Defective B-cell memory in patients with Down syndrome

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Background: Patients with Down syndrome carry immunologic defects, as evidenced by the increased risks for autoimmune diseases, hematologic malignancies, and respiratory tract infections. Moreover, the low numbers of circulating B cells suggest impaired humoral immunity.
Objective: We sought to study how immunodeficiency in patients with Down syndrome results from immunologic defects in the B-cell compartment.
Methods: We studied blood B-cell subset composition, replication history, somatic hypermutation status, and class-switch recombination in 17 children with Down syndrome. Germinal centers and plasma cells were studied in tonsils from 4 additional children with Down syndrome.
Results: Blood transitional B-cell numbers were normal, but naive mature and memory B-cell numbers were reduced despite slightly increased serum B cell–activating factor levels. Germinal centers and plasma cells in tonsils appeared normal, as were serum immunoglobulin levels. $CD27^+IgD^+IgM^+$ "natural effector" B cells showed reduced proliferation and somatic hypermutation levels, whereas these were normal in $CD27^+IgD^-$ memory B cells. Furthermore, IgM^+ and IgA^+, but not IgG^+, memory B cells showed impaired molecular signs for antigen selection. The B-cell pattern was highly similar to that of patients with common variable immunodeficiency and a defect in B-cell activation and proliferation.
Conclusion: Children with Down syndrome seem capable of normal germinal center and plasma cell formation. Still, blood memory B-cell numbers were reduced and showed impaired molecular maturation of IgA and IgM, which are important for mucosal immunity. The observed molecular defects in circulating IgA and IgM B-cell memory could reflect impaired local responses, which underlie the increased susceptibility to respiratory tract infections of patients with Down syndrome. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Down syndrome, common variable immunodeficiency, antibody, B cell, selection, somatic hypermutation, IgM, IgA, plasma cell*

Down syndrome is the most common genetic cause of developmental delay in human subjects and is associated with health issues that include hypotonia, congenital heart disease, and gastrointestinal malformations. Furthermore, high incidences of recurrent respiratory tract infections, hematologic malignancies, and autoimmune diseases are reported,[1-3] which suggest an immune defect. Indeed, laboratory studies have demonstrated abnormalities in the blood B- and T-cell compartments, as well as decreased IgM, IgG_2, and IgG_4 levels and poor immunoglobulin responses to vaccines.[4,5]

On antigen stimulation, naive mature B cells differentiate into memory B and plasma cells. Activated B cells induce somatic hypermutations (SHMs) in the variable regions of their immunoglobulin heavy and light chains. The mutated immunoglobulin molecules are subsequently selected for antigen affinity. In addition, the B cells can induce class-switch recombination to change the IgH isotype region from IgM to IgG, IgA, or IgE. On the basis of their IgH isotype and expression of CD27, 6 memory B-cell subsets can be identified in blood that have been derived from 3 distinct pathways (Fig 1, *A*).[6]

Some of the clinical and immunologic features found in patients with Down syndrome resemble common variable immunodeficiency (CVID). CVID is a primary immunodeficiency characterized by sinopulmonary infections and idiopathic hypogammaglobulinemia.[7] CVID has a heterogeneous pathophysiology, which can be visualized by means of flow cytometric analysis of the blood B-cell compartment.[8] In addition to memory B-cell defects, a subset of patients with CVID have increased $CD21^{low}CD38^-$ B-cell numbers. These $CD21^{low}$ B cells are mostly naive, express highly autoreactive antibodies, are more prone to die through apoptosis, and display functional anergy.[9]

To study how immunodeficiency in children with Down syndrome results from immunologic defects in the B-cell compartment, we performed detailed cellular and molecular analysis of their B lymphocytes. The results were compared with those of age-matched healthy control subjects and the previously described CVID subgroups.[8]

METHODS

Patients

Clinical data, blood samples, and tonsils were collected from children with Down syndrome after written informed consent was obtained from

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Abbreviations used

AID: Activation-induced cytidine deaminase
BAFF: B cell–activating factor
CDR: Complementarity-determining region
CVID: Common variable immunodeficiency
CVID-3: CVID pattern 3
FR: Framework region
IgκREHMA: Igκ restriction enzyme hot-spot mutation assay
SHM: Somatic hypermutation
TACI: Transmembrane activator and CAML interactor

their parents. In addition, we collected blood from healthy age-matched control subjects and tonsils from otherwise healthy children undergoing tonsillectomy for medical reasons. This study was performed according to the Declaration of Helsinki and the guidelines of the Medical Ethics Committees used by the Jeroen Bosch Hospital and the Erasmus MC.

Flow cytometric analysis of lymphocytes in blood and tonsils

Absolute counts of blood T, B, and natural killer cells were obtained with a diagnostic lyse-no-wash protocol. Furthermore, 8-color flow cytometric immunophenotyping was performed, as previously described, to detect transitional cells, naive mature cells, $CD21^{low}$ cells, 6 memory B-cell subsets, and plasma cells on a 3-laser FACS LSR II (BD Biosciences, San Jose, Calif; see Fig E1 in this article's Online Repository at www.jacionline.org).[6] Germinal center B cells ($CD19^{+}CD38^{+}IgD^{-}$) and plasma cells in tonsils ($CD38^{hi}$) were defined as previously described.[10]

Transitional, naive mature, natural effector, and $CD27^{+}IgD^{-}$ memory B cells were high-speed cell sorted to greater than 95% purity on a FACSAria I (BD Biosciences), as described previously.[10]

Histology and immunohistochemistry of tonsils

The tonsil specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Immunohistochemistry was performed according to standard procedures involving mAbs against CD3 (SP7), CD21 (2G9; both from Thermo Fisher Scientific, Waltham, MA), CD20 (L26), CD138 (MI15; both from DAKO, Glostrup, Denmark), and programmed cell death 1 (NAT105; Abcam, Cambridge, United Kingdom).

Quantification of B cell–activating factor serum levels

B cell–activating factor (BAFF) serum levels were measured by means of ELISA and analyzed in duplicate, according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn).

Molecular analysis of replication history and immunoglobulin gene rearrangement

DNA was isolated from each sorted subset for analysis of the replication history with the kappa-deleting recombination excision circle assay and the Igκ restriction enzyme hot-spot mutation assay (IgκREHMA), as described previously.[10,11]

IGA and *IGG* transcripts were amplified and cloned from cDNA prepared from mononuclear cells by using *IGHV3* and *IGHV4* leader primers and consensus Cα or Cγ reverse primers.[6,12] In addition, *IGHV3/4-IGHJ* rearrangements were amplified from DNA of sorted natural effector B cells.[13] Sequences were generated on an ABI PRISM 3130XL (Applied Biosystems, Foster City, Calif) and analyzed with the IMGT (http://imgt.cines.fr/), JOINSOLVER (http://joinsolver.niaid.nih.gov), and BASELINe programs (http://selection.med.yale.edu/baseline/).[14-16]

In vitro plasma cell differentiation of purified B cells from tonsils

Resting B cells from tonsils were purified and cultured with combinations of anti-IgM F(ab′)2, anti-CD40, CpG ODN2006, and IL-21, as described previously.[12] Cells were harvested after a 6-day culture for TaqMan-based quantitative RT-PCR on a StepOnePlus (Applied Biosystems). Target gene expression levels were determined with intron-spanning primes and fluorogenic probes (see Table E1 in this article's Online Repository at www.jacionline.org), corrected for *ABL* expression levels, and expressed as the fold difference from uncultured naive cells.[17] All quantitative RT-PCR reactions were performed in duplicate.

Statistics

Statistical analyses were performed with the Mann-Whitney test (SPSS version 18.0; SPSS, Chicago, Ill) or χ^2 test, as indicated in the figure legends. A P value of less than .05 was considered statistically significant.

