Retinoic Acid Improves Defective TLR9/RP105-Induced Immune Responses in Common Variable Immunodeficiency – Derived B Cells

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Retinoic Acid Improves Defective TLR9/RP105-Induced Immune Responses in Common Variable Immunodeficiency–Derived B Cells

Randi L. Indreævar,* Kristine L. Holm,* Pål Aukrust,†‡ Liv T. Osnes,§ Elin H. Naderi,* Børre Fevang,†‡ and Heidi K. Blomhoff*†

Common variable immunodeficiency (CVID) is a disease that is characterized primarily by low levels of serum Igs, resulting in a high incidence of infections. It also has been associated with impaired B cell signaling via TLR9 and reduced serum levels of vitamin A. Given the established link between vitamin A deficiency and increased susceptibility to infections, we investigated the ability of the vitamin A metabolite all-trans retinoic acid (RA) to restore the defective immune responses in CVID-derived B cells activated through the TLRs TLR9 and RP105. We demonstrate that RA almost normalizes proliferation and IL-10 secretion in patient-derived B cells. IgG secretion is also partially restored, but to a more moderate extent. This can be explained by impaired RA-mediated isotype switching in TLR9/RP105-stimulated CVID-derived B cells owing to reduced induction of activation-induced deaminase. Accordingly, these B cells secreted higher levels of IgM than did normal B cells, and RA augmented IgM secretion. The ability of RA to improve critical immune parameters in CVID-derived B cells stimulated through TLR9 and RP105 support the possibility of combining RA with TLR stimulation for the treatment of CVID. The Journal of Immunology, 2013, 191: 3624–3633.

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ommon variable immunodeficiency (CVID) is the most commonly occurring primary immunodeficiency of clinical significance (1). It is characterized by decreased serum Igs and by absent or poor responses to vaccines. Because of diminished Ig production, CVID patients suffer from recurrent bacterial infections, particularly of the respiratory tract. There is also an increased risk for developing noninfectious complications potentially related to a general immune dysregulation in these patients. The most frequent noninfectious complications linked to CVID are autoimmune disorders, noninfectious gastrointestinal and granulomatous disease, lymphoid hyperplasia, and malignancies (2, 3).

CVID is generally believed to result from an attenuation of normal B cell function. Genetic mutations affecting B or T lymphocytes have been identified in subsets of CVID patients (4). However, most of these defects eventually result in interrupted B cell maturation and signaling, resulting in reduced secretion of cytokines and isotype-switched Igs. In the majority of patients, no disease-associated mutation can be identified. There have been many attempts to identify the causes of CVID, but its etiology remains largely unknown, and it is not curable. Patients are usually subjected to IgG replacement therapy to reduce the infection rate (1). However, some in vitro studies suggested that B cells from CVID have the potential to restore at least some of their functions, but this has not resulted in novel treatment options (5).

Vitamin A is a group of compounds possessing the biological activity of retinol, and it is important for a well-functioning immune system (6). All-trans retinoic acid (RA) is the main vitamin A metabolite involved in immune regulation (6), where its primary function is to regulate gene transcription via binding to the nuclear receptors retinoic acid receptors (RARs) and retinoic X receptors (7). Although it is well established that vitamin A is crucial for an optimal immune defense, its effects on different cells of the immune system vary, depending on the type of costimulation involved (8, 9). Our group demonstrated previously that, although RA inhibits B cells stimulated via the BCR (9–11), it can enhance immunologic responses in B cells when the cells are stimulated through TLR9 alone (9) or in combination with the TLR RP105 (12).

TLRs are receptors belonging to the innate immune system, and they respond to pathogen-associated molecular patterns. TLR9 is an intracellular TLR located in the endoplasmic reticulum. Upon activation with its ligand, unmethylated bacterial CpG DNA, the receptor is translocated to the endosomal compartment where it induces activation of its downstream signaling pathways, resulting in proinflammatory responses (13). Activation of TLR9 can be achieved in vitro by the use of synthetic CpG oligonucleotides mimicking the CpG-rich bacterial DNA (14). Multiple studies reported defective TLR9 signaling in CVID-derived B cells (15–17); recently, defective signaling from TLR9 in combination with RP105 (CD180) was reported in three CVID patients (18). The natural ligand for RP105 has not been identified, but anti-RP105 Abs are potent activators of RP105 both in vitro (19) and in vivo (20).

