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IRF4 Is a Critical Gene in Retinoic Acid–Mediated Plasma Cell Formation and Is Deregulated in Common Variable Immunodeficiency–Derived B Cells

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In the present study, we aimed at identifying the mechanisms whereby the vitamin A metabolite all-trans retinoic acid (RA) promotes the formation of plasma cells upon stimulation of B cells via the innate immunity receptors TLR9 and RP105. Most often, differentiation of B cells involves the sequential events of class switch recombination and somatic hypermutations characteristic of germinal center reactions, followed by plasma cell formation. By studying the regulatory networks known to drive these reactions, we revealed that RA enhances the expression of the plasma cell–generating transcription factors IFN regulatory factor (IRF)4 and Blimp1, and paradoxically also activation-induced deaminase (AID) involved in somatic hypermutations/class switch recombination, in primary human B cells. IRF4 was identified as a particularly important protein involved in the RA-mediated production of IgG in TLR9/RP105-stimulated B cells. Based on kinetic studies, we present a model suggesting that the initial induction of IRF4 by RA favors AID expression. According to this model, the higher level of IRF4 that eventually arises results in sustained elevated levels of Blimp1. Regarded as a master regulator of plasma cell development, Blimp1 will in turn suppress AID expression and drive the formation of IgG-secreting plasma cells. Notably, we demonstrated IRF4 to be deregulated in B cells from common variable immunodeficiency patients, contributing to the observed aberrant expression of AID in these patients. Taken together, the present study both provides new insight into the mechanisms whereby RA induces differentiation of B cells and identifies IRF4 as a key to understand the defective functions of B cells in common variable immunodeficiency patients. The Journal of Immunology, 2015, 195: 000–000.
transcriptional repressors or enhancers to stabilize one of the two transcriptional programs (15). IRF4 has been identified as a particularly important factor in determining whether B cell maturation should result in GC reactions such as SHM and CSR or proceed to plasma cell formation (20–22). Depending on its concentration, IRF4 will form homo- or heterodimers that regulate distinct sets of genes determining the fate of the cells. Whereas low to intermediate concentrations of IRF4 favor AID expression and GC reactions, higher concentrations of IRF4 will eventually form homodimers that drive plasma cell formation (20–22).

We have previously shown that RA is capable of promoting both AIDCA (encoding the AID protein) expression and Ig production in TLR9/RP105-stimulated normal B cells (9, 12), and in the present study, we sought to reveal the impact of RA on the regulatory protein networks that respectively drive the GC reactions and plasma cell formation. We have previously explored RA-mediated responses in B cells from patients with common variable immunodeficiency (CVID), a condition characterized by B cell defects and inadequate Ab responses. Although RA generally was found to induce a limited production of IgG in CVID-derived B cells, the response varied in B cells from different patients (12). In light of these results, we therefore compared the regulatory protein networks in B cells from CVID patients and healthy donors, with the purpose of 1) uncovering defects in the regulatory protein networks in B cells from CVID patients, as well as to 2) identify subgroups of patients that might benefit from supplementation of vitamin A.

We were able to identify IRF4 as a critical factor in RA-mediated plasma cell formation as well as in GC reactions in TLR9/RP105-activated B cells. Interestingly, we found IRF4 and AIDCA to be deregulated in B cells from CVID patients, and we used these data to identify a subgroup of patients that might respond favorably to vitamin A supplementation.

**Materials and Methods**

**B cell isolation and cell culturing**

Peripheral human CD19+ B cells were isolated from buffy coats obtained from healthy blood donors from the Blood Bank (Oslo University Hospital, Oslo, Norway) using anti-CD19 Ab-coated Dynabeads (Invitrogen Dynal, Oslo, Norway) as previously described (23). The cells were detached either by overnight incubation at 37°C in a CO2 incubator or by mixing the beads (5 × 10^6/ml whole blood) were not included in the study. None of the patients suffered from acute infections at the time of blood collection, and the blood samples were collected prior to Ig infusion. The normal whole blood donors were recruited from the Blood Bank (Oslo University Hospital, Oslo, Norway). The study was approved by the Regional Committee for Medical and Health Research Ethics in the South-Eastern Norway Regional Health Authority. The investigation conforms to the principles outlined in the Declaration of Helsinki.