RESULTS

Clinical and basic immunologic characterization

Blood samples were obtained from 17 patients (6 male) aged 7 to 17 years at their routine annual visit to the outpatient clinic for Down syndrome. Tonsils were obtained from 4 children (3 male, age 3-5 years) with karyotype-confirmed diagnosis of Down syndrome after scheduled tonsillectomy. Basic clinical and immunologic information of these patients is shown in Table E2 in this article's Online Repository at www.jacionline.org. Patients were selected on the absence of malignancies and serious respiratory tract infections that required admission to a pediatric intensive care unit. Ear, nose, and throat problems; virus-induced wheezing; and autoimmune-mediated hypothyroidism were common, as expected.

Circulating T-cell and natural killer cell numbers were in the low-to-normal range, whereas B-cell numbers were less than the fifth percentile of the normal range in 10 of 17 patients (see Table E2). The distribution of immunoglobulin serum levels was altered, as previously described in patients with Down syndrome.[4,5,18] Thus the clinical and basic immunologic parameters of the patients in our study population were in line with those of previous studies and representative for children with Down syndrome.[1,4,5,19,20]

Composition of the blood B-cell compartment in children with Down syndrome

To study the nature of reduced total B-cell numbers, we performed detailed flow cytometric analysis in 13 patients with Down syndrome and compared results with those of 43 age-matched healthy control subjects (Fig 1, *B*, and see Fig E1). The children with Down syndrome had normal numbers of $CD27^{-}IgM^{+}IgD^{+}CD24^{high}CD38^{high}$ transitional B cells, whereas $CD27^{-}IgM^{+}IgD^{+}CD24^{dim}CD38^{dim}$ naive mature B-cell numbers were significantly decreased (Fig 1, *B*). Because soluble BAFF is a critical survival factor for mature B cells,[21] we quantified serum BAFF levels. These were slightly increased in patients with Down syndrome, rather than decreased, compared with levels seen in age-matched control subjects (Fig 1, *D*). This indicates that serum BAFF levels are not limiting and might even be increased as a result of reduced use by the low numbers of mature B cells.

Of the 6 memory B-cell subsets, $CD27^{+}IgD^{-}IgM^{+}$ "IgM-only" B cells were present in normal numbers, but both

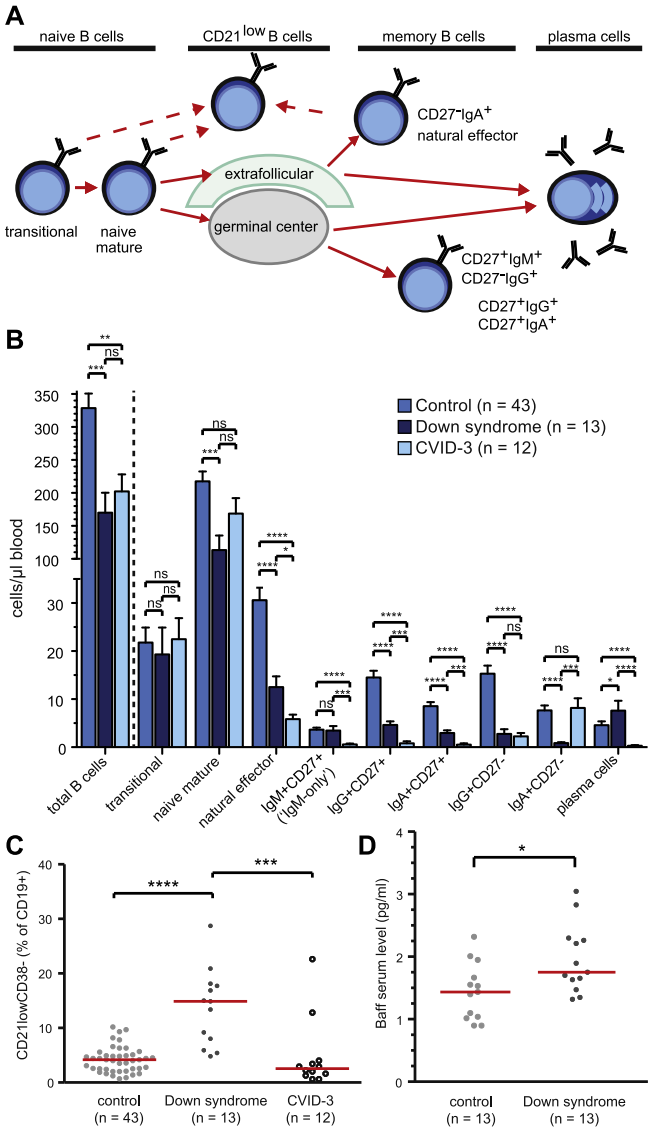

FIG 1. Composition of the blood B-cell compartment in children with Down syndrome. **A,** Model for B-cell maturation. **B,** Absolute numbers of B-cell subsets. **C,** Frequencies of CD21^{low}CD38^{-} B cells. **D,** BAFF serum levels. Panels *B* through *D* include patients with Down syndrome (P1, P4-P8, and P11-P17). Statistical analysis was done with the Mann-Whitney test: **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. *ns*, Not significant.

natural effector B cells and CD27^{+}IgG^{+}, CD27^{+}IgA^{+}, CD27^{-}IgG^{+}, and CD27^{-}IgA^{+} class-switched memory B-cell numbers were significantly lower in patients with Down syndrome than in healthy control subjects. In contrast, circulating plasma cell numbers were increased in patients with Down syndrome. Together with normal-to-high levels of serum IgG and IgA (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org), this indicates that immunoglobulin responses can take place in patients with Down syndrome, but these patients seem to have defective generation, maintenance, or both of T cell–dependent and T cell–independent memory B cells.

Of the 13 patients with Down syndrome, 10 had natural effector and/or CD27^{+}IgD^{-} memory B-cell numbers that were less than the fifth percentile of the normal range of age-matched control subjects (see Table E4 in this article's Online Repository at www.jacionline.org). These 10 patients could be assigned to one of the previously identified B-cell patterns in patients with CVID: 4 patients displayed pattern 2 (defect in early B-cell maturation or survival), 2 patients displayed pattern 3 (defect in B-cell activation and proliferation), and 4 patients displayed pattern 4 CVID (defect in germinal center function). The other 3 patients showed a normal B-cell composition (pattern 5).[8] There was no association between age and type of B-cell pattern. The average B-cell subset composition of patients with Down syndrome was most similar to CVID pattern 3 (CVID-3; Fig 1, *B*), with the exception of the additional reduction in CD27^{-}IgA^{+} B-cell numbers and the normal numbers of IgM-only B cells and circulating plasma cells.

Interestingly, CD21^{low}CD38^{low} B-cell numbers were increased in all patients with Down syndrome, irrespective of age (Fig 1, *C*). This clearly contrasted with the CVID-3 group, which had mostly normal frequencies of CD21^{low} B cells.[8] Thus patients with Down syndrome seem to carry defects in B-cell activation similar to those with CVID-3, but their B-cell phenotype is unique, especially with regard to circulating plasma cells and CD21^{low} B cells.

Germinal center formation and plasma cell maturation

To study whether the defects in blood memory B cells resulted from impaired immune responses, we analyzed the tonsils of children with Down syndrome. The tonsil sections were unremarkable and demonstrated a normal make-up of the lymphoid tissue, with variable numbers of well-proportioned secondary lymph follicles (Fig 2, *A-C*). The germinal centers harbored light and dark zones containing proliferating B-cell blasts, as well as networks of CD21^{+} follicular dendritic cells and programmed cell death 1–positive follicular helper T cells.

