Persistent subclinical immune defects in HIV-1-infected children treated with antiretroviral therapy

Diana van den Heuvel[a], Gertjan J.A. Driessen[b], Magdalena A. Berkowska[a,*], Mirjam van der Burg[a], Anton W. Langerak[a], Dan Zhao[a], Halima Charif[a], Nico G. Hartwig[b,†], Annemarie M.C. van Rossum[b], Pieter L.A. Fraaij[b,c], Jacques J.M. van Dongen[a] and Menno C. van Zelm[a]

Objectives: With the introduction of combined antiretroviral therapy (cART), HIV-infected children can reach adulthood with minimal clinical complications. However, long-term HIV and cART in adults are associated with immunosenescence and end-organ damage. Long-term consequences of HIV and cART in children are currently unknown.

Design and method: We studied 69 HIV-infected children and adolescents under cART (0–23 years) for the occurrence of subclinical immunological aberrations in blood B and T cells, using detailed flow cytometric immunophenotyping and molecular analyses.

Results: Children with undetectable plasma HIV viral loads for more than 1 year showed near-normal to normal $CD4^+$ T-cell numbers and near-normal numbers of most class-switched memory B cells. Furthermore, expansions of aberrant $CD21^{low}$ B cells contracted in patients with virus suppression. In contrast, $CD8^+$ effector T cells were increased, and $CD4^+$ memory T cells, $V\gamma9^+V\delta2^+$ T cells and $CD27^-IgA^+$ memory B cells were decreased and did not normalize under ART. Moreover, $V\gamma9^+V\delta2^+$ T cells showed defects in their T-cell receptor repertoire selection.

Conclusion: Our results show the effectiveness of current cART to enable the build-up of phenotypically diverse B-cell and T-cell memory in HIV-infected children. However, several subclinical immune abnormalities were detected, which were partially caused by defective immune maturation. These persistent abnormalities were most severe in adolescents and therefore warrant long-term follow-up of HIV-infected children. Early identification of such immune defects might provide targets for monitoring future treatment optimization.

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[a]Department of Immunology Erasmus MC, University Medical Center, [b]Department of Pediatrics, Division of Infectious Diseases, Immunology and Rheumatology, Erasmus MC, University Medical Center-Sophia, and [c]Department of Viroscience, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.

Correspondence to Dr Menno C. van Zelm, PhD, Department of Immunology, Central Clinical School, Monash University, 89 Commercial Road, Melbourne, Victoria 3004, Australia.

Tel: +61 3 9903 0834; fax: +61 3 9903 0038; e-mail: menno.vanzelm@monash.edu

[*] Present address: Department of Experimental Immunohematology, Sanquin Research, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

[†] Present address: Sint Franciscus Gasthuis, Rotterdam, the Netherlands.

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Introduction

Current combined antiretroviral therapy (cART) protocols have been successful in the treatment of HIV-infected children, who can now reach adulthood with minimal clinical complications [1]. cART effectively induces viral suppression, and in most patients, plasma HIV-RNA levels are below the detection limit within 1 year after the start of treatment [2]. Furthermore, treatment restores total $CD4^+$ T-cell numbers to levels within the normal range [3], suggesting that the treatment is effectively inhibiting viral replication and inducing immune recovery [4,5]. Signs of immune activation in HIV-infected adults and children, that is, the $CD8^+$ T-cell expansions and hypergammaglobulinemia, resolve after the initiation of cART [1]. Thus, cART changes HIV infection from a lethal disease into a chronic, treatable disease.

Long-term HIV infection in adults has, however, been associated with clinical complications, such as cardiovascular complications, neurological diseases and malignancies [6–8]. Both HIV-associated immune aberrations and immunosenescence, as well as long-term cART, are important contributors to the development of these diseases [6–8]. Immune aberrations in HIV-infected adults include an expansion of $CD8^+$ T cells with an effector memory phenotype: $CD45RA^-CCR7^-$. These HIV-specific cells display reduced cytolytic potential and are more prone to apoptosis [9–11], mechanisms that could contribute to impaired clearing of HIV [12,13]. Furthermore, the Vδ2-expressing subset of T-cell receptor (TCR)$\gamma\delta^+$ T cells is contracted both in mucosal tissues and in blood. This defect is associated with increased microbial translocation in the intestine [14–16]. Finally, HIV infection affects B-cell responses leading to hypergammaglobulinemia [17,18], an expansion of the aberrant, anergic $CD21^{low}$ B-cell population [19–22], and a reduction of $CD27^+$ class-switched memory B cells [18,23], of which especially the latter persists despite ART [18,24–26].

Long-term effects of HIV infection and cART in children currently remain unclear. The first few years of life are crucial for the proper human immune maturation, and thereby to prepare them for adulthood [27]. Even though cART successfully suppressed HIV, it is unclear whether the dynamic adaptive immune maturation is normal in HIV-infected children. Early detection of immune aberrations could be predictive of future complications. Some persistent aberrancies in T-cell and B-cell memory have been described in HIV-infected children [28–30], and their nature seems to depend on the child's age at the start of ART [30–37]. Still, an in-depth study into immune aberrations that might have long-term pathogenic effects has, to our knowledge, not been performed.

We, therefore, studied the blood B and T-cell compartments in a cohort of 69 perinatally HIV-infected children and adolescents (0–23 years). The vast majority of these individuals responded well to ART with HIV levels below the detection limit and total $CD4^+$ T-cell numbers within the normal range. However, several persistent subclinical immune abnormalities could be identified in both B and T cells that warrant long-term follow-up of these perinatally HIV-infected children and might be predictive for future complications.

Methods

Study participants

Blood was obtained from 69 HIV-positive children between 0 and 23 years of age during routine outpatient clinic visits (Supplemental Table 1, http://links.lww.com/QAD/A725) [1,5,38]. HIV-infected individuals were stringently treated and monitored every 3–6 months at Erasmus MC-Sophia Children's Hospital and part of the Dutch vertically HIV-infected pediatric population cohort as registered by the Dutch HIV Monitoring Foundation [1,5,39]. Except for one child who did not give consent, all children of the cohort who were below 23 years of age and visited the outpatient clinic between December 2009 and December 2012 were included in the study. One hundred and forty-eight age and sex-matched HIV-negative healthy individuals were recruited in the same hospital [1,5,38]. Included were children undergoing orthopedic, ophthalmic, urologic or other noninfectious surgical procedures in otherwise healthy condition. Excluded were children having fever, burns or using antibiotics at the moment of sampling, and children having a known immunodeficiency, cardiovascular disease, coagulopathy, renal or hepatic impairment, and children undergoing organ transplantation. Patients and controls were included following informed consent from the parents according to the guidelines of the Medical Ethics Committee of Erasmus MC.

