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Divergent chemokine receptor expression and the consequence for human IgG4 B cell responses

Peter-Paul A. Unger[*,1], Laura C. Lighaam[*,1], Ellen Vermeulen[1], Simone Kruithof[1], Mateusz Makuch[1], Emma L. Culver[2], Robin van Bruggen[3], Ester B.M. Remmerswaal[4], Ineke J.M. ten Berge[4], Reindert W. Emmens[5], Hans W.M. Niessen[5], Eleanor Barnes[2], Gerrit J. Wolbink[1,6], S Marieke van Ham[1,7,†],Theo Rispens[1,†]

[1]Sanquin Research, Department of Immunopathology, Amsterdam, The Netherlands, and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

[2]Translational Gastroenterology Unit, John Radcliffe Hospital, Oxford and Nuffield Department of Medicine, University of Oxford, Oxford, UK

[3]Sanquin Research, Department of Blood Cell Research, Amsterdam, The Netherlands, and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

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[4]Renal Transplant Unit, Department of Internal Medicine, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

[5]Department of Pathology and Cardiovascular Surgery, ACS, VU Medical Center, Amsterdam, The Netherlands

[6]Department of Rheumatology, Amsterdam Rheumatology and Immunology Centre, Reade, Amsterdam, The Netherlands

[7]University of Amsterdam, Swammerdam Institute for Life Sciences, The Netherlands

[*]Both authors contributed equally

[†]Both authors contributed equally

Corresponding author:

S. Marieke van Ham

Plesmanlaan 125, 1066 CX Amsterdam

Telephone: +31-(0)20-512 3158

Fax: +31-(0)20-512 3474

Email: m.vanham@sanquin.nl

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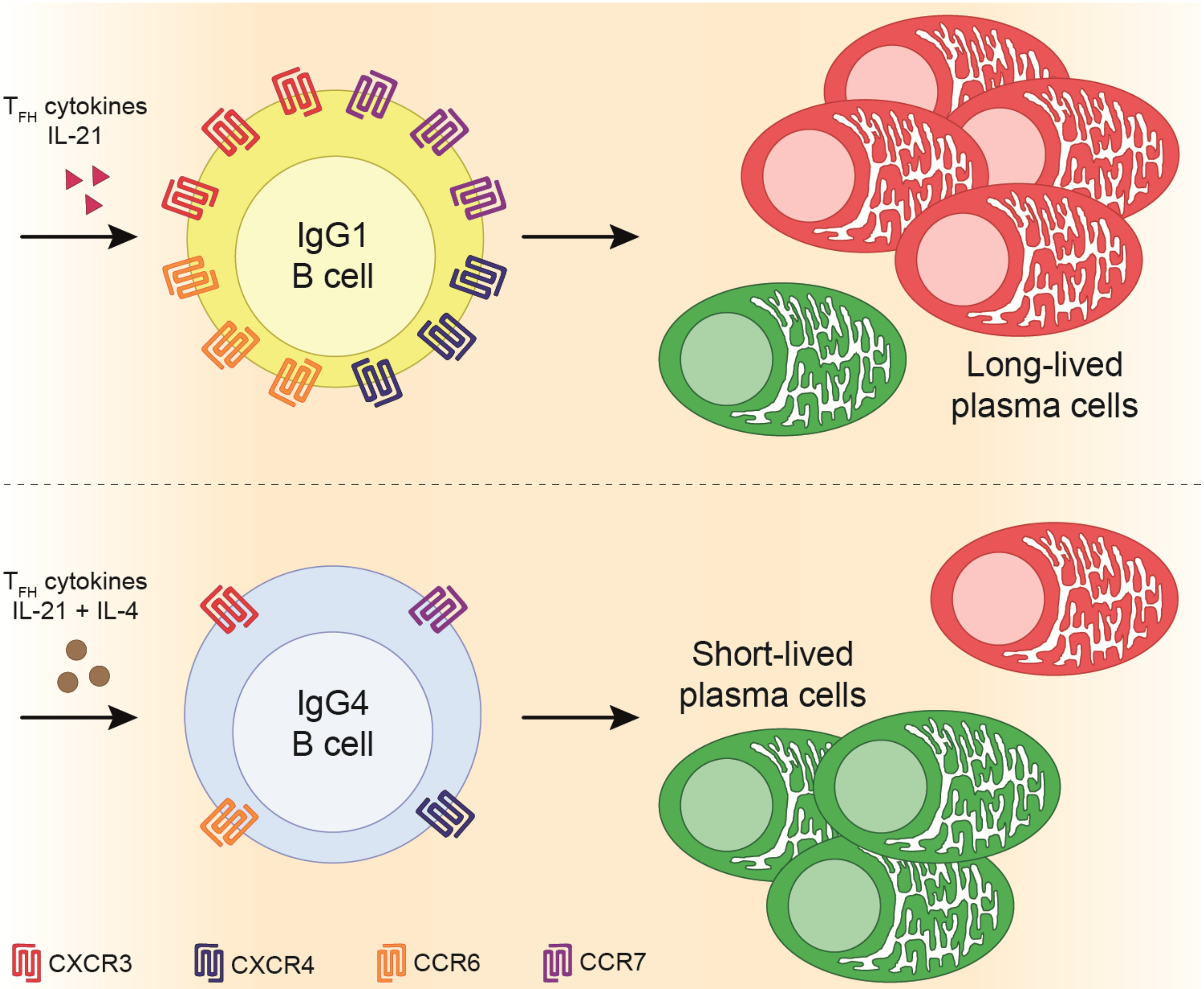
Abbreviations: IgG4-RD: IgG4-related disease; T_{H2}: T helper 2; T_{FH}: follicular T helper cells; FCS: fetal calf serum; PBMCs: Peripheral blood mononucleated cells; LNs: lymph nodes; PBS: phosphate-buffered saline; IgG4-RI: IgG4-responder index; HPE: high-performance ELISA buffer; SD: standard deviation; UC: ulcerative colitis; RA-patients: rheumatoid arthritis-patients; GC: germinal center.

Abstract

IgG4 antibodies are unique to humans. IgG4 is associated with tolerance during immunotherapy in allergy, but also with pathology, as in pemphigus vulgaris and IgG4-related disease. Its induction is largely restricted to non-microbial antigens, and requires repeated or prolonged antigenic stimulation, for reasons poorly understood. An important aspect in generating high-affinity IgG antibodies is chemokine receptor-mediated migration of B cells into appropriate niches, such as germinal centers. Here, we show that compared to IgG1 B cells, circulating IgG4 B cells express lower levels of CXCR3, CXCR4, CXCR5, CCR6, and CXCR7, chemokine receptors involved in germinal center reactions and generation of long-lived plasma cells. This phenotype was recapitulated by *in vitro* priming of naive B cells with an IgG4-inducing combination of T_{FH}/T_{H2} cytokines. Consistent with these observations, we found a low abundance of IgG4 B cells in secondary lymphoid tissues *in vivo,* and the IgG4 antibody response is substantially more short-lived compared to other IgG subclasses in patient groups undergoing $CD20^+$ B cell depletion therapy with rituximab. These results prompt the hypothesis that factors needed to form IgG4 B

cells restrain at the same time the induction of a robust migratory phenotype that could support a long-lived IgG4 antibody response.



