European Journal of Immunology

Adaptive immunity

Basic

Research Article

9-*cis* Retinoic acid and 1.25-dihydroxyvitamin D_3 drive differentiation into IgA^+ secreting plasmablasts in human naïve B cells

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Calcitriol and 9-cis retinoic acid (9cRA) play a fundamental role in shaping the adaptive immune response by altering the Ig profile and the differentiation of B cells, controlled by their corresponding nuclear receptors, VDR and RAR. Herein, after the establishment of a plasmablast differentiation culture, we investigated how both ligands modulate human naïve B cell differentiation and to which extent VDR/RXR and RAR/RXR signaling interferes. Calcitriol and 9cRA mediated activation of purified naïve B cells resulted in a strong differentiation of $CD27^+$ $CD38^+$ plasmablasts and antibody secretion. The significant IgA response was preceded by a strong induction of α-germline transcription (GLT). Induction of αGLT and consecutively IgA secretion driven by calcitriol is a novel observation and we show by magnetic chromatin IP that this was mediated by recruitment of the VDR to the TGF-β promoter thus inducing TGF-β expression. Finally, as revealed by transcriptomic profiling calcitriol and 9cRA modulate several signals required for differentiation and isotype switching in a noncompeting but rather additive manner. Calcitriol and 9cRA participate in the control of the IgA response in human activated naïve B cells. The balance between both ligands may be an important factor in channeling humoral immune responses toward a protective direction.

Keywords: 9-*cis* retinoic acid · B cell · calcitriol · IgA · TGF-β

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

B lymphocytes are firmly established targets of the vitamin D receptor agonist calcitriol (chem. 1α,25-dihydroxycholecalciferol) and 9-*cis* retinoic acid (9cRA), both representing hormonally active entities. The tight association between nuclear receptor (NR) signaling, in particular vitamin D receptor (VDR) and retinoic acid receptor (RAR) and the B cell compartment is underscored by the expression of VDR, RARs, and retinoic X receptors (RXRs) itself, and the abundance of proteins involved in ligand metabolism and function [1–6]. Hence, B lymphocytes are capa-

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ble of controlling calcitriol and 9cRA specific actions and further limit potentially undesirable effects.

At the molecular level, calcitriol mediates its immunomodulating functions through activation of the VDR that heterodimerizes with RXR thus increasing DNA binding and transcriptional activity. Following binding to vitamin D responsive elements (VDREs), the stimulation of transcription by VDR is mediated through recruitment of cofactors [7]. Accordingly, we have previously shown that calcitriol inhibits IgE CSR in human B lymphocytes by recruiting a transrepressive complex to the ε-germline gene promoter [3,6]. In contrast, with its transactive action the calcitriol activated VDR can trigger expression of the immunosuppressive cytokine IL-10 in human B cells and other APCs such as DCs [8,9].

Alike VDR, RAR activation forms RAR-RXR heterodimers leading to transcriptional regulation of target genes [10]. For example, 9cRA enhances expression of the gut homing integrin α4β7 and CCR9 in mesenteric murine B cells or IL-10 secretion in human B cells in an activation signal dependent manner. Moreover, 9cRA can modulate the allergic immune response by promoting an IgA response [4]. In addition, RA augments IgA_1 and IgA_2 production in TLR7/8 or TLR9 stimulated mature B cells [11].

IgA antibodies provide the first line of immune protection at mucosal surfaces. Classical, T-cell-dependent signals to promote class switch recombination (CSR) to IgA in B cells by inducing activation-induced cytidine deaminase (AICDA) and the Cα germline transcript (αGLT) are CD40L and TGF-β1. To maintain immune homeostasis only low concentrations of TGF-β1 initiate C_α gene transcription, whereas high concentrations suppress B lymphocyte proliferation and differentiation [12]. Naïve B cells require antigen-dependent activation to diversify their antibody repertoire by undergoing CSR and somatic hypermutation (SHM) resulting in further differentiate into long-lived memory B cells and antibody-secreting plasma cells [13].

Both nuclear receptors are intertwined, as they share the common heterodimeric binding partner RXR. Numerous studies demonstrated that VDR and RAR alone can affect similar signaling pathways [14,15,4,8], but their interaction has not been addressed in detail. To our knowledge, their interplay has only been analyzed in human innate lymphoid cells (ILCs) showing largely antagonistic effects on the expression of effector cytokines and gut-homing integrin [16]. These findings spurred further interest in the modulatory effect exerted by both, calcitriol and 9cRA, on human naïve B lymphocytes. Focusing on their role in gene expression and differentiation of human naïve B lymphocytes, we now report that both, calcitriol and 9cRA profoundly modulate human naive B lymphocyte gene expression via the induction of several signals that are required for differentiation and isotype switching. In human B lymphocytes both receptor ligands show rather an additive signaling behavior than antagonistic effects. Both hormone receptor ligands induced the formation of activated B cells and plasmablasts. Moreover, we observed a strong IgA response, not only via 9cRA, but also as a novelty calcitriol, which was TGF-β-dependent.

Results

Activation of human naïve B cells with CD40L/IL-4 induces VDR and RARα expression

We have previously shown that VDR activation results in several immune modulatory effects in both mouse and human B cells [15,3,8,6]. However, it remains unknown how and to what extent VDR, RARα, and their corresponding heterodimeric binding partner RXRβ interfere at the molecular level. To analyze these interactions in more detail, we first studied the expression of the according receptors. For this purpose $CD19^+$ $CD27^-$ naïve B cells were activated with CD40L and IL-4 for 3–72 h. We observed a strong upregulation of *VDR* (17.9-fold) and a modest upregulation of *RAR*α (5.1-fold) starting 6 h post stimulation. VDR expression displayed a biphasic pattern within 48 h with a second peak expression at 24 h. RARα expression decreased to initial values after 16 h of stimulation. By contrast *RXR*β expression was more variable and did not show a significant increase after stimulation (Fig. 1A–C), suggesting a constitutive expression pattern. These findings were confirmed by protein expression analysis (Fig. 1D). The other nuclear hormone receptors RXRγ, RARβ, and RARγ were absent in naïve B cells (data not shown).

Stimulation of human naïve B cells with hormone receptor ligands affects target gene expression

Next, we studied the expression patterns of direct response genes of calcitriol and 9cRA, namely *CYP24A1, TGM2*, and *CD38* [14,15,3, 4, 8]. Calcitriol stimulated human naïve B cells show a strong increase of *CYP24A1* expression after 8 h (nil: 0; calcitriol: 0.003505 median values mRNA ratio; $p = 0.0458$; Supporting Information Fig. S1A). This response was not altered by additional stimulation with 9cRA (Supporting Information Fig. S1B). Significant *TGM2* induction was detectable after 6 h of 9cRA stimulation (nil: 0.0008; 9cRA: 0.2172; $p = 0.0346$) and shows in contrast to *CYP24A1* no time- and dose response (Supporting Information Fig. S1A). Addition of calcitriol has no interfering effects (Supporting Information Fig. S1B). *CD38* displayed, in comparison to *CYP24A1* and *TGM2*, a synergistic induction when both ligands were applied together (nil: 0.008; calcitriol [50 nM]: 0.1511; 9cRA [50 nM]: 0.138; both [50 nM]: 0.2952; Supporting Information Fig. S1B). Taken together, both calcitriol and 9cRA induced a stable response gene expression in naïve B cells. A concentration of 50 nM from both ligands was optimal for response gene induction.