Total plasma cell frequencies were slightly increased (Fig 2, *C*), and in each tonsil subsets expressing IgM, IgA, or IgG were found (not shown). Naive B cells obtained from tonsils were stimulated with anti-IgM and IL-21 in combination with anti-CD40 or CpG to mimic T-cell help and T cell–independent Toll-like receptor signaling, respectively, to study B-cell activation and plasma cell differentiation. B cells from children with Down syndrome normally upregulated activation-induced cytidine deaminase *(AID)* gene expression levels. Anti-CD40 stimulation resulted in normal upregulation of interferon regulatory 4 *(IRF4)* and X-box binding protein 1 *(XBP1)*, whereas PR domain containing protein 1 *(PRDM1)* transcription was lower than in control subjects. CpG induced more transcription of all 3 genes in control subjects but not in patients with Down syndrome. Thus naive B cells in patients with Down syndrome can induce plasma cell differentiation, although this might be slightly less efficient than in control subjects, especially after T cell–independent stimuli.

Distinct phenotypic alterations in IgM^{+}, IgA^{+}, and IgG^{+} memory B cells

To further study defects in B-cell memory, we first analyzed the expression of typical memory markers on B-cell subsets of the patients. Naive mature B cells from patients with Down syndrome seemed normal, with low expression of CD80, CD95,

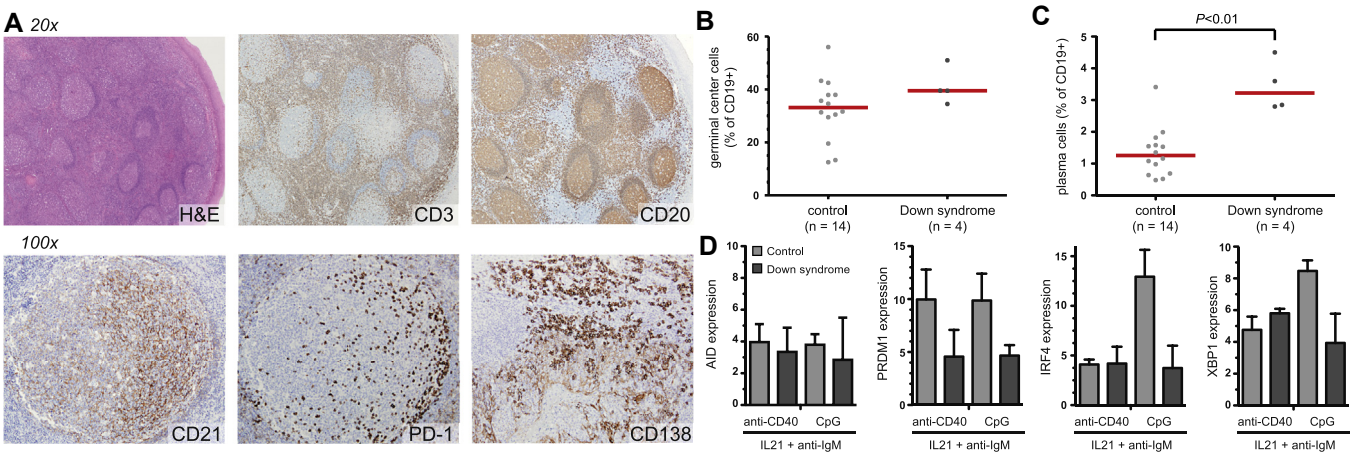

FIG 2. Germinal center composition and plasma cell maturation. **A,** Tonsil sections of a representative patient with Down syndrome. **B** and **C,** Frequencies of CD38^{+}IgD^{-} germinal center B cells (Fig 2, *B*) and CD38^{hi} plasma cells (Fig 2, *C*). **D,** Gene expression levels in naive B cells after 6 days of stimulation expressed as the fold difference from uncultured naive cells. Statistical analysis was done with the Mann-Whitney test. *H&E*, Hematoxylin and eosin; *PD-1*, programmed cell death 1.

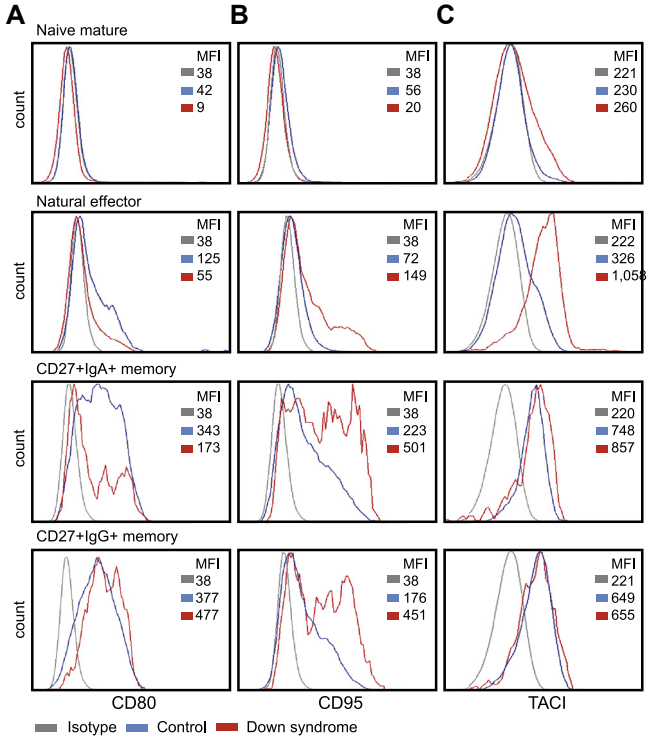

FIG 3. Abnormal immunophenotypes of memory B-cell subsets of patients with Down syndrome. Expression levels of CD80 (L307.4; **A**), CD95 (DX2; **B**), and TACI (goat polyclonal; **C**) on naive mature, natural effector, CD27^{+}IgA^{+}, and CD27^{+}IgG^{+} memory B cells. Data are shown from 4 patients with Down syndrome (P7, P13, P16, and P17) and 4 healthy control subjects. *MFI*, Median fluorescence intensity.

and transmembrane activator and CAML interactor (TACI; Fig 3). In contrast to CD27^{+}IgG^{+} memory B cells, natural effector and CD27^{+}IgA^{+} memory B cells showed impaired upregulation of CD80. All 3 memory subsets show increased upregulation of CD95. TACI was higher on natural effector B cells of patients with Down syndrome than those of control subjects, but it was normally upregulated on IgG and IgA memory B cells. These phenotypic profiles demonstrate that IgM memory, represented by natural effector B cells, is mostly affected in patients with Down syndrome, whereas IgA and IgG are less affected.

Molecular analysis of antigen-driven B-cell maturation in patients with Down syndrome and CVID-3 patients

In addition to phenotypic analysis of memory B-cell subsets, we performed molecular analysis of their replication history, SHMs, and immunoglobulin class-switch profiles. Transitional B cells of patients with Down syndrome, CVID-3 patients, and control subjects showed no proliferation or SHM (Fig 4, *A* and *B*), confirming their status as recent bone marrow emigrants.[10] Naive mature B cells of patients with Down syndrome and CVID-3 patients showed homeostatic proliferation of 1 to 2 cell divisions in the absence of SHM, which was also not significantly different from that seen in healthy control subjects.

Natural effector B cells of patients with Down syndrome and CVID-3 patients showed proliferation in conjunction with SHM. Still, these levels were significantly lower than those of healthy control subjects. Proliferation of CD27^{+}IgD^{-} B cells of patients with Down syndrome and CVID-3 patients was similar to that seen in control subjects, but these cells showed reduced frequencies of mutated *IGKV3-20* alleles in patients with Down syndrome. IgA and IgG subclass analysis of rearranged *IGH* transcripts of patients with Down syndrome revealed no difference in use compared with healthy control subjects. The normal use of *IGHM* downstream IgG_2, IgG_4, and IgA_2 indicates that immunoglobulin class-switching to downstream constant regions was not impaired (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). The effects on proliferation, SHM, and class switching were not age related. Thus antigen-dependent B-cell maturation is clearly impaired in patients with Down syndrome, especially in natural effector B cells.