Interestingly, it was shown that CVID patients are frequently deficient in vitamin A (21, 22). Taken together with our recent findings that RA is able to markedly enhance TLR-mediated B cell responses (9, 12), we wished to explore the possibility that RA could restore the frequently defective immune parameters...
in CVID patients. The present study demonstrates a remarkable ability of RA to enhance several of the deficiencies in CVID-derived B cells, such as proliferation and secretion of IgM and IL-10, when stimulated through TLR9 and RP105. The less pronounced, but still significant, induction of IgG by RA could be explained by the reduced induction of activation-induced deaminase (AID) involved in isotype switching of IgG.

Materials and Methods

Patients and healthy blood donors

Thirty-five CVID patients (17 males and 18 females) and 35 age- and sex-matched healthy donors were included in the study after informed consent. The patients were recruited from the Section for Clinical Immunology and Infection Medicine at Oslo University Hospital, Rikshospitalet, and were diagnosed according to the World Health Organization expert group on primary immunodeficiencies: the International Union of Immunological Societies scientific committee (23). Patients who were known to possess very low levels of B cells were excluded because of the numbers of B cells required to perform the analyses. The median age of the patients was 49 y (range: 23–65 y). All patients were regularly receiving s.c. and/or i.v. Ig-replacement therapy and did not suffer from acute infections at the time of blood collection. The blood samples were collected prior to Ig-replacement therapy and did not suffer from acute infections at the time of blood collection. The blood samples were collected prior to Ig-replacement therapy. The healthy donors were recruited from the Department of Biochemistry, Institute of Basic Medical Sciences, University of Oslo. The study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway Regional Health Authority. The investigation adhered to the principles outlined in the Declaration of Helsinki.

Reagents and Abs

Purified anti-human CD180 (RP105) (clone MHR73-11) and FITC-conjugated goat anti-mouse IgG (clone Poly4053) were from BioLegend (San Diego, CA). Purified mouse IgG1 control Ab (clone P5) was from eBioscience (San Diego, CA). The IgG oligodeoxynucleotide phosphorothionate 2006 and Ro 41-4253 were obtained from Enzo Life Science (Farmingdale, NY), and TTNPB was from Sigma-Aldrich (St. Louis, MO). RA was kindly provided by the Department of Nutrition at the University of Oslo and was originally obtained from Sigma-Aldrich. In all experiments, the concentrations of anti-RP105 and CpG were 1 μg/ml, whereas the concentration of RA was 100 nM, if not otherwise specified. PE-conjugated anti-CD20 (clone LT20) and PE-conjugated isotype-matched mouse IgG1 (clone ISS-21F5) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), and PE-conjugated anti-human CD38 (clone HIT2) and FITC-conjugated anti-human CD80 (clone L307.4) were from BD Biosciences (Franklin Lakes, NJ).

Sample preparation and cell culturing

Whole blood (36 ml) from CVID patients and healthy donors was collected into sterile sodium heparin tubes (BD Vacutainer) and transferred to sterile 75-ml cell culture flasks containing 15 ml RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 125 U/ml penicillin and 125 μg/ml streptomycin. Isolation and culturing of CD19⁺ B cells were performed as previously described (11).

Proliferation assay

Assessment of B cell proliferation by DNA synthesis was performed as previously described (12), and the incorporated radioactivity was counted on a liquid scintillation counter (Topcount; Packard Instrument, Meriden, CT).

ELISA assays

Supernatants were harvested after 5 d of incubation and stored at −80°C until analysis by ELISA, according to the manufacturer’s protocols. IgG and IgM ELISA Kits were from Bethyl (Montgomery, TX), and the IL-10 ELISA Kit was from eBioscience. To determine the concentration of IgG and IgM specific for Streptococcus pneumoniae, the same ELISA assays and procedures were used as for the determination of overall IgG and IgM, except that the original coating Abs were replaced by 100 μl Pneumovax (Sanofi Pasteur MSD, Brussels, Belgium). As described by Yamazaki et al. (18), the concentrations of Abs against S. pneumoniae in pooled serum was arbitrarily set as 20,000 U/ml, and the lower detection limit was 1.4 U/ml for S. pneumoniae–specific IgG and 2 U/ml for S. pneumoniae–specific IgM.