Calcitriol and 9cRA shape B cell differentiation and Ig class switching in a bidirectional manner

After having confirmed the abundance of functionally active nuclear hormone receptors responsive to calcitriol and 9cRA in

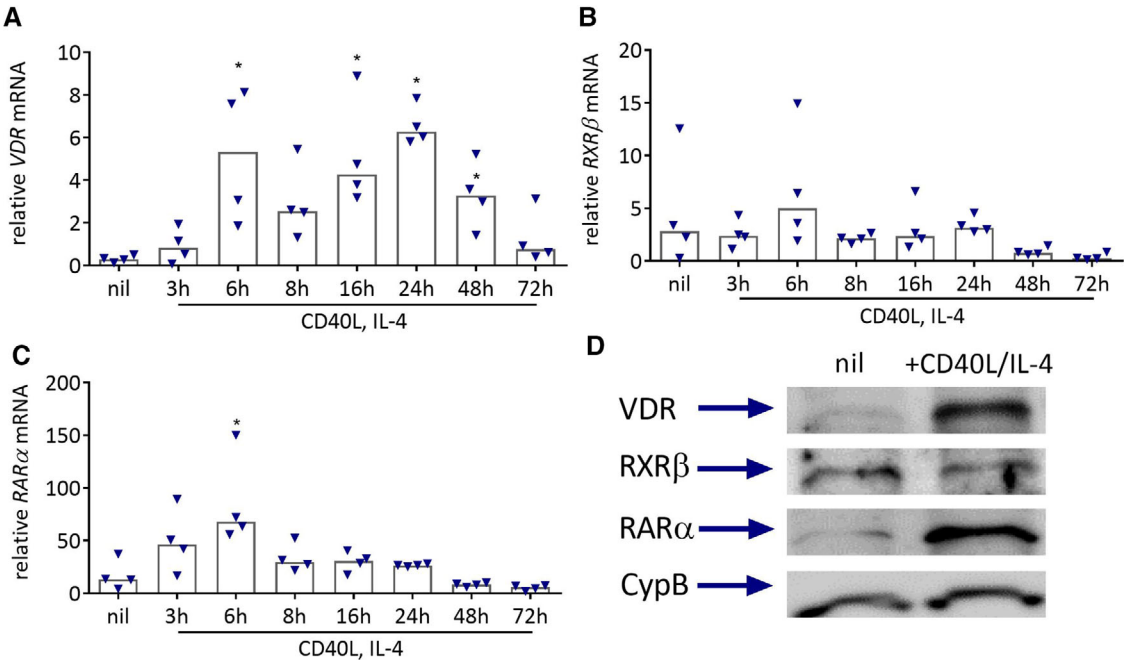

Figure 1. Expression of vitamin D receptor retinoic x receptor and retinoic acid receptor in activated human B cells. (A–C) Naïve B cells were stimulated with CD40L/IL-4 for 3 h to 3 days. *VDR* (A), *RXR*β (B), and *RAR*α (C) expression was determined by means of RT-PCR and normalized to *HPRT* expression. $N = 4$ (pooled independent biological replicates) in each graph. Results are expressed as median and pooled from two independent experiments performed separately. $^{*}p < 0.05$, Kruskal–Wallis test with Dunn–Bonferroni post hoc correction. (**D**) MACS sorted naïve B cells were stimulated with CD40L/IL-4 for 48 h. Whole cell extracts were subjected to western blot analysis for VDR, RXRβ, and RARα (one out of two independent experiments is shown). Cyclophilin B served as loading control.

naïve B cells, we set out to investigate their potential to shape gene expression. For both nuclear hormone receptor ligands, a concentration of 50 nM and a treatment time of 8 h were selected as suitable for potent induction of response genes as dose response and time course studies proofed. To elucidate the molecular downstream events associated with calcitriol and 9cRA stimulation, we performed a global transcriptome analysis of activated $CD19^{+}$ $CD27^{-}$ naïve B cells with stimulation conditions based on target gene expression. We identified 853 significantly regulated genes for calcitriol, 813 for 9cRA and 995 for both with a difference in expression of >1.2-fold and a significance score of >100. Fourty-six genes were identified as being synergistically induced by both vitamins (Supporting Information Fig. S2). Gene expression profiling revealed an impact of both nuclear hormone receptor ligands on genes involved in B cell differentiation and Ig class switch. We could dissect genes regulated by calcitriol from genes regulated by 9cRA or both. A detailed analysis of the transcriptome showed that 9cRA seems to have a stronger effect on the regulation of B cell differentiation (Fig. 2A), whereas calcitriol displayed a response pointing to genes involved in the switching machinery (Fig. 2B). Interestingly, these gene signatures were not antagonistically regulated by both ligands. *BCL6* (nil: 2300; calcitriol: 1746; 9cRA: 1793 probe signal intensity), a well-known transcription factor induced during GC formation, and *CD20* (nil: 9400; 9cRA: 7373; both: 7952), were downregulated. *CD74* and CD79A/B, both crucial for forming new B-cell antigen receptors, were upregulated. Calcitriol induced CD38 expression by 1.4-fold as also shown by us previously [15], whereas the combination of both ligands showed synergistic effects with a sixfold induction (Fig. 2C).

Calcitriol and 9-*cis* retinoic acid promote plasmablast formation

To assess the long-term effects of calcitriol and 9cRA on naïve B cell differentiation, we first applied previously published protocols using $CD40L^{+}$IL-4 [2,3]. As this stimulation did not result in sufficient survival and differentiation, we optimized the protocol by introducing additional stimuli. The data show 11% more viable $CD19^{+}$ B cells after 4 days and 7% more viable cells after 7 days of cultivation using our new established protocol (Fig. 3A). The frequencies of $CD27^{+}$ $CD38^{+}$ plasmablasts increased 17-fold after 7 days via stimulation of naïve B cells with anti-IgM-F(ab2)′, CD40L, IL-4, and IL-21 for 96 h followed by IL-10 and IL-21 treatment for 72 h (Fig. 3A). After a 7-day cultivation period both, calcitriol and 9cRA promoted naïve B cells toward a more mature differentiation state (Fig. 3B–D). The administration of calcitriol, 9cRA, or combinations thereof to this culture system resulted in the formation of fourfold higher frequencies of $CD27^{+}$ $CD38^{+}$ activated B cells and 14–16-fold higher frequencies of $CD27^{high}$ $CD38^{+}$ plasmablasts (Fig. 3B; Supporting Information Fig. S3A). The stimulation of naïve B cells with calcitriol and 9cRA resulted in an additive formation of activated B cells (calcitriol:

