**Research Article**

Modulation of the humoral immune response by constitutively active STAT6 expression in murine B cells

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The transcription factor STAT6 regulates gene expression in response to IL-4 and IL-13. To further investigate how activated STAT6 modulates B cells development and function in vivo, we characterized mice that express a constitutively active version specifically in B cells. CD19Cre STAT6vt mice show spontaneous phosphorylation and nuclear translocation of STAT6 in B cells. About 80 genes were more than twofold up- or downregulated in splenic B cells from CD19Cre STAT6vt as compared to control mice. B cell development, tissue localization, and populations of follicular and marginal zone B cells, B1 B cells, GC B cells, and plasma cells (PCs) appeared to be normal. However, the number of IgE+ and IgG1+ GC B cells and PCs as well as serum IgE and IgG1 levels were increased in CD19Cre STAT6vt mice. Infection with Lymphocytic choriomeningitis virus associated with high levels of TNF and IFN-γ did not prevent the development of a significantly increased IgE and IgG1 response against the virus in CD19Cre STAT6vt mice. These results suggest that prolonged STAT6 activation during chronic allergic inflammation may result in IgE responses during subsequent viral or bacterial infection that could further stimulate mast cell activation even in the absence of the initial allergic response.

**Keywords:** allergy · germinal center · helminths · IgE · LCMV

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

**Introduction**

The transcription factor signal transducer and activator of transcription (STAT6) mediates signaling downstream of the receptors for IL-4 and IL-13 [1]. After receptor activation, monomeric STAT6 gets recruited by its SH2 domain and binds to phosphotyrosine residues in the cytoplasmic domain of the IL-4Rα chain. STAT6 gets phosphorylated at Tyr 641 by Janus kinases Jak1 or Jak3, forms homodimers and translocates to the nucleus where it binds to the spaced palindromic sequence TTCN₃₋₄GAA (also named GAS-4) and thereby regulates gene expression [2]. In B cells, STAT6 is required for class switch recombination to IgG1 and IgE [3–6]. We recently performed a comparative analysis of the STAT6-regulated transcriptome and proteome in primary mouse B cells and revealed that upon stimulation with IL-4 more than 400 genes were up- or downregulated more than threefold in a STAT6-dependent manner [7]. Using mixed BM chimeras, we could further show that STAT6 expression in B cells was required for GC formation during type 2 immune responses against helminths, model allergens, or sheep RBCs [8].
A constitutively active version of STAT6 (STAT6vt) was generated by alanine scanning mutagenesis of the SH2 domain [9]. The exchange of Val 547 and Thr 548 to Ala resulted in spontaneous Jak1/Jak3-independent phosphorylation of the critical Tyr 641 residue required for dimerization [10]. To further investigate the in vivo effects of STAT6vt in lymphocytes, transgenic mice were generated that express STAT6vt under control of the human CD2 promoter and locus control region that leads to expression in T and B cells. These CD2:STAT6vt mice showed reduced numbers of peripheral T cells, a Th2 bias, more B cells and higher serum levels of IgG1 and IgE [11]. Since in this model STAT6vt is expressed in all lymphocytes, it was impossible to dissect T and B cell intrinsic effects of STAT6vt. Furthermore, it has not been investigated whether pSTAT6 in B cells impairs the usual Th1-driven and IgG2a/c-biased humoral immune response to a viral infection.

To address these points, we first generated mice that express STAT6vt under control of a loxP-flanked STOP cassette from the Rosa26 locus [12] and crossed them to CD19Cre mice [13] so that expression is restricted to the B cell lineage. We show that STAT6 is constitutively phosphorylated and detectable in the nucleus of B cells from CD19Cre_STAT6vt mice. RNAseq analysis revealed that 57 genes were more than twofold upregulated and 21 genes were more than twofold downregulated. B cell development, B cell numbers, and their localization in B cell follicles and in the marginal zone in the spleen appeared to be normal. All splenic B cells expressed more CD23 while CD86 expression was only increased in GC B cells. Serum IgE and IgG1 levels were increased in CD19Cre_STAT6vt as compared to control mice. Infection with lymphocytic choriomeningitis virus (LCMV) revealed that constitutively active STAT6 in B cells promotes an IgG1/IgE response to the virus but does not inhibit the IgG2c response.