IgG4 B cells express fewer chemokine receptors involved in germinal center reactions and generation of long-lived plasma cells.This might restrain induction of a robust migratory phenotype that could support a long-lived IgG4 antibody response.

Introduction

IgG4 antibody responses are important in a broad range of clinical settings as the unique structure of IgG4 confers few immunological effector functions. As such IgG4 has been coined as the ‘tolerance-inducing’ antibody. On the one hand, induction of IgG4 is associated with a successful outcome of tolerance-inducing immunotherapies in allergic patients [1]–[3]. On the other hand, IgG4 antibodies can also represent a major component of the neutralizing anti-drug antibodies response to therapeutic proteins [4] such as the TNF-inhibitor adalimumab in rheumatoid arthritis patients, which impairs clinical efficacy [5]. In addition, IgG4 is a biomarker in the family of systemic fibro-inflammatory conditions coined IgG4-related disease (IgG4-RD) [6], which is characterized by abundant IgG4 plasma cell infiltration in inflamed/fibrotic tissues, and by increased IgG4 serum levels in most patients [7]–[11].

It is not well understood how IgG4 responses are regulated. The IgG4 antibody response appears to require repeated or prolonged antigenic stimulation, as demonstrated in novice bee keepers [2],[3] and in allergic patients undergoing specific immunotherapy (reviewed in [12]). *In vivo,* IgG4 antibody levels have been positively associated with T helper 2 (T_{H2}) cytokines IL-4 and IL-13 and IL-10, with regulatory T cells and with follicular T helper cells (T_{FH}) and their hallmark cytokine IL-21 [8],[13]–[17]. Finally, studies with the B cell depleting antibody rituximab in IgG4-RD patients indicate that the IgG4 antibody response is less persistent than IgG1 antibody responses in these patients [18],[19]. Whether this occurs specifically in IgG4-RD or is a general feature of IgG4 responses is not known.

Mechanistic studies of IgG4 antibody responses are hampered by the fact that IgG4 cannot be easily studied in most animal models due to lack of functional homologue in these species, and by difficulties to identify IgG4 B cells. We recently successfully identified and isolated human memory $IgG4^+$ B cells and demonstrated the existence of phenotypical differences between circulating $IgG4^+$ and $IgG1^+$ memory B cells [20]. In addition, we showed that serum IgG4 levels correlate with the number of circulating memory $IgG4^+$ B cells, both in healthy donors as well as in IgG4-RD patients, a finding recently confirmed by others [21]. The question remains if a direct functional association exists between the phenotypic traits of $IgG4^+$ B cells and the unique dynamics of the IgG4 B cell responses.

An important aspect of B cell responses is the migration of B cells towards the appropriate niches. Isotype switching [22] and differentiation of naïve B cells into memory B cells and long-lived antibody-secreting plasma cells [23],[24] requires CD4 T cell help, via co-stimulatory signals (e.g. CD40L) and secretion of cytokines (e.g. IL-4 and IL-21), and depends on induction and maturation of germinal centers in secondary lymphoid organs [25]–[27]. Only well-established and matured germinal centers generate long-lived plasma cells that emigrate from the secondary lymphoid organs and successfully establish themselves in the bone marrow niches [25],[28]. Temporal expression of specific chemokine receptors on the differentiating B cells and plasma cells control their migratory behavior. This includes CXCR5 expression and CCR7 down-regulation, which are required for B cell localization to B cell follicles in secondary lymphoid organs [29]–[31], while temporal up- and down-regulation of CXCR4 mediates dark zone to light zone migration of B cells within a germinal center [32],[33]. In addition, CXCR4 allows antibody-secreting cells to migrate into bone marrow niches and become long-lived plasma cells [34],[35]. CXCR7 acts as a CXCL12 scavenger to repress CXCR4-mediated migration, is expressed on human tonsillar memory B cells and plasmablasts and is proposed to facilitate egress from germinal centers [36]. In addition, CCR6 positions memory B cells in close proximity to CD4 T helper cells within germinal centers during a secondary infection [37]. Migration into inflamed tissue is mediated via CXCR3 expression [38],[39]. Furthermore, B cells present in inflamed joints have higher expression of CCR1 and CCR5 compared to their peripheral blood counterpart, suggesting they facilitate migration into inflamed sites [40]. Absence of CXCR3, CXCR5, CCR6 and CCR7 disrupts the production of high-affinity IgG antibodies [37],[41]–[45]. This demonstrates the importance of migratory receptors and localization in appropriate niches in the generation of high-affinity antibodies.

The distinctive traits of the IgG4 antibody response might, in part, be regulated by the temporal and dynamic expression of chemokine receptors and tissue localization. In this study we identify a distinct chemokine receptor expression pattern on IgG4 B cells. Furthermore, we analyzed tissue localization of IgG4 B cells and show that IgG4 B cells are not abundantly present in secondary lymphoid organs. Our findings provide insight into the possible role of the IgG4 B cell migratory phenotype and the distinct dynamics of IgG4 antibody responses.

Results

Chemokine receptor expression is low on circulating IgG4 B cells and is partly imprinted by IL-4

We assessed the *ex vivo* chemokine receptor expression of IgG1 B cells and IgG4 B cells isolated from peripheral blood. Both the frequency of chemokine receptor-positive IgG4 B cells and expression levels of chemokine receptors on circulating IgG4 B cells were significantly lower for CXCR3, CXCR4, CCR6 and CCR7 compared to IgG1 B cells. Frequency of CXCR5$^+$ cells was similar between IgG1 and IgG4 B cells, whereas the expression levels were lower on IgG4 B cells. On the other hand, CCR1, CCR5 and CXCR7 were expressed at similar levels (Fig. 1; Suppl. Fig. 1; Suppl. Fig. 7a).

Next, chemokine receptor expression was analyzed on *in vitro*-induced IgG1 and IgG4 B cells to investigate a possible role of factors driving naïve B cell priming and IgG4 isotype switching in shaping of the migratory phenotype. In a culture system using a CD40L-expressing fibroblast cell line, *in vitro* isotype switching of human naïve B cells into IgG1 was induced with IL-10 and/or IL-21 alone, but not into IgG4. IL-4 induced IgG4 B cells, increased the relative frequencies of IgG1 B cells and IL-4-mediated isotype switching was enhanced with additional IL-21 and/or IL-10 (Fig. 2a,b). IFNγ did not affect isotype switching to IgG1 or IgG4, either alone or in presence of other cytokines (Fig. 2c). Interestingly, IgG1 and IgG4 B cells generated with the T_{FH}/T_{H2} cytokine mix (IL-4 + IL-21) had significantly lower expression of CXCR3, CXCR4, CXCR5, CCR6 and CCR7 when compared to CD40 co-stimulation and IL-21 alone that allowed induction of IgG1 B cells (Fig. 2d; Suppl. Fig. 2a), but not of IgG4 B cells (Fig. 2a,b). CXCR4, CXCR5, CCR6 and CCR7 expression was down-regulated also in comparison with unstimulated naïve, non-switched cells (Fig. 2e; Suppl. Fig 2b). By contrast, presence of only the T_{FH} cytokine IL-21 upregulated CXCR3, CXCR4 and CCR7 expression on naïve B cells during culture, but overall, resulted in a less pronounced change in the chemokine receptor profile (Fig. 2e; Suppl. Fig. 2b). IFNγ enhanced expression of CXCR3 on *in vitro*-induced IgG1 B cells and IgG4 B cells (Fig. 2f; Suppl. Fig. 2c,d), in line with previous observations [39], and not of CXCR4 and CXCR5. These *in vitro* findings corroborate the *ex vivo* observed chemokine profiles and suggest that the T_{FH}/T_{H2} spectrum of cytokines increase the relative frequencies of IgG1 and IgG4 B cells and concomitantly down-regulate expression of chemokine receptors, including those needed for germinal center reactions and migration to peripheral tissues.