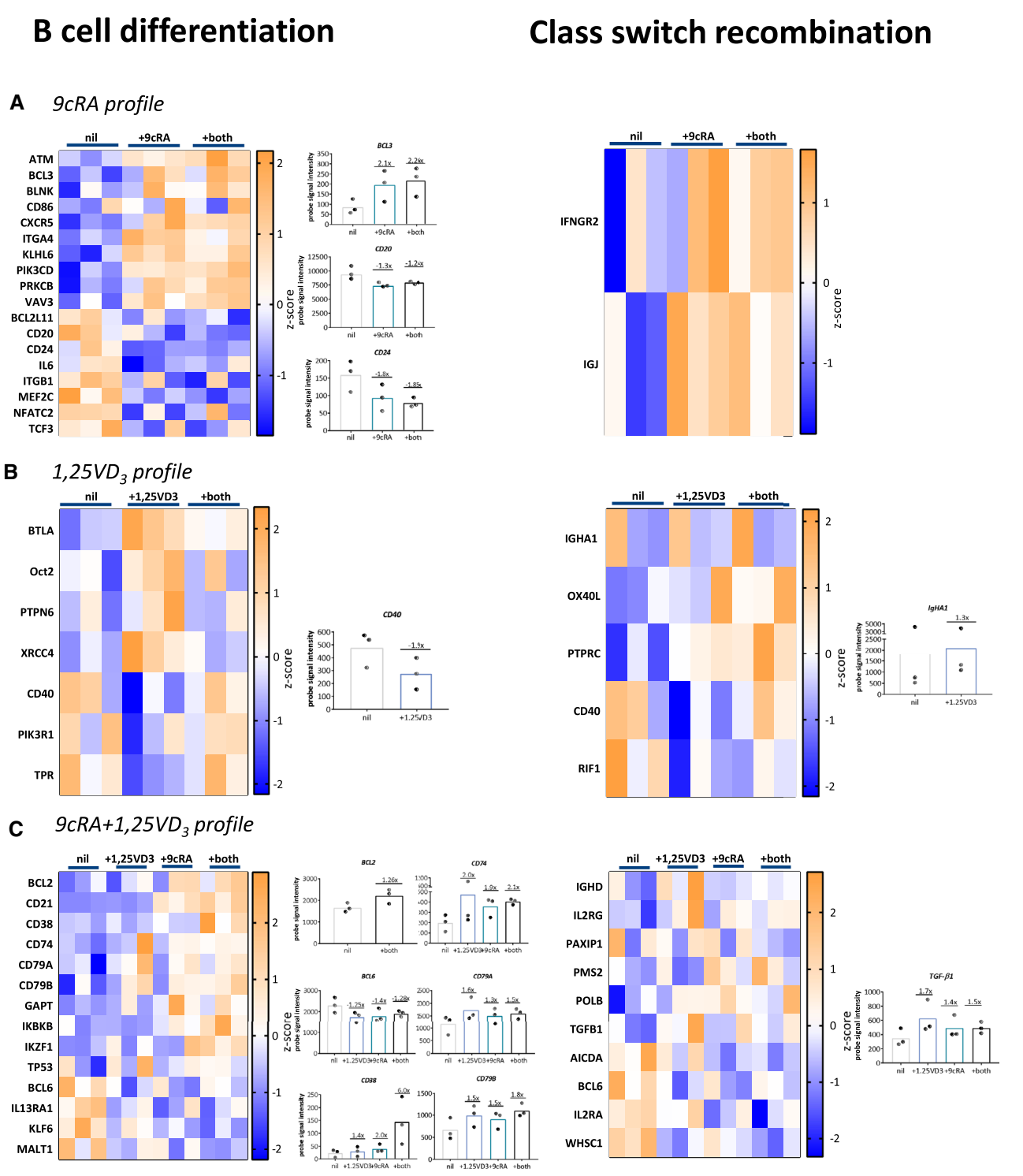

Figure 2. Calcitriol and 9-cis retinoic acid affect human B cell differentiation and Ig class switching. Naïve B cells were stimulated with CD40L/IL-4 for 24 h. During the last 8 h of cultivation calcitriol or/and 9cRA were added. Affymetrix Microarray analysis was performed. *(Left)* Heat maps of genes involved in B cell differentiation and Ig class switching regulated by calcitriol or/and 9cRA. $N = 3$ (independent biological replicates), Microrarray analysis was performed in one independent experiment. Differentially regulated genes were filtered according to high-performance chip data analysis (HPCDA). *(Right)* Selected genes with a known function in plasmablast generation or Ig class switch to IgA. Data shown as median with each symbol representing one donor ($N = 3$ (independent biological replicates), one independent experiment) and numbers denoting fold change (FC).

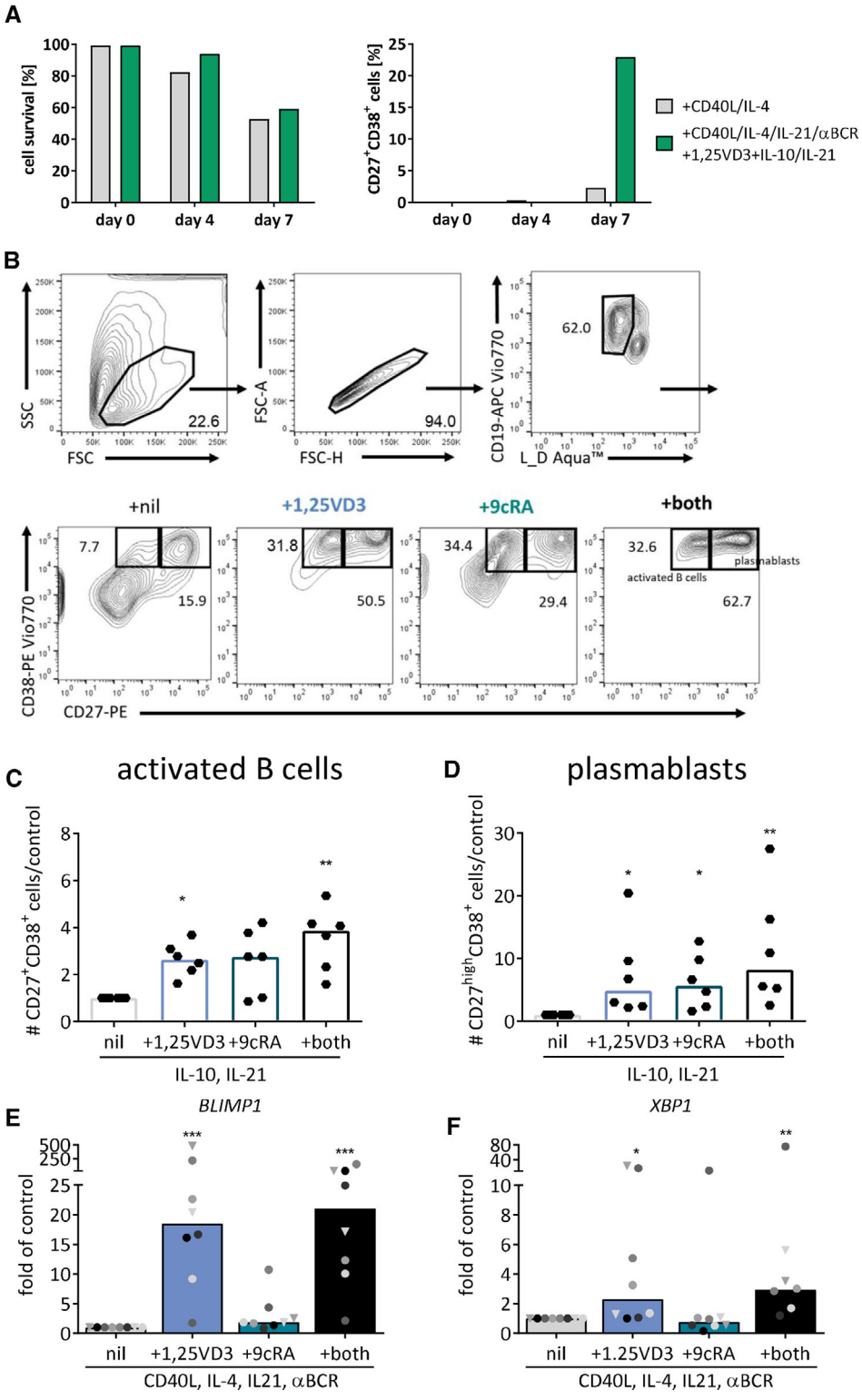

Figure 3. Calcitriol and 9-cis retinoic acid increase $CD27^{high}CD38^{+}$ and $CD27^{+}CD38^{+}$ cell numbers. (A) Influence of different stimulation conditions on plasmablast formation. Naïve B cells were stimulated with CD40L/IL-4 or CD40L/IL-4/IL-21/αBCR for 4 days and with IL-4/IL-21 or IL-10/IL-21 for further 3 days. One representative experiment out of two independent experiments is shown with two samples per experiment. (B–D) Naïve B cells were stimulated with CD40L/IL-4/IL-21/αBCR with or without calcitriol or/and 9 cis retinoic acid for 4 days and with IL-10/IL-21 for further 3 days CD27/CD38 cell numbers were determined by flow cytometry. $N = 6$ (pooled independent biological replicates). (B) One representative out of three independent experiments is shown. (C and D) Results are shown as median compared to stimulated control and pooled from three independent experiments performed separately. (E and F) *Blimp1* and *XBP1* expression was determined by quantitative Real Time-PCR and normalized to *HPRT* expression. $N = 8$ (pooled independent biological replicates). Data are shown as median compared to stimulated control and pooled from four independent experiments. $^{\cdot}p < 0.05$; $^{\cdot\cdot}p < 0.01$, Kruskal–Wallis test with Dunn–Bonferroni post hoc correction.