Targeting of SHMs

More detailed mutation analysis in *IGHV* genes showed decreased SHM frequencies in natural effector B cells (Fig 4, *C*). In contrast to the IgκREHMA assay, SHM frequencies were normal

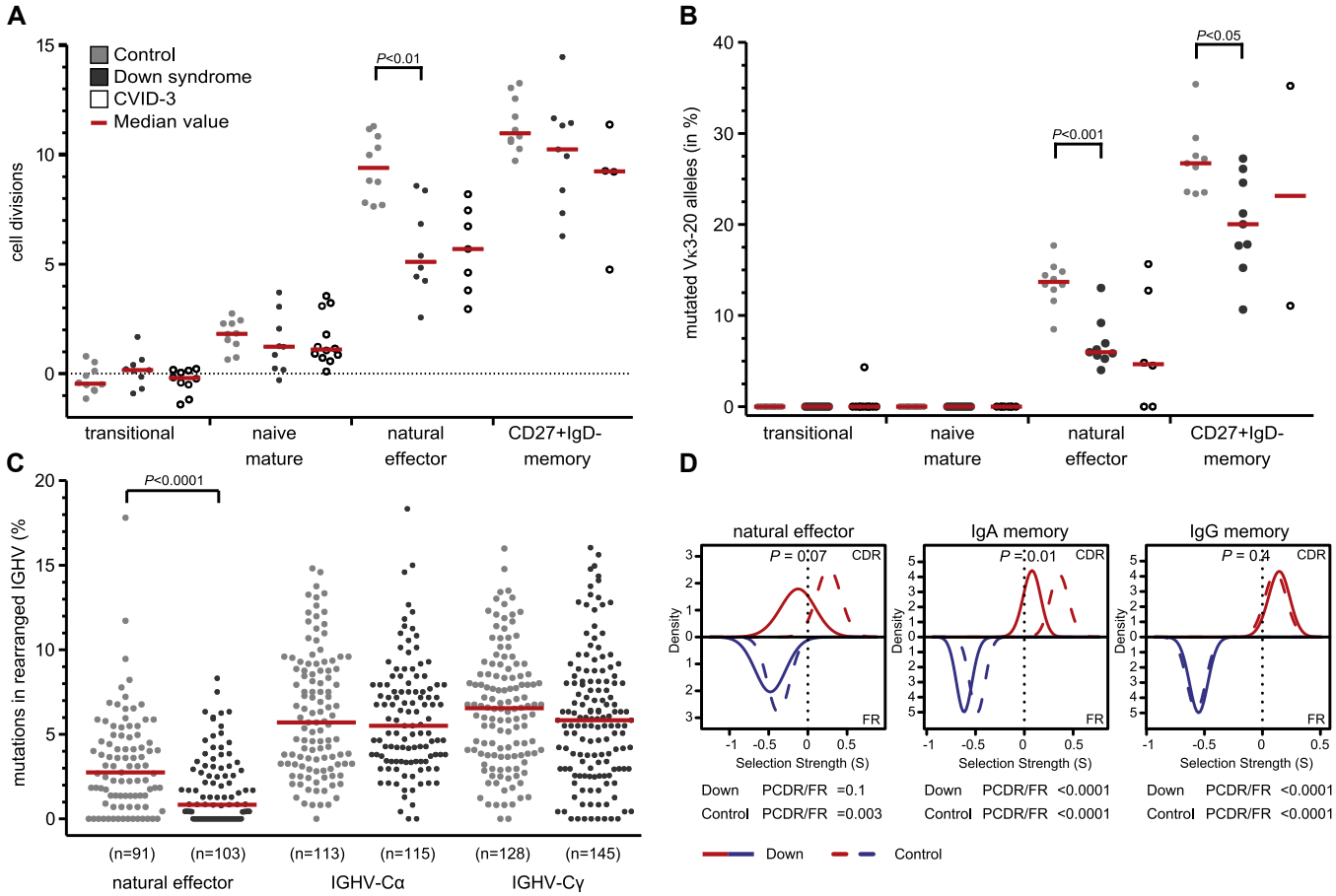

FIG 4. Replication history and SHM. **A** and **B,** *In vivo* replication history (Fig 4, *A*) and frequencies of mutated *IGKV3-20* alleles (Fig 4, *B*) in B-cell subsets (patients P1, P4-6, P8, P11-12, and P14-15). **C** and **D,** SHM frequencies in rearranged *IGHV* genes (Fig 4, *C*) and selection for replacement mutations in CDR *(red)* and FR regions (*blue*; Fig 4, *D*).[14,15] Each subset includes 18 to 52 sequences from 4 to 6 subjects. Statistical analysis in Fig 4, *A-C*, was done with the Mann-Whitney test.

in IgA and IgG memory B cells (Fig 4, *C*), and no differences were seen in SHM frequencies for the various IgA and IgG subclasses (see Fig E2, *B* and *C*).

To study whether the SHM processes were induced normally in memory B cells of patients with Down syndrome, we analyzed targeting of mutations to sequence motifs.[22] Natural effector B cells of patients with Down syndrome had significantly decreased targeting of the RGYW DNA motifs (R, purine; Y, pyrimidine; and W, A or T), which are direct targets of AID (see Table E5 in this article's Online Repository at www.jacionline.org). IgA and IgG transcripts showed normal RGYW targeting. Furthermore, natural effector and immunoglobulin class-switched memory B cells showed normal transition/transversion ratios, as well as normal WA/TW targeting. Thus memory B cells of patients with Down syndrome showed normal repair of AID-induced lesions, and the only defect appears to be reduced AID activity in natural effector B cells.

Molecular analysis of immunoglobulin selection processes

In healthy subjects the use of inherently autoreactive *IGHV4-34* genes and long complementarity-determining regions (CDRs) in *IGH* (IGH-CDR3) are counterselected in memory B cells and therefore less frequent than in naive B cells.[6,23-26] Natural effector B cells of control subjects and patients with Down syndrome showed smaller IGH-CDR3 than naive mature B cells of healthy control subjects (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). However, we did not observe decreased use of *IGHV4-34* in natural effector B cells of either control subjects or patients with Down syndrome (see Fig E3, *B*). IGH-CDR3 sizes and *IGHV4-34* use were decreased in IgA and IgG transcripts of both control subjects and patients compared with those seen in naive mature B cells (see Fig E3). Still, the median IGH-CDR3 size of IgA transcripts in patients with Down syndrome was significantly larger than that seen in control subjects. Furthermore, use of *IGHV4-34* was slightly, although not significantly, increased in IgA and IgG transcripts of patients with Down syndrome compared with that seen in control subjects. Thus despite minor differences with healthy control subjects, natural effector and immunoglobulin class-switched memory B cells of patients with Down syndrome showed normal molecular signs of immunoglobulin repertoire selection.

Both control subjects and patients with Down syndrome revealed increased replacement/silent mutation ratios in regions compared with those seen framework regions (FRs; see Table E5 and Fig E4 in this article's Online Repository at www.jacionline.org).[14,15] However, the increased replacement/silent mutation

ratios in CDRs versus FRs do not necessarily reflect selection processes because the codon use in CDRs differs from that in FRs in their nature to be more susceptible to replacement mutations.[27,28] Therefore we analyzed the *IGH* sequences with the BASELINe program, which determines whether the mutation patterns differed from what can be expected from random targeting.[14] Similar to previous observations, we found positive selection for CDRs and negative selection for FRs in natural effector, IgA, and IgG memory B cells in healthy control subjects (Fig 4, *D*, dotted lines). The differences between selection in CDRs and FRs were highly significant. In contrast, natural effector B cells of patients with Down syndrome did not show positive selection of replacement mutations in CDRs. Although IgA and IgG transcripts of patients with Down syndrome showed significant selection for replacement mutations in CDR, the selection strength of IgA was significantly lower than that seen in healthy control subjects. Thus in addition to the phenotypic profiles, natural effector and IgA memory B cells showed defects in molecular maturation, whereas IgG memory B cells appeared normal.