Flow cytometric immunophenotyping and isolation of B and T-cell subsets from peripheral blood

Absolute counts of blood $CD4^+$ and $CD8^+$ T cells were obtained with a diagnostic lyze-no-wash protocol. Detailed flow cytometric immunophenotyping was performed on fresh blood samples after red blood cell lysis with ammonium chloride. $CD4^+$ T cells, $CD8^+$ T cells, TCR$\gamma\delta^+$ T cells and B cells were characterized using the antibodies described in Supplemental Table 2 (http://links.lww.com/QAD/A725). Flow cytometric analyses were performed on an LSRII or CantoII (both from BD Biosciences, San Jose, California, USA) using standardized measurement settings [40].

Post-Ficoll peripheral blood mononuclear cells (PBMCs) were stored in liquid nitrogen and later thawed for sorting of $CD24^{dim}CD38^{dim}CD27^-CD21^{low}$ ($CD21^{low}$) B cells and three $CD24^{dim}CD38^{dim}CD21^+$ subsets: $CD27^-IgD^+$

naive mature, $CD27^+IgD^+$ natural effector and $CD27^+IgD^-$ memory B cells on a FACS Aria I (BD Biosciences).

Sequence analysis of rearranged *TCR* and *IGH* transcripts

The presence of the invariant-T nucleotide within the Vδ2-Jδ1 junctional region [41] was determined in rearranged transcripts amplified from PBMC cDNA, using the BIOMED-2 Vδ2 forward and Jδ1 reverse primers [42]. Rearranged Vγ9-Jγ1.2 transcripts were amplified with the BIOMED-2 Vγ9 forward primer and the fluorescently labeled BIOMED-1 Jγ1.2 reverse primer [42,43]. Amplified products were subjected to GeneScan analyses using an ABI PRISM 3130XL fluorescent sequencer (Applied Biosystems, Foster City, California, USA) to determine the frequencies of canonical rearrangements [41,44].

IgM and IgG transcripts were amplified and cloned from cDNA of sorted B-cell subsets, immunoglobulin heavy chain V genes, *IGHV3* and *IGHV4/6* leader forward primers and *IGHM* and *IGHG* reverse primers [45,46]. Sequences were generated on an ABI PRISM 3130XL and analyzed with the IMGT (http://imgt.org/), and BASELINe (http://selection.med.yale.edu/baseline/) software [47,48].

B-cell replication history using the Kappa-deleting Recombination Excision Circle assay

DNA was isolated from sorted B-cell subsets with the GenElute Mammalian Total DNA Miniprep Kit (Sigma-Aldrich, St Louis, Missouri, USA) to determine the replication history with the Kappa-deleting Recombination Excision Circle (KREC) assay as described previously on a StepOnePlus Real-Time PCR system (Applied Biosystems) [49].

Statistical analyses

Statistical analyses were performed using the Mann–Whitney *U* test, unpaired *T* test, Fisher's exact test or chi-square test. *P* values less than 0.05 were considered statistically significant.

Results

Patient characteristics

In this study, we included 69 children and adolescents who were infected with HIV in early childhood (Supplemental Table 1, http://links.lww.com/QAD/A725). Of these, four patients had plasma HIV viral loads above 15 000 copies/ml at the moment of sampling (HIVhigh). Of the remaining 65 patients (HIVlow), 26 had detectable viral load below 900 copies/ml at the time of first sampling or detectable viral load at least once in the preceding year (suboptimal viral suppression; HIVdetect) and 39 patients had undetectable viral load for more than 1 year (stable viral suppression; HIVund) (Supplemental Table 1, http://links.lww.com/QAD/A725).

All HIVlow patients received ART for an average of 7.3 years (HIVund) and 5.1 years (HIVdetect). Two of the four HIVhigh patients received ART for 9.9 and 12.3 years; the other two patients were clinically well and treatment was postponed according to guidelines at that time. Disease severity scores at diagnosis, according to Center for Disease Control and Prevention (CDC) guidelines [50], varied between N1 and C3. The nadir $CD4^+$ T-cell counts were not significantly different between the three patient groups, as was the age at which the patients reached their nadir $CD4^+$. During the study follow-up, patients were clinically well; only eight children needed a short-term hospitalization within the cumulative cohort follow-up. Total $CD4^+$ T-cell numbers at the moment of inclusion were normal in 81.5% of HIVlow patients (87.2% HIVund; 73.1% HIVdetect) and in 50% of HIVhigh patients (one treated, one untreated) [27]. Serum IgA, IgG, and/or IgM levels above the normal range were detected in only 17.9% of HIVund patients, but were significantly more frequent in HIVdetect (53.8%) and HIVhigh (75%) patients [51]. Vaccinations against hepatitis A and B induced a protective response [>100 International units/l (IU/l)] in 29 of 29 vaccinated HIVlow children and one of two vaccinated HIVhigh patients. Within the HIVlow group, increasing age of the patients correlated to significantly lower nadir $CD4^+$ cell counts, which were furthermore reached at significantly later age. Also, treatment was initiated later in older patients, and children aged 16–23 years more frequently showed reduced $CD4^+$ T-cell counts at the moment of sampling, suggesting persistent $CD4^+$ T-cell defects. Clinical severity score, high serum immunoglobulin, and plasma HIV RNA levels were, however, independent of the age of the child at inclusion or treatment duration.

Altogether, cART appeared effective in most patients as evidenced by the limited clinical complications, the normal response to vaccinations, and the increase in $CD4^+$ T-cell counts.

Low $CD4^+$ memory T-cell numbers in antiretroviral therapy-responsive adolescents

$CD4^+$ T-cell numbers were within the normal range in most children aged below 16 years, but were reduced in many older children and adolescents (Supplemental Table 1, http://links.lww.com/QAD/A725). When divided over five age groups (<2 years, 2–4 years, 5–9 years, 10–15 years, and 16–23 years), $CD4^+$ T-cell numbers in HIVlow patients were comparable to age-matched controls, except for a significant reduction in adolescents aged 16–23 years (Fig. 1a).