2.6-fold; 9cRA: 2.7-fold; both: 3.8-fold) and plasmablast cells (calcitriol: 4.9-fold; 9cRA: 5.6-fold; both: 8.2-fold; Fig. 3C and D). To confirm that the above described cells are of plasmablast origin the expression levels of plasmablast transcription factors *BLIMP1* and *XBP1* were determined. Both transcription factors were significantly increased after calcitriol treatment (*BLIMP1*: calcitriol: 18.5-fold; both: 21-fold; XPB1: calcitriol: 2.3-fold; both: 2.9-fold) (Fig. 3D and E), suggesting that VDR activation is the driver for plasmablast differentiation. 9cRA stimulation resulted in modest increase of *BLIMP1* expression (1.8-fold), but was, more

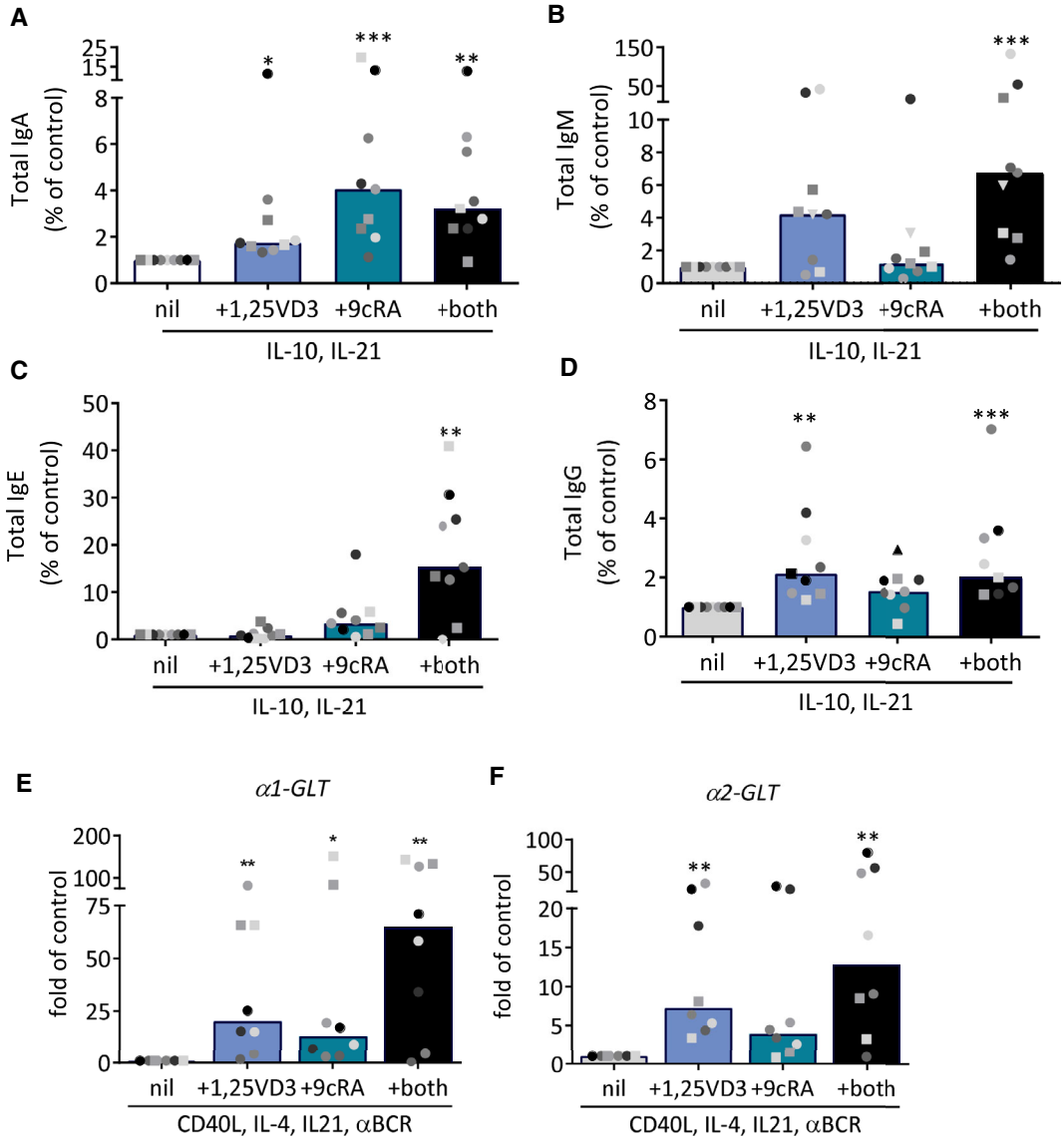

Figure 4. Calcitriol and 9-*cis* retinoic acid induce IgA secretion and αGLT expression. (A–D) MACS sorted naïve B cells were stimulated with CD40L/IL-4/IL-21/αBCR in the presence or absence of calcitriol or/and 9cRA for 4 days and with IL-10/IL-21 for further 3 days. IgA (A), IgM (B), IgE (C), and IgG (D) secretion was determined by ELISA. $N = 9$ (pooled independent biological replicates) in each graph. The median concentration after 7 days of cultivation were 106.5 ng/mL [nil]; 307.4 ng/mL [1.25VD3]; 472.0 ng/mL [+9cRA], and 416.5 ng/mL [both] for IgA; 1108 ng/mL [nil]; 2476 ng/mL [1.25VD3]; 1130 ng/mL [+9cRA]; and 4371 ng/mL [both] for IgM; 163.1 ng/mL [nil]; 324.1 ng/mL [1.25VD3]; 222.2 ng/mL [+9cRA] and 265.3 ng/mL [both] for IgG and 12.5 ng/mL [nil]; 6.2 ng/mL [1.25VD3]; 70.3 ng/mL [+9cRA] and 185.4 ng/mL [both] for IgE. Results are shown as median compared to stimulated control and pooled from five independent experiments performed separately. (E and F) Naïve B cells were stimulated with CD40L/IL-4/IL-21/αBCR in the presence or absence of calcitriol or/and 9cRA for 4 days. *α1/2GLT* expression was determined by quantitative Real Time-PCR and normalized to *HPRT* expression. $N = 8$ (pooled independent biological replicates). Data are expressed as median compared to stimulated control and pooled from four independent experiments performed separately. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Kruskal–Wallis test with Dunn–Bonferroni post hoc correction.

importantly not negatively interfering with the calcitriol induced expression of both transcription factors.