**Patients and healthy blood donors**

Ten CVID patients (6 males and 4 females; median age, 55 y; age range, 28–77 y) and 11 age- and sex-matched healthy donors were recruited in the study after informed consent. The clinical and immunologic characteristics of the patients are presented in Table I. The patients were recruited from the Section of Clinical Immunology and Infection Medicine at Oslo University Hospital Rikshospitalet, Oslo, Norway, and were diagnosed according to the World Health Organization expert group on primary immunodeficiencies, that is, the International Union of Immunological Societies scientific committee (24). Because of the requirement of a sufficient number of B cells to perform the analyses, patients with low B cell numbers (<1.0 × 10^7/35 ml whole blood) were not included in the study. None of the patients suffered from acute infections at the time of blood collection, and the blood samples were collected prior to Ig infusion. The normal whole blood donors were recruited from the Blood Bank (Oslo University Hospital, Oslo, Norway). The study was approved by the Regional Committee for Medical and Health Research Ethics in the South-Eastern Norway Regional Health Authority. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Reagents and Abs

CpG-ODN phosphorothionate 2006 and Ro 41-5253 were purchased from Enzo Life Sciences (Farmingdale, NY). Purified anti-human CD180 (RP105) (clone MHR73-11) was from BioLegend (San Diego, CA), and RA and 4-[(E)-2-(5,6,7,8-tetrahydro-5,8,8-trimethyl-2-naphthalenyl)-l-propanoyl]benzoic acid (TTNPB) were from Sigma-Aldrich (St. Louis, MO). In all experiments, the final concentrations of CpG-ODNs and anti-RP105 were 1 μg/ml and the final concentration of RA was 100 nM unless otherwise indicated. PE-conjugated anti-human CD38 (clone HIT2) and anti-human PerCP-conjugated mouse IgG1 were from BD Biosciences (Franklin Lakes, NJ), and anti-human PerCP-conjugated anti-CD20 (clone LT20) and PE-conjugated mouse IgG1 (clone BS5-21F5) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). SMARTpool ON-TARGETplus small interfering RNA (siRNA) targeting IRF4 and PRDM1, and control non-targeting pool siRNA were obtained from Dharmaco (Lafayette, CO). Abs against IRF4, XBP1s (spliced isoform), BCL6, and calnexin were purchased from Cell Signaling Technologies (Danvers, MA), whereas the Abs against AID and Blim1 were from Invitrogen (Carlsbad, CA) and R&D Systems (Minneapolis, MN), respectively.

**Surface expression assessed by flow cytometry**

For analysis of surface expression of CD20 and CD38 the cells were stained with PE-conjugated anti-human CD38 and PerCP-conjugated anti-human CD20 according to the manufacturer’s protocols. Isotype-matched Abs were used as controls. The cells were analyzed using a FACSCalibur (BD Biosciences).

**ELISA assays**

Freshly isolated CD19+ B cells were cultured with various combinations of CpG-ODNs, anti-RP105, and RA. After 72 h, supernatants were collected and stored at −80°C until analysis according to the manufacturer’s protocols. IgG ELISA kits were obtained from Bethyl Laboratories (Montgomery, TX).

**Gene expression analysis**

Freshly isolated CD19+ B cells were cultured (3.0–5.0 × 10^6 cells/ml) with various combinations of CpG-ODNs (1 μg/ml), anti-RP105 (1 μg/ml), and RA (100 nM). After 72 h the cells were washed in PBS supplemented with 0.5% FBS, and the cell pellets were collected and stored at −80°C. RNA was isolated from the cells using RNeasy Plus mini kit (Qiagen, Valencia, CA) and subjected to reverse transcription using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturers’ protocols. Reverse transcriptase quantitative PCR (RT-qPCR) was performed in the 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA) using the TaqMan custom array (Applied Biosystems). Information on the primers used for the custom arrays can be obtained upon request. ExpressionSuite software (Applied Biosystems) was used to analyze the data. The expression of target mRNA was quantified using the cycle threshold (Ct) normalized against endogenous controls and the data are presented as the mean 2^−ΔΔCt values.