Results

Expression of constitutively active STAT6 in B cells of CD19Cre_STAT6vt mice

B cell-intrinsic expression of STAT6 plays an important role for class switch recombination to IgG1 and IgE but also for GC formation during type 2 immune responses. To further investigate the impact of STAT6 signaling on B cell biology, we generated CD19Cre_STAT6vt mice that express a constitutively active form of STAT6 specifically in B cells. The phosphorylation status of STAT6 was determined by western blot analysis with untouched splenic B cells of WT, STAT6-deficient, and CD19Cre_STAT6vt mice after purification by magnetic cell sorting and culture for up to 4 h either with LPS alone or with LPS and IL-4. The expression level of total STAT6 was comparable between B cells of WT and CD19Cre_STAT6vt mice and did not change over time in response to LPS alone or with LPS and IL-4 (Fig. 1A). As expected, phosphorylated STAT6 (pSTAT6) was detected in unstimulated and LPS-stimulated samples of CD19Cre_STAT6vt mice but not in WT and STAT6-deficient mice (Fig. 1A; Supporting Information Fig. 1).

Next, we performed histological staining to determine whether STAT6 spontaneously localizes to the nucleus of B cells in CD19Cre_STAT6vt mice. In comparison to WT B cells, we observed a similar cytoplasmic but more pronounced nuclear staining in samples from CD19Cre_STAT6vt mice (Fig. 1B).

Transcriptome analysis of splenic B cells from naïve CD19Cre_STAT6vt and control mice

Based on the finding that STAT6vt accumulates in the nucleus of naïve splenic B cells in CD19Cre_STAT6vt mice, we next determined the changes of the global transcriptome by RNAseq analysis. Therefore, we isolated naïve B cells by cell sorting (CD19⁺ CD3⁻CD4⁻ CD8⁻, purity >95%) from three age-matched
CD19Cre,STAT6vt and three CD19Cre control mice, purified total RNA, and performed transcriptome analysis using the Illumina platform. Setting a p-value cutoff at 0.05 we revealed 973 differentially expressed genes (Fig. 2A). Fifty-seven genes were more than twofold upregulated and 21 genes were more than twofold downregulated (Fig. 2B). Among the 36 upregulated genes with a combined expression level $>1$ were IgE and IgG1, the LIF receptor, Cathepsin E, PIANP (a ligand for the inhibitory receptor PILRa), PTPRV (a tyrosine phosphatase), and the signaling adaptor SLP-76 (Fig. 2C; Supporting Information Fig. 2). Some of the 11 more than twofold downregulated genes with a combined expression level $>1$ included two miRNAs (Mir421 and Gm27663), KLRA1 (an inhibitory receptor), and a few uncharacterized genes (Fig. 2B and C). Results were compared to published data on gene expression of in vitro IL-4-stimulated primary mouse B cells from WT and STAT6ko mice [7, 14]. Gene set enrichment analysis provides evidence that a similar pattern of genes is upregulated by activated WT STAT6 and STAT6vt (Fig. 2D). Gene set enrichment analysis was also performed to screen for changes in hallmark pathways of the MSigDB database and all eight gene sets with a false discovery rate $<0.05$ were upregulated. Included were cell cycle related gene sets as well as Myc, MTORC, glycolysis, angiogenesis, and unfolded protein response related gene sets (Fig. 2E).