Calcitriol and 9-*cis* retinoic acid promote IgA production

To determine the functional consequences of calcitriol- and 9cRA-driven mature plasmablast formation, we assessed Ig production next. Isolated human naïve B cells were cultivated for 7 days and the total Ig levels were measured from supernatants thereof. We observed significantly elevated levels of IgA, with each NHL alone or in combination (calcitriol: 1.7-fold; 9cRA: 4.0-fold; both: 3.2-fold; Fig. 4A). Calcitriol induced total IgM secretion by 4.2-fold and 9cRA cooperated with calcitriol in the induction of IgM (both: 6.7-fold; Fig. 4B). Regarding IgE, calcitriol stimulation resulted in a diminished IgE response while 9cRA led to a slight induction. Notably, IgE secretion increased by interference with

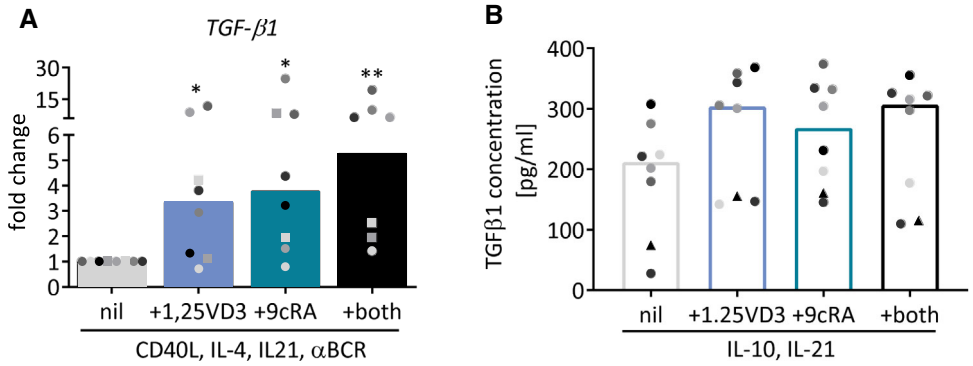

Figure 5. Calcitriol and 9-cis retinoic acid induce *TGFβ1* expression and secretion of bioactive TGF-β1. (A) Naïve B cells were stimulated with CD40L/IL-4/IL-21/αBCR in the presence or absence of calcitriol or/and 9cRA for 4 days. *TGFβ1* expression was determined by quantitative Real-Time PCR and normalized to *HPRT* expression. Values are shown as median compared to stimulated control. (B) Naïve B cells were stimulated with CD40L/IL-4/IL-21/αBCR in the presence or absence of calcitriol or/and 9cRA for 4 days and with IL-10/IL-21 for further 3 days. Human TGF-β1 secretion was determined by ELISA. (A and B) $N = 8$ (pooled independent biological replicates) in each graph. Results are shown as median and pooled from four independent experiments performed separately. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to stimulated control, Kruskal–Wallis test with Dunn–Bonferroni post hoc correction.

calcitriol and 9cRA significantly (Fig. 4C). IgG levels were only significantly enhanced by calcitriol (1.9-fold), but only marginally altered in the presence of 9cRA (Fig. 4D). To clarify next whether both IgA isoforms are sensitive to nuclear receptor stimulation *α1GLT* and *α2GLT* expression was analyzed. In this setting *α1GLT* expression was increased upon both, calcitriol and 9cRA treatment, whereas, *α1GLT* was synergistically induced by concurrent application of both nuclear hormone receptor ligands (calcitriol: 20.2-fold; 9cRA: 12.9-fold; both: 64.5-fold; Fig. 4E). In addition, calcitriol robustly triggered *α2GLT* expression (7.2-fold) and 9-*cis* RA by trend (3.9-fold). *α2GLT* expression was more pronounced when the receptor routes were jointly activated (both: 12.7-fold; Fig. 4F).

Calcitriol and 9-*cis* retinoic acid induce bioactive TGF-β1

Both, the transcriptome and the functional B cell differentiation analysis showing a dominant IgA response suggest *TGF-β1* as a candidate mediator for the strong IgA induction. We speculated that both calcitriol and 9cRA can drive naïve B cells to produce *TGF-β1*. Experimental blocking of surface TGFR1-3 using antibodies in the presence of calcitriol and/or 9cRA did not provide valid data, as the B cell survival was directly negatively affected (data not shown). Therefore, we determined *TGF-β1* expression and production in our culture system. Indeed, the data show a baseline expression of *TGF-β1* by naïve B cells but, both, calcitriol and 9cRA alone induce *TGF-β1* expression in a significant manner (calcitriol: 3.3-fold; 9cRA: 3.7-fold). However, targeting both receptors simultaneously resulted in enhanced expression compared to individual induction (both: 5.2-fold; Fig. 5A). In accordance to the transcriptional upregulation of *TGF-β1*, calcitriol and 9cRA led to an increased secretion of bioactive TGF-β1 in cultivated naïve B cells. In comparison to the *TGF-β1* expression the secreted TGF-β1 was not enhanced significantly but we were able to observe an 1.43-fold increase of TGF-B1 secretion in the presence of calcitriol, 1.27-fold increase in the presence of 9cRA, and a 1.45-fold increase in the presence of both ligands.

Identification of VDR responsive binding sites in the TGF-β promoter

The above results suggested that VDR/RXR signaling may converge on the *TGF-β1* promoter. We next screened the *TGF-β1* promoter region 10.000 kb upstream of the transcription start site with the in silico approach using MatInspector (www.genomatix.com) for the presence of potential VDR binding sites (Fig. 6A). We identified five binding sites (A–E) that are phylogenetically conserved between man and mice. To verify these putative VDR binding sites within the *TGF-β1* promoter a ChIP assay was performed. $CD19^{+}$ $CD27^{-}$ human B cells (1×10^{6} cells/mL) were cultivated 48 h with CD40L/IL-4/IL-21 and αBCR. Calcitriol was added the last 16 h of cultivation. Three of the selected regions (B, D, and E) displayed binding of the ligand/VDR complex in calcitriol stimulated B cells (Fig. 6B). Binding of RNA-polymerase-II to the *TGF-β1* promoter was induced by calcitriol stimulation within region B, D, and E (Fig. 6C). The VDR is mostly acting as heterodimer and is known to recruit cofactors, like SRC-1, RAC3, CBP, and p300 to form a transactivator complex. Next, we performed ChIP analysis using antibodies against RXRβ, SRC-1, RAC3, CBP, and p300 to analyze the composition of the VDR complex in region B, D, and E. Our results demonstrate that RXRβ is forming heterodimers with VDR in the preselected regions. The VDR/RXRβ complex recruits p300 to region B, SRC-1, CBP, and p300 to region D and SRC-1, RAC3, CBP, and p300 to region E. The heterodimeric binding partner RXRβ and the cofactors SRC-1, RAC3, CBP, and p300 were recruited to the TRPV6 promoter but not the negative control region TRPV6-orf (Fig. 6D).