**Immunoblot analysis**

Cells were lysed in RIPA buffer for 20 min on ice, and the protein concentrations were determined using the BCA protein assay kit (Pierce, Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (15–25 μg) were separated by SDS-PAGE gel (Bio-Rad) and transferred to an Immobilon-P polyvinylidene difluoride membrane (0.45 μm; Millipore, Billerica, MA) using a wet blot system. Proteins were detected and quantified as described previously (25).

**In silico analysis of retinoic acid receptor/retinoic X receptor binding sites**

In the search for putative retinoic acid receptor (RAR)/retinoic X receptor (RXR) binding sites upstream of the PRDM1, AIDCA, and IRF4 genes, in silico analyses were performed on 2-kb sequences 5’ of the transcription start sites using MatInspector version 8.1 (Genomatix, Munich, Germany) (26), and the Matrix Family Library version 9.1, with a Matrix similarity cut-off at 0.80.

**Chromatin immunoprecipitation–PCR analysis**

Chromatin immunoprecipitation (ChIP) was performed using a ChIP-IT high-sensitivity kit from Active Motif (La Hulpe, Belgium, catalog no. 53040). Briefly, 6.0 × 10^6 freshly isolated CD19+ B cells were cultured (5.0 × 10^6 cells/ml) with CpG-ODNs (1 μg/ml) and anti-RP105 (1 μg/ml) in...
the presence or absence of RA (100 nM) for 72 h before fixation and lysis of cells as recommended by the manufacturer. Chromatin was fragmented by sonication in a Diagenode Bioruptor (Diagenode, Seraing, Belgium) with four cycles of 10 min 30 s on/off at high power. Cross-linked chromatin was subjected to immunoprecipitation with anti-RAR Abs (ab41934; Abcam, Dundee, Scotland) or control IgG before purification of DNA. Equal volumes of purified DNA were analyzed by PCR using Promega PCR master mix (M7505) and the following conditions: 95˚C for 3 min and equal volumes of purified DNA were analyzed by PCR using Promega PCR master mix (M7505) and the following conditions: 95˚C for 3 min and 40 cycles of 95˚C for 30 s, 57˚C for 30 s, and 72˚C for 30 s. Primers were designed using the HG18 human genome assembly with the following sequences: 5’-CCGGTGGAGGACGGAATAG-3’ (CD38-1), 5’-CCGG-CAAAAAGAATCGGAGT3’ (CD38-2), 5’-GATCGAAAGCTCAACGG-3’ (CD38-3), 5’-AGTTGACCGAGTTATCATATTCT3’ (IRF4-2).

**Transfection of siRNA into B cells**

CD19+ B cells were isolated using DETACHaBEAD as described above and immediately transfected with 1.6 μM siRNA targeting IRF4, PRDM1, or matching control siRNA, using the human B cell Nucleofector kit (Amaxa Biosciences, Basel, Switzerland) and a Nucleofector device (Amaxa Biosciences) according to the manufacturer’s protocols.

**Statistical analysis**

Linear regression analysis was used for correlating two variables from the gene profiling data and was performed using Microsoft Excel (Microsoft, Redmond, WA). Statistical analyses of the data were performed using IBM SPSS Statistics 22 (IBM, New York, NY). A paired sample t test was used to analyze the results obtained in the experiments using cells from normal buffy coats, whereas a Mann–Whitney U test and Wilcoxon signed-rank test were used when analyzing the patient material. The Mann–Whitney U test was used to compare different groups, and the Wilcoxon signed-rank test was used to compare different stimuli within groups. A p value <0.05 was considered statistically significant.