Relatively low abundance of IgG4 B cells in secondary lymphoid organs

As the chemokine receptors CXCR4, CXCR5 and CCR7 are required for secondary lymphoid organ homing and migratory behavior essential for B cell memory formation and plasmablast/plasma cell differentiation during germinal center reactions, the localization of IgG4-switched B cells within these organs was investigated. Paired human peripheral blood and lymph node or spleen samples were analyzed for frequencies of IgG1 and IgG4 B cells. In line with the chemokine receptor expression pattern, IgG4 B cells were present in lymph nodes and spleen in frequencies similar to the paired peripheral blood samples, in contrast to the observed (and expected) relative enrichment of IgG1 B cells in these secondary lymphoid organs (Fig. 3).

Plasticity in chemokine receptor expression upon recall response

Next, we investigated if regulation of chemokine receptor expression also occurred in IgG1 and IgG4 memory B cells differentiating *in vitro* into plasma cells, representing a secondary immune response. Plasmablast ($CD38^{+}CD138^{-}$; Fig. 4a,b) and plasma cell ($CD38^{+}CD138^{+}$; Fig. 4a,c) differentiation from human memory B cells after six days of culture required IL-21 in addition to CD40 co-stimulation, as observed before [46],[47], and was not affected by additional IL-4 and/or IL-10 (Suppl. Fig. 3). Of note, the frequency of IgG4 plasma cells was higher compared to IgG1 plasma cells at day 11 (Fig. 4c). IgG1 and IgG4 plasmablasts and plasma cells showed higher CXCR3 expression compared to the undifferentiated population in culture ($CD27^{-/+}CD38^{-}$) (Fig. 4d; Suppl. Fig. 4b), as well as to *ex vivo* analyzed memory B cells (Suppl. Fig. 4c), with IgG1 cells expressing more CXCR3 than IgG4 cells. In contrast, CXCR4, CXCR5 (Fig. 4d; Suppl. Fig. 4b), CCR6 and CCR7 expression (Suppl. Fig. 4a) was lower in comparison to the undifferentiated population. Addition of IFNγ did not affect chemokine receptor expression (Suppl. Fig. 5). Taken together, these data show that – similar to IgG1 – differentiation of IgG4 memory B cells into antibody-secreting cells requires the T_{FH} cytokine IL-21, and that this process of plasma cell differentiation promotes a similar regulation of chemokine receptors for both IgG1 and IgG4 B cells.

IgG4^{+} cells localize in inflamed colon of patients with ulcerative colitis and in bone marrow

The observed CXCR3 and CXCR4 expression on *in vitro*-generated IgG4 plasma cells during a ‘recall response’ implies the potential for homing to inflamed tissue or bone marrow [28]. Analysis of colonic resection specimens from 9 patients suffering from ulcerative colitis (Fig. 5a) revealed a significantly higher number of IgG4^{+} cells per mm^{2} in inflamed sections of the colon compared to non-inflamed tissue sections of the same donor (Fig. 5b), indicating that IgG4 plasma cells can indeed localize to sites of inflammation. Furthermore, analysis of 7 human bone marrow samples demonstrated that IgG1^{+} and IgG4^{+} cells (CD19^{int}CD38^{hi}) were both present in the bone marrow, albeit with considerable inter-donor variation (Fig. 5c). The variation was not explained by the age of the donor (ranging from 15 – 70 years).

IgG4 antibody response is more short-lived than responses of other IgG subclasses

The altered migratory phenotype and low abundance of IgG4 B cells in secondary lymphoid organs might suggest that the IgG4 B cell response is not supported by well-established germinal center reactions. This is likely to affect IgG4 B cell differentiation and formation of long-lived IgG4 memory B cells and IgG4 plasma cells and thus reduce longevity of the IgG4 antibody response. This notion is supported by the observation that IgG4-RD patients treated with rituximab –

depleting short-lived $CD20^+$ B cells/plasmablasts but not long-lived $CD20^-$ plasma cells – show a selective decrease in serum IgG4 levels in comparison to other IgG subclasses after 8 weeks [18],[19], a result recapitulated in the present study (Fig. 6a). Here, we also analyzed IgG subclasses in serum samples of 26 rituximab-treated rheumatoid arthritis patients. This disease is not primarily linked to aberrant IgG4 production. IgG4 serum levels selectively and significantly decreased by week 12 (Fig. 6b left) and even more so by week 28 (Fig. 6b right) after rituximab treatment, suggesting a major contribution of short-lived plasma cells to steady-state IgG4 serum levels (see Discussion for relationship with antibody half-life). By contrast, adalimumab (anti-TNFα) treatment did not affect IgG4 serum levels by week 12 and week 24 (Suppl. Fig. 6), demonstrating that the effect is specific for B cell depletion rather than a general immunosuppressive effect. These data indicate that a substantial part of the IgG4 antibody production is carried out by short-lived plasmablasts/plasma cells requiring continuous input from newly differentiating B cells.

Discussion

To date, the factors that control formation of human IgG4 B cells and regulate the dynamics of the IgG4 response remain poorly understood. This in spite of a clinical need in immunotherapy, asthma, autoimmunity, and cancer (melanoma) [12],[48]–[51] to identify factors that can specifically modulate IgG4-skewed B cell responses. Our finding that the IL-4 requirement for IgG4 B cell formation prohibits expression of CXCR4 and CXCR5 on the B cells is striking. CXCR4 is involved in homing to lymph nodes and CXCR5 for entry into the B cell follicle [29],[52]. The observed low relative abundance of IgG4 B cells in secondary lymphoid organs *in vivo* suggests an association with the reduced expression of CXCR4 and CXCR5. Moreover, both receptors are involved in the establishment of germinal center reactions (GCs) and the cycling of B cells between the dark and light zone of the GCs [32],[33]. After these cyclic GC reactions, B cells differentiate into memory B cells and long-lived plasma cells. Both memory B cell formation and generation of long-lived plasma cells thus depends on effective germinal center formation *in vivo* [24],[25],[53]–[56]. As our data indicate that one of the outcomes of GC reactions, namely generation of long-lived plasma cells, is hampered and skewed more to the generation of short-lived IgG4 plasma cells this might suggest suboptimal GC reactions for IgG4 B cells. Whether this is linked to the IgG4 migratory phenotype remains to be investigated. It is important to note that serum half-life of IgG1, IgG2 and IgG4 is very similar and short (~ three weeks) in comparison to the time points examined in this study. Already after 12 weeks, less than 6% of circulating IgG would remain if production of new IgG were to be completely blocked, and this would drop to less than 0.1% after

28 weeks. Therefore, the results in Figure 6 reflect differences in antibody production rather than clearance.