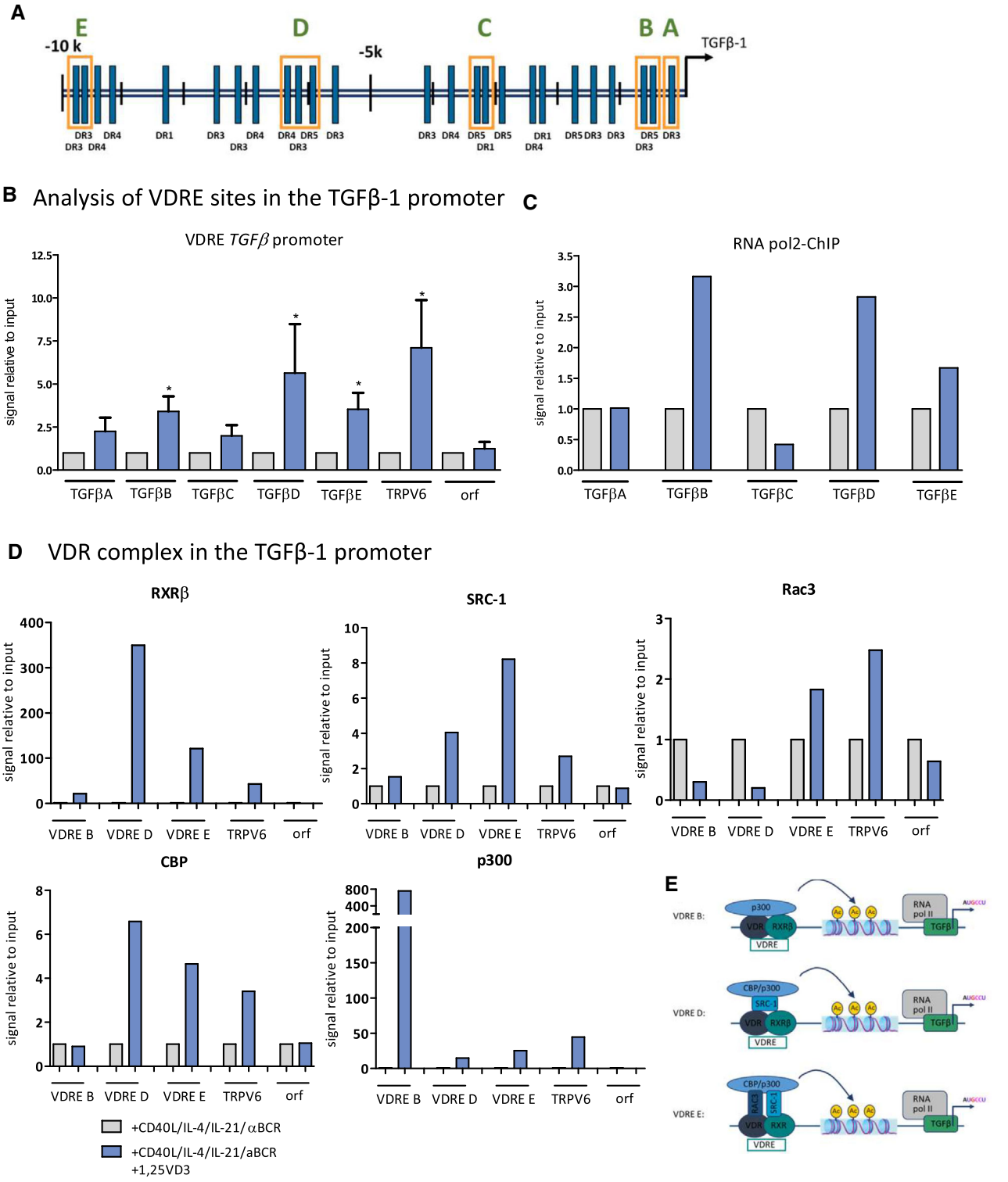

Figure 6. The VDR binds to distinct TGFβ promoter regions. (A) Schematic diagram of the TGF-β1 promoter. (B) MACS sorted naïve B cells were stimulated with CD40L/IL-4/IL-21/αBCR for 48 h and with or without calcitriol for 16 h and chromatin IP (ChIP) was performed with an anti-VDR antibody. TGFβ, TRPV6, and orf regions were determined by RT-PCR and output DNA was normalized to input DNA. $N = 4$ (pooled independent biological replicates). Data are shown as the relative amount of antibody bound to unprecipitated DNA as means ± SEMs and pooled from four independent experiments performed separately. $^{*}p < 0.05$, Mann–Whitney test. (C) RNA-polymerase-II binding in the tgf-β promoter region of activated B cells analog to (B). (D) ChIP was performed with anti-RXRβ, -SRC-1, -CBP –p300, and anti-Rac3 antibody. TGF-β, TRPV6, and orf regions were determined by RT-PCR and output DNA was normalized to input DNA. One representative experiment out of four independent experiments performed separately is shown with one sample per experiment. (E) Schematic model of co-activator complexes at different VDRE sites within the TGF-β1 promoter.

Discussion

Our data show that calcitriol and 9cRA are potent modulators of human naive B cell gene expression via the induction of several signals that are required for differentiation and isotype switching. We observed no competing but a rather additive signaling behavior. The data suggest that VDR is a major driver for B cell activation and differentiation.

The pleiotropic functions of calcitriol and 9cRA are mainly mediated through binding to the VDR and RAR. Naïve human B cells respond to the activation with CD40L/IL-4 with a significant upregulation of the VDR and the RAR. RXRβ, their central interaction partner is constitutively expressed. Upon activation, the molecular basis for calcitriol and 9cRA signaling is given in human naïve B cells. $CD19^+$ peripheral blood cells and tonsil B cells constitutively express low levels of VDR upregulated upon activating stimuli [1,3,8,17,18]. We identified that VDR is expressed in a biphasic manner, whereas RARα expression quickly declines after an initial induction. These findings indicate that the susceptibility of B cells to VDR and RAR signaling strongly depends on their activation and differentiation status. The absence of other heterodimeric binding partners such as RXRγ, RARβ, and RARγ underlines that VDR, RARα, and RXRβ are sufficient to mediate calcitriol and 9cRA signaling in human naive B cells. *CYP24A1* is known to be the most inducible gene responsive to calcitriol [19–21] and was strongly upregulated in a dose and time dependent manner upon calcitriol stimulation but, interestingly not upon activation by CD40L/IL-4 opposing to VDR regulation. 9cRA did not influence *CYP24A1* expression neither in the presence nor the absence of calcitriol indicating an exclusive regulation via the VDR/RXR complex. *CYP26A1* and *RAR*β, well known response genes to RA stimulation [22] could neither be detected in resting human naïve nor in activated B cells (not shown), suggesting their multifactorial regulation in a cell-specific manner. *TGM2* was confirmed as a direct response gene of the RAR/RXR complex in human naïve B cells as well and is in line with previous findings [23–25]. Concurrent treatment of B cells with calcitriol and 9cRA *TGM2* emerged as exclusively regulated by RAR/RXR.

We observed a coordinated induction of CD38 upon naïve B cell exposure to calcitriol and 9cRA implying a functional crosstalk between both ligands in an in vitro environment. The expression of *CYP24A1* and *CYP27b1* [8,5] enables B cells to control their own calcitriol metabolism, whereas *CYP26A1* and *RALDH* (retinaldehyde dehydrogenase) are absent, suggesting that RA is delivered on demand by other cells [26].

Additionally, impurities within the B cell culture due to the isolation process might also affect the immediate effects of calcitriol and 9cRA on the donor response. The presence of other cells in the culture medium could lead to indirect B cell effects due a change of the cytokine milieu.

Having uncovered the immediate functions of calcitriol and 9cRA on B cell activation, suggesting an impact on the differentiation into antibody secreting cells, we turned our attention to study their long-term effects. As previously published protocols using $CD40L^+$IL-4 [15,1] did not display sufficient survival and differentiation of naïve human B cells we optimized the protocol by introducing additional stimuli. A three phase model as shown by others for the differentiation of memory B cells [27,28] was established. The combination of αBCR/CD40L/IL-4/IL-21 as activation signals allowed obtaining a maximum number of viable activated B cells within 4 days. BCR cross-linking and CD40 engagement allows high cell proliferation even at low cell densities [29]. IL-21 is thereby crucial to trigger proliferation, a prerequisite for ASC formation, and to mediate adoption to a PC fate [27]. From day 4 onward, CD40L was removed because it partially blocks PC differentiation [30]. IL-10 was added in phase II to support B-cell blast Ig production [29]. After a 7-day cultivation period of $CD19^+$ $CD27^-$ B cells, we observed higher plasma blast cell frequencies and numbers in both calcitriol and 9cRA stimulated cells even with an additive effect when applied together. *BLIMP1* and *XBP1* are two well-known transcription factors regulating B cell maturation and plasmablast differentiation [31]. The increase of both genes after calcitriol treatment revealed that these phenotypically defined plasmablasts were also genotypically modulated. *BLIMP1* and *XBP1* levels were not affected during concurrent application of 9cRA and calcitriol, compared to calcitriol alone, again suggesting no interfering cascades. However, data from previous studies revealed an inhibitory effect of calcitriol on plasmablast differentiation when added directly after culture start [2]. Interestingly, the same trend as with our study was achieved adding calcitriol 5 days after culture start [32]. A key requirement for plasma blast differentiation is the initial cell proliferation [33,34]. Such bidirectional results may indicate a higher susceptibility of memory than naïve B cells regarding the inhibitory effects of calcitriol on cell proliferation. Although we observed similar promoting activity of 9cRA on plasmablast generation, for calcitriol the crosstalk between 9cRA signaling and specific B cell activation pathways seems to be of importance. The comparison between T-cell independent activation of human B cell subsets with *Staphylococcus aureus* and CpG revealed that 9cRA exerts both inhibiting and promoting effects on cell differentiation [35,36]. Noteworthy, both, naïve and mature human B cells stimulated with TLR7/8 or TLR9 significantly upregulated CD38 in the presence of RA [11]. That RA signaling is essential for B cell maturation has also been demonstrated in a mouse model with silenced RA signaling in the B cell lineage resulting in developmental defects of MZ and B1 B cells [37].

