

Letter to the Editor

Phenotypic differences between IgG₄⁺ and IgG₁⁺ B cells point to distinct regulation of the IgG₄ response

To the Editor:

IgG₄ antibodies provide humoral immune tolerance on chronic antigenic stimulation. Specific IgG₄ antibodies are frequently observed during repeated antigenic stimulation such as specific immune therapy or treatment with therapeutic proteins (factor VIII, adalimumab).¹ Furthermore, asymptomatic helminth infections induce strong and specific IgG₄ antibodies.² Besides IgG₄-dominated antigen-specific antibody responses, the so-called IgG₄-related diseases³ are characterized by highly elevated serum levels of polyclonal IgG₄. In all of these situations, it remains unclear which factors drive the development and maintenance of the IgG₄ response. In particular, the characteristics of B cells that produce these antibodies are unknown.

IgG₄ responses often become prominent only after a prolonged period of repetitive stimulation with the antigen.¹ How the delay in IgG₄ production is regulated is not known. The T_H2 cytokines IL-4 and IL-13 are deemed necessary to induce isotype switching to IgG₄. The tolerance-associated cytokine IL-10 may preferentially promote class switch toward IgG₄ over IgE.⁴ IL-10 production becomes prominent only in those situations in which immune tolerance is required. Interestingly, in both SIT and helminth infections, IgG₄ serum levels also correlate with immune tolerance,

which can at least in part be explained by the poor immune-activating properties of IgG₄ antibodies.¹ A regulatory role for IL-10 in the induction of the tolerogenic IgG₄ response would thus coordinate cellular and humoral tolerance. Indeed, sorted IL-10-producing B cells produce increased amounts of IgG₄ after culture.⁵ It is not known whether established IgG₄⁺ B cells produce IL-10 to promote tolerance in an autocrine or paracrine fashion. B-cell intrinsic factors may also play a role in the slow appearance of IgG₄ antibodies, such as the possibility of indirect isotype switch, or a differential readiness of IgG₄⁺ and IgG₁⁺ memory B cells to differentiate into antibody-secreting cells.

The need to understand how IgG₄⁺ B-cell formation is regulated and related to immune tolerance is hampered by complete lack of information on the phenotype and function of IgG₄⁺ B cells. Until now, attempts to positively isolate and characterize IgG₄⁺ B cells have been unsuccessful. To optimize isolation by cell sorting, we tested several commonly used anti-IgG₄ clones (HP6025, MH164.4) for their ability to stain a B-cell-line-expressing IgG₄ (CRL2632) and found that they very poorly recognized the membrane-bound form of IgG₄ (Fig 1, A; for methods, see the Online Repository at www.jacionline.org). These antibodies bind to the CH3 domain of IgG₄, which may be less accessible in its membrane-bound form. In contrast, a clone that is directed against the more membrane-distal CH2 domain (MH164.1) did bind to the membrane-bound form of IgG₄ on the CRL2632 cell line (Fig 1, A). No cross-reactivity to other

