Brief Communication

Pathogenic NFKB2 variant in the ankyrin repeat domain (R635X) causes a variable antibody deficiency

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ABSTRACT

Genetic studies are identifying an increasing number of monogenic causes of Common Variable Immunodeficiency (CVID). Pathogenic variants in the C-terminus of *NFKB2* have been identified in the subset of CVID patients whose immunodeficiency is associated with ectodermal dysplasia and central adrenal insufficiency. We describe 2 unrelated CVID pedigrees with 4 cases of pathogenic stop gain variants (c.1903C > T) in the ankyrin repeat domain (ARD) of NF-κB2, leading to a premature truncation of the protein at p.Arg635Term (R635X). By immunophenotyping and functional *ex vivo* B- and T-cell experiments we characterized the variant by reduced class-switched memory B-cell counts and immature plasmablasts, unable to produce IgG and IgA. Features of a poor proliferative T-cell response and reduced expansion of CD4$^+$CXCR5$^+$ T cells was only observed in the two clinically affected index cases without any clear clinical correlate. In conclusion, pathogenic stop variants in the ARD of *NFKB2* can cause 'infection-only' CVID with an abnormal B-cell phenotype and a variable clinical penetrance.

1. Introduction

Common variable immunodeficiency (CVID [MIM 607594]) is a heterogeneous disorder characterized by sinopulmonary infections, hypogammaglobulinemia and poor vaccine responses with a prevalence of 1:25,000–1:50,000 [1], frequently complicated by autoimmunity, lymphoproliferation and granulomatous disease [2]. Although initially characterized as late-onset with a complex genetic etiology, an increasing number of monogenic gene defects have been found leading to CVID [3]. Dominant-negative heterozygous *NFKB2* mutations have been reported that result from variants in the C-terminus of the protein causing immunodeficiency with characteristic features that include ectodermal dysplasia and adrenal insufficiency [4–11].

Recently, Kuehn et al. described a novel case-series of patients with more proximal *NFKB2* mutations leading to an altered phenotype, without endocrine or ectodermal manifestations [12]. This series included 3 cases with a stop-variant c.1903C > T, p.Arg635Term (R635X), in the ankyrin repeat domain (ARD), which lead to a gain-of-function (GOF) disease mechanism and presented with the clinical phenotype of combined immunodeficiency (CID) with defective B- and T-cell function.

In most *NFKB2*-defective patients, the immunophenotypic and functional data are not described in detail. Here we describe the clinical and cellular phenotype of an additional 4 individuals of 2 unrelated families with the R635X mutation, identified by Whole Genome Sequencing (WGS) as part of the NIHR BioResource – Rare Diseases study.