Surface expression assessed by flow cytometry

For analysis of surface expression of CD105 (CD180), CD20, CD38, and CD80, the cells were stained with anti-human CD180, followed by FITC-conjugated goat anti-mouse IgG, by PE-conjugated anti-CD20 Ab, by PE-conjugated anti-CD38 Ab, or by FITC-conjugated anti-CD80, according to the manufacturer’s protocols. Isotype-matched Abs were used as controls. The cells were analyzed using a FACSCalibur (BD Biosciences).

Analysis of AID and Blimp-1 by real-time quantitative PCR

Freshly isolated CD19⁺ B cells (0.7–1.0 × 10⁶ cells) were cultured in the presence of appropriate stimuli for 3 d and washed in PBS, and the pellets were stored at −80°C. RNA was isolated from the cell pellets (RNAeasy Plus Mini Kit; QIAGEN, Valencia, CA) and subjected to reverse transcription (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA), according to the manufacturers’ protocols. Real-time quantitative PCR (RT-qPCR) was performed using the CFX96 Real-Time PCR Detection system and SsoFast EvaGreen Supermix (both from Bio-Rad). Primers toward AID (AICDA), TBP, and Blimp-1 (PRDM1) were from QIAGEN; Quantitect Primer Assay and β-actin (forward, 5′-CTGAAACCCCAAGCCCAACAG-3′; reverse, 5′-CCAGGAAAGAGGAGTGGC-3′) were from Sigma-Aldrich.

Statistical analysis

Statistical analysis of the data was performed using PASW Statistics 18 (IBM, Armonk, NY). 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. The p values < 0.05 were considered significant.

Results

Characterization of B cells isolated from CVID patients and healthy donors

To assess the purity of the isolated B cells from CVID patients and healthy donors, the cells were stained with PE-conjugated anti-CD20 Ab or isotype-matched control Ab, and the cells were analyzed by flow cytometry. The CD20 B cell marker was used instead of CD19, because the CD19 protein on B cells is internalized during the purification procedure (24). As shown in Fig. 1, the purity of the isolated B cells from both healthy donors and patients was >98%, and there was no significant difference in the expression of CD20 between normal B cells and CVID-derived B cells (data not shown).

FIGURE 1. The purities of isolated CD19⁺ B cells from CVID patients and healthy donors. Freshly isolated CD19⁺ B cells from healthy donors (left panels) and CVID patients (right panels) were stained with PE-conjugated anti-CD20 Ab or isotype-matched control Ab, as described in Materials and Methods. The plots are based on gating on viable cells in the forward and side scatter plots, and one representative image of three is shown.


RA enhances the proliferation of B cells from CVID patients

There have been many reports on reduced TLR9-mediated immune responses in B cells from CVID patients (15, 25, 26) but few and conflicting reports with regard to the proliferative abilities of such cells (16, 18, 25). Having recently reported that RA enhances TLR9-mediated B cell responses (9), as well as up-regulation of surface CD80 (Fig. 2C), we wished to assess the proliferative response of CVID-derived B cells to CpG and anti-RP105 and to explore the ability of RA to enhance the proliferative response in CVID-derived B cells. Accordingly, B cells were isolated from CVID patients and healthy donors, and proliferation was assessed by measuring thymidine incorporation in response to various combinations of CpG, anti-RP105, and RA.