Based on our results, we hypothesize the following model: Formation of IgG4 B cells during primary immunization is supported by the hallmark T_{FH} cytokine IL-21 and CD40 co-stimulation, but depends on conjunct expression of IL-4. The IgG4 B cell priming process represents a bottleneck for the IgG4 B cell response, as the efficiency of the process (at least *in vitro*) is reduced compared to IgG1 B cell priming and is accompanied by a chemokine receptor expression pattern suboptimal for induction of long-lived plasma cell differentiation. This might explain why IgG4 responses are associated with repeated or prolonged antigen exposure, which could eventually result in a highly matured response, representing the accumulation of multiple incremental rounds of maturation. In the memory recall phase, the limited number of IgG4 memory B cells that has been formed upon previous antigen exposure differentiate more rapidly into IgG4 plasma cells that home to inflamed tissues and bone marrow, allowing the generation of a more long-lived component of the IgG4 antibody response. The fact that a substantial part of the IgG4 antibody response is still derived from short-lived antibody-secreting cells may be the result of the low abundance of IgG4 B cells in secondary lymphoid organs.

These new insights on the regulation of the IgG4 B cell response may contribute to optimizing current immunotherapies in allergy in which induction of IgG4 antibodies is desired, for example with the transient use of fingolimod that traps lymphocytes in lymph nodes, or in prevention of detrimental IgG4-skewed antibody production with the use of, among others, mTOR inhibitors [57], for example in hemophilia A patients treated with recombinant FVIII [4] or in auto-immune diseases like myasthenia gravis [49].

Materials & Methods

Cell lines

NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) [58] were cultured in IMDM (Lonza) containing 10% FCS (Bodinco), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2mM L-glutamine (Invitrogen), 50 μM β-mercaptoethanol (Sigma Aldrich) and 500 μg/ml G418 (Life Technologies). 3T3-CD40L cells were harvested, irradiated with 30 Gy and were seeded in B cell medium (RPMI 1640 (Gibco) without phenol red containing 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 50 μM β-mercaptoethanol and 20 μg/ml human apotransferrin (Sigma Aldrich; depleted for human IgG with protein G sepharose)) on 96-well flat-bottom plates (NUNC) to allow adherence overnight.

Isolation of B cells from human healthy donors

Buffycoats of healthy human donors were obtained from Sanquin Blood Supply upon written informed consent in accordance with the protocol of the local institutional review board, the Medical Ethics Committee of Sanquin Blood Supply, and conforms to the principles of the Declaration of Helsinki. Peripheral blood mononucleated cells (PBMCs) were isolated using a Lymphoprep (Axis-Shield PoC AS) density gradient.

Afterwards, $CD19^+$ B cells were separated using magnetic Dynabeads (Invitrogen) according to manufacturer's instructions.

Human material

Lymph nodes

Matched PBMCs and lymph nodes (LNs) derived from surgical residual material were collected from kidney transplant recipients on informed consent. Cell suspensions were obtained by grinding LN pieces through a flow-through chamber.

Spleen

As described previously[59], spleens were collected from living organ transplant donors without clinical signs of infection or inflammation. Written informed consent for organ donation was obtained according to national regulations regarding organ donation. Splenic tissue of the organ donor was obtained during transplantation surgery, as part of the standard diagnostic procedure for HLA-typing, and was transported in University of Wisconsin Fluid (100mM Potassium lactobionate, 25mM KH_2PO_4, 5mM $MgSO_4$, 30mM Raffinose, 5mM Adenosine, 3mM Glutathione, 1mM Allopurinol, 50g/L Hydroxyethyl starch) at 4^oC. In case there was an excess of splenic tissue for diagnostic procedures, the splenic tissue was used in an anonymous fashion for research in this study, in accordance with the Dutch law regarding the use of left over material for research purposes. Human splenocytes were isolated as described previously [60] using digestion of spleen material with collagenase buffer (Collagenase CLSP 100 U/ml, DNAse, Deoxyribonuclease I, bovine recombinant 2 Kunitz Units/ml, Aggrastat 0.5 µg/mL, Glucose 1mg/ml, Calcium Chloride 1mM) for 30 minutes at 37°C . Isolated cells were obtained after filtration through a 100µm filter. Subsequently, erythrocytes were lysed with an isotonic ammoniumchloride buffer for 5 minutes at 4°C, followed by washing with phosphate-buffered saline (PBS).

Bone marrow

Bone marrow was obtained from vertebrae of deceased patients (n=9) at autopsy within 24 hours of death and consent of patients upon admission in hospital (VU University Medical Centre (VUmc), Amsterdam, the Netherlands), This follows the guidelines of the ethics committee of the VUmc, Amsterdam, and conforms to the principles of the Declaration of Helsinki. Bone marrow was stored in PBS at 4°C. The resected bone marrow was fragmented using tweezers (within 4 hours following resection) and bone marrow cells were collected by filtration using a dripping-filter chamber (HPF7024, Beldico BV) using PBS. Mononucleated cells were subsequently isolated using a Lymphoprep (Axis-Shield PoC AS) density gradient.

Colon

Colon tissue was obtained from patients (n=9) who underwent colectomy as treatment option for severe ulcerative colitis at the VU University Medical Centre (VUmc, Amsterdam, the Netherlands). Material used in this study consisted of excess fixed tissue after completion of regular pathological examination in agreement with informed consent from the patient. This follows the guidelines of the ethics committee of the VUmc, Amsterdam, and conforms to the principles of the

Declaration of Helsinki. From each removed colon, a tissue section was obtained from an inflamed area and an area with normal histology (control). These sections were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. The criteria for inflamed tissue were acute and chronic inflammation of the mucosa and/or crypt disarray and/or crypt abscesses.

Immunohistochemical analysis of colon tissue sections

Colon tissue sections (sliced 4µm thick) were obtained from 9 patients with ulcerative colitis. They were deparaffinised in xylene for 10 minutes, dehydrated in 100% ethanol for 10 minutes and endogenous peroxidase was blocked in methanol + 0.3% H_2O_2 for 30 minutes. Antigen retrieval was done by boiling of the sections in 0.01M Tris/EDTA buffer, pH9 for 10 minutes. Sections were subsequently stained with mouse α-human IgG4 (Zymed #05-3800, Invitrogen, ThermoFischer Scientific, diluted 1:2000) for 60 minutes. As secondary step, sections were treated with BrightVision post-antibody blocking reagent for 15 min, followed by BrightVision poly-HRP for 30 minutes (#DPVB110HRP, Immunologic). Antibody-HRP complexes were visualized with diaminobenzidine (Dako), for 10 minutes in darkness. Sections were counterstained with haematoxylin, and covered.

The number of IgG4-positive cells was counted manually using a light microscope in the mucosal layer of the colon. The surface areas of the mucosal layers were measured with Qprodit v3.2 (Leica Microsystems), using a Leica DM/LM microscope.

Serum of RA-patients and IgG4-RD-patients

Twenty-six rheumatoid arthritis patients treated with Rituximab or adalimumab were included from previously reported studies [61],[62]. The study protocol was approved by the ethics committee of the participating centers; all patients gave written informed consent.

IgG4-RD patients were referred to the John Radcliffe Hospital, Oxford, UK; a tertiary referral center. The diagnosis of IgG4-RD was made using the HISORt criteria (2006/7) for IgG4-related pancreatic and biliary disease [63], and the Japanese Comprehensive Diagnostic Criteria (2011) for systemic disease [64]. Ethical approval was obtained from the Research Ethics Committee Oxfordshire (10/H0604/51) and the study was registered on the NIHR UK portfolio (10776).