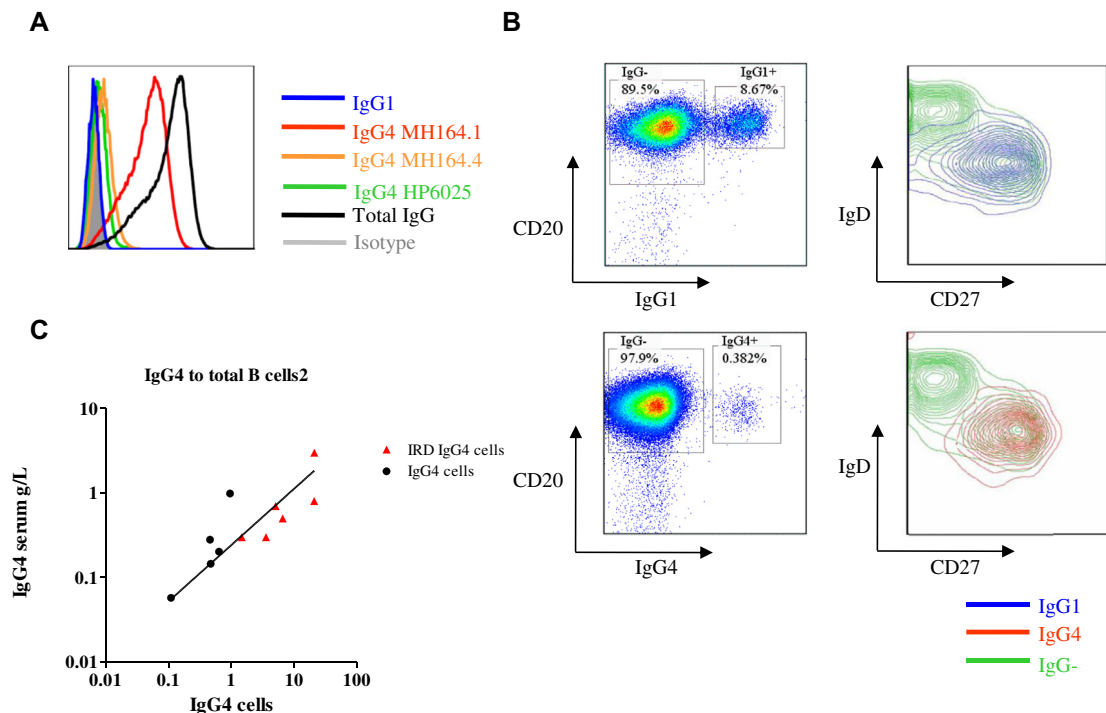


FIG 1. The IgG₄ antibody MH164.1 can be used to specifically stain and sort IgG₄⁺ B cells. **A**, Binding of different anti-IgG₄ antibodies to the IgG₄-expressing cell line CRL2632. **B**, IgG₁ (upper panel) and IgG₄ staining (lower panel) on B cells isolated with anti-CD19 magnetic beads from PBMCs of a healthy donor. Cells were costained for CD20, IgD, and CD27. **C**, IgG₄ serum levels and number of B cells that express IgG₄ of healthy controls (circles) and of patients with IgG₄-related diseases (IRDs) (triangles), expressed as a percentage of total IgG.