As shown in Fig. 2A, the proliferation of CVID-derived B cells compared with normal B cells was significantly reduced (p = 0.035) in response to costimulation with CpG and anti-RP105. In agreement with our recent report (12), RA markedly enhanced this proliferative response in B cells from healthy donors (p = 0.001, Fig. 2B), but even more interesting was the clear ability of RA to enhance this proliferation in CVID-derived B cells, as well. Thus, RA enhanced the uptake of [3H]thymidine in CpG/anti-RP105–stimulated CVID-derived B cells from 42,393 cpm to 69,857 cpm (p = 0.001, Fig. 2B), reaching levels comparable to those of normal B cells that were activated by CpG/anti-RP105. No significant reduction in the proliferative response to CpG alone was noted in CVID-derived B cells, but RA also enhanced the CpG-mediated response in B cells from both healthy donors and CVID patients. Still, the extent of this proliferation was negligible compared with that obtained by CpG in the presence of anti-RP105 and RA. The ability of RA to enhance proliferation of TLR9/RP105-activated CVID-derived B cells was further supported by the increased surface expression of the activation marker CD80 (Fig. 2C).

To rule out the possibility that the differential proliferation seen in normal and CVID-derived B cells stimulated via TLR9 and RP105 was simply due to differences in the proportion of viable cells, the extent of apoptosis was determined in B cells from five healthy donors and five CVID patients. There was a tendency toward reduced survival of CVID-derived B cells for 72 h compared with cells from healthy blood donors, but this difference was <10% and was not statistically significant (data not shown). RA only marginally (not statistically significantly) improved the viability of the stimulated cells, and its effects were the same in normal and CVID-derived B cells (data not shown). Taken together, we showed that the proliferative response to costimulation with CpG and anti-RP105 is diminished in CVID-derived B cells and that RA is able to nearly normalize this response.

RA enhances Ig production in CVID-derived B cells

CVID is hallmark by reduced serum levels of IgG and IgM and/or IgA (27). With the capability of RA to partially restore the reduced CpG/anti-RP105–mediated proliferation of CVID-derived B cells, we were anxious to see whether it could also enhance IgG and IgM production.

As expected, IgG secretion from CVID-derived B cells was significantly (p = 0.001) lower than in cells from the healthy donors when costimulated with CpG and anti-RP105 (Fig. 3A). In agreement with our recent report (12), RA potentiated IgG production in normal B cells (p = 0.001, Fig. 3B). The effect of RA in CVID-derived B cells was more modest, but it was still able to enhance the CpG/anti-RP105–mediated production of IgG in the CVID-derived B cells (p = 0.001, Fig. 3B). Although none of

![FIGURE 2](http://link.springer.com/content/pdf/10.1088/1355-5114/164/10/3626.pdf)
the stimulants could fully restore the defective IgG production in CVID-derived B cells, RA enhanced the IgG secretion by a factor of two compared with cells costimulated with TLR9 and RP105 alone, and the combination of CpG, anti-RP105, and RA induced a 6-fold increase in the production of IgG compared with cells cultured in medium alone.

Reduced levels of IgM are found in approximately 50% of CVID patients (27). Furthermore, reduced secretion of IgM has been reported in CVID-derived B cells stimulated through TLR9 (17) or TLR9 in combination with RP105 (18) in vitro. To our surprise, we did not find reduced production of IgM in CVID-derived B cells stimulated through TLR9 and RP105 (Fig. 3C). On the contrary, CVID-derived B cells generally secreted more IgM than did B cells from healthy donors, although the differences did not reach statistical significance (p = 0.072). Again, RA further enhanced the effects of CpG in the presence of anti-RP105 in normal B cells (p = 0.002) and in CVID-derived B cells (p = 0.001) (Fig. 3D). RA also significantly (p = 0.001) enhanced the effect of CpG alone, but the effects of RA together with CpG were marginal compared with cells treated with RA and CpG in the presence of