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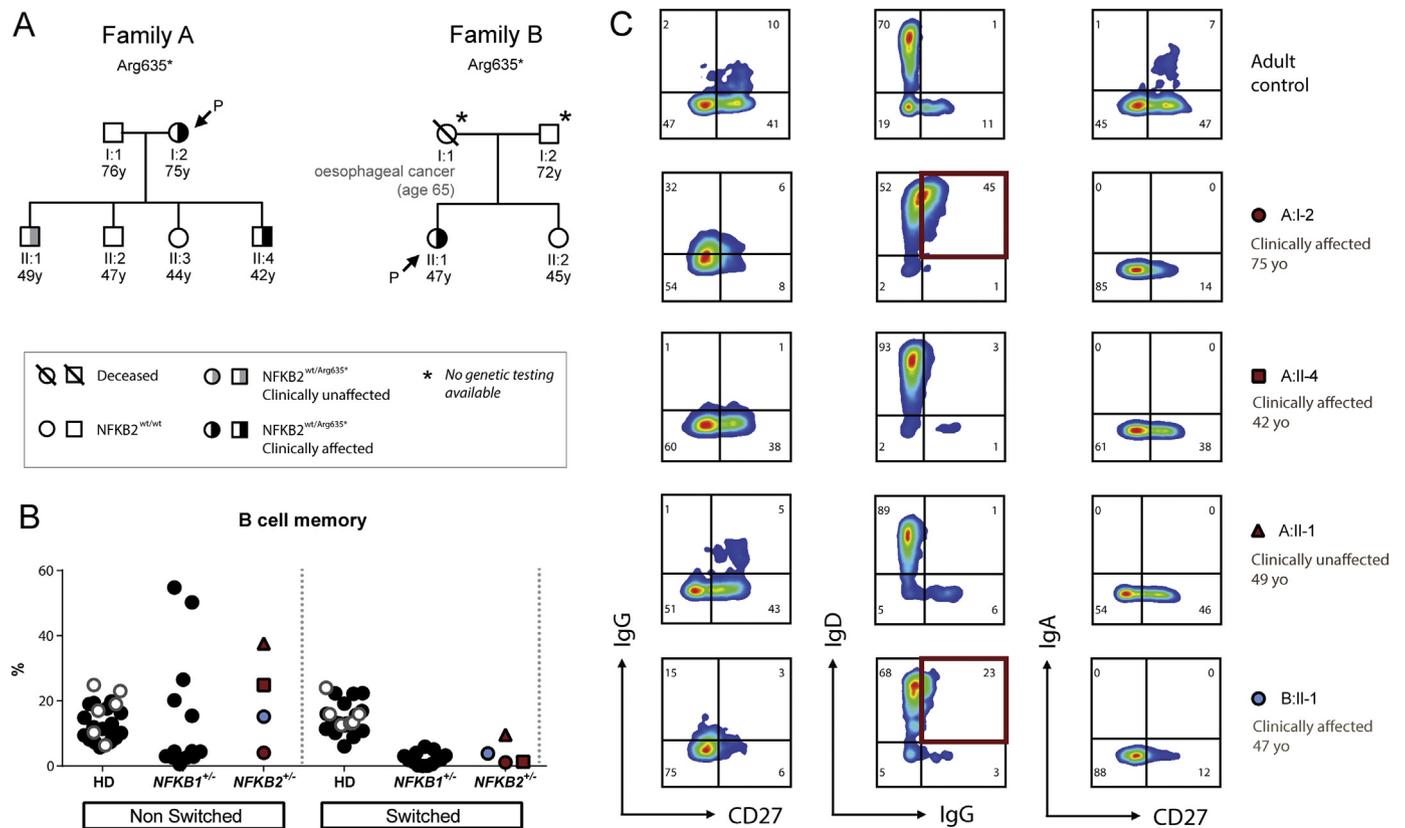
Fig. 1. Reduced class-switched memory B cells in two families of *NFKB2* R635X cases. (A) Pedigrees of two *NFKB2* R635X families, symbol legend included. Proband/index cases indicated with *P*. (B) Percentages non-switched and switched memory B cells within CD19$^+$CD20$^+$ B cells. *HD:* healthy donor, open dots are day controls within this study, *NFKB1*$^{+/−}$: pathologic heterozygous *NFKB1* cases published previously by our group [13], *NFKB2*$^{+/−}$: *NFKB2* R635X cases within this study. (C) Flow cytometry plots of CD27$^+$IgG$^+$ (left), IgG$^+$IgD$^+$ (middle), CD27$^+$IgA$^+$ (right) within CD19$^+$CD20$^+$ B cells.

2. Material and methods

2.1. Patient samples

All participants provided written informed consent and the study was approved by the East of England Cambridge South national institutional review board (13/EE/0325) and the Medical Ethics Committee of the Academic Medical Center (NL40331.078.12) in accordance with the Declaration of Helsinki.

2.2. Genetic analysis

WGS and sequence data analysis were performed as described previously [13]. Pathogenic *NFKB2* variants were identified by screening all genes previously associated with PID [14]. No other potential genetic causes of CVID were identified in the two pedigrees described here. Variants were confirmed by Sanger sequencing using standard protocols.