To address whether $CD4^+$ T-cell numbers were related to plasma HIV-RNA levels, children aged 5–20 years were

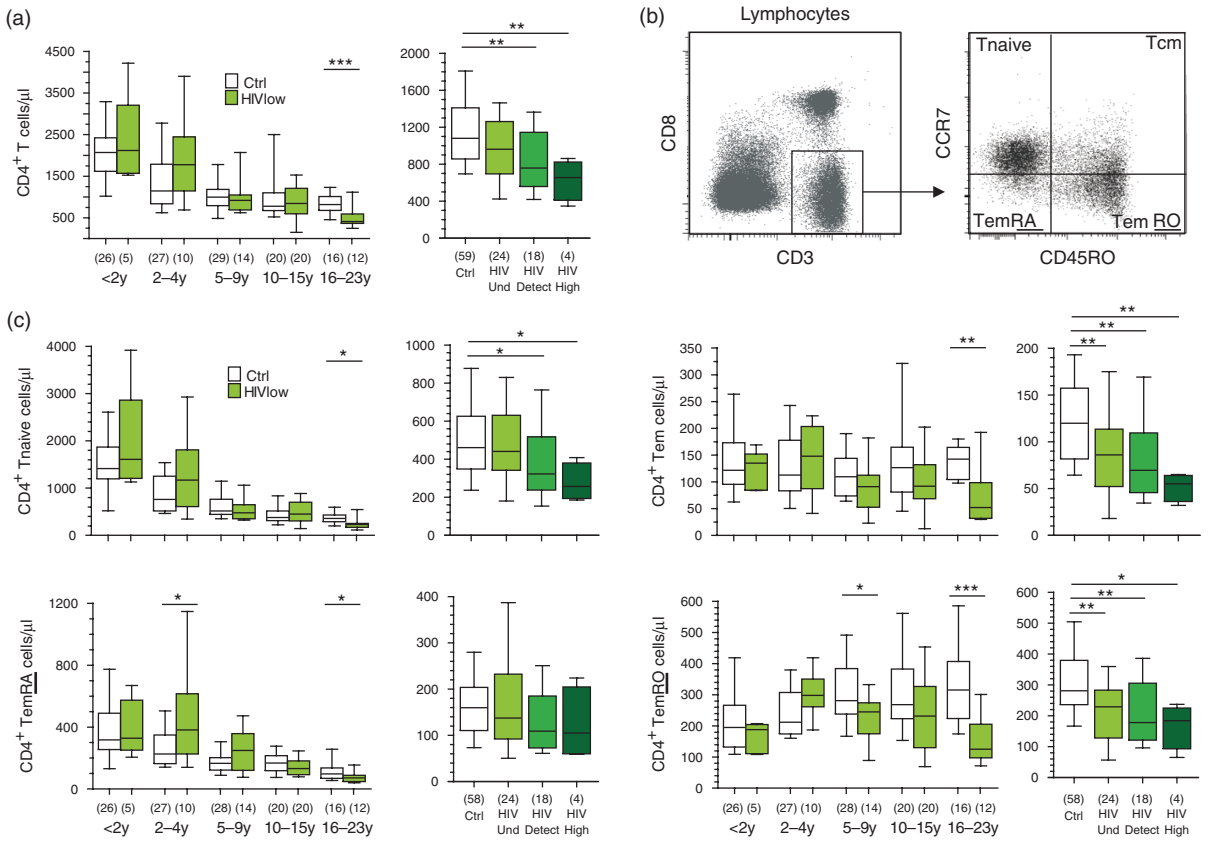

Fig. 1. The CD4$^+$ T-cell compartment in HIV-infected children and adolescents. (a) CD4$^+$ T-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect, or HIVhigh patients of 5–23 years old (right). The number of individuals per group is indicated in parentheses. Plots depict 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). (b) Definition of naive (TNAIVE), central memory (TCM), and CD45RO$^-$ effector memory (TEMRO), and CD45RO$^-$ effector memory (TEMRA) subsets within CD3$^+$CD8$^-$ T cells. (c) Absolute numbers of CD4$^+$ TNAIVE, TCM, TEMRO, and TEMRA subsets presented similarly as in panel (a). Statistical significance was determined using the Mann–Whitney *U* test; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (****) $P < 0.0001$. ART, antiretroviral therapy.

separated into HIVund, HIVdetect, and HIVhigh (Supplemental Table 1, http://links.lww.com/QAD/A725). CD4$^+$ T-cell numbers were only slightly, but not significantly, reduced in HIVund patients, and more severely reduced in HIVdetect and HIVhigh patients (Fig. 1a).

To study the nature of the CD4$^+$ T-cell reduction, we dissected total CD4$^+$ T cells into CD45RO$^-$CCR7$^+$CD27$^+$CD28$^+$ naive, CD45RO$^+$CCR7$^+$CD27$^+$CD28$^+$ central memory (TCM), CD45RO$^+$CCR7$^-$ effector memory (TEMRO), and CD45RO$^-$CCR7$^-$ effector memory (TEMRA) (Fig. 1b). These four subsets were all significantly reduced in 16–23-year-old HIVlow patients (Fig. 1c). In younger children, these subsets were normally present, except for significantly increased TEMRA in children aged 2–4 years, and decreased TEMRO in children aged 5–9 years. Within TEMRO, the early differentiated CD27$^+$CD28$^+$ subset was not only significantly reduced in the 16–23-year-olds but also in all age groups above 5 years (Supplemental Fig. 1a, http://links.lww.com/QAD/A725). The reductions in TCM and TEMRO numbers were similar in HIVund or HIVdetect patients, but slightly stronger in patients with high viral load (Fig. 1c and Supplemental Fig. 1a, http://links.lww.com/QAD/A725). Thus, viral suppression seems to restore the CD4$^+$ T-cell compartment to a large extent in HIVlow patients, but especially adolescents showed reduced TCM and TEMRO numbers that are typically seen in viremic patients.

CD8$^+$ effector memory T-cell expansions in young children

Of the 65 HIVlow patients, only four showed the increase in total CD8$^+$ T-cell numbers that is typically associated with HIV infection. Still, direct comparison of patient groups with age-matched controls showed significantly increased numbers of CD8$^+$ T cells in children of aged 2–4 and 5–9 years (Fig. 2a). Subset analysis revealed that this increase was mainly due to significantly high numbers of TEMRA and to a lesser extent of TEMRO (Fig. 2b and c). Within these TEMRO and TEMRA populations especially the CD27$^-$CD28$^-$ late effector cells were significantly increased in HIV-infected children

