are physiologically significant. Indeed, we and others have reported that the level of $\gamma 1$ GLT does not necessarily reflect the level of class switch recombination and subsequent antibody production [29,48,49]. In this study and our previous work [29], a reduction in the expression of $\gamma 1$ GLT by RA could be detected very early in the B-cell differentiation process (e.g., by 1.5 h in Fig. 1A). It was shown that $\gamma 1$ GLT expression is regulated by the NF κ B family of transcription factors [50]; thus, the suppression of NF κ B activity by RA may play a role in the negative regulation of $\gamma 1$ GLT expression level [37,51]. We were, however, surprised that the IgG1 GLT expression increased very shortly, as early as 1 h, after B-cell stimulation. Based on our experimental results, GLT is a rapid signal that appears to sense the stimulation of the B-cell and promotes the class switch recombination (IgG expression), observed at later times.

Our experiments also showed that the effect of RA on B-cell proliferation is stimulus dependent. Ligation of CD40 alone increased B-cell proliferation and division (Fig. 2), while BCR engagement combined with CD40 and IL-4 stimulation significantly heightened B cell proliferation. In this model, RA inhibited B-cell proliferation induced by ligation of CD40 and BCR, but RA did not inhibit proliferation induced by IL-4. Previous reports have shown that the transmission of signals from CD40 and the BCR mainly involves the mitogen-activated protein (MAP) kinase and nuclear factor kappa-B (NF- κ B) pathways [33,35,52-54], while signal transduction from IL-4 and its receptor is mediated primarily through the activation-induced phosphorylation of STAT-6, by which STAT-6 becomes competent as a nuclear transcription factor [34]. Through shared and/or different pathways, the signals generated through CD40 and the BCR, and by IL-4, may stimulate B-cell activation/differentiation to a state that cannot be achieved by each alone. It is plausible that RA selectively reduces MAP and NF- κ B signaling, while having little or no effect on STAT-6 activation. It is also worthy of mention that the reduction in B-cell growth by RA was not due to induction of apoptosis (data not shown). Overall, the ability of RA to regulate cell cycle progression may play a role in inhibiting cell proliferation while promoting differentiation [28,55].

To further investigate B-cell responses, we used flow cytometric analysis to distinguish how CD40 ligation, IL-4 stimulation, and both of these stimuli combined with anti- μ alter the characters of B cells in the absence and presence of RA (Fig. 3). Analysis at this level is important because proliferation measured by 3 H-thymidine incorporation and germline gene

recombination measured by PCR assays can provide information only the average response of the B-cell population as a whole, whereas differences within the population might be critical regarding cell differentiation and IgG production. Previously we have shown that B-cell subsets analyzed according to forward angle light scatter, a parameter determined mainly by cell size, are representative of differences in B-cell differentiation stages [29]. Our current results showed that the B-cell population became relatively more diverse after triple stimulation (Fig. 3B) and that discernable clusters of B cells were formed after stimulation that differed in their forward angle light scatter-side scatter profiles. Cells categorized as being in the B gate (representing larger cells), increased in percentage after triple stimulation, and increased further in response to triple stimulation plus RA (Fig. 3B). Triple stimulation also increased B cells in the A gate and presence of RA decreased this population. While more cells in B gate were sIgG1 positive and less CFSE dilution, cells in A gate contained fewer sIgG1-positive cells, and higher CFSE dilution. Together, these data support the hypothesis that the regulation of B-cell activation by RA is developmental stage-specific and quantitative, as not all cells within the total B cell population are equally responsive to stimulation or equally affected by RA.