**Results**

**RA enhances the expression of genes important for plasma cell differentiation**

Having previously demonstrated that physiological concentrations of RA enhance proliferation and differentiation of B cells stimulated through TLR9 alone (10) or in combination with RP105 stimulation (9, 12), we now sought to further unravel the mechanisms involved. The differentiation of B cells into plasma cells is associated with enhanced expression of the cell surface marker CD38, concomitant with reduced expression of CD20 (27). To determine the proportion of plasma cells induced by RA, we therefore assessed the expression of CD38 and CD20 after 5 d of coculturing TLR9/RP105-stimulated B cells in the presence or absence of RA (100 nM). As shown in Fig. 1, the fraction of CD38highCD20low cells was increased from 6.7 to 29.4% when RA was added to the TLR9/RP105-stimulated B cell cultures, consistent with an increased frequency of cells gaining a plasma cell phenotype. Although reporting this study we use the term “plasma cell” when describing the differentiated B cells, importantly note that there are several subgroups of cells belonging to this cell compartment. The terminally differentiated plasma cell correctly describes a cell that is no longer proliferating. In vivo, plasmablasts are supposed to represent an early stage of plasma cell, whereas in vitro most likely include most of the Ab-secreting cells (16, 27). The CD38highCD20low cells we refer to as plasma cells in the present study probably include both plasmablasts and terminally differentiated plasma cells. To further elucidate this issue, we also determined the surface expression of the plasma cell marker CD138. Interestingly, although the numbers of CD138-expressing cells were relatively low (1–5%), RA enhanced the expression of CD138 in all three experiments performed (Supplemental Fig. 1).

Plasma cell differentiation is highly dependent on specific gene expression programs. Upon differentiation into plasma cells, genes such as PRDM1 (encoding the Blimp1 protein), IRF4, and XBP1 are induced, whereas genes associated with a GC phenotype such as Bcl6 are repressed (15). Accordingly, we explored whether the RA-mediated induction of IgG and cell surface plasma cell markers involved changes in relevant gene expressions. Freshly isolated B cells were stimulated with CpG-ODNs and anti-RP105 for 3 d in the presence or absence of RA, and isolated RNA was subjected to RNA sequencing. After processing the data, we observed that RA induced expression of several genes, including IRF4, XBP1, and PRDM1, consistent with its ability to promote plasma cell differentiation.
to RT-qPCR. As shown in Fig. 2A, RA induced a 2-fold \((p = 0.006)\) induction of IRF4 in TLR9/RP105-activated B cells and a 3-fold induction \((p = 0.003)\) of PRDM1. The expression of XBP1s was also enhanced \((p = 0.13)\) but not to the same extent as IRF4 and PRDM1. In accordance with our previous results (12), RA also increased the mRNA level of AICDA in TLR9/RP105-stimulated cells, which is considered to be crucial for the GC-associated processes SHM and CSR (28). Consistent with the upregulation of the typical plasma cell–generating genes, RA reduced the expression of the GC-associated gene, Bcl6, by 40% \((p = 0.01)\). Taken together, the results of the gene expression profiles strengthen our hypothesis that RA promotes the differentiation of B cells into plasma cells by inducing the expression of transcription factors known to be driving the process.

**Correlation between RA-mediated mRNA and protein expressions**

Changes in mRNA expressions revealed by RT-qPCR do not always translate into changes in protein expression. We therefore performed Western blot analysis on cell lysates from freshly isolated B cells stimulated via TLR9 and RP105 for 3 d in the presence or absence of RA. By comparing the results of the Western blot analysis in Fig. 2B with the mRNA expression profiles in Fig. 2A, we noted a general correlation between RA-mediated changes in mRNA and protein levels. Hence, RA significantly \((p < 0.02)\) enforced a 2- to 3-fold induction of IRF4, Blimp1, XBP1s, and AID proteins. The levels of BCL6 protein were not significantly altered by RA after 3 d of stimulation, but after 5 d of culturing, RA was also able to significantly decrease BCL6 protein levels in TLR9/RP105-activated B cells (data not shown).

**RA-mediated changes in gene expressions are mediated via RARs**

Although RA primarily acts as a transcription factor via RARs, also nontranscriptional effects of RA have been documented (29, 30). To elucidate the involvement of RARs in RA-mediated induction of IRF4 and Blimp1 in TLR9/RP105-stimulated B cells, we made use of the RAR-specific agonist (TTNPB) and the RAR-specific antagonist (Ro 41-5253). As shown in Fig. 3A and 3B, TTNPB (100 nM) induced IRF4 and Blimp1 in the same manner as did
The 1 nM concentration of RA was included in the experiment to obtain a 500:1 ratio of the antagonist to RA, without reaching toxic levels of the antagonist. As shown in Fig. 3, the RA-mediated induction of IRF4 and Blimp1 were abolished by Ro 41-5253, indicating that RA induces transcription of plasma cell–driving factors via RARs.