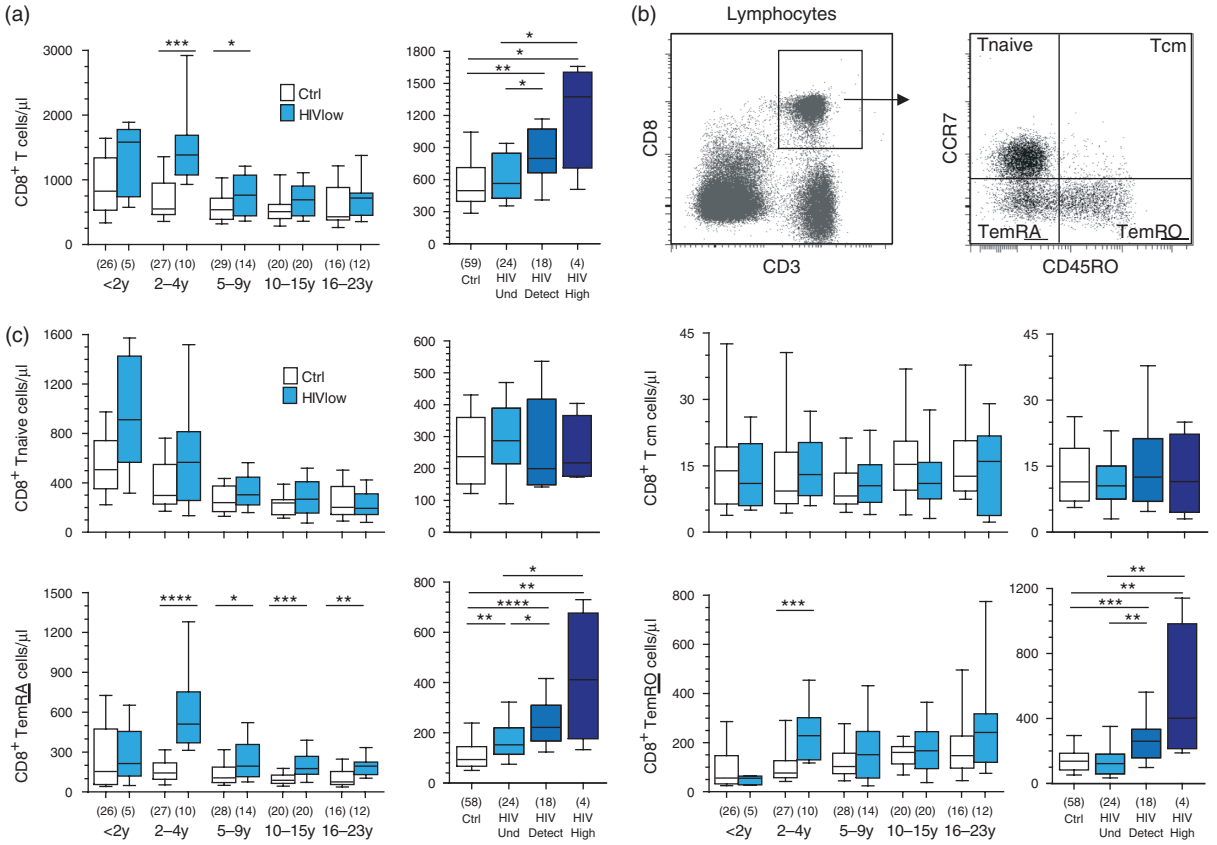

Fig. 2. The CD8$^+$ T-cell compartment in HIV-infected children and adolescents. (a) CD8$^+$ T-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect, or HIVhigh patients of 5–23 years old (right). The number of individuals per group is indicated in parentheses. Plots depict 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). (b) Definition of naive (TNAIVE), central memory (TCM), CD45RO$^-$ effector memory (TEMRO), and CD45RO$^-$ effector memory (TEMRA) subsets within CD3$^+$CD8$^+$ T cells. (c) Absolute numbers of CD8$^+$ TNAIVE, TCM, TEMRO, and TEMRA subsets presented similarly as in panel (a). Statistical significance was determined using the Mann–Whitney *U* test; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (****) $P < 0.0001$. ART, antiretroviral therapy.

(Supplemental Fig, 1b and c, http://links.lww.com/QAD/A725). In line with this, more CD8$^+$ T cells expressed CD57 and human leukocyte antigen DR (HLA-DR) (not shown) [52–54]. The expansions of total and TEMRO CD8$^+$ effector T cells significantly related to the presence of virus, whereas TEMRA T-cell expansions were found in all three viral load groups (Fig. 2c and Supplemental Fig. 1b and c, http://links.lww.com/QAD/A725). Thus, good control of viremia seems to inhibit the CD8$^+$ effector memory T-cell expansions, but abnormalities can still be found, especially in young children.

Cellular and molecular TCRγδ$^+$ T-cell repertoire changed in HIV patients

TCRγδ$^+$ T-cell numbers were low to normal in our patients with the typically increased ratio of Vδ1$^+$ over Vδ2$^+$ T cells (Fig. 3a) [15,16]. In young children, this was mainly due to significantly increased numbers of Vδ1$^+$ T cells, whereas older children and adolescents had significantly reduced Vδ2$^+$ T-cell numbers compared to controls (Fig. 3b and c). Moreover, Vγ9$^+$ T-cell numbers were slightly reduced in children aged 5–9 and 16–23 years, suggestive of a reduced number of Vγ9$^+$Vδ2$^+$ T cells, the dominant population in healthy adults (Supplemental Fig. a and b, http://links.lww.com/QAD/A725) [44].

To study the nature of the Vγ9$^+$Vδ2$^+$ T-cell defects, we analyzed two typical selection determinants in these cells: selection for the invariant-T nucleotide in the Vδ2–Jδ1 junctional regions, normally occurring within the first year of life and present in 90% of healthy adults; and the canonical Vγ9–Jγ1.2 rearrangement, formed through homology-mediated repair and yielding a canonical complementarity-determining region 3 (CDR3) of 14 amino acids [41,44]. In 15 patients, we did not find the invariant-T (Fig. 3d). These mainly concerned young children aged below 2 years, but their frequency was also significantly reduced in the patients aged 2–23 years. Furthermore, patients showed a trend towards reduced usage of the canonical Vγ9–Jγ1.2 rearrangements, even