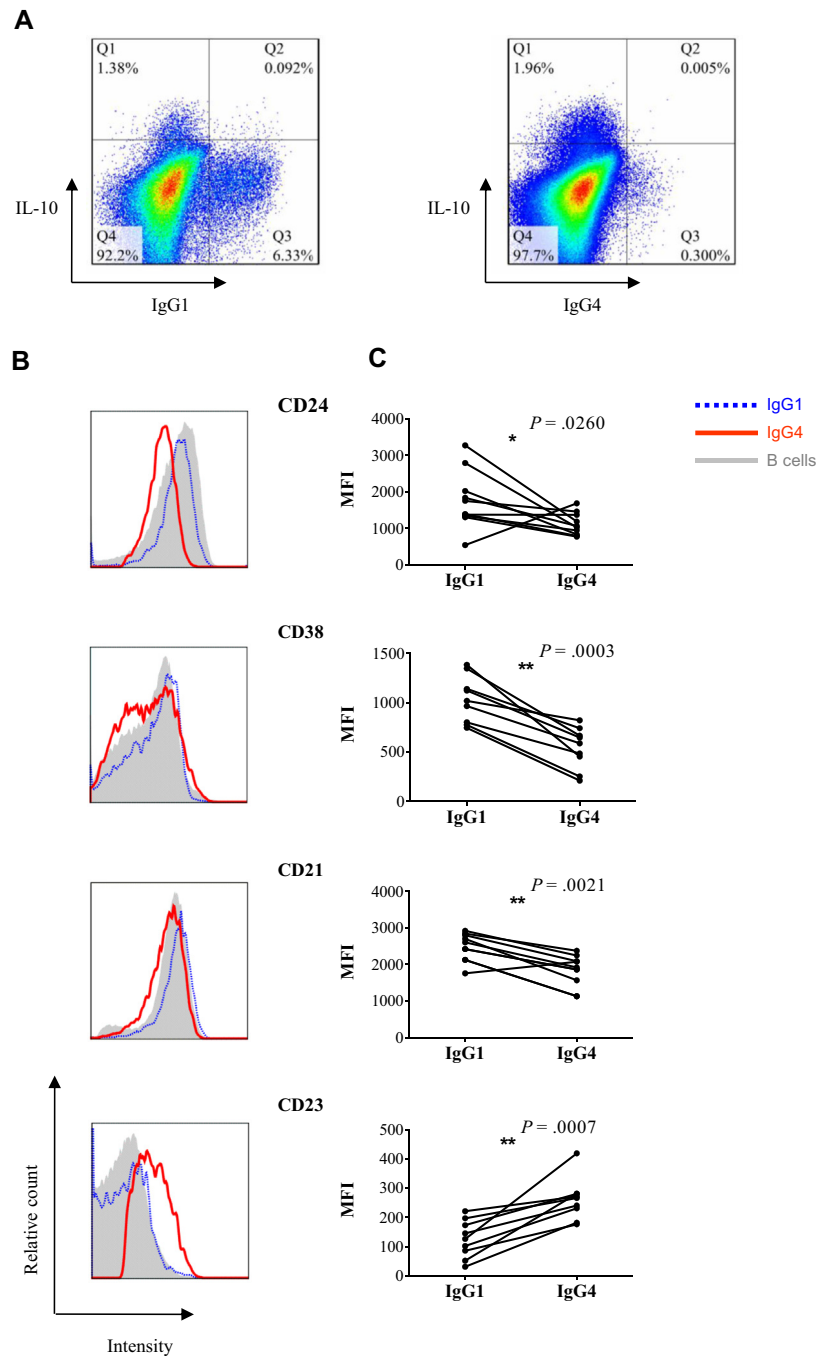


FIG 2. IgG₄⁺ B cells have a phenotype distinct from that of IgG₁⁺ B cells and regulatory B cells. **A**, B cells were isolated and stimulated with CpG. IL-10 production was analyzed by using intracellular staining on flow cytometry. Dotplot of a representative experiment is shown. Cells were first gated on CD20 positivity. IgG₄⁺ B cells do not have increased IL-10 production compared with IgG₁⁺ B cells. A total of 0.2% to 8.8% of the cells were IL-10 positive relative to CD20⁺ B cells, while 0.6% to 4.4% of CD20⁺IgG₁⁺ cells and 3.5% to 8.9% of CD20⁺IgG₄⁺ cells were positive for IL-10 ($n = 5$). **B**, CD20⁺IgG₁⁺ and CD20⁺IgG₄⁺ B cells were assessed for surface expression of regulatory B-cell markers CD24 and CD38, complement receptor 2 (CD21), and Fc ϵ RII receptor (CD23). In all cases, histograms for a representative experiment and **(C)** median fluorescence intensities from 8 or 9 healthy donors are shown. *MFI*, Median fluorescence intensity.

subclasses was found by ELISA (not shown), and no binding to membrane-bound IgG₁ was observed (see Fig E1 in this article's Online Repository at www.jacionline.org). Using MH164.1, IgG₄⁺ B cells were subsequently identified from primary B-cell

isolates from healthy donors (Fig 1, B, lower panels) and found to represent a small but distinct population of cells, which are largely IgD⁻, CD27⁺, similar to IgG₁⁺ memory B cells (Fig 1, B, upper panels; also see Fig E3 in this article's Online Repository

at www.jacionline.org). In single-cell cultures, cells sorted for IgG₄ produced exclusively IgG₄ antibodies, demonstrating that sorting was subclass specific (Fig E1). Combined, these data show that we now can specifically identify and isolate IgG₄⁺ memory B cells.

Next, we compared the frequency of IgG₄⁺ B cells to the serum levels of IgG₄ in a group of both healthy donors and patients with IgG₄-related diseases (who typically have serum IgG₄ levels up to 50 times normal). A correlation between serum IgG₄ levels and IgG₄⁺ B cells was found (Fig 1, C). Furthermore, there is an overall correspondence between the relative abundance of IgG subclasses for serum IgG and circulating IgG B cells (see Fig E2 in this article's Online Repository at www.jacionline.org). These data suggest that the number of subclass-specific memory B cells correlates with the level of IgG subclass-specific antibody-secreting cells.