2.3. Immunophenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation techniques using Lymphoprep (Nycomed, Oslo, Norway). PBMCs were resuspended in PBS, containing 0.5% (*w/v*) BSA and 0.01% sodium azide and incubated with saturating concentrations of fluorescently labeled conjugated monoclonal antibodies. Analysis of cells was performed using a FACSCanto-II flowcytometer and FlowJo software. Patient samples were analyzed simultaneously with PBMCs from healthy controls. The following directly conjugated monoclonal antibodies were used: CD3 APC-R700, CD4 PE-Cy7, CD8 PerCP-Cy5.5, CD19 APC-R700, CD20 PerCP-Cy5.5, CD21

FITC, CD27 APC, CD38 PE-Cy7, CXCR5 Alexa Fluor 488 and IgD PE from BD (San Jose, USA), CD3 Alexa Fluor 700, CD19 Alexa Fluor 700 and CD27 APC-eFluor 780 from eBioscience (San Diego, USA), CD27 FITC from Sanquin (Amsterdam, the Netherlands), CD45RA (2H4-RD1) PE from Beckman Coulter (Brea, USA), IgM FITC and IgG FITC from Dako (Glostrup, Denmark), IgA FITC from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.4. B- and T-cell functional assay

To analyze the *ex vivo* activation of T and B cells as described previously [13,15], PBMCs were resuspended in PBS at a concentration of 5–10 × 10^6 cells/ml and labeled with 0.5 µM CFSE (Molecular Probes) in PBS for 10 min at 37 °C under constant agitation. Cells were washed and subsequently resuspended in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (BioWhittaker), antibiotics, and 3.57 × 10$^{−4}$%(*v/v*) β-mercaptoethanol (Merck). Labeled PBMCs containing a fixed number of 2 × 10^4 B cells per well, were plated in a flat-bottom 96-well plate for 6 days at 37 °C and stimulated with saturating amounts of anti-IgM mAb (clone MH15; Sanquin), anti-CD40 mAb (clone 14G7; Sanquin) and 20 ng/ml IL-21 (Invitrogen), or 1 µg/ml CpG oligodeoxynucleotide 2006 (Invivogen) and 100 U/ml IL-2 (R&D Systems), or soluble anti-CD3 (clone 1xE), immobilized anti-CD3 (clone 16A9T), anti-CD2 (combination of clone 6G4, 4B2H4 and Hik27) alone or in combination with anti-CD28 (clone 15E8), or IL-15 (R&D Systems). Proliferation of B and T cells was assessed by measuring CFSE dilution in combination with the same mAbs used for immunophenotyping and analyzed using a FACSCanto-II flowcytometer and FlowJo software.

2.5. ELISA

The secretion of immunoglobulins by mature B cells was assessed by testing supernatants for secreted IgM, IgG and IgA with an in-house ELISA using polyclonal rabbit anti-human IgM, IgG and IgA reagents and a serum protein calibrator all from Dako (Glostrup, Denmark).

3. Results and discussion

In a cohort of 447 unrelated CVID patients sequenced as part of the NIHR BioResource - Rare Diseases study, we identified two index cases who had the recently reported *NFKB2* GOF mutation c.1903C > T, leading to a premature stop p.Arg635Term (R635X) [12]. The index case in Family A was sequenced as part of an apparently autosomal-dominant pedigree with whole genome sequence data available for both parents and four children (Fig. 1A). The R635X variant was found to be inherited, as expected, from the index case (A:I-2) by her clinically affected son (A:II-4); unexpectedly, however, it was also inherited by one of the three clinically unaffected children (A:II-1). No other pathogenic variants co-segregating with the disease phenotype were identified.