**IRF4 is involved in RA-mediated plasma cell development**

Having demonstrated that RA exerts its effects on plasma cell–generating genes in a transcriptional manner via RARs, we elucidated whether IRF4, suggested as being the upstream factor in a chain of events ultimately resulting in generating plasma cells (20, 22), could be a primary target of RA. To this end, in silico analysis of a 2-kb sequence upstream of the IRF4 transcription start site (TSS)
was performed by using MatInspector and the Matrix Family Library, identifying three potential DR1, DR3, and DR5 binding sites for the RAR/RXR heterodimer upstream of the TSS, as shown in Fig. 3C. Interestingly, the same analysis of 2 kb upstream of the TSS in the PRDM1 and the AICDA genes did not reveal any putative RAR/RXR binding sites. To confirm binding of RAR to the IRF4 promoter, we performed ChIP-PCR analysis of a sequence including the DR element with the highest similarity to the RAR/RXR matrix of binding sites according to the in silico analyses (DR5, Fig. 3C). RAR was successfully immunoprecipitated from isolated and fragmented B cell chromatin (Fig. 3D), and subsequent PCR analyses of immunoprecipitated material with primers against the DR5-like element in IRF4 yielded an amplicon of expected size (343 bp). The CD38 promoter with a DR5-like element that has previously been shown to bind RAR (31) was included as a positive control.

To further establish a vital role of IRF4 in mediating the effects of RA on TLR9/RP105-induced B cell differentiation, we performed knock down of IRF4 by siRNA. To choose an optimal time point for assessing the effects of siRNA treatment, we had to make a compromise between the optimal time point for knocking down the gene (24 h, see Fig. 4A, 4B) and for assessing the effects of RA on IgG production (4 d, see Fig. 4C). Hence, freshly isolated B cells were transfected with siRNA against IRF4 or with a control siRNA and stimulated with CpG-ODNs, anti-RP105, and RA for 3 d, before the supernatants were collected and subjected to IgG ELISA assays. As depicted in Fig. 4D, the RA-induced IgG secretion was significantly reduced in the cells transfected with IRF4 siRNA as compared with cells transfected with control siRNA ($p = 0.002$). We also transfected the cells with siRNA against PRDM1, but the RA-induced IgG production was not reduced (data not shown). Moreover, IRF4 siRNA reduced the levels of CpG/anti-RP105/RA-induced Blimp1, whereas siRNA against PRDM1 did not reduce the level of CpG/anti-RP105/RA-induced IRF4 (Fig. 4E–G). For unknown reasons, we were unfortunately not able to determine either the protein levels of AID or the mRNA levels of AICDA in the transfected cells. In our experience, the stress imposed on the B cells when exposing them to transfection reagents and the electroporation procedure can occasionally by itself interfere with the detection of certain proteins, such as in this case AID.

To further elucidate the link between IRF4, Blimp1, and AID, we performed kinetic experiments measuring the levels of the proteins at different time points between 0 and 96 h of stimulation (see Fig. 5A, 5B). The results revealed that IRF4 was the first of the proteins to be induced by CpG-ODNs and anti-RP105 both in the presence and absence of RA. Importantly, however, the cells stimulated with RA expressed higher levels of IRF4 at all