**Constitutively active STAT6 does not affect B cell development and homeostasis**

To further investigate whether expression of STAT6vt changes B cell development in the BM or B cell homeostasis in spleen and peritoneum, we performed flow cytometric analysis. In the BM, the frequency of pre/pro B cells (B220$^+$IgM$^+$), immature B cells (B220$^+$IgM$^-$), and mature B cells (B220$^+$IgM$^+$) was comparable between CD19Cre,STAT6vt and CD19Cre control mice (Fig. 3A). In the spleen, the total B cell frequency and the fraction
of marginal zone B cells (IgDloIgMhi) was not affected by expression of STAT6vt and the B1 cell populations in the peritoneum were also comparable (Fig. 3A).

Next, we determined the expression levels of the low-affinity IgE receptor CD23, the co-stimulatory molecule CD86 and MHC class II (I-Ab) as these proteins have been shown to be upregulated on B cells from CD2:STAT6vt mice that express constitutively active STAT6 in all lymphocytes under control of the human CD2 promoter [11]. Ex vivo isolated splenic B cells from naïve CD19Cre STAT6vt mice showed strongly increased expression of CD23 on all B cells while CD86 was only upregulated on B cells with a GC B cells phenotype (B220+CD38loGL-7hi) (Fig. 3B; Supporting Information Fig. 3). However, the expression level of MHC-II was comparable between CD19Cre STAT6vt and CD19Cre control mice (Fig. 3B). Similar results were obtained with B cells from LCMV infected mice (Supporting Information Fig. 4). Upon stimulation with LPS+IL-4 splenic B cells from CD19Cre STAT6vt mice showed more pronounced upregulation of CD23, CD86, and MHC-II on the cell surface as compared to B cells from CD19Cre control mice (Supporting Information Fig. 5).

Histological analysis of cryosections from the spleen by immunofluorescence staining revealed no obvious structural differences of B cell follicles and marginal zone B cells (Fig. 3C). Overall, we did not observe major effects on B cell development and homeostasis by expression of STAT6vt in B cells.

**GC B cells and PCs express more IgG1 and IgE in naïve and LCMV-infected CD19Cre STAT6vt mice**

Since STAT6 signaling is not only important for CSR to IgG1 and IgE, but also required for GC formation during type 2 immune responses, we next analyzed the GC B cell and PC compartments and expression of IgE, IgG1, and IgG2c within these cells. The analysis was performed with naïve and LCMV-infected CD19Cre STAT6vt and CD19Cre control mice. The LCMV infection was chosen to determine whether an IgE/IgG1 response could be observed even during a strong type 1 immune response with high IFN-γ levels that usually inhibit CSR to IgE and IgG1.

Naïve CD19Cre STAT6vt mice showed no spontaneous expansion of the splenic GC B cell population (B220+CD380GL-7hi).
However, the frequency of IgG1+ cells within the GC B cell compartment was increased from 10 to 30% while IgG2c+ and IgE+ GC B cells were comparable in both strains (Fig. 4A and E). The GC B cell population increased from below 1% in naïve mice to 4–5% on day 14 after LCMV infection in both CD19Cre_STAT6vt and CD19Cre control mice (Fig. 4B). Within the GC B cell compartment of LCMV-infected mice the IgG1+ cells constituted 4% in CD19Cre and 8% in CD19Cre_STAT6vt mice. The frequency of IgE+ GC B cells was generally very low but still increased in CD19Cre_STAT6vt mice, while the frequency of IgG2c+ GC B cells was around 17% in both strains of mice (Fig. 4B and E; Supporting Information Fig. S6A and B).
Within the PC compartment (B220<sup>+</sup> CD138<sup>+</sup> TACI<sup>+</sup>) of naive mice IgG1<sup>+</sup> cells increased from 0.5% in CD19Cre mice to 2.5% in CD19Cre<sub>STAT6vt</sub> mice (Fig. 4C and F). Likewise IgE<sup>+</sup> PCs increased from 0.2 to 0.5% while IgG2c<sup>+</sup> PCs were not significantly different (Fig. 4C and F; Supporting Information Fig. 6C). After LCMV infection IgG1<sup>+</sup> cells constituted 20% of all PCs in CD19Cre mice and about 32% in CD19Cre<sub>STAT6vt</sub> mice (Fig. 4C and F). The frequency of IgE<sup>+</sup> PCs increased from 0.1% in CD19Cre mice to 0.8% in CD19Cre<sub>STAT6vt</sub> mice while IgG2c<sup>+</sup> PCs were comparable with about 17% in both strains of LCMV infected mice (Fig. 4C and F; Supporting Information Fig. 6D).