When investigating whether calcitriol and 9cRA impact Ig production, we determined that IgA secretion significantly differed between receptor ligand stimulated cells and non-stimulated cells. For the induction of CSR to IgA the micromilieu is essential. Human studies show stable IgA production under 9cRA treatment in hand eczema [38] and in αCD40 plus IL-4 activated cells [4], whereas data from tonsillar B cells activated with αBCR, αCD40 and IL-4 support our findings [39]. In pigs, the intramuscular immunization using calcitriol as immunomodulator resulted in a significant induction of an antigen-specific IgA serum response [40]. The administration of both ligands alone strongly induced IgA polarization, whereas the combination showed no

synergistic effects. Yet, it seems plausible that naïve B cells are more prone to RAR/RXR signaling in terms of IgA induction. The finding, that both receptor ligands induce IgA is essential at mucosal sites, where IgA is the predominant Ig for neutralizing toxins and pathogens. There, B cells come in close contact to RA producer cells like IECs (intestinal epithelial cells) and DCs. Both cell types are able to provide RA by expressing RALDH which is crucial for the oxidation of retinaldehyde. A third source of RA represent lamina propria stromal cells (LPSCs), a cell subset underlying IECs [41]. The concurrent stimulation of B cells with both ligands and the associated increase of IgA secretion provide the possibility of a strengthened mucosal immunity.

Additionally, the analysis of AID expression by microarray and qPCR (data not shown) confirmed that calcitriol stimulation alone negatively modulates AICDA. Therefore, it is very likely that calcitriol affects rather germline Cα gene transcription than AID-mediated Sμ-Sα DNA recombination. The modulation of AICDA by 9cRA is strongly donor and concentration dependent and probably a result of different receptor expression.

The human Ig heavy chain locus possesses two different Cα regions resulting in the production of two IgA isotypes [12]. Our data confirm that both isotypes are transcriptionally regulated in a comparable manner by calcitriol and 9cRA, showing a synergism for both αGLTs and not a specific preference for IgA_1 or IgA_2. In our model, we show that calcitriol fosters IgM and IgG production without interfering actions of 9cRA. However, several reports illustrate a decrease of IgM and IgG in calcitriol stimulated human B cells originating from healthy individuals and inactive SLE patients [32,42,43]. Activating stimuli and calcitriol promote the survival of non-switched IgM plasmablasts in vitro. VDR/RXR facilitated inhibition of the εGLT and the associated reduction of IgE [3,6] is circumvented when both ligands stimulate B cells simultaneously indicating an interfering pathway of calcitriol and 9cRA.

TGF-β is an essential IgA promoting factor [44] exemplified by the loss of IgA secretion in mice with a B cell–specific blockade of TGF-β signaling [45]. We determined in activated naive B cells basal TGF-β1 mRNA that was enhanced after calcitriol and 9cRA administration. Validation by protein detection rendered comparable results. Basal TGF-β1 expression has been reported previously [46]; however, human studies on B cell activation are heterogeneous. It has been proposed that exclusively $CD19^+CD5^+$ regulatory B cells produce autocrine TGF-β [47]. Stimulation of $CD19^+$ human B cells with anti-CD40 induced TGF-β [48] and and switching to IgA [49]. Calcitriol/9cRA mediated elevated TGF-β1 suggests a synergistic action of CD40 receptor and nuclear hormone receptor signaling. As calcitriol and 9cRA are tightly regulated and present at inflammatory sites this pathway for IgA induction gains in importance. To elucidate whether the elevated expression of TGF-β is a direct consequence of the transactive action of the VDR/DNA complex, we exploited ChIP analysis at five putative binding sites. We found that VDR binds as a heterodimer with RXRβ at three different sites within the TGF-β promoter. Following calcitriol stimulation the co-repressor complex dissociates and co-activators like SRC-1, TIF2, RAC3, CBP, and p300 are recruited [7]. Moreover, we demonstrate that distinct co-activators associate with their respective binding site (B: p300; D: SRC-1, CBP, p300; E: SRC-1, RAC3, CBP, p300). Most likely, the joint activation of all three VDR binding sites contributes to a robust TGF-β regulation. The ligand-based activation of VDR has the potential to directly affect centrocytes by inhibiting the CSR to IgE through binding to the ε germline promoter [6]. Our results support the idea that VDR indirectly shapes the GC reaction via inducing the early TGF-β-dependent phase of IgM-to-IgA class switching. Thus, calcitriol is among numerous other factors of great importance for CSR during the GC reaction and thereby crucial for the regulation of Ig responses.

In conclusion, we have shown that calcitriol and 9cRA regulate gene expression in naïve human B cells in an unidirectional manner by inducing IgA-secreting plasmablasts in an in part TGF-β dependent manner. Our data provide evidence that a tightly regulated administration of the nuclear hormone receptor ligands calcitriol and 9cRA has the potential to channel humoral immune responses towards a protective direction. To strengthen these data set it would be advantageous to analyze larger cohorts and dissect individual changes among the individual donors.

Materials and methods

Cell isolation and cell culture

All studies were approved by the Charité ethical board and written informed consent was obtained from all Donors. PBMCs were isolated from healthy donors (Charité blood donation) by density gradient centrifugation using ficoll hypaque and B cells were purified by magnetic cell sorting using magnetically labeled CD19-multisort beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) as described previously [6]. Subsequently $CD19^+$ B cells were purified into naïve B cells by depletion of $CD27^+$ and $CD14^+$ cells resulting in a purity of 85–95% of the naïve B cell fraction. For nuclear receptor expression analysis and global transcriptome analysis $CD27^-$ naïve B cells were cultured in Advanced RPMI 1640 (Invitrogen, Darmstadt, Germany) supplemented with 5% charcoal-stripped FCS (Invitrogen), 4 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (all from Biochrom). Cells were stimulated with 1 μg/mL CD40 ligand (Miltenyi Biotec), 5 ng/mL IL-4 (Miltenyi Biotec), and with/out 50 nM calcitriol and/or 50 nM 9cRA (both Sigma–Aldrich, St Louis, MO). For the analysis of B cell differentiation and immune globulin secretion, $CD27^-$ naive B cells were activated in Iscove modified Dulbecco medium (supplemented with 4 mmol/L L-glutamine nonessential amino acids, 5 μg/mL transferrin, and 5 μg/mL insulin; Sigma–Aldrich) for 4 days with anti–IgM-F(ab2)′ fragments (2 μg/mL; Jackson ImmunoResearch, Dianova, Hamburg, Germany) to cross-link the antigen receptor (B-cell receptor [BCR]), CD40 ligand (1 μg/mL, Miltenyi Biotec), IL-4 (5 ng/mL, Miltenyi Biotec), and IL-21 (50 ng/mL, Miltenyi Biotec),

followed by IL-10 and IL-21 (50 ng/mL, Miltenyi Biotec) treatment for 72 h. At the beginning of cell culturing calcitriol and/or 9cRA were added (Sigma–Aldrich, both at 100 nM). Cultures were carried out at 37°C in humidified air and 5% CO_2.