Because IgG₄ production is increased in cultures of isolated IL-10-producing B cells,⁵ we investigated whether established IgG₄⁺ B cells are still IL-10-producing regulatory B cells. Stimulation of B cells from healthy donors demonstrated that IL-10 was predominantly produced by cells not expressing IgG₄ (Fig 2, A). Furthermore, the expression of both CD24 and CD38—described to be present on human regulatory B cells⁶—was lower on IgG₄⁺ B cells than on IgG₁⁺ B cells (Fig 2, B). Taken together, class-switched IgG₄⁺ B cells are not “classical” regulatory B cells. There were also no differences in expression of IL-10Ra or IL-10Rb between IgG₄⁺ and IgG₁⁺ B cells (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting that the responsiveness toward IL-10 is similar for both cell types.

During an ongoing immune response, immune complexes can exert regulatory functions via complement and Fc receptors. We therefore studied the expression of complement and Fc receptors on IgG₄⁺ B cells and compared this with that on IgG₁⁺ B cells. Notably, IgG₄⁺ B cells expressed significantly less of the complement receptor 2 (CD21; Fig 2, B), suggesting a decreased sensitivity to complement components. CD21 is part of the B-cell receptor complex and CD21 coligation lowers the threshold for B-cell activation and clonal expansion.⁷ Larger amounts of antigen may be needed to induce the production of IgG₄ antibodies, which might contribute to the need for prolonged stimulation with antigen. Furthermore, there was a higher expression of the Fcε receptor (CD23) on IgG₄⁺ cells (Fig 2, B). Because many allergens are good inducers of IgG₄ as well as IgE and CD23 can be upregulated by IL-4,⁸ it is interesting that IgG₄⁺ B cells appear to have increased capacity to capture IgE molecules. Signaling via IgE might trigger IgG₄⁺ cells and contribute to dampening of allergic IgE responses. Besides IgE antibodies, CD23 binds to CD21 and several integrins.⁸ Taken together, IgG₄⁺ B cells may stand out in the way they receive signals via the B-cell receptor complex. For complement

receptor 1 (CD35) and the inhibitory Fcγ receptor IIb (CD32), no significant differences were observed (Fig E3).

In summary, we identified an IgG₄-specific antibody suited for staining B cells and isolating IgG₄⁺ B cells. Our data suggest that although IgG₁⁺ and IgG₄⁺ cells are similar in many ways, there are differences in the phenotype of IgG₄⁺ B cells with regard to their potential to react to complement activation and immune complexes. These results may help explain the differential regulation of IgG₄ antibody response.

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METHODS

Labeling of mAbs with fluorescent dye

Antibodies (anti-IgG₁, HP6188; anti-IgG₂, HP6014; anti-IgG₃, HP6095; and anti-IgG₄: MH164.1 (HP6098) and MH164.4 (HP6196) [Sanquin Blood Supply, Amsterdam, The Netherlands]; and HP6025 [Invitrogen, Paisley, United Kingdom])^{E1} were labeled with DyLight488 or DyLight649 fluorophore (Pierce/Thermo Scientific, Rockford, Ill). Excess dye was removed by repeated dilution/concentration by using Amicon Centrprep centrifugal filter devices (Millipore, Billerica, Mass) until dye could no longer be detected in the filtrate. The average degree of labeling was between 4 and 6.

Cell lines

CESS human myelomonocytic leukemia cells expressing IgG₁,^{E2} CRL2632 human diffuse large-cell lymphoma cells expressing IgG₄,^{E3,E4} and murine 3T3 fibroblast cells transfected with CD40L^{E5} were cultured in Iscove's modified Dulbecco's medium (Lonza, Basel, Switzerland) containing 10% FCS (Bodinco, Alkmaar, The Netherlands), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany), and 20 µg/mL human apo-transferrin (Sigma-Aldrich, depleted for human IgG with protein A Sepharose [GE Healthcare, Buckinghamshire, United Kingdom]).