### B cell-intrinsic STAT6 signaling promotes LCMV-specific IgE and IgG1 production

The significantly increased frequencies of IgE<sup>+</sup> and IgG1<sup>+</sup> PCs after LCMV infection in CD19Cre<sub>STAT6vt</sub> mice suggested that this difference results in higher serum IgE and IgG1 levels as compared to control mice. ELISA analyses for total serum concentrations of different isotypes revealed that naive CD19Cre<sub>STAT6vt</sub> mice produced about threefold more total IgE while IgG1 levels were only slightly increased (Fig. 5A). IgM and IgG2c levels were comparable between CD19Cre<sub>STAT6vt</sub> and control mice. Interestingly, the increased serum IgE and IgG1 levels were also detected after LCMV infection (Fig. 5A). To further determine whether the IgE/IgG1 response included LCMV-specific antibodies, we performed ELISAs specific for LCMV GP. Indeed, significantly higher serum levels of LCMV-GP-specific IgE and IgG1 antibodies were detected in CD19Cre<sub>STAT6vt</sub> as compared to control mice (Fig. 5B).

These results indicate that specific expression of constitutively active STAT6 in B cells promotes the humoral type 2 immune response even during a strongly type 1 immunity biased response to LCMV infection.

### Discussion

The requirement of STAT6 for IL-4-induced CSR to IgE and IgG1 in B cells is well established [2–4, 7, 14, 15]. In addition, B cell intrinsic expression of STAT6 appears to be critical for GC formation during type 2 immune responses [8]. These findings are mainly based on analyses of STAT6-deficient B cells. However, it remained unclear whether ectopic expression of active STAT6 in B cells would be sufficient for spontaneous IgE/IgG1 CSR and GC formation. Therefore, we investigated here the phenotype of CD19Cre<sub>STAT6vt</sub> mice in which mature B cells express a constitutively active form of STAT6 from the Rosa26 locus.

Analysis of B cells isolated from naive CD19Cre<sub>STAT6vt</sub> mice revealed the spontaneous phosphorylation and nuclear translocation of STAT6<sub>vt</sub>. However, only 47 genes with a decent expression level were significantly regulated (36 up and 11 down). This stands in contrast to several hundred STAT6-regulated genes observed in ex vivo isolated B cells cultured for 4 days with LPS and anti-CD40 in presence or absence of IL-4 [7]. One possible explanation for this difference could be linked to the effects of LPS that provides a strong proliferative signal and probably makes chromatin more accessible for STAT6. In addition, some STAT6-regulated genes may require additional transcription factors for their expression and LPS- or anti-CD40-induced activation of NF-κB could play an important role here. In fact, NF-κB activity has been shown to be required for DNA binding of STAT6 [16, 17].

The upregulated genes in B cells of CD19Cre<sub>STAT6vt</sub> mice included the heavy chains for IgE and IgG1 indicating enhanced CSR to these isotypes. Other upregulated genes such as Gatm, Lcp2, Lfr, and Nfil3 that had been previously identified as STAT6-regulated genes in B cells [7, 18]. Nfil3 has been described to promote IgE CSR while the function of most other STAT6-regulated genes in B cells remains to be analyzed [19].