FIGURE 3. The effect of RA on Ig secretion in normal and CVID-derived B cells. CD19+ B cells (1.75 × 10^5 cells/ml) were cultured in various combinations of CpG (1 μg/ml), anti-RP105 (αRP; 1 μg/ml), and RA (100 nM). After 5 d, the supernatants were collected and subjected to total or S. pneumoniae–specific IgG and IgM ELISA in duplicates, as described in Materials and Methods. (A) The horizontal lines represent the median IgG values, boxes represent 50% of the measured values, and the whiskers represent ± 1.5 Interquartile range (n = 14). *p < 0.01, Mann–Whitney U test. (B) Unstimulated cells and cells costimulated with CpG and anti-RP105 in the presence or absence of RA. Each symbol represents the IgG measurement from an individual patient or healthy donor, and the horizontal lines indicate the median values (n = 14). *p < 0.01, Wilcoxon signed-rank test. (C) The lines represent the median IgM values, boxes represent 50% of the measured values, and the whiskers represent ± 1.5 interquartile range (n = 13). (D) Unstimulated cells and cells costimulated with CpG and anti-RP105 in the presence or absence of RA. Each symbol represents the IgM measurement for an individual patient or healthy donor, and the lines indicate the median values (n = 13). *p < 0.01, Wilcoxon signed-rank test. S. pneumoniae–specific IgG (E) and IgM (F) secreted from cells costimulated with CpG and anti-RP105 in the presence or absence of RA. Each symbol represents data from an individual patient or healthy donor, and the lines indicate the median values.
anti-RP105 (Fig. 3C). The effect of RA on IgA secretion from TLR9/RP105-activated cells was also evaluated in B cells from six CVID patients and healthy donors. In accordance with a previous report (17), the levels of IgA secreted by the CVID-derived B cells varied extensively but were significantly lower than the levels secreted from the normal B cells. RA was able to enhance TLR9/RP105-mediated IgA secretion in both the normal and CVID-derived B cells from 75.0 μg/ml to 147.0 μg/ml (SEM: 16.1 and 27.8) and from 16.2 μg/ml to 23.2 μg/ml (SEM: 14.0 and 19.0), respectively (data not shown).

To evaluate the ability of RA to increase specific Ab production, Abs against S. pneumoniae were measured in cells stimulated with CpG and anti-RP105 in the presence or absence of RA. The production of S. pneumoniae–specific IgM was reduced in CVID-derived B cells, and RA enhanced this production by a factor of two in both CVID-derived B cells and in normal B cells (Fig. 3F). RA also enhanced the secretion of S. pneumoniae–specific IgG in normal B cells (Fig. 3E). Unfortunately, however, S. pneumoniae–specific IgG levels were below the detection limit in the CVID-derived B cells.

RA restores the deficient production of IL-10 in CVID-derived B cells

Diminished TLR9-induced production of IL-10 is one of the B cell defects frequently linked to the reduced IgG production in B cells from CVID patients (15). We showed previously that IL-10 is in involved in both RA-induced proliferation and IgG synthesis in B cells stimulated with CpG alone (9) or CpG in combination with anti-RP105 (12). Hence, we considered it important to determine whether RA could restore the production of IL-10 in CVID-derived B cells stimulated via TLR9 and RP105.

As shown in Fig. 4A, we revealed a reduced production of IL-10 in CVID-derived B cells compared with normal B cells (p = 0.039). RA alone was not able to induce an appreciable production of IL-10 in either the normal or CVID-derived B cells; importantly, however, it was able to restore the CpG/anti-RP105-mediated production of IL-10 by B cells from CVID patients. In fact, the fold induction induced by RA was even greater in CVID-derived B cells than in normal B cells (3.3-fold and 2.1-fold, respectively) (Fig. 4B). It should be noted that in our previous study (12), RA’s ability to enhance IL-10 secretion in normal B cells was due to increased production of IL-10 per cell and not the result of an increased frequency of IL-10–producing cells.

RA normalizes differentiation of CVID-derived B cells into CD38+ and Blimp-1–expressing plasma cells but only partially restores the expression of AID

In an attempt to explain the mechanisms behind the limited ability of RA to restore IgG production in CVID-derived B cells, we focused on the process of isotype switching of IgGs catalyzed by AID. We show in this study that RA caused a 5.9-fold induction of AID expression in normal B cells costimulated via TLR9 and RP105, whereas only a 1.9-fold induction was observed in CVID-derived B cells (Fig. 5A).