PBMC isolation from healthy donors and patients with IRD

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS, Dundee, United Kingdom) from a buffy coat or whole blood obtained from healthy donors or patients with IgG₄-related diseases (Sanquin Blood Supply). All healthy donors provided written informed consent in accordance with the protocol of the local institutional review board, the Medical Ethics Committee of Sanquin Blood Supply. The Medical Ethics Committee of Sanquin approved the study. All patients with IgG₄-related diseases were prospectively recruited in the John Radcliffe Hospital in Oxford. Diagnosis was based on Histology, Imaging, Serology, Organ manifestations, Response to steroid treatment criteria for autoimmune pancreatitis and IgG₄-related cholangitis and the Japanese Consensus Diagnostic Criteria for IRC. Ethical approval for the study was granted by the Oxfordshire Research Ethics Committee A, and funding was provided by a Wellcome Trust Research Fellowship. Ten patients, 90% men, median age 62 years (41-75 years) with elevated serum IgG₄ levels (>1.4 g/L), were included. All had histologic confirmation of disease.

Isolation, proliferation, and identification of IgG₄⁺ and IgG₁⁺ B cells

B cells were purified by using anti-CD19 Dynabeads and DETACHaBEAD (Invitrogen, Paisley, United Kingdom), according to the manufacturer's instructions. IgG₄⁺ and IgG₁⁺ memory B cells were isolated by fluorescence-activated cell sorting (FACS) sorting (BD FACSaria II) using anti-CD20-PerCP-Cy5, anti-CD27-APC, anti-IgD-PE (BD Bioscience, Franklin Lakes, NJ), and anti-IgG₄-DyLight488 (MH164.1) or anti-IgG₁-DyLight488. Isolated B cells were sorted 1 cell per well in 96-well flat-bottom plates. Cells were cultured for 12 days in RPMI (Gibco, Carlsbad, Calif) containing 5% FCS (Bodinco), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich), 20 µg/mL human apo-transferrin (Sigma-Aldrich, depleted for human IgG with protein A sepharose [GE Healthcare]), 1 ng/mL IL-1β, 50 ng/mL IL-21, 0.3 ng/mL TNF-α, and 0.5 µg/mL R848 (Alexis, Heidelberg, Germany) in the presence of 10,000 irradiated (30Gy), CD40L-expressing 3T3 cells. After 12 days, supernatants were tested in an IgG subclass ELISA to determine total IgG production as well as IgG₁, IgG₄, and IgM production.

IgG subclass ELISAs

Maxisorp ELISA plates (NUNC, Roskilde, Denmark) were coated overnight with 1 µg/mL anti-IgG (MH16-1), anti-IgG₁, anti-IgG₂, anti-IgG₃, or anti-IgG₄ (Sanquin Reagents, Amsterdam, The Netherlands) in PBS. After

washing 5 times with PBS-Tween, plates were incubated with culture supernatant or serum (diluted appropriately in high-performance ELISA [HPE] buffer, Sanquin Blood Supply). Subsequently, the plates were washed and incubated for 1 hour with horseradish peroxidase-conjugated mouse-anti-human-IgG (0.5 µg/mL in HPE) or horseradish peroxidase-conjugated mouse-anti-human-IgM (0.5 µg/mL in HPE). After washing, the ELISA was developed with 100 µg/mL tetramethylbenzidine in 0.11 mol/L sodium acetate (pH 5.5) containing 0.003% (v/v) H₂O₂. The reaction was stopped with 2M H₂SO₄. Absorption at 450 nm was measured with a multiscan (Multiskan; Titertek, Elfab Oy, Finland). Results were related to a titration curve of purified recombinant IgG₁ or IgG₄ or a serum pool in each plate.