We also observed that cells in B gate, which had increased in response to RA during the 5-day culture, were nearly exclusively the cells that expressed Blimp-1, AID mRNA and $\gamma 1$ GLT. In contrast, cells in A gate that had undergone more division events, as evidenced by CFSE dilution, but fewer sIgG1 positive cells, $\gamma 1$ GLT, AID, and Blimp-1 gene expression were extremely low or undetectable. These results further support the interpretation that B gate contains B cells that have progressed to more differentiated stages of B-cell activation and progression. Although RA markedly reduced $\gamma 1$ GLT expression level, it did not repress, or more or less increased, the levels of Blimp-1 and AID in triple-stimulated B cells, supporting the hypothesis that RA can promote B cell differentiation. It is known that AID and Blimp-1 are responsible for B cell differentiation and/or class switch recombination; especially Blimp-1 gene expression is rapidly up-regulated in B cells that have become terminally differentiated to antibody-secreting cells and plasma cells [48,56]. Under the conditions we have used in our model, such as triple stimulation through CD40, BCR and IL-4, B cells were exposed to several signals known to be important for their activation, therefore, these B cells might go through an early differentiation stage into antibody-producing plasma cells [9,57]. This could explain the co-expression of $\gamma 1$ GLT [29], Blimp-1 and AID gene expression in B gate (Fig. 4), which was enriched after triple stimulation and RA for sIgG1-expressing cells. Moreover, the induced expression of CD138/syndecan-1 in B gate of stimulated B cells with/without RA also supports the idea that these B cells have become more differentiated, having acquired a marker of plasmacytic cells. In other studies of B cell activation *in vitro* using anti- μ , anti-CD40, and anti-CD38 plus T-cell derived cytokines to simulate somatic hypermutation, Berghorsdottir et al. [58] noted differences in the signals required for cell differentiation and IgG gene mutation, as compared to proliferation. In our studies, RA differentially promoted the differentiation process while reducing cell proliferation, also an indication that these processes are, in part, distinct.

Taken together, our data suggest that at early times RA represses B-cell proliferation and $\gamma 1$ GLT expression, which could be important for allowing some of the stimulated, RA-exposed B-cells to “escape” from cell cycling into a differentiating pool in which cells may further mature into sIgG1-expressing cells, having undergone class switch recombination and further differentiation on the pathway to becoming antibody-secreting cells. Overall, the ability of RA to promote a subset of B cells towards a more differentiated phenotype may override the inhibitory effect of RA on B-cell proliferation which has been observed previously and in the present studies at the whole population level, and it may explain at least in part why RA can enhance the production of antibodies after vaccination *in vivo* [25,26]. In B-cells stimulated with anti- μ combined with CD40 and IL-4, as an *in vitro* model of T-cell-dependent B-cell activation, RA effectively promoted cell differentiation and increased the total sIgG expression

(number of cells times intensity per cell). The in vivo correlate of these in vitro results may be increased antibody production, which would be consistent with the generally positive effect of RA on the antibody response that has been observed experimentally in animal models, and as implied from epidemiological studies in human populations.

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Abbreviations used:

BCR, B-cell receptor; CFSE, carboxyfluorescein diacetate succinimidyl ester; GLT, germline transcript; RA, retinoic acid.

References

1. Baumgarth N. A two-phase model of B-cell activation. *Immunol Rev* 2000;176:171–180. [PubMed: 11043776]
2. Schonbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. *Cell Mol Life Sci* 2001;58:4–43. [PubMed: 11229815]
3. Bishop GA, Hostager BS. The CD40-CD154 interaction in B cell-T cell liaisons. *Cytokine Growth Factor Rev* 2003;14:297–309. [PubMed: 12787567]
4. Neron S, Racine C, Roy A, Guerin M. Differential responses of human B-lymphocyte subpopulations to graded levels of CD40-CD154 interaction. *Immunology* 2005;116:454–463. [PubMed: 16313359]
5. Noelle RJ, Erickson LD. Determinations of B cell fate in immunity and autoimmunity. *Curr Dir Autoimmun* 2005;8:1–24. [PubMed: 15564715]
6. Benson MJ, Erickson LD, Gleeson MW, Noelle RJ. Affinity of antigen encounter and other early B-cell signals determine B-cell fate. *Curr Opin Immunol* 2007;19:275–280. [PubMed: 17433651]
7. van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol* 2000;67:2–17. [PubMed: 10647992]
8. Chan WL, Pejnovic N, Liew TV, Hamilton H. Predominance of Th2 response in human abdominal aortic aneurysm: mistaken identity for IL-4-producing NK and NKT cells? *Cell Immunol* 2005;233:109–114. [PubMed: 15963967]
9. Huntington ND, Xu Y, Puthalakath H, Light A, Willis SN, Strasser A, Tarlinton DM. CD45 links the B cell receptor with cell survival and is required for the persistence of germinal centers. *Nat Immunol* 2006;7:190–198. [PubMed: 16378097]
10. Haxhinasto SA, Bishop GA. A novel interaction between protein kinase D and TNF receptor-associated factor molecules regulates B cell receptor-CD40 synergy. *J Immunol* 2003;171:4655–4662. [PubMed: 14568940]
11. Haxhinasto SA, Bishop GA. Synergistic B cell activation by CD40 and the B cell antigen receptor: role of B lymphocyte antigen receptor-mediated kinase activation and tumor necrosis factor receptor-associated factor regulation. *J Biol Chem* 2004;279:2575–2582. [PubMed: 14604983]
12. Ross AC. Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic Acid oxidation. *J Nutr* 2003;133:291S–296S. [PubMed: 12514312]
13. Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 2004;328:1–16. [PubMed: 15019979]
14. Ballow M, Wang X, Xiang S, Allen C. Expression and regulation of nuclear retinoic acid receptors in human lymphoid cells. *J Clin Immunol* 2003;23:46–54. [PubMed: 12645859]
15. Morikawa K, Nonaka M. All-trans-retinoic acid accelerates the differentiation of human B lymphocytes maturing into plasma cells. *Int Immunopharmacol* 2005;5:1830–1838. [PubMed: 16275619]
16. Ross, AC. Vitamin A, retinoids and immune responses. In: Livrea, MA., editor. *Vitamin A and retinoids: an update of biological aspects and clinical application*. Birkhauser Verlag Basel; Switzerland: 2000.