The Family A index case (A:I-2) experienced frequent infections, mainly sinopulmonary and gastrointestinal (*Giardia lamblia*) infections with concomitant nodular lymphoid hyperplasia. Serum immunoglobulins (IgG, IgM and IgA) were low at presentation. Treatment with intravenous immunoglobulin (IVIG) was successfully initiated. Of the two children who inherited the R635X variant, only one son was diagnosed with CVID (A:II-4) because of frequent sinopulmonary infections and hypogammaglobulinemia, whereas the eldest son remained clinically unaffected at the time of the study (A:II-1). Both genetically affected sons were deficient for circulating IgA in plasma like the mother, although also the genetically unaffected son (A:II-2) was found to be IgA-deficient.

In Family B, the index patient (B:II-1) suffered from recurrent sinopulmonary infections (including pneumonia), with low vaccine responses and hypogammaglobulinemia. All three major serum immunoglobulin isotypes (IgG, IgM and IgA) were low at presentation, whereas the unaffected sibling showed normal levels (data not shown). No warts or other frequent viral infections nor any autoimmunity was present in B:II-1. Since IVIG treatment was initiated the patient is clinically stable. Her clinically unaffected sister was confirmed not to carry the R635X variant. EBV viral loads were undetectable in all described cases (A:I-2, A:II-4, A:II-1 and B:II-1). To summarize, we found that in these patients the R635X variant leads to CVID, but without any of the previously described clinical signs of T-cell dysfunction, autoimmune disease or adrenal insufficiency in these two unrelated families.

Upon phenotyping of the B- and T-cell compartment in members of both pedigrees, B-cell lymphopenia (0.08 and 0.02×10^9/L) was seen in both *NFKB2*-deficient index cases. Similar to *NFKB1* loss-of-function mutations, a reduction in class-switched memory B cells was observed (Fig. 1B). Both index cases showed skewed B-cell differentiation with an atypical distribution of IgG$^+$ memory B cells and a complete absence of memory IgA$^+$ B cells (Fig. 1C). In contrast to *NFKB1* loss-of-function B cells [13], there was no increase in CD21^{low} B cells (< 10% [16], data not shown), irrespective of clinical phenotype.

There was normal B-cell proliferation upon T cell-independent and -dependent activation and plasmablast formation also seemed unaffected in all four *NFKB2* R635X carriers (Fig. 2A, representative plots). However, whilst plasmablast differentiation appeared normal as indicated by CD38 expression (and CD138, data not shown), there was reduced upregulation of CD27 in some R635X cases. Although proliferation and plasmablast formation seemed largely normal, there was no production of class-switched IgG and IgA, the latter expected since there was a complete absence of surface IgA-expressing B cells (Fig. 2B). There was IgM production in all cases (Fig. 2C), albeit very low in case

B:II-1, showing the functional inability to produce class-switched immunoglobulins *ex vivo*.

Since Kuehn et al. found a combined B- and T-cell defect in 2 of their *NFKB2* R635X cases based on the clinical phenotype [12], the T cells in our genetically affected cases were examined in detail. We observed in both the CD4$^+$ and CD8$^+$ T cells a highly differentiated T-cell phenotype irrespective their clinical status or age (Fig. 2D). By challenging the T cells with soluble anti-CD3/anti-CD28, an impaired response of mainly CD8 T cells and B cells was observed in the index patient of Family A (Fig. 2E), which may have been caused by the lack of proliferation in the large more terminally differentiated CD28-negative T-cell population. Although proliferation of CD4$^+$ T cells was seemingly normal in case A:I-2, the induction of CD4$^+$CXCR5$^+$ T cells upon stimulation was less compared to healthy controls (Supplemental Fig. 1A, B), which might explain the poor indirect B-cell proliferation upon T-cell activation. This could indicate that the low number of class-switched B cells is (at least partially) caused by a concomitant T follicular helper cell defect. When T-cell receptor (TCR) stimulation was applied by immobilized anti-CD3 or alternative stimulation with anti-CD2 [17], the poor proliferative response could be rescued in both CD4 and CD8 T cells (Supplemental Fig. 1B and data not shown, respectively). In contrast to patients with C-terminal mutations in *NFKB2* [9,18], proliferation of NK cells was normal upon IL-15 stimulation (data not shown). Overall, we observed a highly differentiated T-cell memory compartment with intact proliferative capacity, which is compatible with the lack of an *in vivo* T-cell defect.