DISCUSSION

Through cellular and molecular analysis of the B-cell compartment, we showed specific defects in B-cell memory in patients with Down syndrome. $CD27^+$ memory B cells were reduced in number and displayed impaired proliferation and antibody maturation. The B-cell pattern was reminiscent of a subgroup of patients with CVID with a potential defect in B-cell activation (CVID-3).[8] Still, in contrast to those with CVID, patients with Down syndrome had normal germinal center cells, high numbers of plasma cells, and normal immunoglobulin serum levels. Thus in addition to their anatomic and physiologic abnormalities of the respiratory tract, patients with Down syndrome carry B-cell memory defects that might contribute to the increased frequency of respiratory tract infections.

Our detailed analysis of the blood B-cell compartment in patients with Down syndrome revealed a decrease in naive mature B-cell numbers. This decrease can be the result of reduced output from bone marrow, reduced homeostatic proliferation, or impaired survival. Patients with multisystem DNA repair disorders, such as Nijmegen breakage syndrome or ataxia telangiectasia, display a humoral immunodeficiency with reduced B-cell output caused by impaired DNA repair during V(D)J recombination in bone marrow.[29-31] This is reflected by reduced transitional B-cell numbers and increased homeostatic proliferation of naive mature B cells reminiscent of CVID pattern 1.[8] However, none of our patients with Down syndrome had this B-cell pattern. Therefore it is less likely that their reduced B-cell compartment is the result of impaired bone marrow output.

Homeostatic proliferation of naive mature B cells in patients with Down syndrome seemed normal, with approximately 2 cell cycles in the absence of SHM. This leaves impaired survival as the most likely cause of the reduced naive B-cell compartment. This is supported by previous studies that showed increased apoptosis of B cells in patients with Down syndrome.[32,33] However, we found that levels of the critical cytokine for naive B-cell survival, BAFF, were slightly increased in the serum of patients. Unfortunately, we were unable to study BAFF receptor expression on B cells. Low BAFF receptor expression could contribute to the observed decreased survival. B-cell survival is also dependent on macrophage migration inhibitory factor produced by bone marrow–resident dendritic cells.[34] Circulating dendritic cells are decreased in patients with Down syndrome,[35] which could be associated with impaired production of migration inhibitory factor and underlie the reduced naive B-cell numbers seen in patients with Down syndrome.

In addition to decreased naive mature B-cell numbers, circulating $CD27^+$ memory B-cell numbers were also reduced in patients with Down syndrome. Both natural effector and IgD^- memory B-cell subsets showed increased expression levels of the FAS receptor (CD95), which is also known as the death receptor that induced apoptosis.[36] Increased CD95 expression levels can tip the balance between BCR-induced survival and CD95-induced cell death, thereby negatively affecting memory B-cell numbers.[37,38]

In addition to more CD95 expression, natural effector B cells showed impaired proliferation and SHM levels and defective selection for replacement mutations in CDR. Thus in addition to a potential survival defect, patients with Down syndrome are defective in generation of IgM^+ B-cell memory. Because IgM responses are important for clearing blood-borne pathogens,[39] this defect could underlie the increased susceptibility of patients with Down syndrome to blood-borne infections.[40] Children with genetic defects in myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP) and interleukin 1 receptor-associated kinase 4 (IRAK4) Toll-like receptor signaling experience invasive bacterial infections.[41] Recently, MyD88-TIRAP-IRAK4–dependent signaling was found to be critical for generation of homeostasis of natural effector B cells.[42] However, the natural effector B cells in these immunodeficient children carry normal SHM frequencies.[41] Thus patients with Down syndrome might have a Toll-like receptor signaling defect that could contribute to the observed deficiency in natural effector B cells in patients with Down syndrome, but it is unlikely that this is the single cause.

A specific subset of neutrophils was recently shown to support splenic marginal zone B-cell responses.[43] Because neutrophil abnormalities have been described in patients with Down syndrome,[44,45] it is possible that these contribute to our observed defects in circulating natural effector B cells. Because these B-helper neutrophils do not circulate in blood, this would require further study of spleen tissue, which we were unable to obtain.

In addition to natural effector B cells, numbers of IgA and IgG memory B cells were reduced in patients with Down syndrome. Despite normal SHM levels, IgA transcripts showed impaired molecular selection. Although serum IgA and IgM levels were found to be normal, these defects might impair local mucosal immunity considering their role in the respiratory and intestinal tract. Despite reduced numbers, IgG B-cell memory seemed quite normal in phenotype and molecular maturation in the patients we studied. This fits in with the generally mildly affected responses to vaccinations in patients with Down syndrome.[4,46-49] These features might at least partly explain why patients in this age range (7-17 years) are less susceptible to recurrent respiratory tract infections than very young children less than 5 years old.[5,50]

We identified increased $CD21^{low}$ B-cell numbers in patients with Down syndrome. Because numbers of these cells are also increased in patients with autoimmune diseases, as well as in patients with CVID with autoimmune phenomena,[9,51] this could be related to the increased risk for autoimmune disease in

patients with Down syndrome. However, we did not observe increased *IGHV4-34* use or long IGH-CDR3 in the memory B cells of our patients, indicating that not all autoimmunity selection checkpoints are affected in B cells of patients with Down syndrome.[24,25]

Most of the patients with Down syndrome we studied showed abnormalities in their blood B-cell compartment that fit with one of the previously published CVID patterns.[8] Despite the memory B-cell defects, germinal centers, plasma cells, and serum immunoglobulin levels were normal in patients with Down syndrome. Because defects in immunoglobulin levels are the hallmark of CVID, patients with Down syndrome do not meet the CVID criteria. Still, the reduced memory B-cell numbers in patients with Down syndrome were suggestive of a defect in B-cell activation and proliferation (CVID-3). Together with the impaired molecular maturation of IgM and IgA memory, these defects in B-cell memory might have implications for subsequent encounters with the same pathogen and hence the susceptibility to recurrent infections. Thus our study indicates that analysis of immune competence in patients with Down syndrome should include analysis of (mucosal) IgA and IgM responses.

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Key messages

- **Despite normal germinal center formation and plasma cell maturation, patients with Down syndrome have reduced numbers of memory B cells.**
- **These memory B cells with reduced numbers showed impaired proliferation similar to that seen in patients with CVID with a defect in B-cell activation and proliferation.**
- **Impaired B-cell memory and IgM and IgA antibody maturation might contribute to the increased susceptibility to respiratory tract infections in patients with Down syndrome.**