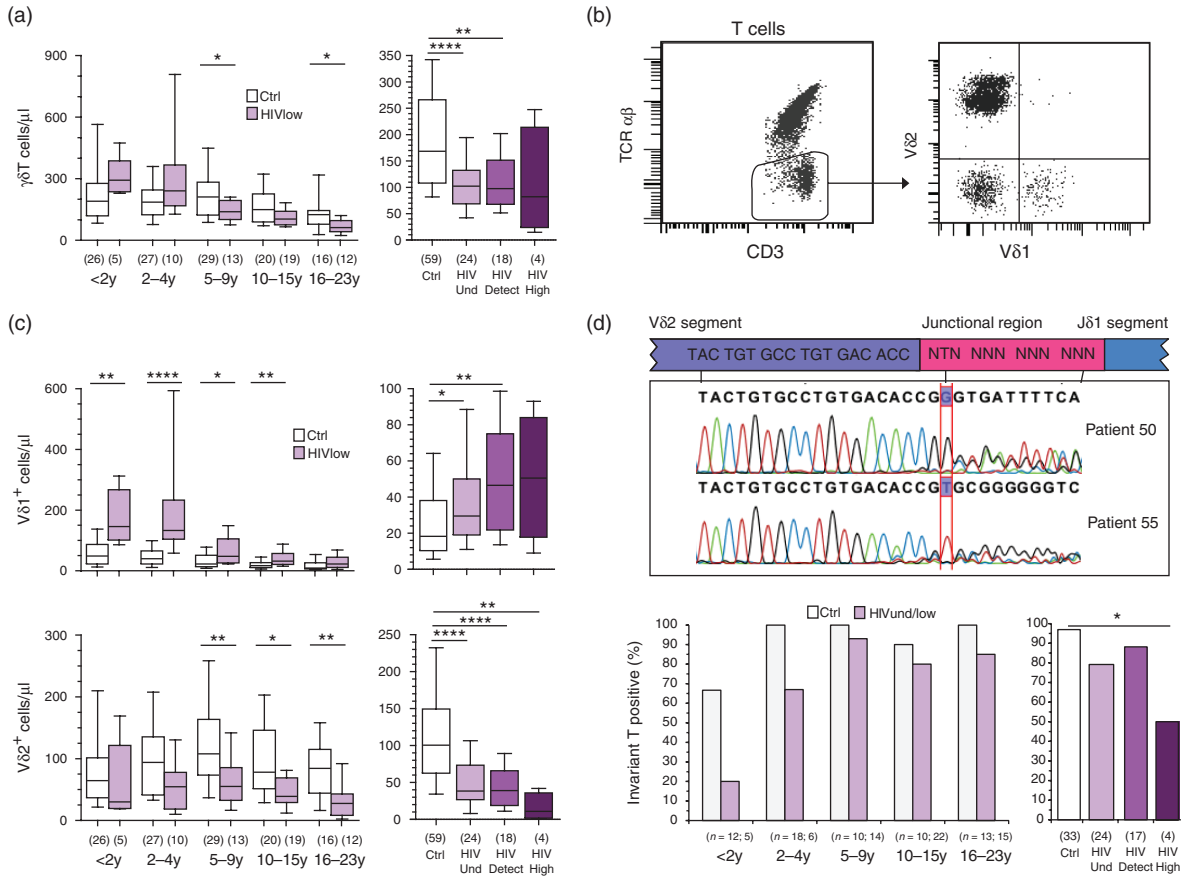

Fig. 3. Cellular and molecular defects in TCRγδ$^+$ T cells of HIV-infected children and adolescents. (a) TCRγδ$^+$ T-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect, or HIVhigh patients of 5–23 years old (right). The number of individuals per group is indicated in parentheses. Plots depict 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). (b) Definition of Vδ1 and Vδ2-expressing T-cell subsets. (c) Absolute numbers of Vδ1 and Vδ2-expressing T-cell subsets presented as in panel (a). (d) Molecular analysis of TCRDV2-TCRDJ1 rearrangements for detection of the invariant-T nucleotide in the first codon of the complementary-determining region (CDR)3. (a/c) Plots depict 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). Statistical significance was determined using the Mann–Whitney *U* test; (*) $P<0.05$; (**) $P<0.01$; (***) $P<0.001$; (****) $P<0.0001$. (d) Statistical significance was determined using the chi-square test. ART, antiretroviral therapy.

despite effective virus suppression in HIVund patients (Supplementary Fig. 2c, http://links.lww.com/QAD/A725). Thus, in adolescents, Vγ9$^+$Vδ2$^+$ T cells were impaired in numbers and in their TCR maturation and repertoire selection, irrespective of ART.

Reductions in IgA$^+$ B-cell memory despite antiretroviral therapy

Total B-cell numbers, including the transitional and naive mature B-cell subsets, were normal in HIV patients of all age groups (Fig. 4a and data not shown). Within the memory B-cell compartment, all subsets were decreased in number, except for a slight increase in IgM-only B cells (Fig. 4b and c). Partial normalization of CD27$^-$IgG$^+$, CD27$^+$IgG$^+$ and CD27$^+$IgA$^+$ memory B-cell numbers was related to viral suppression, whereas the IgM$^+$ populations were equally affected in HIVund, HIVdetect, and HIVhigh patients. The strongest effect was observed in the CD27$^-$IgA$^+$ subset that is thought to originate from T-cell-independent responses in the gut [46]. This population was consistently reduced in all age categories and to the same extent in HIVhigh, as well as HIVdetect or HIVund children (Fig. 4c). Thus, even though ART seems to partially restore class-switched memory B-cell defects, increased IgM-only and decreased CD27$^-$IgA$^+$ memory B-cell numbers persist during ART in HIV-infected children.

CD21^{low} B cells are associated with high HIV loads

HIV-infected adults show expansions of atypical CD21^{low} B cells with molecular signs of antigen maturation and reactivity to HIV [55]. They are functionally impaired due to reduced replication history and somatic hypermutation (SHM) levels, and reduced responsiveness to stimulation [55]. Our HIVhigh children also carried