Despite these differences in gene expression and several reports that identified activating STAT6 mutations in B cell lymphomas [20–22], we neither found obvious alterations in the composition of the major peripheral B cell populations in the spleen or peritoneal cavity nor did we observe mice that developed lymphomas. B cell development in the BM also appeared to be normal, which could have been expected based on the fact that Cre recombinase is expressed rather late during B cell development in CD19Cre mice [13]. However, we observed upregulation of CD23 on the surface of B cells from CD19Cre<sub>STAT6vt</sub> mice. Similar findings were described in CD2:STAT6vt mice and in a B cell line transduced with a construct where STAT6 can be induced to dimerize by adding tamoxifen to the culture [11, 23]. In contrast to CD2:STAT6vt mice where T and B cells express STAT6vt, we did not observe upregulation of MHC-II and slightly higher levels of CD86 were only detected in GC B cells. This indicates that additional signals that may be derived from activated T cells are required for MHC-II and CD86 upregulation in B cells.

Since B cell-intrinsic STAT6 appears to be important for GC formation during type 2 immune responses [8], we expected to see more GC B cells in CD19Cre<sub>STAT6vt</sub> as compared to control mice. However, this was not the case. Thus, activated STAT6 alone is not sufficient to drive the GC B cell differentiation program. Consistent with the RNAseq data, we observed more IgE- and IgG1-expressing GC B cells and PCs as well as higher serum IgE and IgG1 levels in naive CD19Cre<sub>STAT6vt</sub> as compared to control mice. Therefore, we conclude that constitutively active STAT6 does not modulate the population size of total GC B cells and PCs in naive mice but promotes expression of IgE and IgG1.

Surprisingly, the enhanced IgE and IgG1 response was also observed after LCMV infection, which is characterized by a type 1 immune response with high levels of IFN-γ [24]. IFN-γ activates the STAT1 signaling pathway and inhibits CSR to IgE and IgG1 [25–27]. Using an LCMV-GP-specific ELISA, we could demonstrate that the IgE and IgG1 response is in part directed against the LCMV GP on the viral particle. On the other hand, we did not observe a reduction of the IgG2c response in LCMV-infected
CD19Cre\_STAT6\textit{vt} mice, which suggests that activated STAT6 is not sufficient to suppress IgG2c CSR in B cells.

Taken together, we show here that selective expression of constitutively active STAT6 in mouse B cells leads to an enhanced IgE and IgG1 response that can even be directed against a viral pathogen. This suggests that chronic allergic inflammation with prolonged activation of the STAT6 signaling pathway in B cells might further be promoted by IgE responses to viral or bacterial antigens that would not occur under non-allergic conditions.

### Materials and methods

#### Mice

Rosa26\textsuperscript{LSL-STAT6\textit{vt}} mice were generated by inserting a constitutively active version of STAT6 with a C-terminal FLAG-tag (STAT6\textit{vt} [9]) behind a loxP-flanked Stop cassette into the genomic Rosa26 locus by standard ES cell technology using the pBigT/pROSA26-PA targeting vector system [28] and BALB/c-1 ES cells [29]. Homologous recombination was identified by Southern blot analysis of EcoR1-digested genomic DNA.

Mice were infected with 200 pfu of LCMV strain WE intravenously and analyzed at day 14 after infection.
Flow cytometric analysis