Prior reports on the C-terminal NF-κB2 mutations described the characteristic clinical phenotype of immunodeficiency, (mild) ectodermal dysplasia and central adrenal insufficiency. The R635X variants in the ARD of the protein as described by Kuehn et al. [12], were demonstrated to behave as GOF mutation. The interrupted p100 was shown to be less effective in exerting its inhibitory function on both the non-canonical as the canonical NF-κB signaling pathway. Both pathways (activating TLR9 for the canonical and CD40 for non-canonical signaling) were studied in our functional B-cell experiments. Although a GOF mutation, we did not observe a hyperactive B-cell response and only found a reduction in immunoglobulin production coinciding with CD27^{dim} plasmablasts instead of the usual CD27^{++} plasmablasts. Clinical manifestations of T-cell-dependent infection (*i.e.* warts, uncommon/severe viral or fungal infections), autoimmunity or autoinflammation were absent, as opposed to the R635X symptomatic cases described previously [12]. Mouse models have shown that defective p52/p100 signaling leads to immunodeficiency without clear T-cell pathology [19,20]. Although patients described with homozygous mutations in *RELB* show both B- and T-cell defects [21], the degree of T-cell dysfunction in NFKB2 deficiency remains unclear and seems at least variable in its clinical penetrance. Instead of the T-cell lymphocytosis in the 2 previously reported symptomatic cases with R635X GOF mutations [12], we observed mild T-cell lymphopenia in 2 out of the 3 genetically affected subjects in Family A (one being clinically unaffected). Although CID was not present in our families, we confirm the absence of endocrine and ectodermal manifestations.

In conclusion, *NFKB2* mutations in ARD can present as a classic 'infection-only' CVID. Being environmental, epigenetic or otherwise, the clinical and cellular phenotype in NFKB2 deficiency has - for as yet unknown reasons - a highly variable penetrance and expressivity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.03.010.