17. Stephensen CB. Vitamin A, infection, and immune function. *Annu Rev Nutr* 2001;21:167–192. [PubMed: 11375434]
18. Semba RD, Farzadegan H, Vlahov D. Vitamin A levels and human immunodeficiency virus load in injection drug users. *Clin Diagn Lab Immunol* 1997;4:93–95. [PubMed: 9008289]
19. Kirkwood BR, Arthur P, Ross DA, Morris SS, Gyapong JO, Tomkins AM. Vitamin A supplementation in Northern Ghana: effects on clinic attendances, hospital admissions, and child mortality. *Lancet* 1993;342:7–12. [PubMed: 8100345]
20. Ross AC, Stephensen CB. Vitamin A and retinoids in antiviral responses. *FASEB J* 1996;10:979–985. [PubMed: 8801180]
21. Villamor E, Fawzi WW. Effects of vitamin a supplementation on immune responses and correlation with clinical outcomes. *Clin Microbiol Rev* 2005;18:446–464. [PubMed: 16020684]
22. Glasziou PP, Mackerras DE. Vitamin A supplementation in infectious diseases: a meta-analysis. *BMJ* 1993;306:366–370. [PubMed: 8461682]
23. Cui D, Moldoveanu Z, Stephensen CB. High-level dietary vitamin A enhances T-helper type 2 cytokine production and secretory immunoglobulin A response to influenza A virus infection in BALB/c mice. *J Nutr* 2000;130:1132–1139. [PubMed: 10801909]
24. Hoag KA, Nashold FE, Goverman J, Hayes CE. Retinoic acid enhances the T helper 2 cell development that is essential for robust antibody responses through its action on antigen-presenting cells. *J Nutr* 2002;132:3736–3739. [PubMed: 12468615]
25. Ma Y, Chen Q, Ross AC. Retinoic acid and polyriboinosinic:polyribocytidylic acid stimulate robust anti-tetanus antibody production while differentially regulating type 1/type 2 cytokines and lymphocyte populations. *J Immunol* 2005;174:7961–7969. [PubMed: 15944302]
26. DeCicco KL, Youngdahl JD, Ross AC. All-trans-retinoic acid and polyriboinosinic: polyribocytidylic acid in combination potentiate specific antibody production and cell-mediated immunity. *Immunology* 2001;104:341–348. [PubMed: 11722649]
27. Ma Y, Ross AC. The anti-tetanus immune response of neonatal mice is augmented by retinoic acid combined with polyriboinosinic:polyribocytidylic acid. *Proc Natl Acad Sci U S A* 2005;102:13556–13561. [PubMed: 16157890]
28. Blomhoff HK. Vitamin A regulates proliferation and apoptosis of human T- and B-cells. *Biochem Soc Trans* 2004;32:982–984. [PubMed: 15506942]
29. Chen Q, Ross AC. Vitamin A and immune function: retinoic acid modulates population dynamics in antigen receptor and CD38-stimulated splenic B cells. *Proc Natl Acad Sci U S A* 2005;102:14142–14149. [PubMed: 16093312]
30. Blomhoff HK, Smeland EB, Erikstein B, Rasmussen AM, Skrede B, Skjonsberg C, Blomhoff R. Vitamin A is a key regulator for cell growth, cytokine production, and differentiation in normal B cells. *J Biol Chem* 1992;267:23988–23992. [PubMed: 1429735]
31. Lomo J, Smeland EB, Ulven S, Natarajan V, Blomhoff R, Gandhi U, Dawson MI, Blomhoff HK. RAR-, not RXR, ligands inhibit cell activation and prevent apoptosis in B-lymphocytes. *J Cell Physiol* 1998;175:68–77. [PubMed: 9491782]
32. Deaglio S, Capobianco A, Bergui L, Durig J, Morabito F, Duhrsen U, Malavasi F. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. *Blood* 2003;102:2146–2155. [PubMed: 12763926]
33. Andjelic S, Hsia C, Suzuki H, Kadowaki T, Koyasu S, Liou HC. Phosphatidylinositol 3-kinase and NF-kappa B/Rel are at the divergence of CD40-mediated proliferation and survival pathways. *J Immunol* 2000;165:3860–3867. [PubMed: 11034392]
34. Linehan LA, Warren WD, Thompson PA, Grusby MJ, Berton MT. STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *J Immunol* 1998;161:302–310. [PubMed: 9647237]
35. Mizuno T, Rothstein TL. B cell receptor (BCR) cross-talk: CD40 engagement creates an alternate pathway for BCR signaling that activates I kappa B kinase/I kappa B alpha/NF-kappa B without the need for PI3K and phospholipase C gamma. *J Immunol* 2005;174:6062–6070. [PubMed: 15879100]
36. De Leenheer AP, Lambert WE, Claeys I. All-trans-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. *J Lipid Res* 1982;23:1362–1367. [PubMed: 7161564]