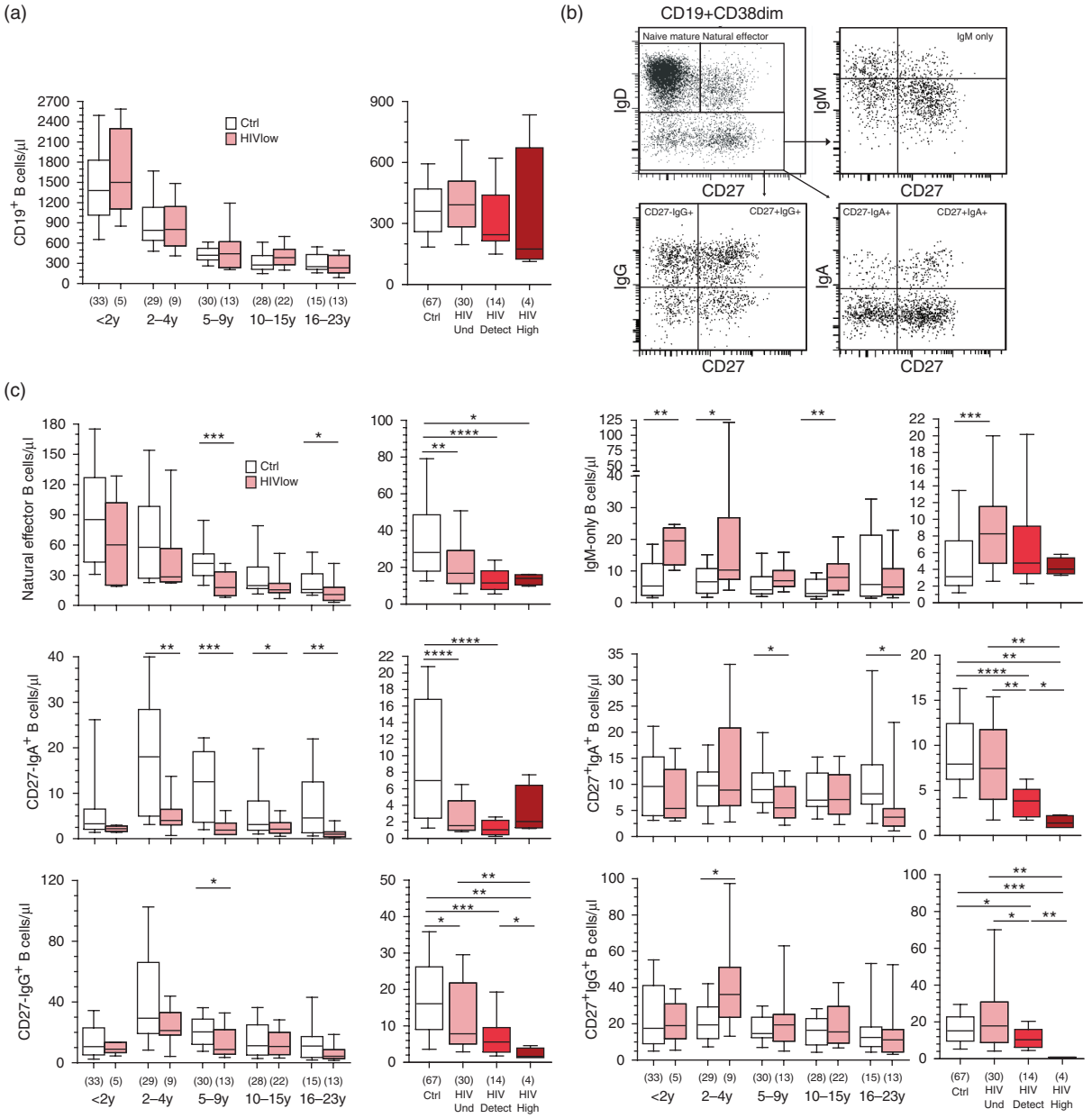

Fig. 4. The memory B-cell compartment in HIV-infected children and adolescents. (a) $CD19^+$ B-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect, or HIVhigh patients of 5–23 years old (right). The number of individuals per group is indicated in parentheses. Plots depict 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). (b) Definition of six memory B-cell subsets: $CD27^+IgD^-IgM^+$ (IgM-only), $CD27^+IgD^+IgM^+$ (natural effector), $CD27^-IgA^+$, $CD27^+IgA^+$, $CD27^-IgG^+$ and $CD27^+IgG^+$ [46]. (c) Absolute numbers of six memory B-cell subsets in HIV-infected children presented similarly as in panel (a). Statistical significance was determined using the Mann–Whitney *U* test; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (****) $P < 0.0001$. ART, antiretroviral therapy.

significant expansions of $CD38^{dim}CD27^-CD21^{low}$ B cells (Fig. 5a and b). Importantly, numbers of these $CD21^{low}$ B cells were near-normal in HIVlow patients in all age groups (Fig. 5a–c). To study whether $CD21^{low}$ B-cell numbers were directly related to HIV viral loads, we performed longitudinal analysis (1.7–2.9 year) of 14 patients (Fig. 5d and Supplemental Fig. 3, http://links.lww.com/QAD/A725). In all four patients with high viral loads, we observed increased $CD21^{low}$ B-cell numbers. In two patients, the $CD21^{low}$ B-cell numbers increased within 0.5 year following the rise of plasma HIV-RNA levels. Patients with consistently low plasma HIV-RNA levels carried a persistently small $CD21^{low}$ population of less than 20% of total B cells. Finally, patients who successfully started ART showed a concomitant decrease in $CD21^{low}$ B-cell numbers within