Single cell suspensions of BM and spleen were generated by mechanical disruption, the peritoneal cavity was washed with PBS to retrieve the cells. Erythrocytes were lysed with ACK-buffer (0.15 M NH₄Cl, 1 mM KHO₃, 0.1 mM Na₂EDTA) and washed with FACS buffer (PBS, 2% FCS, 1 mg/mL NaCl). For staining of IgE-expressing cells cytoplasmic IgE was efficiently removed by short treatment with acetate buffer as described [30]. Fc receptors were blocked with anti-mouse CD16/CD32 mAb (clone 2.4G2; BioXcell, West Lebanon, NH, USA) for 5 min at RT and the primary antibodies were incubated for 25 min at 4°C. Antibodies were bought from BD bioscience (San Jose, USA), Biolegend (San Diego, USA), eBioscience (Thermo Fisher; Waltham, USA), or Southern Biotech (Birmingham, USA) and were used were conjugated to AF488, AF647, APC, APC-Fire™ 750, biotin, BV421, eF450, eF506, eF660, eF780, FITC, PE, PE Cy7, PerCP Cy5.5, V450, or V500. The following antibodies were used: CD5 (53-7.3), CD11b (M1/70), CD23 (B3B4), CD38 (90), CD45R/B220 (RA3-6B2), CD49b (HM alpha2), CD86 (GL1), CD138 (281-2), CD267/TACI (ebio8F10-3), GL-7 (GL-7), I-A^B (MS114.15.2), IgA [11-26c (11-26)], and IgM (eB121-15F9). Dead cells were excluded by staining with ECDsbio™ Fixable Viability Dye eFluor™506 or eFluor™780. As secondary staining streptavidin conjugated to Pacific Blue or PE CF594 was added and the cells were incubated for 20 min at 4°C. For intracellular staining cells were fixed with 4% paraformaldehyde (Roth) and permeabilized using a commercial permeabilization & wash buffer (BioLegend). Afterward they were intracellularly stained for IFN-γ (XMG1.2), IgE (R35-72), IgM (eB121-15F9), IgG1 (A85-1), IgG2c (goat-polyclonal), or TNF-α (MP6-XT22). The cells were analyzed with a BD LSRFortessa™ (BD Bioscience) according to the guidelines for the use of flow cytometry and cell sorting in immunological studies [31].

Western blot analysis

Untouched B cells were isolated from spleen by magnetic cell purification (MojoSort™ Mouse Pan B Cell Isolation Kit II, BioLegend) and 4 × 10⁶ cells were stimulated for 15, 30, 120, and 240 min with LPS (2 µg/mL) or LPS plus IL-4 (25 ng/mL; R&D Systems, Minneapolis, USA) in 1 mL culture medium (RPMI1640, 10% FCS, 5 × 10⁻⁵ M β-mercaptoethanol, Pen/Strep). Cells were lysed in RIPA buffer (1% NP-40, 50mM Tris pH7.4, 0.15 M NaCl, 1 mM EDTA pH 8.0, 0.25% deoxycholic acid), incubated on ice for 30 min and centrifuged at 22 000 × g for 20 min. The protein concentration of the supernatant was measured by BCA assay (Thermo Fisher) and 20 µg protein lysates were separated on Mini/Mini TGX precast protein gels (4–12%) at 120 V. Gels were transferred to Immuno-Blot polyvinylidene difluoride Mini/Midi membranes using the Trans-Blot Turbo System (all Bio Rad; Hercules, USA). After blocking, the membranes were then first incubated overnight at 4°C with anti-pSTAT6 (rabbit-polyclonal; Tyr641; Danvers, USA) or anti-beta-actin (13E5, Cell Signaling), followed by horse-radish peroxidase-coupled donkey-anti-rabbit IgG (Donkey-polyclonal; Jackson Immuno Research; Cambridge, UK) and Signal Fire™ Elite ECL substrate (Cell Signaling Technology). The membrane was then stripped and incubated overnight at 4°C with anti-STAT6 (rabbit-polyclonal;M-20; Santa Cruz; Dallas, USA) followed by horse-radish peroxidase-coupled donkey-anti-rabbit IgG and Signal Fire™ Elite ECL substrate. All signals were detected using the ChemiDoc™ Imaging System (Bio Rad).

In vitro B cell culture

B cells were isolated and stimulated with LPS and IL-4 as described for Western blot analysis. After 18 h cells were analyzed via flow cytometry.