37. Chen Q, Ma Y, Ross AC. Opposing cytokine-specific effects of all trans-retinoic acid on the activation and expression of signal transducer and activator of transcription (STAT)-1 in THP-1 cells. *Immunology* 2002;107:199–208. [PubMed: 12383199]
38. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000;102:553–563. [PubMed: 11007474]
39. Hasbold J, Corcoran LM, Tarlinton DM, Tangye SG, Hodgkin PD. Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat Immunol* 2004;5:55–63. [PubMed: 14647274]
40. Hasbold J, Lyons AB, Kehry MR, Hodgkin PD. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur J Immunol* 1998;28:1040–1051. [PubMed: 9541600]
41. Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. *Nat Rev Immunol* 2002;2:982–987. [PubMed: 12461571]
42. Shinagawa T, Nunoi H, Nonoyama S, Kanegasaki S. Induction of CD40 in promyelocytic HL60 cells cultured with retinoic acid and/or various cytokines. *J Biochem (Tokyo)* 1995;118:534–540. [PubMed: 8690713]
43. Park HY, Park JY, Kim JW, Lee MJ, Jang MJ, Lee SY, Baek DW, Park YM, Lee SW, Yoon S, Bae YS, Kwak JY. Differential expression of dendritic cell markers by all-trans retinoic acid on human acute promyelocytic leukemic cell line. *Int Immunopharmacol* 2004;4:1587–1601. [PubMed: 15454112]
44. Moodycliffe AM, Shreedhar V, Ullrich SE, Walterscheid J, Bucana C, Kripke ML, Flores-Romo L. CD40-CD40 ligand interactions in vivo regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. *J Exp Med* 2000;191:2011–2020. [PubMed: 10839815]
45. Worm M, Krah JM, Manz RA, Henz BM. Retinoic acid inhibits CD40 + interleukin-4-mediated IgE production in vitro. *Blood* 1998;92:1713–1720. [PubMed: 9716600]
46. Worm M, Herz U, Krah JM, Renz H, Henz BM. Effects of retinoids on in vitro and in vivo IgE production. *Int Arch Allergy Immunol* 2001;124:233–236. [PubMed: 11306979]
47. Scheffel F, Heine G, Henz BM, Worm M. Retinoic acid inhibits CD40 plus IL-4 mediated IgE production through alterations of sCD23, sCD54 and IL-6 production. *Inflamm Res* 2005;54:113–118. [PubMed: 15883744]
48. Nambu Y, Sugai M, Gonda H, Lee CG, Katakai T, Agata Y, Yokota Y, Shimizu A. Transcription-coupled events associating with immunoglobulin switch region chromatin. *Science* 2003;302:2137–2140. [PubMed: 14684824]
49. Jabara HH, Geha RS. Jun N-terminal kinase is essential for CD40-mediated IgE class switching in B cells. *J Allergy Clin Immunol* 2005;115:856–863. [PubMed: 15806010]
50. Kaku H, Horikawa K, Obata Y, Kato I, Okamoto H, Sakaguchi N, Gerondakis S, Takatsu K. NF-kappaB is required for CD38-mediated induction of C(gamma)1 germline transcripts in murine B lymphocytes. *Int Immunol* 2002;14:1055–1064. [PubMed: 12202402]
51. Carlsen H, Alexander G, Austenaa LM, Ebiara K, Blomhoff R. Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutat Res* 2004;551:199–211. [PubMed: 15225593]
52. Salmon RA, Foltz IN, Young PR, Schrader JW. The p38 mitogen-activated protein kinase is activated by ligation of the T or B lymphocyte antigen receptors, Fas or CD40, but suppression of kinase activity does not inhibit apoptosis induced by antigen receptors. *J Immunol* 1997;159:5309–5317. [PubMed: 9548470]
53. Feng B, Cheng S, Hsia CY, King LB, Monroe JG, Liou HC. NF-kappaB inducible genes BCL-X and cyclin E promote immature B-cell proliferation and survival. *Cell Immunol* 2004;232:9–20. [PubMed: 15922711]
54. Craxton A, Shu G, Graves JD, Saklatvala J, Krebs EG, Clark EA. p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol* 1998;161:3225–3236. [PubMed: 9759836]
55. Chen Q, Ross AC. Retinoic acid regulates cell cycle progression and cell differentiation in human monocytic THP-1 cells. *Exp Cell Res* 2004;297:68–81. [PubMed: 15194426]