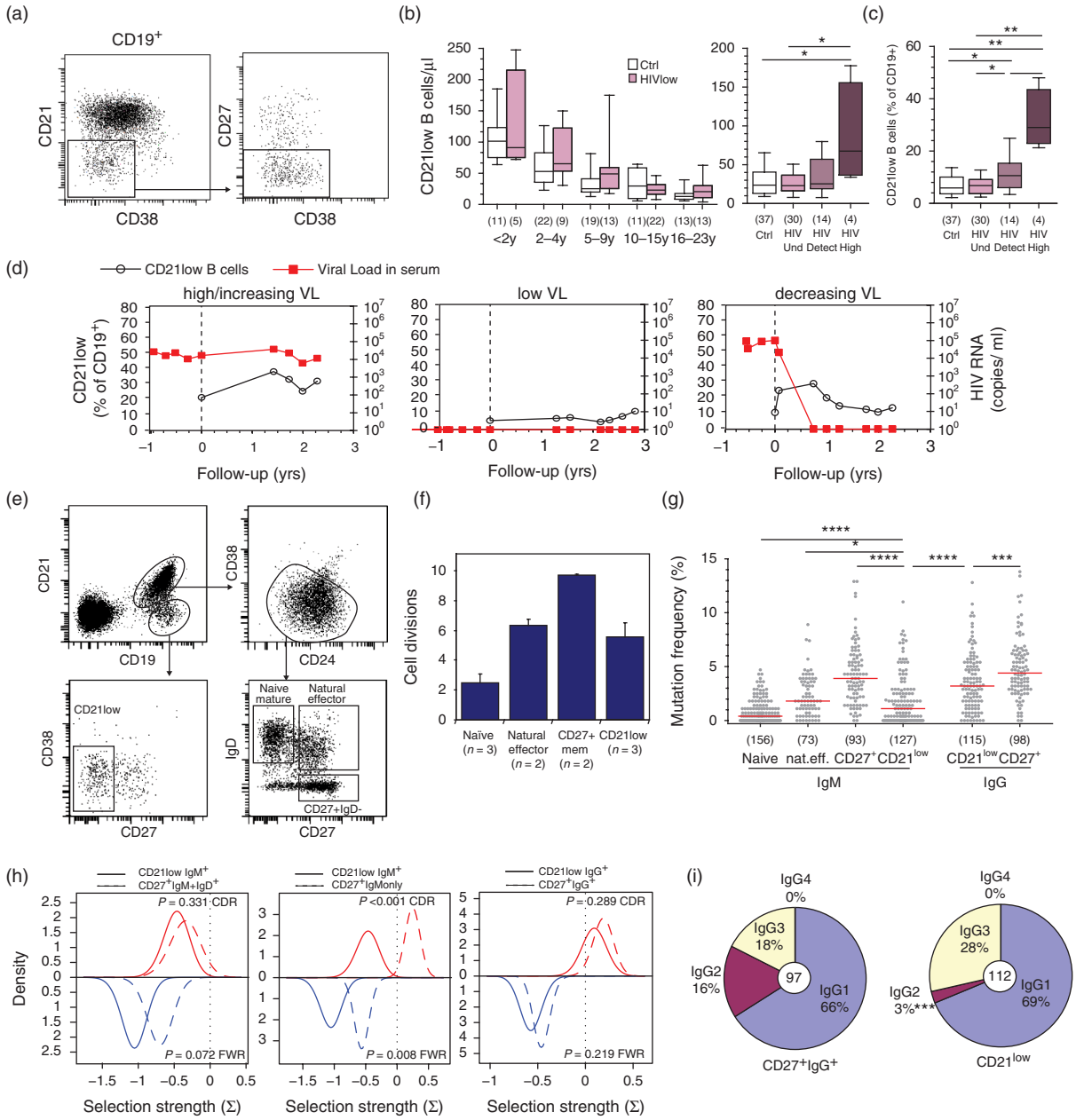

Fig. 5. Kinetics and molecular diversity of CD21^{low} B cells of HIV-infected children. (a) Definition of CD19^{+}CD38^{dim} CD21^{low}CD27^{-} 'CD21^{low} B cells'. (b) CD21^{low} B-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect, or HIVhigh patients of 5–23 years old (right). The number of individuals per group is indicated in parentheses. Plots depict 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). (c) CD21^{low} B-cell frequencies within total CD19^{+} B cells presented similarly as in panel (b). (d) Longitudinal follow-up of HIV plasma loads and CD21^{low} B-cell frequencies in one representative patient with high VL (left column), low VL (middle), and decreasing VL (right). (e) Sorting strategy and definition of CD21^{low}, naive mature, natural effector, and CD27^{+}IgD^{-} memory B cells. (f) Replication history of purified B-cell subsets of three HIV-infected children (patients 28, 29, and 34) as determined with the KREC assay [49]. (g) Mutation frequencies in *IGHV* regions of IgM and IgG transcripts in five HIV-infected children (patients 52, 56, 59, 67, and 68). The number of transcripts analyzed in each category is indicated in parentheses. (h) BASELINe analysis of IgM and IgG transcripts to determine the selection strength for replacement mutations [47,48]. (i) IgG subclass distributions of sequenced IgG transcripts from CD21^{low} and CD27^{+}IgD^{-} B cells. The number of sequences included is depicted in the center of the chart. Statistical significance was determined using the Mann–Whitney *U* test (b/c/g) or chi-square test (i); (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (****) $P < 0.0001$. ART, antiretroviral therapy; KREC, Kappa-deleting Recombination Excision Circle; VL, viral load.

0.5 year of decreased plasma HIV-RNA levels (Fig. 5d and Supplemental Fig. 3, http://links.lww.com/QAD/A725). Thus, $CD21^{low}$ B-cell numbers seem directly related to active HIV infection and can be normalized by ART in children.

$CD21^{low}$ B-cells in HIV patients are a mixture of naive and antigen-experienced B cells

To study the nature of these $CD21^{low}$ B cells, we purified these, as well as the CD21-expressing $CD27^{-}IgD^{+}$ naive B cells, $CD27^{+}IgD^{+}$ natural effector and $CD27^{+}IgD^{-}$ memory B cells from five HIV-infected children aged over 13 years for comparative analysis of replication history and SHM levels (Fig. 5e). Similar to previous observations in adults [55], $CD21^{low}$ B cells had a replication history of approximately six cell divisions, which was comparable with natural effector B cells, but clearly less than $CD27^{+}IgD^{-}$ memory B cells (Fig. 5f). The $CD21^{low}$ population consisted mainly of IgM^{+} (with or without IgD) and IgG^{+} B cells (data not shown). To distinguish between these subsets for SHM analysis, we sequenced *IGHV* genes from separately amplified *IGHM* and *IGHG* transcripts. IgG transcripts of $CD21^{low}$ B cells were nearly all mutated, although the SHM loads were slightly lower than in IgG transcripts from classical $CD27^{+}IgD^{-}$ memory B cells (Fig. 5g). Still, the transcripts of $CD21^{low}IgG^{+}$ and $CD27^{+}IgD^{-}$ cells showed similar selection for replacement mutations in CDRs (Fig. 5h). Furthermore, both subsets showed similar IgG subclass distributions with the IgM-proximal IgG1 and IgG3 mostly used (Fig. 5i). In contrast, about half of the IgM transcripts in $CD21^{low}$ B cells were unmutated. The range of SHM levels and selection for replacement mutations of the mutated clones were more similar to natural effector B cells, but clearly lower than $CD27^{+}IgD^{-}$ memory B cells (Fig. 5g and h). Thus, our molecular analyses indicate that the $CD21^{low}$ population in HIV patients is actually a mixture of naive and memory B cells, and that the molecular signs of antigen maturation in the IgG^{+} subset are more similar to classical $CD27^{+}IgG^{+}$ memory B cells than might have been previously appreciated.

Discussion

We here performed an in-depth study on the effect of perinatal HIV infection and cART on the immune compartment of children and adolescents. Our results show the effectiveness of current cART to enable the build-up of phenotypically diverse B and T-cell memory in HIV-infected children. This included, at least in part, the normalization of $CD4^{+}$ T-cell numbers in the youngest children, normalization of class-switched memory B-cell numbers, and the reduction of $CD21^{low}$ B-cell expansions. However, persistent expansions of $CD8^{+}$ effector T cells, and the reductions in $V\gamma9^{+}V\delta2^{+}$ T-cells and $CD27^{-}IgA^{+}$ memory B cells were not restored by cART.