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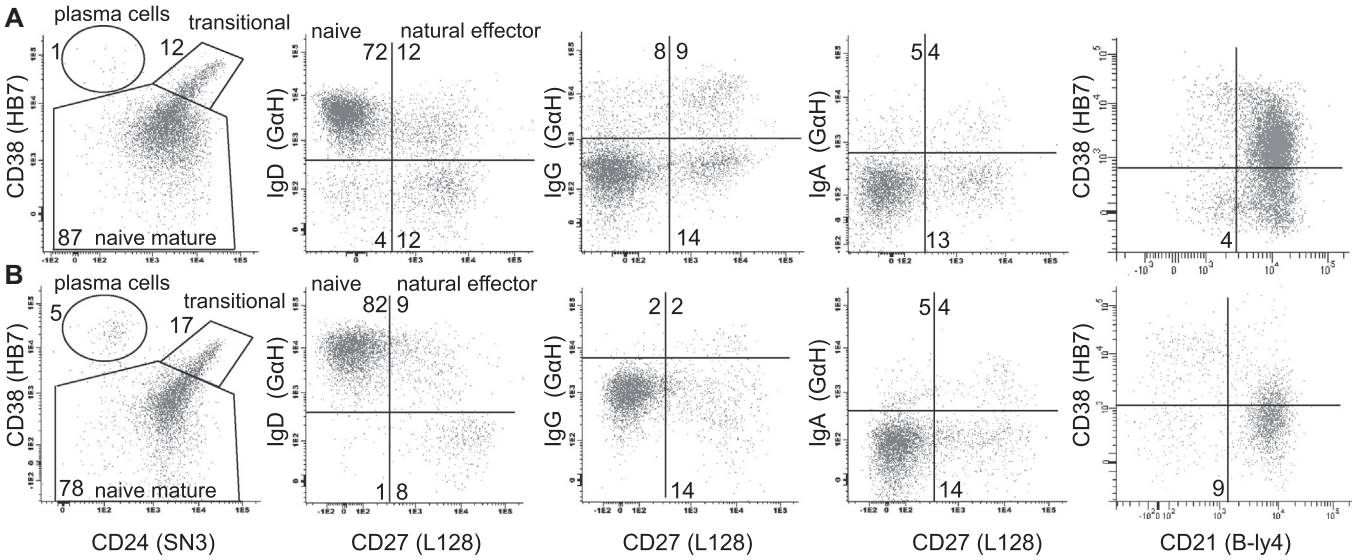

FIG E1. Flow cytometric gating strategy to determine the described B-cell subsets in a representative control subject **(A)** and a patient with Down syndrome **(B)**. Gates were electronically defined on cells that did not express specific markers (ie, $CD19^-$ lymphocytes for IgD, IgD^+ B cells for IgG and IgA, and $CD27^+$ B cells for CD38 and CD24). For each analysis, more than 5,000 to 10,000 $CD19^+$ events were acquired for control subjects and approximately 50% of patients, and greater than 1000 events were acquired in patients with low B-cell counts.

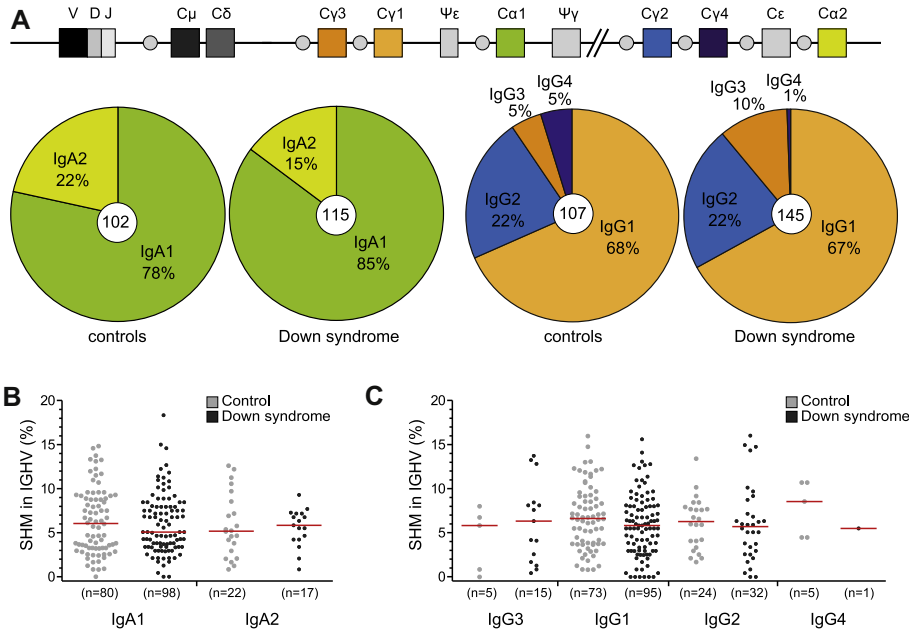

FIG E2. *IGHV* mutation frequencies of distinct IgA and IgG subclass transcripts. **A,** Distribution of IgA and IgG subclass use in *IGH* transcripts. The total number of analyzed sequences is indicated in the center of each plot. No significant differences were seen in the relative use of *IGHM*-proximal IgG_1, IgG_3, and IgA_1 versus *IGHM*-distal IgG_2, IgG_4, and IgA_2 constant regions. **B** and **C,** The frequency of mutated nucleotides in *IGHV* genes are shown for the 2 IgA subclasses (Fig E2, *B*) and IgG subclasses (Fig E2, *C*). Each *dot* represents a transcript from a healthy control subject (*light gray*) or a patient with Down syndrome (*dark gray*), with *red lines* indicating the median value for each category. The total number of analyzed transcripts is shown in brackets. Twenty-seven to 45 sequences from 4 patients (P1 and P9-11) and 18 to 52 sequences from 4 control subjects were obtained for IgA and IgG transcripts.

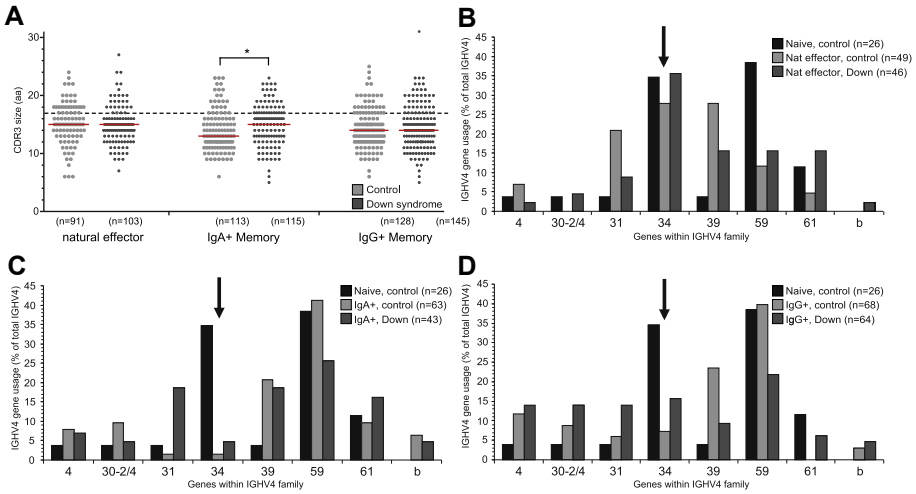

FIG E3. Selection against *IGHV4-34* use and long IGH-CDR3 in natural effector, IgA, and IgG memory B cells. **A,** IGH-CDR3 size distributions. All individual sizes of healthy control subjects *(light gray)* and patients with Down syndrome *(dark gray)* are indicated as *dots*, with *black lines* representing median values. The *dashed line* represents the median value for centroblasts (n = 67). Differences between healthy control subjects and patients with Down syndrome were statistically analyzed with the Student *t* test. $*P < .05$. **B-D,** *IGHV4* gene use in natural effector (Fig E3, *B*), IgA (Fig E3, *C*), and IgG (Fig E3, *D*) memory B cells of patients with Down syndrome. *IGHV4* gene use is displayed as frequency within the total *IGHV4* subgroup, and in each plot the patients are depicted together with the corresponding subset and with naive mature B cells from healthy control subjects. The *arrows* highlight *IGHV4-34* gene use. The total numbers of analyzed transcripts are shown in brackets. Fig E3, *A-D*, include 23 to 28 sequences from 4 patients (P1, P8, P11, and P12) and 10 to 18 sequences from 6 control subjects for natural effector B cells and 27 to 45 sequences from 4 patients (P1 and P9-11) and 18 to 52 sequences from 4 control subjects for IgA and IgG transcripts.