56. Johnson K, Shapiro-Shelef M, Tunyaplin C, Calame K. Regulatory events in early and late B-cell differentiation. *Mol Immunol* 2005;42:749–761. [PubMed: 15829263]
57. Cattoretti G, Angelin-Duclos C, Shaknovich R, Zhou H, Wang D, Alobeid B. PRDM1/Blimp-1 is expressed in human B-lymphocytes committed to the plasma cell lineage. *J Pathol* 2005;206:76–86. [PubMed: 15772984]
58. Bergthorsdottir S, Gallagher A, Jainandunsing S, Cockayne D, Sutton J, Leanderson T, Gray D. Signals that initiate somatic hypermutation of B cells in vitro. *J Immunol* 2001;166:2228–2234. [PubMed: 11160276]

Fig. 1.

Retinoic acid decreased stimulation-induced IgG1 germline transcript expression. B cells were cultured *in vitro* with stimulation as indicated for different times. Total RNA was isolated and subjected to RT-PCR to determine the $\gamma 1$ GLT level. **A.** RA inhibited CD40-ligation-induced $\gamma 1$ GLT. B cells were stimulated with anti-CD40 (1 $\mu\text{g}/\text{ml}$) with and without 20 nM RA for up to 48 h. **B.** B cells were stimulated with anti-CD40, anti- μ (1 $\mu\text{g}/\text{ml}$) or IL-4 (2 ng/ml) with and without 20 nM RA for 48 h. A representative ^{33}P -dATP-labeled PCR gel image is showed alone with the chart. Data shown were normalized to GAPDH mRNA and are presented as mean $\pm$ SEM from four independent experiments. $P < 0.05$.

Fig. 2.