Flow cytometry

Flow cytometry experiments and analyses were conducted according to the recently published guidelines [65]. Flow cytometry gating strategies are depicted in Suppl. Fig. 7. Cells were washed with PBS and stained with LIVE/DEAD Fixable Near-IR (Dead cell stain kit, Invitrogen) for 30 minutes at room temperature in the dark. Then, cells were washed with PBS supplemented with 1% bovine serum albumin (PBS-A). Staining was performed by incubating the cells for 30 minutes at room temperature in the dark with the following antibodies: Anti-CD19 (SJ25-C1), anti-CD20 (L27), anti-CD27 (O323), anti-CD38 (HB7), anti-CD138 (MI15), anti-IgD (IA6-2), anti-CXCR3 (IC6/CXCR3), anti-CXCR4 (12G5), anti-CXCR5 (MU5UBEE), anti-CCR1 (53504), anti-CCR5 (2D7/CCR5), anti-CCR6 (11A9) from BD Bioscience; anti-IgG1

(MH161.1), anti-IgG4 (MH164.1) from Sanquin Reagents; anti-CXCR7 (11G8), anti-CCR7 (150503) from R&D, and anti-CXCR5 (RF8B2) from BD Pharmingen. Cells derived from bone marrow were fixed with 4% paraformaldehyde (Sigma Aldrich) and intracellular staining was performed using PBS-A plus 0.5% saponin (Calbiochem) with the following antibodies: Anti-IgD, anti-IgG1 and anti-IgG4. Samples were measured on LSRII or LSR Fortessa and analyzed using Flowjo software (Treestar).

In vitro cultures of naive, IgG1$^+$ memory and IgG4$^+$ memory B cells

Human CD40L-expressing 3T3 mouse fibroblasts were irradiated (30 Gy) and 10x10^3 cells were seeded overnight in 96-well flat-bottom culture plates (Nunc). Next, naive (CD19$^+$CD27$^-$IgD$^+$), IgG1$^+$ memory (CD19$^+$CD27$^+$IgG1$^+$) and IgG4$^+$ memory (CD19$^+$CD27$^+$IgG4$^+$) B cells were sorted on a FACS Aria II. 25x10^3 naive B cells, 1x10^3 IgG1$^+$ memory and 1x10^3 IgG4$^+$ memory B cells were co-cultured with the irradiated CD40L-expressing 3T3 fibroblasts in presence of IFNγ (50 ng/ml; Peprotech), IL-4 (100 ng/ml; Cellgenix), IL-10 (40 ng/ml; Peprotech) and/or IL-21 (50 ng/ml; Invitrogen) for six and eleven days to assess IgG1 and IgG4 B cell formation, plasma cell differentiation and Ig secretion.

IgG subclass ELISA for serum samples

IgG subclasses were measured as described previously [20].

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7; Graphpad Software). Data were analyzed using a Student's t test, Wilcoxon test, Friedman's test or Repeated Measures one-way ANOVA where appropriate. Results were considered significant at $p<0.05$. Significance is depicted as * ($p<0.05$) or ** ($p<0.01$).

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Conflict of interest:

The authors declare no commercial or financial conflict of interest.

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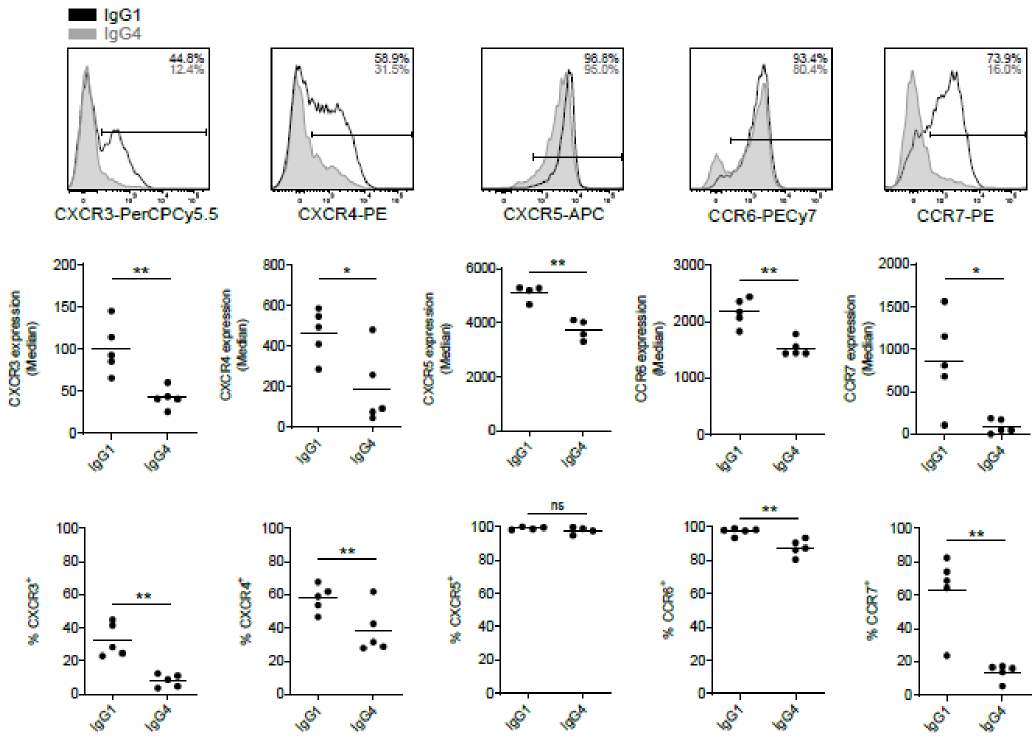
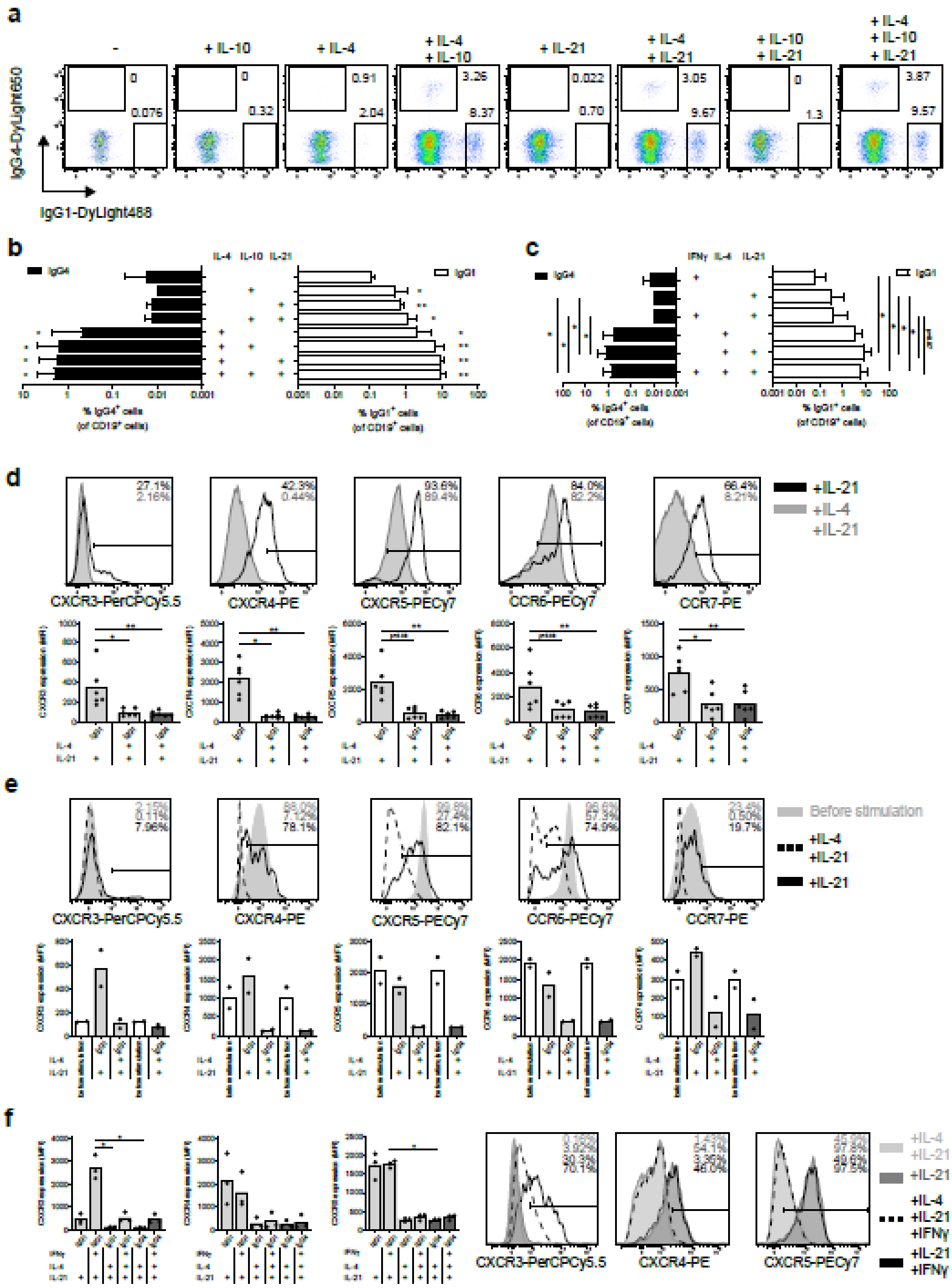