FACS analysis of IgG₁⁺ and IgG₄⁺ B cells

Expression of cell surface molecules was determined by using a Beckton Dickinson LSR II (BD Biosciences, Franklin Lakes, NJ) and analyzed with FACS DIVA software (BD Biosciences) and FlowJo software (v7.6.5; Treestar, Ashland, Ore). B cells were washed with PBS supplemented with 0.1% BSA (Sigma-Aldrich) and incubated with mAb or the appropriate isotype control in the dark, at room temperature, for 20 minutes. The following antibodies were used: DyLight488-conjugated IgG₁ isotype control (purified IgG₁-anti-bet v1) and phycoerythrin (PE)-conjugated IgG_{2a} isotype control (Invitrogen) and PE-conjugated IgG₁ isotype control (Caltag/Life Technologies, Carlsbad, Calif) and allophycocyanin (APC)-conjugated IgG₁ isotype control (MOPC-21), PerCP-Cy5.5-conjugated IgG₁ isotype control (MOPC-21), anti-IgD-PE (IA6-2), anti-CD23-PE (M-L233), anti-CD24-PE (ML5), anti-CD35-PE (E11), anti-CD21-APC (B-ly4), anti-CD27-APC (L128), anti-CD32-APC (FL18-26), anti-CD38-APC (HB-7), anti-CD19-PerCP-Cy5.5 (SJ25C1) and anti-CD20-PerCP-Cy5.5 (L27) (BD Biosciences), and anti-IgG₁ (MH161-1) and anti-IgG₄ (MH164-1) (Sanquin Reagents) labeled with DyLight488 or DyLight649 (Thermo Scientific). Cells were washed twice and resuspended in PBS supplemented with 1% BSA. 4'-6-Diamidino-2-phenylindole, dihydrochloride (Sigma-Aldrich) was added immediately before analysis to stain for cell viability and to exclude dead cells from analysis.

Analysis of IL-10 production by B cells

Cytokine production was measured by intracellular staining after 24 hours of stimulation with 1.25 µM CpG ODN2006 (Invitrogen) and stimulation during the last 5 hours with 0.1 µg/mL phorbol 12-myristate 13-acetate, 1 µg/mL ionomycin, and 10 µg/mL brefeldin A (Sigma-Aldrich). Cells were washed twice with PBS, fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes, and after washing with PBS and PBS containing 1% BSA (Sigma-Aldrich) permeabilized with 0.5% saponin (Calbiochem, Darmstadt, Germany) in PBS containing 1% BSA and incubated with fluorescent antibodies for 30 minutes at room temperature. Staining was analyzed on an LSR II (BD Biosciences) and analyzed with FACS Diva software (BD Biosciences) or FlowJo (v7.6.5, Treestar).

RT-PCR for IL10R and IL10

cDNA from B cells was made as described previously.^{E6} Briefly, RNA was isolated from unstimulated (IL-10R) or stimulated (for IL-10; as described before) IgG₁⁺, IgG₄⁺, or memory B cells and reverse transcribed to cDNA by using random hexamers in combination with Superscript II and an RNase H-reverse transcriptase kit. TaqMan Gene expression assay kit (Applied Biosystems/Life Technologies, Carlsbad, Calif) with primers for 18S rRNA, IL-10 and IL-10RA, and IL-10RB was used according to manufacturer's protocol. Gene expression levels were measured in triplicate reactions for each sample. All results were normalized to the internal control 18S rRNA and are expressed relative to the expression levels found in total B cells.

Statistical analysis

Statistical differences were determined by a 2-sided, paired Student's *t* test, using GraphPad Prism (version 5.01, GraphPad Software, San Diego, Calif). Results were considered significant as follows: **P* < .05, ***P* < .01, and ****P* < .005.

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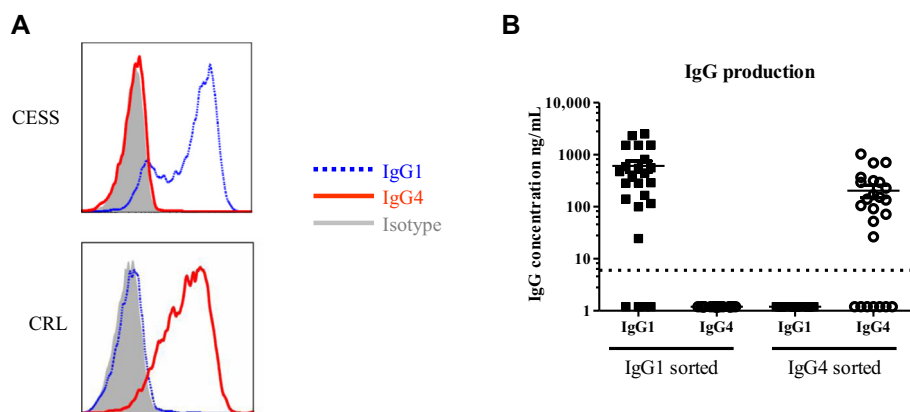