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Knock down of IRF4 reduces the effect of RA on IgG secretion. (A) B cells were transfected with siRNA targeting IRF4 or control siRNA and stimulated with CpG-ODNs (1 μg/ml), anti-RP105 (1 μg/ml), and RA (100 nM) for 24, 48, or 72 h before the cells were harvested and subjected to Western blot analysis. One representative experiment of four is shown. (B) The protein levels were quantitated and normalized to calnexin, and the ratios are displayed. The data represent the mean values ± SEM of four independent experiments. (C) B cells (1.0 × 10^6/ml) transfected with control siRNA were stimulated with CpG-ODNs and anti-RP105 in the presence or absence of RA for the indicated time points, before the supernatants were collected and subjected to IgG ELISA assays. The data represent the mean values ± SEM of two independent experiments. (D) B cells (7.0 × 10^5/ml) were transfected with siRNA targeting IRF4 or control siRNA and stimulated with CpG-ODNs (1 μg/ml), anti-RP105 (1 μg/ml), and RA (100 nM) for 72 h prior to analysis of IgG by ELISA assays. The data represent the mean values ± SEM of five independent experiments. (E) B cells were transfected with siRNA targeting IRF4, PRDM1, or control siRNA and stimulated with CpG-ODNs (1 μg/ml), anti-RP105 (1 μg/ml), and RA (100 nM) for 36 h, before the cells were harvested and subjected to Western blot analysis. One representative experiment is shown. (F) and (G) The protein levels from (E) were quantitated and normalized to calnexin, and the ratios are displayed. The data represent the mean values ± SEM of five independent experiments. *$p < 0.05$, paired sample t test. aRP, anti-RP105.
time points. RA also induced a transient expression of AID, but with a 24 h delay compared with that of IRF4. Hence, RA induced a peak in the expression of AID at 72 h of treatment. In contrast, the expression levels of the typical plasma cell proteins Blimp1 and XBP1s continue to increase as of treatment with CpG-ODNs and anti-RP105 both in the presence or absence of RA. Taken together, these results imply that RA initially, via induction of IRF4, will induce AID responsible for generating isotype switching and affinity maturation in the B cells. This initial phase will be followed by differentiation of B cells into plasma cells characterized by the sustained expression of Blimp1 and XBP1s. In line with the notion that Blimp1 leads to suppression of AID (32, 33), the expression of AID was transient possibly due to the sustained high levels of Blimp1 induced by RA.

To further characterize the different cell populations described in Fig. 1, freshly isolated CD19+ B cells were costimulated via TLR9 and RP105 for 5 d in the presence or absence of RA prior to cell sorting based on the expression of CD20 and CD38. The different cell fractions were analyzed by Western blot analysis as described in Materials and Methods. The blot shows one representative experiment (C) of two. The three fractions that were obtained in both experiments were quantitated and normalized to calnexin, and the ratios are displayed as mean values ± SEM (D). αRP, anti-RP105.
Expressions of IRF4 and AICDA are deregulated in CVID-derived B cells

We previously demonstrated that RA was able to nearly normalize deficient immune responses, such as IL-10 secretion and proliferation, in TLR9/RP105-activated B cells from CVID patients (12). However, the deficient IgG production in CVID-derived B cells was only partially restored by RA, as was also the RA-induced expression of AICDA (12). Based on our previous results, we suggested that the switching to IgG production rather than plasma cell formation per se is defective in most CVID-derived B cells (12). However, in the previous study (12), we had noticed that the response to RA varied in B cells from different patients. In the present study, we took advantage of the gene profiling experiments to 1) further elucidate the relationship between plasma cell formation and switching in CVID-derived B cells, and 2) identify subgroups of CVID patients that would be more likely to benefit from vitamin A supplementation to increase their plasma levels of IgG. B cells were isolated from eight CVID patients and seven healthy controls, and the cells were cultured with CpG-ODNs and anti-RP105 in the presence or absence of RA for 72 h before the isolated RNA samples were subjected to RT-qPCR. As shown in Fig. 6A, the expressions of the typical plasma cell–associated genes IRF4, PRDM1, and XBP1s were all significantly increased by RA in both control- and CVID-derived B cells. Interestingly, the expressions of all three genes were higher in the TLR9/RP105-activated CVID-derived B cells compared with B cells derived from the healthy controls. For IRF4 and XBP1s, the differences were statistically significant (p = 0.046 for both). In accordance with our previous findings (12), RA was able to increase the AICDA expression in CVID-derived B cells (p = 0.012), although not to the same extent as in the normal B cells (Fig. 6A). Furthermore, by linear regression analysis, we also found correlations between the expressions...
of AICDA and IRF4 in the CVID-derived B cells, revealing an inverse correlation between the two genes that was not found in the normal B cell populations (Fig. 6B). Based on these B cell analyses, we were able to identify two distinct subgroups of the patient-derived B cells. One group (group I, marked in blue in Fig. 6B) was identified with higher levels of IRF4 concomitant with lower levels of AICDA as compared with cells from the healthy controls. In the presence of CpG-ODNs and anti-RP105, RA was able to bring the expression of IRF4, but not AICDA, in these cells to the level seen in normal B cells (Fig. 6B). B cells from the other group of patients (group II, marked in red in Fig. 6B) exhibited normal expression levels of both IRF4 and AICDA, and in these cells RA managed to enhance the expression of both genes in the same manner as in control cells in the presence of CpG-ODNs and anti-RP105 (Fig. 6B). Importantly, note that the CVID-derived B cells expressing high levels of IRF4 upon stimulation via TLR9/RP105 (belonging to group I patients) concomitantly expressed elevated levels of PRDM1 compared with cells from the healthy controls (data not shown).