Retinoic acid differentially regulates B-cell growth rate stimulated by the engagement of CD40 and BCR, and cytokine IL-4. Isolated B cells were stimulated with anti-CD40, anti-μ (1 μg/ml) and IL-4 (2 ng/ml), alone or in combination, without and with 20 nM RA for 3 days. [³H]-Thymidine was added to the culture for the last 6 h of incubation. Mean ± SEM. * or +, $P < 0.05$.

Fig. 3.

RA promotes B-cell differentiation after a combined stimulation by CD40, IL-4 and BCR. B cells were labeled with CFSE and then cultured for five days with triple stimulation, including anti-CD40, anti- μ (1 μ g/ml, respectively) and IL-4 (2 ng/ml), with and without 20 nM RA. On day five, cells were stained with PE-Cy7 conjugated anti-mouse CD19 and PE-conjugated anti-mouse IgG1. Propidium iodine was added to cells 5 min before flow analysis. **A.** sIgG1 fluorescence was increased by triple stimulation and further increased by RA. Data presented as total fluorescence [percent of IgG1-positive cells $\times$ mean fluorescence intensity (mean $\pm$ SEM)]. **B.** A representative scatter diagram showing the cell distribution and gating of cells.

Fig. 4.

RA enriched more differentiated B cells in the upper gate. **A, B and C.** Expression of IgG1 GLT, AID and Blimp-1 in B cell populations. B cells were cultured for 5 days with triple stimulation with and without 20 nM RA. B cells were sorted based on their forward and side scatter characters (See Fig. 3B) into A and B populations. Relative expression levels of IgG1 GLT (**A**), AID (**B**) and Blimp-1 (**C**) mRNA were determined by real-time PCR and normalized with GAPDH, for cells of each gates. Data are from one of two independent experiments each conducted in duplicate. Inserts show representative gel images for each gene. **S** and **S+RA** indicate stimulation without and with RA. **ND**, not detectable.

Fig. 5.

RA enriched CD138 expression B cells. Splenic B cells on day zero were stained with anti-CD138-PE and subjected to flow sorting. CD138-negative B cells were then cultured for five days with and without triple stimulation in the presence and absence of 20 nM RA. On day five, cells were stained with anti-CD138-PE antibody. **A.** Total CD138 fluorescence in control cells (Medium), with triple stimulation and triple stimulation with RA. **B.** Distribution of total CD138 fluorescence in A and B gates from triple stimulation plus RA treated B cells. Mean $\pm$ SEM; *, $P < 0.05$.

Table 1

Distribution of B cells in A, B and C gates after 5 days of culture.

Stimulation conditions	A gate (%)	B gate (%)	C gate (%)	Ratio (B/A)
Control	39.9 ± 0.5	7.1 ± 0.4	2.4 ± 0.1	0.18 ± 0.01
RA	27.8 ± 0.9 *	6.7 ± 0.1	2.8 ± 0.1	0.24 ± 0.01 *
Triple Stimulation	61.6 ± 1.4 †	18.9 ± 0.5 †	2.6 ± 0.2	0.31 ± 0.01 †
Triple Stimulation + RA	58.3 ± 1.0 *†	23.7 ± 0.4 *†	2.5 ± 0.2	0.41 ± 0.01 *†

B cells were treated with and without triple stimulation in the presence and absence of RA. Cell distribution as illustrated in Fig. 3B was summarized in the table. The ratio of B gate to A gate cells was derived to show the increase of B cells in the B gate. Numbers showed in the table are mean ± SE.

* indicates significant difference due to RA

† due to triple stimulation, $P < 0.05$.

Table 2

Characteristics of B cells in A and B gates after triple stimulation in the presence and absence of RA.

	<u>CFSE diluted</u>		<u>sIgG1 positive</u>	
	<u>% in A gate</u>	<u>% in B gate</u>	<u>% in A gate</u>	<u>% in B gate</u>
Triple Stimulation	57.2 ± 1.0	49.3 ± 2.2 [†]	52.7 ± 2.1	89.3 ± 0.7 [†]
Triple Stimulation + RA	50.5 ± 0.3 [*] [†]	38.3 ± 0.3 [*] [†]	72.5 ± 0.8 [*] [†]	89.4 ± 0.6 [†]

The percentage of B cells that exhibited CFSE dilution or were positive for sIgG1 in the A gate and B gate were determined.

* indicate $P < 0.05$ due to RA

[†] between the A and B gates respectively.