FIG E1. MH164.1 specifically stains IgG₄⁺ B cells. **A**, *Upper panel*: staining for IgG₁, *lower panel*: staining for IgG₄, *red*: CRL2632 (expresses IgG₄), *blue*: CESS (expresses IgG₁). **B**, Antibody production by sorted single B cells; 24 samples per condition, assay cutoff 6 ng/mL. The percentage of sorted cells that produced antibodies was a little higher for IgG₁-sorted cells (83%) than for IgG₄⁺-sorted (71%) cells, suggesting that different culture conditions may be needed for IgG₄⁺ cells. CRL, CRL2632.

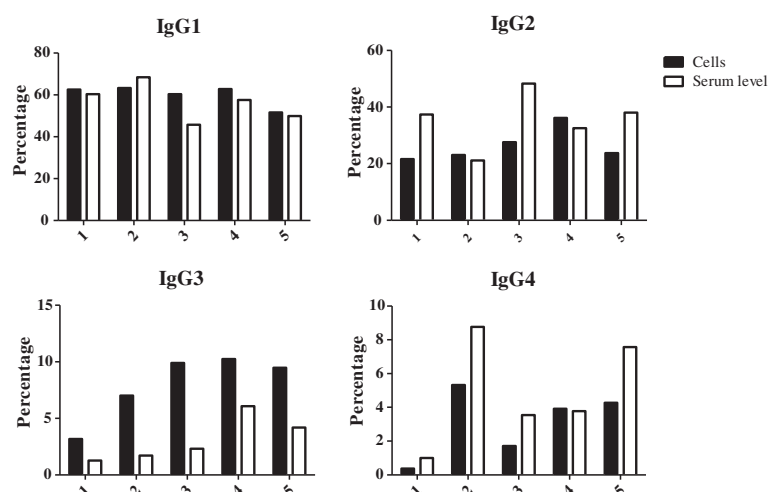


FIG E2. Antibody levels correlate to B-cell frequency expressing IgG isotypes. Serum and EDTA blood were obtained from 5 healthy donors (1-5). In serum, levels of IgG subclasses were measured by using ELISA. Percentages of subclass antibodies relative to total IgG measured are depicted with *black bars*. In the same donors, the percentage of B cells expressing IgG subclasses 1 to 4 relative to cells expressing total IgG was measured (*white bars*). IgG₁⁺ B cells were most abundant (50% to 65%), followed by IgG₂⁺ (20% to 35%), IgG₃⁺ (3% to 10%), and IgG₄⁺ (0.3% to 5%). In the serum, a similar pattern was found, IgG₁ being most abundant (4.2-7.5 mg/mL; 50% to 70%), followed by IgG₂ (2.3-6.4 mg/mL; 20% to 40%), IgG₃ (0.1-0.8 mg/mL; 1.2% to 6%), and IgG₄ (0.1-1.0 mg/mL; 1% to 9%).