<u>Figure 1</u> Circulating IgG4 B cells express lower levels of chemokine receptors than IgG1 B cells. Direct *ex vivo* expression of chemokine receptors on IgG1 B cells and IgG4 B cells isolated from peripheral blood using flow cytometry. Median expression and frequencies of chemokine receptor-positive IgG1 B cells and IgG4 B cells are depicted. Horizontal line depicts mean value and each dot represents an individual donor. Representative histograms are shown ($IgG1^+$ cells: black; $IgG4^+$ cells: grey). Data are combined from 4-5 experiments with 1 donor per experiment. *P* values were calculated using paired t-test. ns: not significant; * $P \leq 0.05$, ** $P \leq 0.01$.



<u>Figure 2</u> IL-4 induces IgG1 and IgG4 isotype switching of naïve B cells *in vitro* and lowers chemokine receptor expression

$25x10^3$ human naïve B cells ($CD19^+CD27^-IgD^+$) were co-cultured with $10x10^3$ 3T3 mouse fibroblasts expressing human CD40L with indicated cytokines (IL-4: 100 ng/ml; IL-10: 40 ng/ml; IL-21: 50 ng/ml; IFNγ: 50 ng/ml). (**a-c**) IgG1 and IgG4 cell surface expression after 6 days in absence (**b**) or presence of IFNγ (**c**) using flow cytometry. Bars depict mean + SD. Representative dot plots are shown in **a**. Significance is compared to CD40 co-stimulated B cells without cytokines. **b-c** are log-transformed data. If observed percentages were zero, 0.01% (as 1 per 10,000 events) was used to log-transform the data. (**d, f**) Expression of chemokine receptors (as indicated) on *in vitro*-generated IgG1 (light grey) and IgG4 subclasses (dark grey) with or without IL-4 (100 ng/ml) and IL-21 (50 ng/ml) (**d**;) and IFNγ (50 ng/ml) (**f**) were analyzed after 6 days of culture with flow cytometry. Representative histograms of *in vitro*-generated $IgG1^+$ cells with the indicated cytokines are shown. (**e**) Chemokine receptor expression (as indicated) on *in vitro*-generated IgG1 (light grey) and IgG4 subclasses (dark grey) with or without IL-4 and IL-21 after 6 days of culture were compared to chemokine receptor expression on IgD^+ naïve B cells before start of the *in vitro* culture by flow cytometry. Representative histograms of naïve B cells (before stimulation; grey) and *in vitro*-generated $IgG1^+$ cells with the indicated cytokines are shown are shown. Data are combined from 4 (**b**), 3 (**c** and **f**), 6 (**d**) and 2 (**e**) experiments with 1 donor per experiment. Each dot represents an individual donor. *P* values were calculated using Friedman test. * $P \leq 0.05$, ** $P \leq 0.01$.