To verify these findings at the protein level, B cells from four CVID patients and four healthy blood donors were stimulated for 3 d with CpG-ODNs and anti-RP105 in the presence or absence of RA, before cell lysates were subjected to Western blot analysis (Fig. 7). Owing to a low number of cells obtained from 35 ml whole blood, we were not able to perform protein analysis on the same patients as we had analyzed for mRNA levels. Still, we were able to see similar trends in the CVID patients when analyzing the protein levels of IRF4, Blimp1, and AID. Hence, we observed markedly higher expression of IRF4 and Blimp1 in TLR9/RP105-stimulated B cells from three CVID patients than in TLR9/RP105-stimulated cells from healthy donors, concomitantly with significantly lower levels of AID. The AID levels were only marginally induced by RA in B cells from these patients, in line with these patients belonging to group I of CVID patients. The fourth patient (CVID7) had lower levels of IRF4 and Blimp1 in the TLR9/RP105-stimulated B cells as compared with cells from healthy donors, and the level of AID was normal to slightly higher. However, RA was able to increase the levels of all three proteins in the stimulated B cells, in line with this patient belonging to group II. Taken together, we have demonstrated that the levels of IRF4, Blimp1, and AID are deregulated in CVID patients, with a general reverse correlation between the expression levels of the genes encoding the switching-inducing protein AID and the plasma cell–generating proteins IRF4 and Blimp1. Furthermore, we have identified a subgroup of patients (group II) that potentially might benefit from vitamin A supplementation to increase their plasma levels of IgG.

Discussion
The present study provides mechanistic explanations for our previous findings that RA promotes differentiation of TLR-stimulated B cells into plasma cells (9, 10, 12). Hence, we reveal that RA in TLR9/RP105-stimulated B cells not only increases the expression of the plasma cell–related transcription factors PRDM1/Blimp1, IRF4, and XBP1, but that RA also enhances the expression of AICDA/AID involved in GC reactions. The genes were induced both at the mRNA and protein levels, and RA was found to induce IRF4 in an RAR-dependent manner. In silico analysis of the IRF4 gene revealed putative DR1, DR3, and DR5 binding sites for the RAR/RXR receptor complex upstream of the TSS, and similar binding sites were not identified in the PRDM1 or AICDA genes. By performing ChIP-PCR analysis of a sequence of the IRF4 promoter including the DR5 element, the ability of RA to regulate the transcription of IRF4 via RAR was further established. Interestingly, less DNA was immunoprecipitated from cells not treated with RA, suggesting that RA promotes the binding of RAR to the DR5-like element. Taken together, these results pointed to a key function of IRF4 in RA-mediated plasma cell formation, and this role was confirmed by knocking down IRF4 versus PRDM1 in the TLR9/RP105-stimulated cells. Furthermore, as siRNA against IRF4 reduced the level of Blimp1 (PRDM1) and not vice versa, our results support the notion that Blimp1 (PRDM1) is a direct target of IRF4 (20).