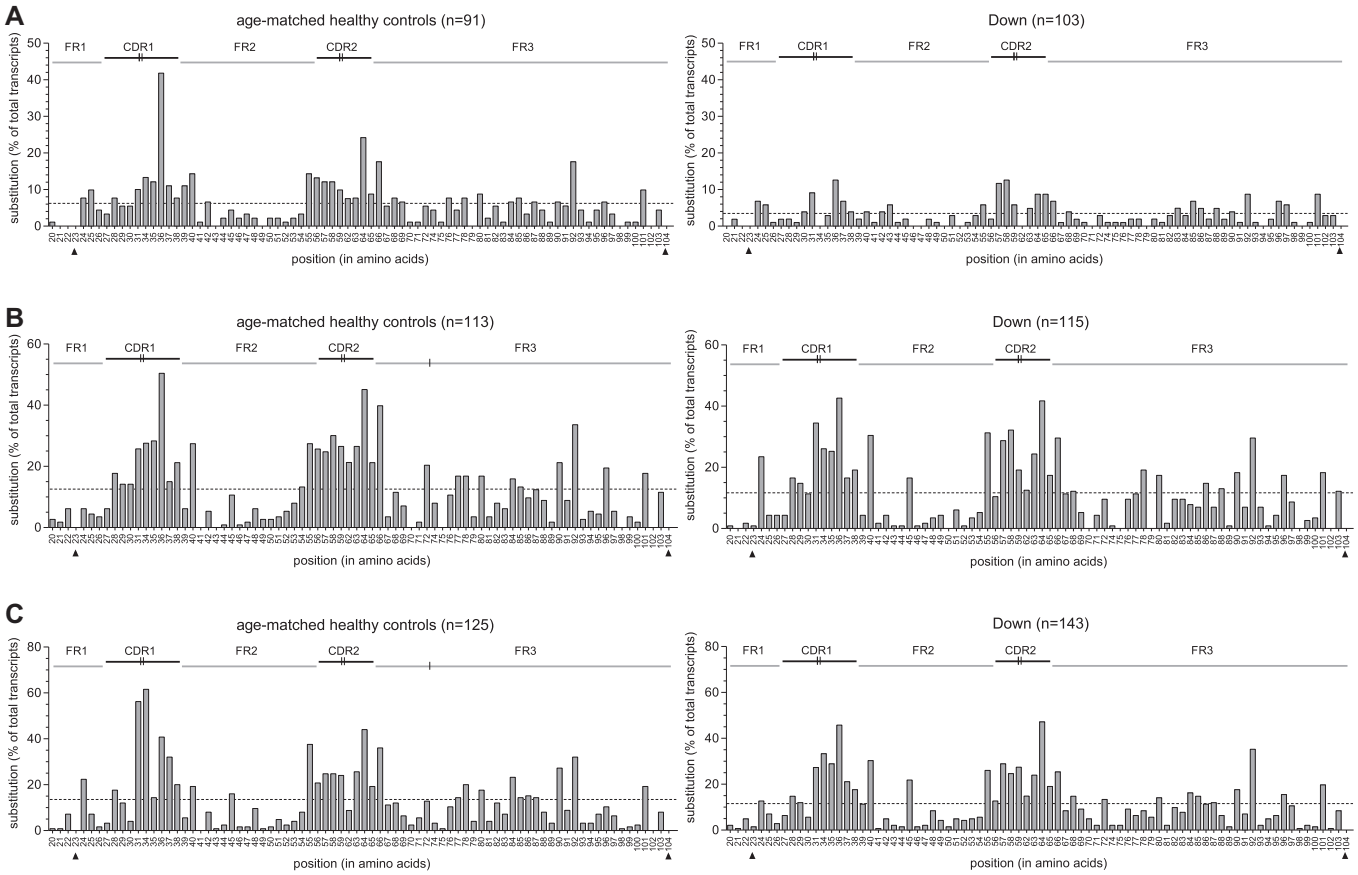

FIG E4. Distribution of replacement mutations in rearranged *IGHV* genes in memory B-cell subsets. Distribution of replacement mutations in rearranged *IGHV* genes is determined for natural effector B cells **(A)**, IgA^+ memory B cells **(B)**, and IgG^+ memory B cells **(C)** from control subjects *(left)* and patients with Down syndrome *(right)*. Each *bar* represents the frequency of replacement mutations at each amino acid position starting from 20 (first codon after the primer sequence) to 104 (last codon of the FR3 region). The total numbers of analyzed transcripts are shown in brackets. Panels *A* through *C* include 23 to 28 sequences from 4 patients (P1, P8, P11, and P12) and 10 to 18 sequences from 6 control subjects for natural effector B cells and 27 to 45 sequences from 4 patients (P1 and P9-11) and 18 to 52 sequences from 4 control subjects for IgA and IgG transcripts.

TABLE E1. PCR primers and UPL FAM-labeled hydrolysis probes for gene expression analysis

Gene	Forward primer (5′-3′)	Position (exon)	Reverse primer (5′-3′)	Position (exon)	UPL probe no.	Reference
AID	ACTCTGGACACCACTATGGACA	1	GCGGACATTTTTGAATTGGT	2	69	[12]
PRDM1	ACGTGTGGGTACGACCTTG	1	CTGCCAATCCCTGAAACCT	2	67	—
IRF4	GTTCCTGAGGGAGCCAAAA	3	GGTGGCTCATGGACATCTG	4	65	—
XBP1	GGAGTTAAGACAGCGCTTGG	3	CACTGGCCTCACTTCATTCC	4	37	—

UPL, Universal probe library (Assay Design Center; Roche, Mannheim, Germany).

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TABLE E2. Clinical and basic immunologic characteristics of patients with Down syndrome

Patient ID	Sex	Age (y)	Recurrent respiratory tract infections	Prophylactic antibiotics	History of inhaled medication*	Tympanostomy tubes	Adenotonsillectomy	Hypothyroid disease†	Lymphocyte subsets (cells/μL)			Immunoglobulin levels (g/L)						
									T cells	B cells	NK cells	IgG	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM
Blood																		
P1	M	7	Yes	Yes	No	Yes	No	No	1830	296	340	11.0	8.3	0.93	0.45	0.19	0.84	0.76
P2	M	7	Yes, until age 6 y	No	No	No	No	No	1160	290	150	11.5	9.4	1.25	0.81	0.23	1.23	0.99
P3	F	7	Yes	No	Yes	Yes	No	Yes	1100	270	120	9.9	8.6	**0.67**	0.45	0.03	0.76	0.63
P4	M	7	No	No	No	No	No	No	ND	310	ND	11.8	8.7	1.09	*1.53*	0.12	1.54	1.03
P5	F	8	No	No	Yes	No	No	No	ND	**153**	ND	11.4	8.5	**0.87**	1.21	0.03	1.99	**0.48**
P6	F	8	Yes	Yes	Yes	No	No	No	ND	219	ND	*14.2*	9.6	2.68	1.50	0.14	1.71	**0.48**
P7	M	9	Yes, until age 6 y	No	Yes	No	No	No	800	**87**	100	*14.8*	*10.8*	1.71	0.89	0.03	1.26	1.36
P8	F	10	Yes, until age 6 y	No	Yes	No	No	No	ND	**126**	ND	11.3	8.1	1.56	1.02	0.23	1.28	**0.41**
P9	F	10	Yes, until age 8 y	No	No	Yes	No	No	980	**140**	200	10.2	8.1	**0.74**	0.74	0.12	1.64	**0.43**
P10	F	11	Yes, until age 8 y	No	No	Yes	No	Yes	950	**110**	110	9.7	7.1	**0.63**	0.72	0.08	1.84	0.57
P11	F	13	No	No	No	No	Yes	No‡	1330	406	210	12.3	8.3	2.08	1.31	0.28	0.97	0.53
P12	M	14	No	No	No	No	No	No	ND	**120**	ND	10.5	7.3	2.17	1.43	0.11	2.39	0.63
P13	M	15	Yes, until age 10 y	No	Yes	Yes	Yes	No	**560**	**50**	80	11.7	8.1	2.02	1.24	0.30	2.43	0.45
P14	F	15	No	No	No	No	No	No‡	ND	**68**	ND	12.5	7.6	3.19	0.52	0.42	1.69	0.58
P15	F	17	No	No	No	Yes	No	Yes	ND	167	ND	12.9	9.0	2.30	0.93	0.37	1.76	0.99
P16	F	17	No	No	No	No	No	No‡	1520	**60**	100	*18.1*	*14.7*	1.79	0.66	<0.01	1.46	0.47
P17	F	17	No	No	No	Yes	No	Yes	1110	**68**	**80**	16.0	*10.7*	2.79	1.23	0.44	1.84	**0.22**
Tonsils																		
T1	F	4	Yes	Yes	Yes	No	Yes	No	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T2	M	4	Yes	Yes	No	No	Yes	No‡	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T3	M	5	Yes	No	No	Yes	Yes	No	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T4	M	3	Yes	No	No	No	Yes	No‡	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