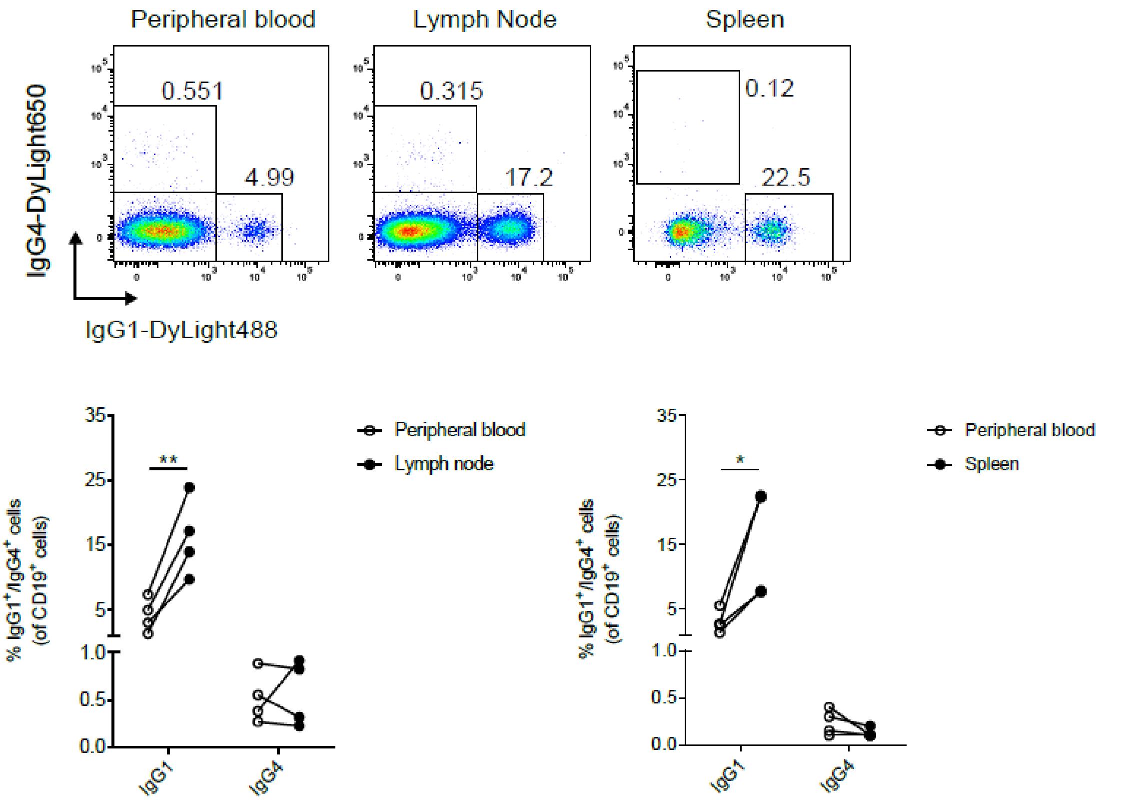
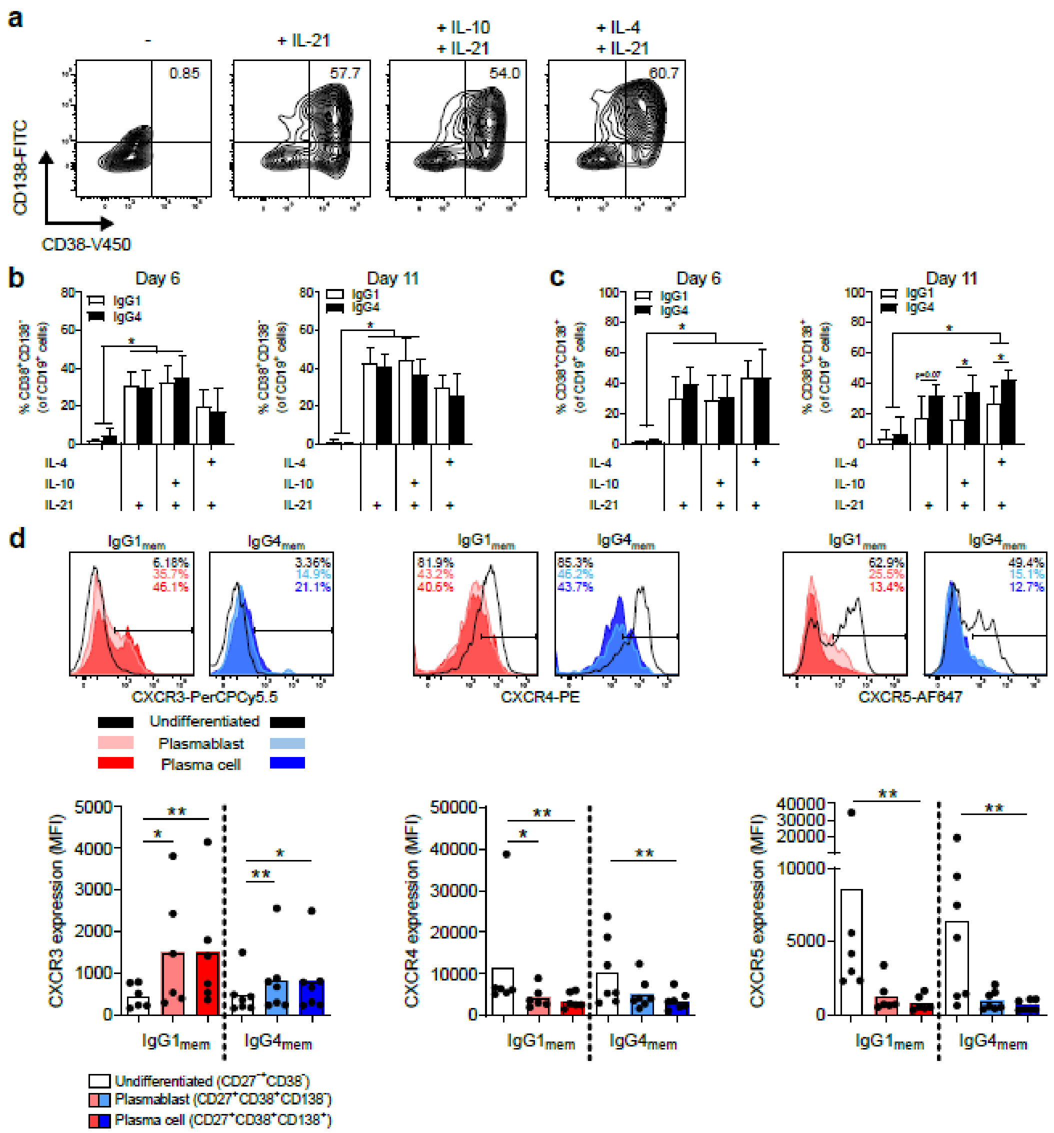


Figure 3 IgG4 B cells do not accumulate in secondary lymphoid organs. Paired peripheral blood and lymph node (left) or spleen (right) samples were analyzed directly *ex vivo* for the frequency of IgG1 and IgG4 B cells within total B cell population using flow cytometry. Data are combined from 4 experiments with 1 donor per experiment. Each dot represents an individual donor. *P* values were calculated using paired t-test. * $P \leq 0.05$, ** $P \leq 0.01$.



<u>Figure 4</u> IgG4 memory reactivation and plasma cell differentiation is induced by IL-21 and leads to up-regulation of CXCR3 and down-regulation of CXCR4 and CXCR5. 1x10^{3} human IgG1 or IgG4 memory B cells (CD19^{+}CD27^{+}IgG1/4^{+}) were co-cultured with 10x10^{3} 3T3 mouse fibroblasts expressing human CD40L for 6 or 11 days with or without the indicated cytokines (IL-4: 100 ng/ml; IL-10: 40 ng/ml; IL-21: 50 ng/ml; IFNγ: 50 ng/ml). (**a-c**) Plasmablast (**b**; CD38^{+}CD138^{-}) and plasma cell differentiation (**c**; CD38$^+$CD138$^+$) was assessed after 6 and 11 days using flow cytometry. Bars depict mean + SD. Representative contour plots of **b** and **c** are shown in **a**. (**d**) Expression of chemokine receptors (as indicated) on *in vitro* IL-21-reactivated IgG1 (red) and IgG4 (blue) memory B cells were analyzed in different subpopulations (as indicated) after 6 days of co-culture by flow cytometry. Bars depict mean. Representative histograms are shown. Data are combined from 5 (**b** and **c**) and 6-7 (**d**) experiments with 1 donor per experiment. Each dot represents an individual donor. *P* values were calculated using Friedman test. * $P \leq 0.05$, ** $P \leq 0.01$.