Histology

Untouched isolated B cells (MojoSort™ Mouse Pan B Cell Isolation Kit II, BioLegend) were attached onto a coverslip using a Cytospin. From the spleen 6 µm thick Cryo-sections were created. All samples were fixed with –20°C Aceton, air-dried, and blocked with donkey- or rat-serum, anti-mouse CD16/CD32 mAb, and a commercial streptavidin/biotin blocking kit (Vector Laboratories; Burlingame, USA). B cells were stained using anti-STAT6 (rabbit-polyclonal, M-20) followed by incubation with Rhodamine Red™. X conjugated anti-rabbit IgG (donkey-polyclonal, Jackson Immuno Research). Spleen sections were stained with biotin-labeled anti-B220 (RA3-6B2, BioLegend), AF488-labeled anti-IgD (11-26c, BioLegend), AF647-labeled anti-CD169 (MOMA-1, Bio Rad) followed by incubation with streptavidin-Cy3 (Jackson Immuno Research). For microscopy the sections were mounted with Fluoroshield™ with DAPI (Sigma–Aldrich; St. Louis, USA). Images were taken with an Axio VERT.A1 using 10× or 40× lenses and analyzed with the Zen software (all Carl Zeiss; Oberkochen, GER).

Serum ELISA

ELISA plates were coated with purified anti-IgE (clone R35–72, BD Biosciences) or goat anti-mouse Ig (Southern Biotech). For LCVM-specific ELISA, Strep-Tactin® plates (IBA Lifescience; Göttingen, GER) were coated with LCMV GP labeled with Strept-tag® II (kindly provided by Daniel Pinschewer). All plates were blocked, coated with serum, and alkaline phosphatase-conjugated goat-anti mouse IgE, IgG1, IgG2c, or IgM (Southern Biotech) were used for detection of respective isotypes. P-nitrophenyl phosphate (Southern Biotech) was added and the absorption at 405 nm was detected. Total serum concentration was calculated using purified unlabeled mouse IgE, IgG1, IgG2c, or IgM (Southern Biotech) standards.

Transcriptome analysis

Single cell suspension from spleen of naive mice were generated by mechanical disruption. Erythrocytes were lysed with ACK-buffer
(0.15 M NH₄Cl, 1 mM KHO₃, 0.1 mM Na₂EDTA) and washed with FACS buffer (PBS, 2% FCS, 1 mg/mL NaN₃). Fc receptors were blocked with anti-mouse CD16/CD32 mAb and the cells were stained at 4°C for 30 min with FITC-labeled CD19 (eBio 1D3, eBioscience) and PE Cy7-labeled CD3 (17A2, BioLegend). Immediately before sorting eBioscience™ Propidium Iodide Staining Solution (Thermo Fisher) was added to the cells. The cells were sorted into 1 mL FCS, pelleted and immediately lysed with RLT-buffer (RNeasy-Kit; Qiagen, Venlo, NL). The lysate was stored at −80°C until RNA isolation. RNA was isolated using the RNeasy-Kit (Qiagen). Purity and Concentration were determined via Nanodrop (Thermofisher) and Qubit (Invitrogen; Thermo Fisher) and (Qiagen). The RNA was sent to Novogene Co. (Hong-Kong) for transcriptome sequencing gene. The following primer pairs were used: Lcp2-forward: GTGACTTTGTT, Hprt-reverse: GAGGGTAGGCTGGCCTATAGGCT.

**Quantitative RT-PCR analysis**

RNA was isolated as described for RNAseq analysis. RT-PCR was then performed with oligo-dT primers, SuperScript™ III (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific), the SYBR Select Master Mix (Thermo Fisher Scientific), and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Ct value results were normalized to Hprt as a reference gene. The following primer pairs were used: Lcp2-forward: GTGACTTTGTT, Hprt-reverse: GAGGGTAGGCTGGCCTATAGGCT.

**Statistical analysis**

The sample size was chosen by preliminary observations. Statistical analysis was performed with SigmaPlot (12.3; Systat Software, San Jose, USA). Data are presented as mean with SE or SD, as indicated. Statistical significance was tested using an unpaired two-tailed Students t-test or Mann–Whitney U-test.

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Abbreviations: AF: Alexa Fluor™ · APC: allophycocyanin · BV: Brilliant Violet™ · CSR: class switch recombination · Ef: eFluor™ · FITC: fluorescein-isothiocyanate · PC: plasma cell · PE Cy7: PE cyanine7 · PercP Cy 5.5: Peridinin-chlorophyll-protein complex Cyanine5.5 · PI: propidium iodide

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