The reduced expression of AID in RA-stimulated CVID-derived B cells could be due to a general failure of these cells to undergo differentiation into plasma cells in the presence of RA. To rule out this possibility, we analyzed the expression of the plasma cell marker CD38 by flow cytometry and the differentiation marker Blimp-1 (PRDM1) by RT-qPCR. CD38 is a cell surface molecule expressed on subsets of B cells, and it is particularly enhanced on Ig-producing plasma cells. As shown in Fig. 5C and 5D, the expression of CD38 and the percentage of CD38+ cells were reduced in CVID-derived B cells activated via TLR9 and RP105 compared with normal B cells. However, RA was able to enhance the expression of CD38 and the percentage of CD38-expressing cells in B cells from CVID patients to nearly normal levels. Furthermore, RA induced the expression of Blimp-1 to the same extent in CVID-derived B cells as in normal B cells (Fig. 5E). Taken together, we believe that the limited ability of RA to enhance IgG levels in CVID-derived B cells can be explained by reduced AID-mediated isotype switching and not by a general differentiation failure.

RAR is required for RA to enhance CpG/anti-RP105–mediated IgG secretion

Although RAR-independent effects of RA have been reported (28, 29) the main mechanism of RA is to regulate gene expression through activation of nuclear receptors, such as RARs (7). To examine the involvement of RARs in CpG/anti-RP105–mediated IgG secretion, normal CD19+ B cells were isolated from buffy coats, as previously described (24). TTNPB and Ro 41-5253, selective agonist and antagonist of RARα, respectively, were added to the cell cultures together with various combinations of CpG, anti-RP105, and RA. The effects of TTNPB were also explored in B cells from whole blood from three patients and three healthy donors. Supernatants were collected after 5 d for analysis of IgG by ELISA.
As shown in Fig. 6A, TTNPB was as efficient as RA in enhancing CpG/anti-RP105–mediated IgG secretion, demonstrating the involvement of RARα in this process, and the RARα receptor agonist was also active in CVID-derived B cells (Fig. 6B). The crucial role of RARα was demonstrated further by the ability of Ro 41-5253 to almost completely block the enhancing effect of RA on CpG/anti-RP105–induced IgG production (Fig. 6A).

Expression of RP105 in normal and CVID-derived B cells
Defective TLR9-mediated responses in CVID-derived B cells has been linked to reduced expression of TLR9 itself (15, 25); hence, we wished to assess whether the reduced responses to anti-RP105 in the presence of TLR9 stimulation could be accounted for by the reduced expression of RP105 in CVID-derived B cells. To this end, the surface expression of RP105 was determined by flow
cytometry of freshly isolated B cells from CVID patients and healthy donors stained with anti-RP105 (CD180). The expression of RP105 on B cells from a healthy donor and a CVID patient is shown in Fig. 7A; as shown in Fig. 7B, there was no statistical difference between the median expression of RP105 in the healthy and CVID-derived B cells. Because the only available Ab clone raised against RP105 is the one that we used to activate the cells, it was not possible to analyze the expression of RP105 in TLR9/RP105-stimulated B cells.

RA improves immunological parameters in CpG/anti-RP105–stimulated B cells from individual CVID patients

To summarize the effects of RA on different immunological parameters, the individual measurements of CpG/anti-RP105–stimulated cells in the presence or absence of RA are shown in Fig. 8. It becomes clear that RA improves the defective proliferation and secretion of IL-10 and IgM in B cells from all but one of the CVID patients. The defect in IgG secretion was more severe, but importantly RA also enhanced IgG secretion in B cells from all the patients.

Discussion

CVID is a heterogeneous group of primary immunodeficiencies predominantly characterized by reduced levels of serum Igs and, in particular, low levels of IgG (1). Interestingly, frequent vitamin A deficiencies also were reported among these patients (21, 22), a condition that is known to increase the risk for infections (7). Based on our recent demonstration of the ability of RA to strengthen immune responses in normal B cells stimulated via TLRs, such as TLR9 and RP105 (9, 12), we addressed the possibility of using RA to restore the immune defects in CVID-derived B cells.