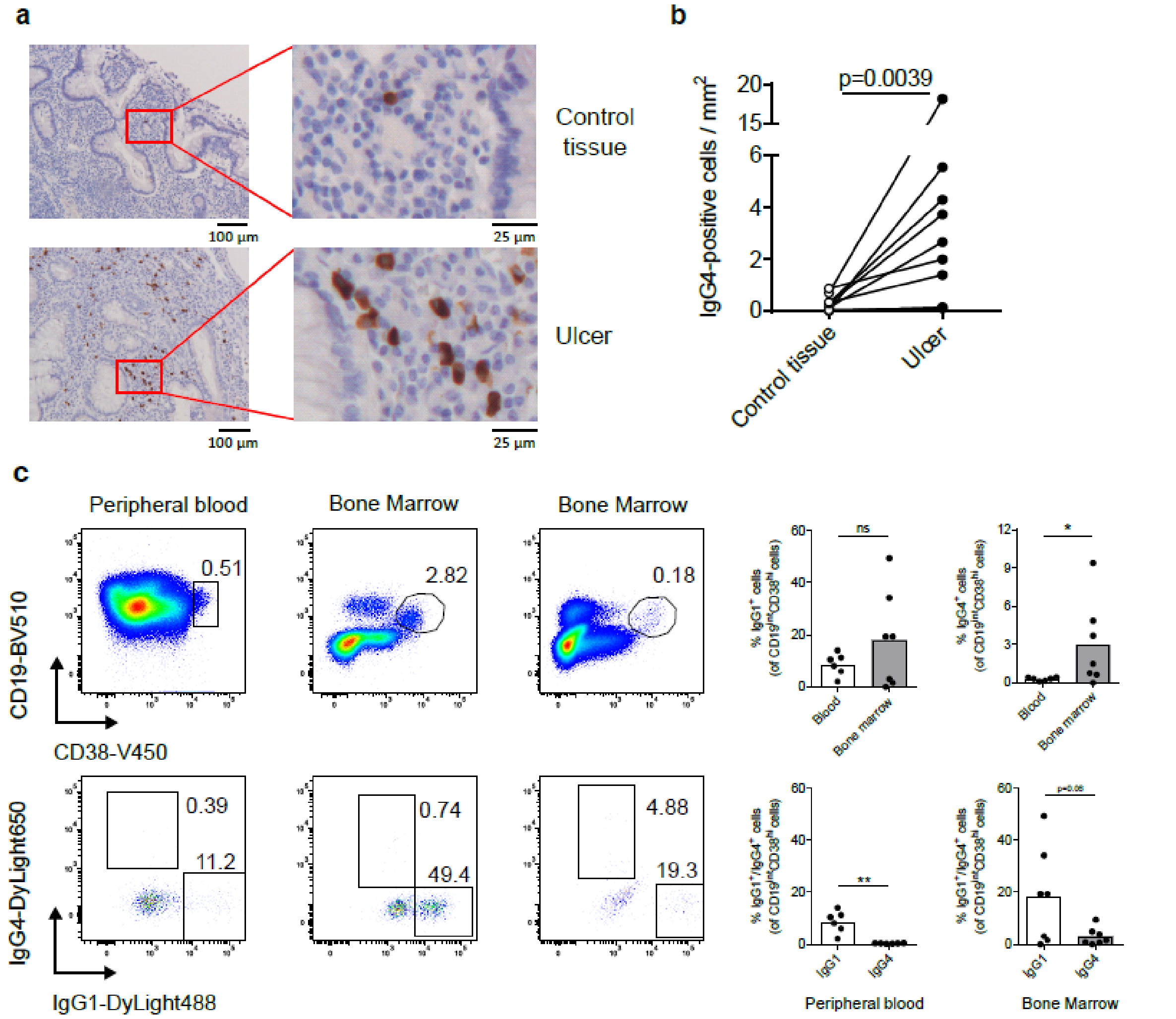
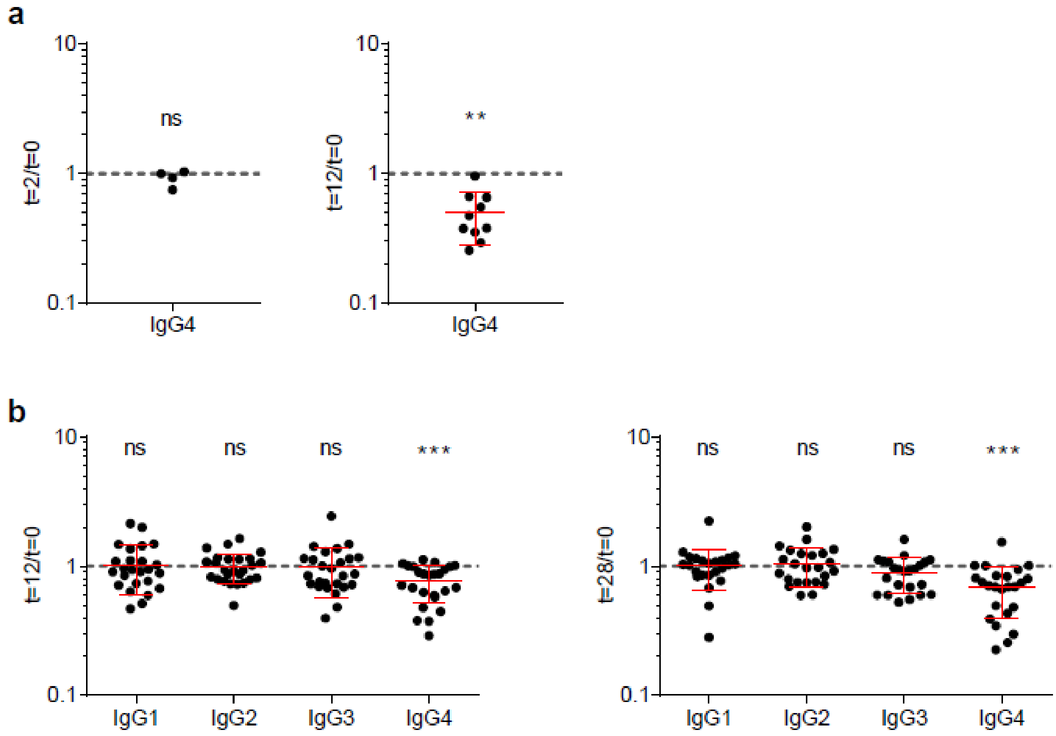


Figure 5 IgG4+ cells localize in bone marrow and in inflamed colon of patients with ulcerative colitis. (**a**) Representative IgG4 immunostainings of a non-inflamed and inflamed section of resected colon of a patient suffering from ulcerative colitis (UC). Microscopic magnification 100x (left) and digital magnification 5.5x (right). Section of picture (left) is enlarged (right) and depicted with associated scale bar, 100µm and 25µm respectively . (**b**) Quantification of number of IgG4^{+} cells/mm^{2} for 9 UC patients in paired non-inflamed and inflamed sections of resected colonic tissue. Data are combined from 9 experiments with 1 donors per experiment. *P* value was

calculated using Wilcoxon test. (**c**) Spinal bone marrow was analyzed directly *ex vivo* for the frequency of $IgG1^{+}$ and $IgG4^{+}$ cells within the $CD19^{int}CD38^{hi}$ population comparing between blood and bone marrow (top graphs) and comparing the frequency of $IgG1^{+}$ and $IgG4^{+}$ cells with each other within blood or bone marrow (bottom graphs) using flow cytometry and compared to non-paired peripheral blood. Representative dot plots are shown. Bars depict mean values and are combined from 6 experiments with 1 donor per experiment. Each dot represents an individual donor. *P* values were calculated using unpaired t-test. * $P \leq 0.05$, ** $P \leq 0.01$.



<u>Figure 6</u> IgG4 antibody response is more short-lived than responses of other IgG subclasses. (**a**) IgG4 subclass levels were measured from serum of 10 rituximab-treated IgG4-RD patients before treatment and 2 weeks (left) and 12 weeks (right) after treatment by ELISA. (**b**) IgG subclass levels were measured from 26 rituximab-treated RA-patients before treatment and 12 weeks (left) and 28 weeks (right) after treatment by ELISA. Serum level ratios of levels after treatment over levels before treatment are depicted. Red line depict mean -/+ SD. Data represent the average of 3 separate experiments with all 26 patient sera/experiment. Each dot represents an individual donor. *P* values were calculated using Wilcoxon test. *** $P \leq 0.001$.