None of the children were ever admitted to a pediatric intensive care unit because of serious respiratory tract infections or showed signs of celiac disease, diabetes mellitus, hematological malignancies, asthma, or allergy. Values of lymphocyte subsets and immunoglobulin levels less than and greater than age-related normal values are marked in boldface and italics, respectively. For normal values of immunoglobulin, see Table E3, according to De Vries et al.[18] For normal values of lymphocyte subsets, see Comans-Bitter et al.[20]

F, Female; *M*, male; *ND*, not determined; *NK*, natural killer.

*History of use of inhaled β_2-adrenergic receptor agonists, corticoids, or both.

†Hypothyroid disease for which thyroid hormone replacement therapy was started and antithyroperoxidase antibodies were increased (>25 U/L).

‡Patients with normal thyroid function but increased antithyroperoxidase antibodies (>25 U/L).

TABLE E3. Normal values of serum immunoglobulin levels

Age group	IgA (g/L)	IgM (g/L)	IgG (g/L)	IgG_1 (g/L)	IgG_2 (g/L)	IgG_3 (g/L)	IgG_4 (g/L)
7-12 y	0.3-2.0	0.5-2.0	6.0-12.3	3.8-10.0	0.9-5.0	0.15-1.5	0.03-2.1
>12 y	0.7-4.0	0.4-2.3	7.0-16.0	3.8-10.0	0.9-5.0	0.15-1.5	0.03-2.1

Data represent 5th and 95th percentiles and were obtained from De Vries et al.[18]

TABLE E4. Absolute numbers of blood B-cell subsets and designation to CVID patterns

Patient ID	Age (y)	Total B cells (cells/μL)	Transitional (cells/μL)	Naive mature (cells/μL)	Natural effector (cells/μL)	$CD27^+IgD^-$ memory (cells/μL)	Plasma cells (cells/μL)	CVID pattern*
P1	7	296	45.6	206.5	18.0	**12.7**	3.5	4
P4	7	310	43.7	167.8	33.7	25.1	*30.3*	5
P5	8	**153**	21.7	**103.4**	**6.9**	**9.5**	8.4	2
P6	8	219	33.5	142.7	**10.5**	14.2	10.3	3
P7	9	**87**	**8.7**	**52.7**	**12.5**	**5.5**	1.2	2
P8	10	**126**	12.9	**87.6**	**7.7**	**5.1**	8.8	2
P11	11	406	61.3	309.9	13.8	**4.5**	6.9	4
P12	14	**120**	4.9	90.6	**4.2**	**7.8**	8.4	3
P13	15	**40**	**2.9**	**21.1**	**2.9**	**5.5**	2.6	2
P14	17	**68**	**1.6**	**34.9**	12.0	**10.8**	6.4	4
P15	17	167	5.7	115.7	20.1	16.2	7.9	5
P16	17	**60**	3.9	100.4	10.2	22.4	2.4	5
P17	17	**68**	4.9	**39.8**	10.1	**5.0**	1.9	4
Control subjects (5th-95th percentiles)	5-10		11-77	111-486	15-88	13-100	1-15	—
Control subjects (5th-95th percentiles)	11-16		4-108	87-390	7-90	10-76	0.5-20	—
Control subjects (5th-95th percentiles)	>16		3-50	57-447	9-88	13-122	1-23	—

Subnormal values are shown in boldface, and increased numbers are shown in italics.

*Definition of CVID subgroups: *2*, decreased naive mature, natural effector, and $CD27^+IgD^-$ memory cell numbers; *3*, normal naive mature, decreased natural effector, and $CD27^+IgD^-$ memory cell numbers; *4*, normal naive mature and natural effector and decreased $CD27^+IgD^-$ cell numbers; and *5*, normal numbers of all indicated B-cell subsets.[8]

TABLE E5. Targeting and selection of individual mutations in rearranged *IGHV*

	Natural effector		IgA memory		IgG memory	
	Control subjects (n = 120)	Patients with Down syndrome (n = 98)	Control subjects (n = 112)	Patients with Down syndrome (n = 115)	Control subjects (n = 129)	Patients with Down syndrome (n = 145)
Mutated rearrangements (%)	100/120 (83.3)	76/98 (77.6)	111/112 (99.1)	113/115 (98.3)	126/129 (97.7)	139/145 (95.9)
Transitions (%)	504/909 (55.4)	281/552 (50.9)	997/1850 (53.9)	921/1792 (51.4)	1241/2438 (50.9)	1191/2296 (51.9)
Transversions (%)	405/909 (44.6)	271/552 (49.1)	853/1850 (46.1)	871/1792 (48.6)	1197/2438 (49.1)	1105/2296 (48.1)
Transitions at C·G (%)	298/543 (54.9)	164/311 (52.7)	563/1076 (52.3)	553/1083 (51.1)	743/1432 (51.9)	728/1401 (52.0)
Targeting of C·G (%)	543/909 (59.7)	311/552 (56.3)	1076/1850 (57.7)	1083/1792 (60.4)	1432/2438 (58.7)	1401/2296 (61.0)
RGYW (%)	244.1/909 (26.9)	**106.5/552 (19.3)***	483.3/1850 (26.1)	486.4/1792 (27.1)	613.7/2438 (25.2)	606/2296 (26.4)
WRCY (%)	132/909 (14.5)	79.7/552 (14.4)	264.6/1850 (14.3)	250.6/1792 (14.0)	351.7/2438 (14.4)	321.3/2296 (14.0)
WA (%)	131.7/909 (14.5)	74.8/552 (13.5)	252.3/1850 (13.6)	207.3/1792 (11.6)	303.7/2438 (12.5)	303.8/2296 (13.2)
TW (%)	45.2/909 (5.0)	39.1/552 (7.1)	151.9/1850 (8.2)	143.7/1792 (8.0)	159.0/2438 (6.5)	137.9/2296 (6.0)
FR (R/S)	379/212 (1.8)	229/135 (1.7)	719/460 (1.6)	677/494 (1.4)	1065/618 (1.7)	903/600 (1.5)
CDR (R/S)	259/59 (4.4)	145/41 (3.5)	535/134 (4.0)	485/136 (3.6)	596/159 (3.7)	642/151 (4.3)

Significant value is shown in boldface.

The number of analyzed sequences is indicated in parentheses next to the population name. All analyses were performed with the JOINSOLVER program, and the differences between control subjects and patients were analyzed with the χ^2 test.

R/S, Ratio between replacement and silent mutations.

*Significant differences: $P < .01$ (also after adjustment for multiple comparisons).