RNA isolation, cDNA synthesis, and quantitative PCR

Isolation of RNA was performed with the Nucleospin RNA II kit (Macherey-Nagel, Dueren, Germany) according to manufacturer´s instruction. cDNA synthesis was carried out with TaqMan Reverse Trancription Reagent (Thermo Fisher Scientific, USA) and quantitative RT-PCR using QuantiTect SYBR Green on Rotorgene Q (Qiagen, Germany). All oligonucleotides were synthezied by TIBMOLBIOL (Berlin, Germany) and given in 5´-3´format: α1GLT for CTCAGCACTGCGGGCCCTCCA and rev GTTCCCATCTGGCTGGGTGCTGCA, α2GLT for CTCAGCACTGCGGGCCCTCCA and rev GTTCCCATCTTGGGGGGTGCTGTC, BLIMP1 for GTGCTCGGTTGCTTTAGACTGCT and rev TAAGCCCATCCCTGCCAACCA, CD38 for TGGCGCGATGCGTCAAGTACA and rev GGGTGAACATGTCCCGCTGGA, RARα for CTATGCTGGGTGGACTCTCC and rev GAACTGCTGCTCTGGGTCTC, RXRβ for GGCTTCTTCAAACGCACCAT and rev GCTGACGCTCCTCCTGTACC, TGFβ1 for GCGTGCTAATGGTGGAAAC and rev CGGTGACATCAAAAGATAACCAC, TGM2 (RT^2 qPCR Primer Assay for Human TGM2 from Qiagen, NM_004613), VDR for ACTTGCATGAGGAGGAGCAT and rev AGGTCGGCTAGCTTCTGGAT, XBP1 for CTGAGTCCGCAGCAGGTG and rev GGAAGGGCATTTGAAGAACA. CYP24A1, HPRT, and VDR as previously described [1,8].

Global transcriptome analysis

$CD27^-$ naïve B cells were activated for 24 h and stimulated with calcitriol and/or 9cRA during the last 8 h of activation. Total RNA of the cells was isolated using RNeasy-Kit including enzymatic digestion of genomic DNA (Qiagen, Germany). RNA isolation and Affymetrix GeneChip hybridization were conducted as previously described [50].

Flow cytometric analysis

Flow cytometry was conducted in accordance with the current flow cytometry guidelines [51]. $CD27^-$ naïve B cells were activated for 4 days with aBCR, CD40L, IL-4, and IL-21 and subsequently for the next 3 days with IL-10 and IL-21. Additionally cultures were treated with 10^{-7} M calcitriol, 9cRA, or both. Staining was conducted using fluorochrome-conjugated monoclonal antibodies and characterized according to the surface expression of CD19 APC-Vio770, CD38 PE-Vio770 (all from Miltenyi Biotec, Bergisch Gladbach, Germany), CD27 PE (BD Biosciences, Franklin Lakes, USA), and Zombie Aqua™ Fixable Viability Kit (BioLegend, San Diego, USA). Data were assessed using FlowJo10 software (TreeStar, Ashland, OR, USA).

Enzyme-linked immunosorbent assay

For Ig assays $CD27^-$ naïve B cells were cultivated with 10^{-7} M calcitriol, 9cRA, or both and activated for 4 days with αBCR, CD40L, IL-4, and IL-21 and subsequently for the next 3 days with IL-10 and IL-21. After 7 days total Ig levels were measured in cell free supernatants by ELISA as described previously [52].

Chromatin immunoprecipitation

$CD27^-$ naïve B cells were fixed and lysed and ChIP was performed as previously described [8]. DNA was shreaded by sonication (Bandelin, Germany). ChIP was carried out with antibodies against VDR (D-6), RXRβ (C-20), SRC-1 (1135/H4), CBP (C-1), p300 (F-4), Rac3 (1A8; all obtained from Santa Cruz Biotechnology, Dallas, USA), and RNA pol2 (4H8; Cell Signalling Technology, Danvers, USA). Subsequently, DNA was purified using NucleoSpin extract II (Macherey–Nagel). The precipitated DNA was quantified using qPCR. *TRPV6* was used as a positive control for VDR binding and Trpv6 open reading frame (ORF) was used as a negative control as described previously [1]. Sequences of specific primers for VDR binding within the *TGF*β promoter were: VDRE TGFβ A for ACCCACACCGCCCGCAAA and rev CGTCCTCATCTCGCGTGG, VDRE TGFβ B for AAATGTATGGGGTCGCAGGG and rev GAGGGTGTCAGTGGGAGGA, VDRE TGFβ C for TGATTTGGAGAGGCTGTGGG and rev GGCCCTTCACAGCAAAGTTC, VDRE TGFβ D for CAGACAGTGGTAAGTGCTAGGA and rev CCCCTCCCTCATCACCCT, VDRE TGFβ E for CAGGGGTGTTGACGAAGCTAG and rev ATACCTGTGTGAATGCCCCA.

Bioinformatics and statistical analysis

Differentially regulated genes were filtered according to high-performance chip data analysis using Bioretis database (www.bioretis-analysis.de) as described in ref. [53]. For additional pattern discovery and multivariate feature selection, Genes@Work software [54] was used including gene vector normalization and Pearson's correlation coefficient with mean as distance measure.

Statistical significance was determined by analysing the data sets with GraphPad Prism, version 7.0 (GraphPad Software, La Jolla, USA). When a non-Gaussian distribution of sample values was assumed nonparametrical two-tailed Kruskal–Wallis test with Dunn-Bonferroni post hoc correction or Mann–Whitney *U*-test for independent groups was applied. Data are shown as scatter plots with bar and median values. A p-value < 0.05 was considered as statistically significant for all tests.

Acknowledgments: We thank Roy-Arne Senkel, M. Sc., for helping with the Western Blot analysis and the TGF-β ELISA; and Dennis Ernst for excellent technical assistance. This work was supported by grants awarded by grants to M.W., A.R., and G.H. of the Deutsche Forschungsgemeinschaft (DFG-TRR130-P19). S.T. was financed by the DFG (TRR130-TP19).
Open access funding enabled and organized by Projekt DEAL.

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202048557.

Conflict of interest: G. Heine and M. Worm have received grants from the German Research Foundation (DFG-TRR130-P19). S. Treptow has received a grant from, was employed by, and has received travel support from the German Research Foundation (TRR130-TP19). The rest of the authors declare that they have no commercial or financial conflict of interest.

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Abbreviations: **9cRA**: 9-*cis* retinoic acid · **AICDA**: activation-induced cytidine deaminase · **BLIMP1**: B lymphocyte-induced maturation protein-1 · **CCR9**: C-C motif chemokine receptor 9 · **CD**: cluster of differentiation · **CSR**: class switch recombination · **CYP24A1**: cytochrome P450 family 24 subfamily A member 1 · **ILC**: innate lymphoid cell · **NR**: nuclear receptor · **ORF**: open reading frame · **RAR**: retinoic acid receptor · **RXR**: retinoic x receptor · **SHM**: somatic hypermutation · **TGM2**: transglutaminase 2 · **VDR**: vitamin D receptor · **VDRE**: vitamin D responsive elements · **XBP1**: X-box binding protein 1 · **α4β7**: integrin-alpha4-beta7

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Received: 25/3/2020
Revised: 4/8/2020
Accepted: 22/10/2020
Accepted article online: 27/10/2020