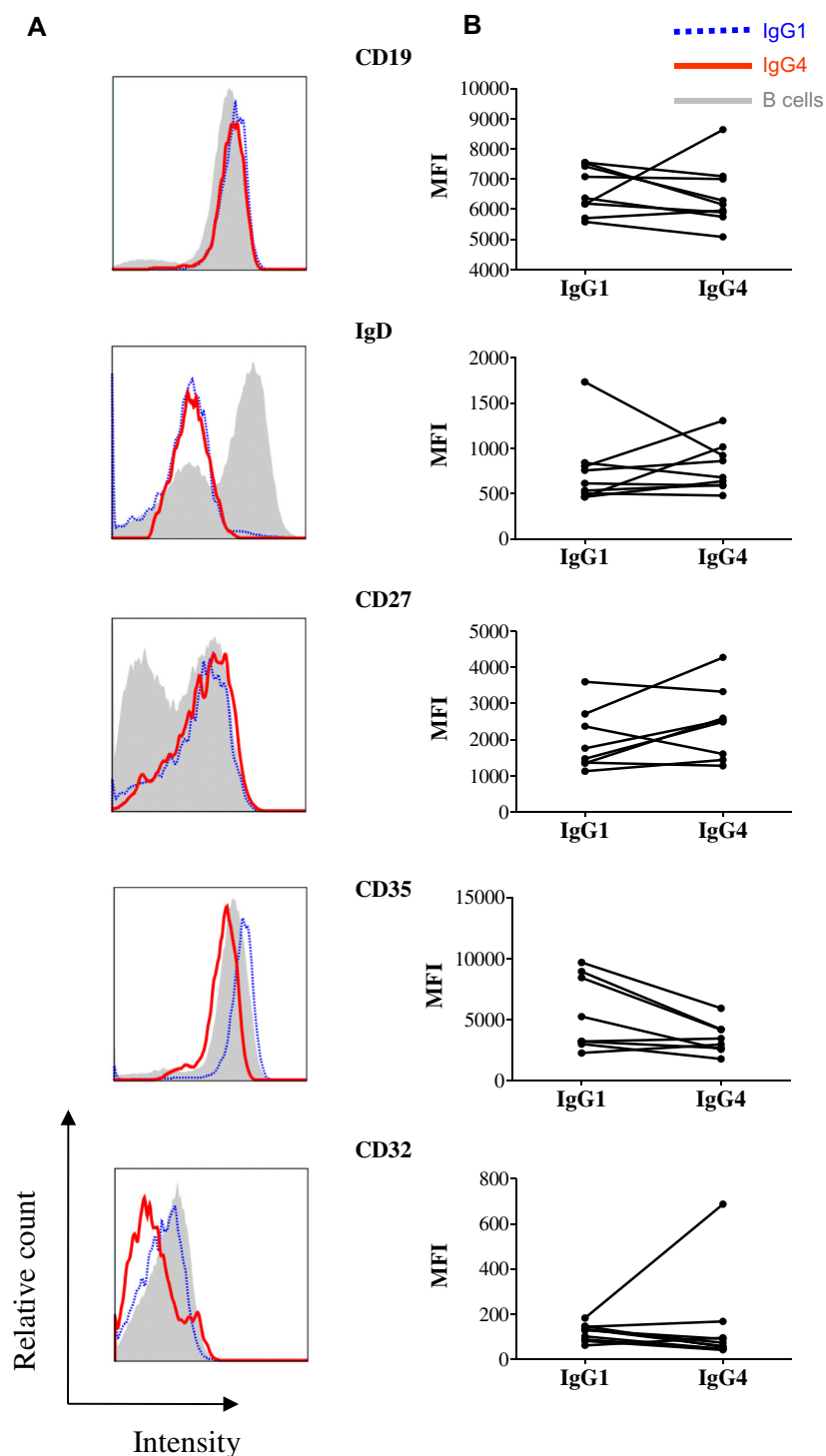


FIG E3. IgG₁⁺ and IgG₄⁺ B cells express similar amounts of CD19, CD27, IgD, CD35, and CD32. CD20⁺IgG₁⁺ and CD20⁺IgG₄⁺ B cells were assessed for surface expression of CD19, CD27, IgD, CD35, and CD32 using flow cytometry. **A**, Histograms for a representative experiment. **B**, Median fluorescence intensities from 8 or 9 healthy donors. *MFI*, Median fluorescence intensity.

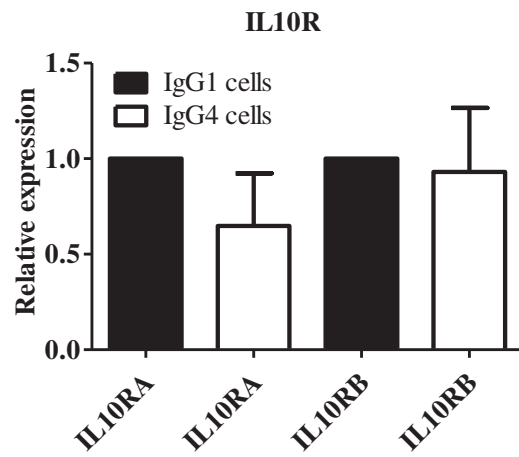


FIG E4. IL-10R expression was assessed by using RT-PCR on mRNA isolated from IgG₁⁺ or IgG₄⁺ sorted B cells (n = 5). Expression is shown relative to 18S.