Authorship contributions

P.T. and H.L.A. wrote the paper, designed and performed the experiments. M.H.J. performed the experiments. G.J.d.B., S.S., C.S. and I.J.M.t.B collected data. I.S. analyzed genetic data. J.E.T. wrote the paper. E.M.M.v.L. collected data and designed the experiments. T.W.K. wrote the paper and designed the experiments.
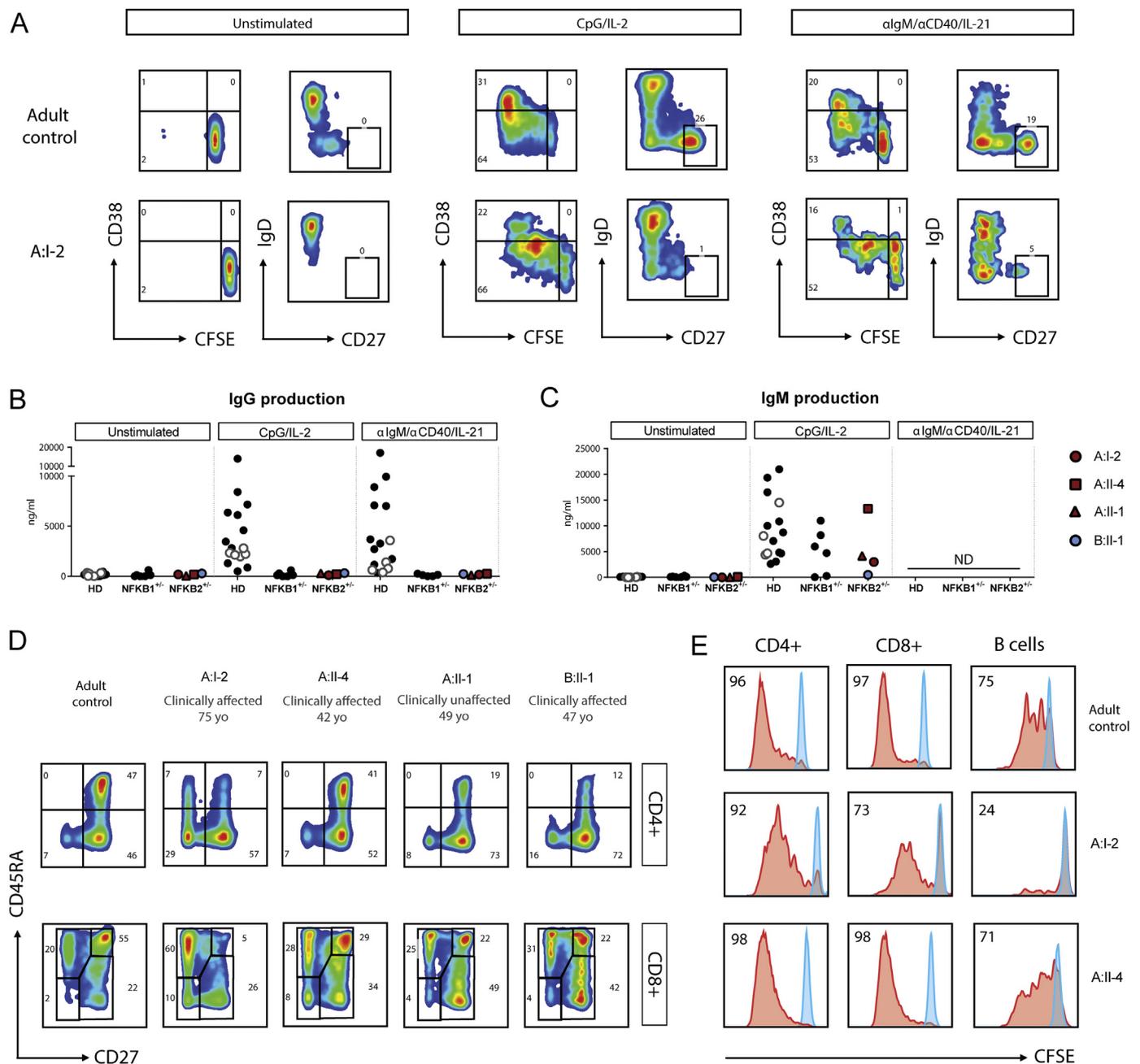
Fig. 2. *NFKB2* R635X cases produce no class-switched immunoglobulins *ex vivo* with a variable T-cell phenotype. (A) Representative flow cytometry plots of CFSE-labeled PBMCs normalized for B cell numbers after 6 days of culture stimulated with CpG/IL-2 (T cell-independent signaling) and anti-IgM/anti-CD40/IL-21 (T cell-dependent signaling) gated on CD19$^+$CD20$^{dim/+}$ B cells. Production of (B) IgG and (C) IgM in the supernatant of before mentioned culture. *HD:* healthy donor, open dots are day controls within this study, *NFKB1*$^{+/-}$: clinically affected pathologic heterozygous *NFKB1* cases published previously by our group [13], *NFKB2*$^{+/-}$: *NFKB2* R635X cases within this study. (D) Flow cytometry plots of CD27/CD45RA memory distribution of CD4$^+$ T cells (upper panel) and CD8$^+$ T cells (lower panel). (E) Histograms of case A:I-2 and A:II-4 alongside a healthy adult control of CFSE-labeled PBMCs normalized for T-cell or B-cell numbers after 6 days of culture stimulated with soluble anti-CD3/anti-CD28 and gated on CD4$^+$ (left) and CD8$^+$ (middle) T cells, or B cells (right). Blue line represents unstimulated samples, Red line represents anti-CD3/anti-CD28 stimulated samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Disclosure of conflicts of interest

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