There have been conflicting reports regarding the proliferative status of B cells from CVID patients (16, 18, 25). We found that the proliferation was significantly reduced in CVID-derived B cells stimulated through TLR9 and RP105. In accordance with a previous report (25), we did not find reduced proliferation of B cells from CVID patients when the cells were stimulated via TLR9 alone. However, in the same report by Escobar et al. (25), diminished B cell proliferation was observed when the cells were stimulated with extracts from *Haemophilus influenzae* and *S. pneumoniae*, which activate several TLRs. Taken together with
The inset shows the data for IgG secretion from CVID-derived B cells. Each line represents the results from one CVID patient or healthy donor.

RA on TLR9/RP105-induced proliferation and secretion of IL-10 and Igs. Individual CVID patients and healthy controls. Summary of the effects of switching of B cells. Accordingly, we found that IgG production was even greater in the CVID-derived B cells than in B cells from healthy donors.

In fact, the stimulating ability of RA was able to almost completely restore the proliferative response of RP105-stimulated B cells from CVID patients, it is noteworthy that the effects in CVID-derived B cells appear when the cells engage several distinct receptors. In light of the diminished proliferation of TLR9/RP105-stimulated B cells from CVID patients, the induction of IgG induced by RA was highly statistically significant. It is worth noting that RA enhanced the levels of IgG in CVID-derived B cells two-fold compared with cells costimulated with CpG and anti-RP105, as well as by six-fold compared with the levels in cells cultured in medium alone. Upon i.v. or s.c. IgG-replacement therapy of CVID patients, serum IgG levels increased only 3–6-fold (30). Thus, any treatment resulting in a 6-fold increase in IgG from the basal levels obviously would be highly clinically relevant.

The impact of RA on specific Ig production was elucidated by the determination of the levels of IgG and IgM specific for S. pneumoniae Ags. We were not able to attain detectable S. pneumoniae-specific IgG levels in CVID-derived B cells, which was not surprising given the extensively reduced levels of total IgG in these cells. However, RA significantly increased the level of S. pneumoniae-specific IgG in normal cells. In contrast to the somewhat elevated levels of total IgM that we uncovered in CVID-derived B cells, we noted that the amount of S. pneumoniae-specific IgM was reduced. IgM memory B cells are considered the primary source of IgM as a first-line defense against encapsulated bacteria, such as S. pneumoniae, and it was reported that some CVID patients have lower levels of IgM memory B cells (31). However, because the proportion of IgM memory B cells in the present patient material was within the normal range (on average 18.8% in CVID patients compared with the normal range of 7.4–32.5%), we can exclude this a possible explanation for the apparent discrepancy between total and S. pneumoniae-specific IgM levels that we observed in CVID-derived B cells. Nevertheless, the important finding in this context was that RA significantly increased S. pneumoniae-specific IgM levels in both normal and CVID-derived B cells. Because infections caused by S. pneumoniae are known to cause chronic lung disease in CVID patients (4), the RA-mediated increased production of S. pneumoniae-specific IgM might help to reduce these serious complications in CVID patients.

To shed light on the mechanisms underlying the discrepancy between the ability of RA to nearly fully restore the proliferative status versus the more limited effect on IgG production in CVID-derived B cells, we addressed the involvement of IL-10 in these processes. There have been reports on defect production of IL-10 in CVID-derived B cells, we noted that the amount of S. pneumoniae-specific IgG was reduced. IgM memory B cells are considered the primary source of IgM as a first-line defense against encapsulated bacteria, such as S. pneumoniae, and it was reported that some CVID patients have lower levels of IgM memory B cells (31). However, because the proportion of IgM memory B cells in the present patient material was within the normal range (on average 18.8% in CVID patients compared with the normal range of 7.4–32.5%), we can exclude this a possible explanation for the apparent discrepancy between total and S. pneumoniae-specific IgM levels that we observed in CVID-derived B cells. Nevertheless, the important finding in this context was that RA significantly increased S. pneumoniae-specific IgM levels in both normal and CVID-derived B cells. Because infections caused by S. pneumoniae are known to cause chronic lung disease in CVID patients (4), the RA-mediated increased production of S. pneumoniae-specific IgM might help to reduce these serious complications in CVID patients.