The genes considered to be the most important for GC reactions include Bcl6, Pax5, AICDA, and Myc, and these genes are able to (directly or indirectly) repress the expression of genes belonging to the plasma cell program, such as PRDM1, IRF4, and XBP1 (15, 16). In line with the ability of RA to induce the plasma cell–generating genes in TLR9/RP105-stimulated cells, Bcl6 was concomitantly reduced. It was therefore a paradox to find that RA in the same settings also induced AICDA, which is a gene associated with the GC program and is being silenced by Blimp1 when cells differentiate into plasma cells (32). However, a key to understand this paradox may lie in the ability of RA to induce IRF4. In a recent report, Sciammas et al. (20) demonstrated that IRF4 is crucial for both plasma cell generation and CSR/SHM in a concentration-dependent manner. Hence, whereas high levels of IRF4 led to plasma cell differentiation, intermediate levels induced the expression of AID (20). According to this report, IRF4 is therefore required for the expression of both AICDA and PRDM1. Supporting these data, Ochiai et al. (22) reported that early in the activation process of B cells, IRF4 cooperates with its partners PU.1 or BATF to induce genes involved in B cell activation and GC reactions including AICDA. In contrast, when higher levels of IRF4 are obtained at later stages of B cell maturation, IRF4 shifts its targets toward IFN sequence response element motifs and induction of genes involved in the plasma cell program (22). The results of our present study might fit with such a model. Hence, it is possible that the moderate levels of IRF4 that are reached in the early phase of TLR9/RP105-mediated stimulation of B cells might be sufficient to induce AID. Upon pro-

![FIGURE 7](http://www.jimmunol.org/) Effect of RA on proteins involved in plasma cell differentiation in CVID-derived B cells. CD19+ B cells were isolated from whole blood from CVID patients and healthy donors and cultured (3.0–5.0 × 10^6 cells/ml) with various combinations of CpG-ODNs (1 μg/ml), anti-RP105 (1 μg/ml), and RA (100 nm). After 72 h of stimulation, the cell pellets were harvested and subjected to Western blot analysis for determining the protein expressions of IRF4, Blimp1, and AID. Calnexin was used as an endogenous control. HD, healthy donor; αRP, anti-RP105.
longed cotreatment with RA, higher levels of IRF4 will result in elevated expression of Blimp1, which in turn represses AID and hence the transient induction of AID. Although the levels of IRF4 eventually also decline, the sustained levels of Blimp1 (often referred to as the master regulator of plasma cell development) and XBP1s result in commitment to the plasma cell–generating program.

In contrast to the pronounced RA-mediated stimulation of IgG levels in normal B cells, we previously showed that RA was only able to induce limited IgG production in B cells from patients with CVID (12). CVID is the most frequent of the primary immune deficiencies and is hallmarked by reduced serum levels of IgG, causing recurrent infections, particularly of the respiratory tract (34). There have been previous reports on defective TLR9 signaling in CVID-derived B cells (35–37), and our group (12) and others (38) have demonstrated diminished immune responses in CVID-derived B cells costimulated via TLR9 and RP105. However, although RA generally showed limited ability to restore the levels of IgG in CVID-derived B cells, we had noted that the responses to RA appeared to vary somewhat in the different patient-derived cells (12). In the present study we therefore hypothesized that the variations we observed in the CVID-derived B cells might be due to differences in their regulatory protein networks. Interestingly, we identified a group of CVID patients that potentially might take advantage of vitamin A supplementation. Future studies should examine whether this subgroup of CVID patients might benefit from the combined treatment of RA and TLR ligands to enhance their plasma levels of IgG.

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Disclosures

R.B. and H.K.B. have an interest in Vitas, Oslo, Norway. The remaining authors have no conflicts of interest.

References

9. Eriksen, A. B., R. L. Indrevær, K. L. Holm, J. Landskron, and H. K. Blomhoff. 2012. TLR9-signaling is required for turning retinoic acid into a potent stimulator of CD40L/IL-4–stimulated PBMCs, and they linked the levels of AICDA to distinct clinical features such as IgE/IgA levels and autoimmunity (39).