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Nevertheless, it is worth noting that enhanced secretion of IL-10 per se might be beneficial in CVID patients, because of its general...
anti-inflammatory effects. It was shown that regulatory B cells are an important source of IL-10 and that, upon activation, these cells can suppress inflammatory and autoimmune responses (34). Hence, the enhanced secretion of IL-10 induced by RA in TLR9/RP105-stimulated CVID-derived B cells might aid in the defense against autoimmune and inflammatory complications frequently associated with CVID.

To explain the differential production of IgG in CVID-derived B cells and normal cells, it is important to take into consideration the various subsets of B cells in the CD19+ populations used in the current study. In a previous study by our group (12), we compared the effects of RA and TLR9/RP105 stimulators on naïve (CD19+ CD27−) versus memory (CD19+CD27+) B cells from healthy blood donors. Although RA was able to enhance the TLR9/RP105-mediated IgG production in both subsets of cells, its effect on memory cells was much stronger. However, in the current study, the percentage of naïve B cells in the CD19+ population from CVID patients (68.8%) was within the normal range (42.6–82.3%). In contrast, we found a lower proportion of class-switched B cells (range, 0.8–1.5) compared with previous reports (2, 35). Because of the very limited number of CD19+ B cells (range, 0.8–1.5 × 10^6) that could be obtained from the 35 ml of whole blood taken from each patient, it was impossible to subdivide the CD19+ fraction of cells into numbers that allow functional studies on IgG secretion.

However, diminished isotype switching catalyzed by AID is one of the events that has been used to explain the deficient IgG production in CVID-derived B cells (16, 26). In this study, we demonstrated a limited ability of RA to restore the expression of AID in CVID-derived B cells compared with normal cells, supporting the notion that reduced production of IgG in RA-treated B cells from CVID patients can be attributed to diminished induction of AID. We ruled out the possibility of a general failure of CVID-derived B cells to differentiate into plasma cells. Hence, by analyzing the expression of the plasma cell marker CD38, we showed that RA was able to efficiently restore the expression of CD38 in TLR9/RP105-mediated differentiation of CVID B cells into plasma cells. Furthermore, RA enhanced the differentiation marker Blimp-1 to the same extent in normal and CVID-derived B cells. To further substantiate our conclusion, we found that the total levels of secreted IgM were higher in TLR9/RP105-stimulated B cells from CVID patients than from healthy blood donors. These observations are consistent with diminished AID-mediated isotype switching and not a general failure of the cells to differentiate into plasma cells.

The effects of RA on the immune parameters in normal and CVID-derived B cells are summarized in Fig. 8. It becomes clear that RA has a remarkable ability to almost completely normalize the deficient proliferation and IL-10 secretion in TLR9/RP105-stimulated B cells from all patients with CVID, as well as to improve Ig production in almost all the patients. Based on these encouraging results, it is tempting to suggest that CVID patients would benefit from treatment with a combination of RA, CpG, and anti-RP105. CpG is already in clinical use as a vaccine adjuvant and is in clinical trials for cancer treatment (36). Anti-RP105 has not been tested in humans; however, it was recently shown in a mouse model that i.v. anti-RP105 Abs induced Ig production of several subclasses and was well tolerated by the mice (20). mAbs are now being used routinely in the treatment of diseases such as cancer and rheumatoid arthritis, and there is no obvious reason why humanized anti-RP105 Abs should not be tested for treatment of CVID. In our recent study (12), we showed that 100 nM RA was the optimal concentration to synergize with CpG and anti-RP105 to enhance the immunological responses in normal B cells, and this concentration was used in the current study. Such pharmacological doses of RA are achieved in serum of cancer patients treated with high doses of retinoids (37). However, we noticed stimulating effects of RA at concentrations as low as 0.1 nM, suggesting that the levels normally obtained with a vitamin A–rich diet (5–10 nM) could result in biologically significant effects in CVID patients. The benefit of vitamin A supplementation was proved in two studies of CVID patients (21, 38); it resulted in elevated levels of serum IgA and IL-10 in vivo and increased production of IgG and IgM from PBMCs cultured in vitro. We hope that the current study will encourage further research on the potential use of RA together with CpG and anti-RP105 in the treatment of this severe and common immunodeficiency.

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Disclosures
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References