Nearly all children in our cohort responded well to ART; they hardly had clinical complaints, mounted protective vaccination responses, showed low to undetectable HIV counts in plasma, and carried near-normal blood $CD4^{+}$ T-cell counts [4,5]. Adolescents more often showed a persistent reduction in their $CD4^{+}$ T-cell numbers. Various reasons might underlie this age-associated effect, including a lack of medication adherence, waning of the T-cell compartment over time due to prolonged HIV infection [56], or the change in treatment protocols over the past 10 years (Supplemental Table 1, http://links.lww.com/QAD/A725) [57]. The late initiation of ART in children above 10 years of age was associated with significantly lower nadir $CD4^{+}$ T-cell counts than in children below 10 years, suggesting a more disrupted immune compartment in the adolescents. The near-normal $CD4^{+}$ T-cell compartment in the young children might therefore highlight a strong improvement in ART regimens in recent years and might further emphasize the importance of an early initiation of ART. Still, future longitudinal studies will be needed to address whether the waning of $CD4^{+}$ T cells over time is stably inhibited in these young children.

While the depletion of $CD4^{+}$ T cells in adolescent patients concerned all naive, memory, and effector subsets, TCM and early TEMRO cells were most severely affected. Even in adolescents with undetectable HIV for more than 1 year, $CD4^{+}$ TCM and TEMRO cells were lower than in uninfected controls, indicating that, once lost, these populations might be impossible to fully restore by ART. Loss of TCM cells was reported to correlate with rapid disease progression [58], whereas a small HIV reservoir in TCM cells and stable TCM functionality were observed in long-term nonprogressors and natural HIV controllers [59,60]. The higher numbers of TCM cells in young HIVlow patients in our study might, therefore, again indicate an important immunological improvement due to ART, suggesting that early diagnosis and treatment might be important to prevent the initial loss of these populations.

All patients aged above 2 years had increased numbers of effector memory $CD8^{+}$ T cells, which might reflect ongoing response caused by residual HIV replication or might potentially be an early sign of immunesenescence. It will be important to longitudinally follow up these $CD8^{+}$ TEMRA expansions, to address whether they indeed correlate to chronic immune activation or possibly indicate early signs of immunosenescence.

The HIV-infected children in our cohort had increased numbers of $V\delta1^{+}$ T cells and decreased $V\delta2^{+}$ T cells, extending previous $TCR\gamma\delta^{+}$ T-cell aberrations found in blood and the intestinal mucosa of HIV-infected adults [15,16,61]. The increase in $V\delta1^{+}$ T cells did not result from clonal expansion (data not shown), but could still be the result of chronic stimulation [15]. The low $V\delta2^{+}$ T-cell numbers were mainly due to a reduction in the

$V\gamma9^+V\delta2^+$ subset. On top of the depletion that had previously been described in adults [62], we here showed that in children, $V\gamma9^+V\delta2^+$ T cells showed defective repertoire selection. Because the defects in $V\gamma9^+V\delta2^+$ T cells were not restored by ART, their levels could be good biomarkers to monitor future treatment optimization. Still, longitudinal follow-up of our patients will be important to define potential clinical complications that are associated with defects in $V\gamma9^+V\delta2^+$ T cells.

In our cohort, we observed severely reduced numbers of blood $CD27^-IgA^+$ memory B cells, which are derived from T-cell-independent responses in the intestinal lamina propria [46]. Because these responses do not critically depend on $CD4^+$ T-cell help, this defect is most likely independent of any impaired $CD4^+$ T-cell responses, but likely depend on other, possibly HIV-targeted, mechanisms [63,64]. In line with the increased monocyte activation and intestinal microbial translocation observed by others [37], the persistent loss of $CD27^-IgA^+$ B cells might result from persistent intestinal complications and ongoing HIV replication. However, we cannot exclude a possible effect of the oral intake of antivirals, which might affect B-cell responses in the intestine. Studies in HIV-negative individuals receiving postexposure prophylaxis treatment might help to unravel the effect of treatment on the intestinal immune responses.

$CD27^-IgG^+$, $CD27^+IgG^+$, and $CD27^+IgA^+$ memory B-cell numbers were near-normal in children under ART. Immunoglobulin serum levels and memory responses to common vaccination antigens, such as measles, pneumococci, influenza, and tetanus antigens, are reported to be reduced in HIV-infected patients, and it is debated whether ART is able to restore these [65–68]. The normalization of these T-cell-dependent memory B-cell subsets in our patients, coinciding with a normalization of $CD4^+$ memory T cells and protective hepatitis A/B vaccination responses, suggests maintenance or recovery of T-cell-dependent humoral responses in our cohort.

We found that $CD21^{low}$ B-cell expansions correlated with plasma HIV RNA levels. We, moreover, showed that this subset was composed of both naive and memory IgM^+ B cells and IgG^+ B cells. This could explain the low replication history of the total subset. The $CD21^{low}IgG^+$ subset contained SHM, and IgG1 and IgG3 usage that was more similar to $CD27^-IgG^+$ than to $CD27^+IgG^+$ memory B cells [46,69]. Considering that $CD21^{low}$ B cells display poor response to antigen and contain high frequencies of HIV-reactive B cells [55], down-regulation of CD21 could be a mechanism for HIV to impair the host's protective immunoglobulin responses, which, we here showed for the first time, could affect both naive and memory B-cell responsiveness. The direct and stable contraction in $CD21^{low}$ B-cell numbers following declines in plasma HIV-RNA levels after cART, therefore, indicates an important beneficial effect of cART and makes it a good candidate marker for successful cART.

Altogether, our study showed the effectiveness of current ART to enable the build-up of phenotypically diverse B and T-cell memory in HIV-infected children, especially in the younger children in our study, who receive the most recent treatment protocols and in whom treatment was started significantly earlier than in adolescents. However, even with undetectable viral loads, subclinical defects in $CD4^+$ TEMRO and TCM, $CD8^+$ TEMRA, $V\delta2^+$ T cells and $CD27^-IgA^+$ memory B cells persisted, which were partially caused by defective immune maturation. Careful prospective monitoring of these persistent defects will be important for the early detection of clinical complications, ongoing virus replication or immunosenescence that might arise from these defects when these children grow older [37,56].

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D.v.d.H., M.A.B., J.J.M.v.D., and M.C.v.Z. designed the experiments; G.J.A.D., M.A.B., M.v.d.B., A.W.L., N.G.H., A.M.C.v.R., and P.L.A.F. provided conceptual advice; D.v.d.H., D.Z., and H.C. performed and analyzed most of the experiments and contributed to data analyses; G.J.A.D., N.G.H., A.M.C.v.R., and P.L.A.F. provided material necessary for performing experiments; D.v.d.H. and M.C.v.Z. wrote the manuscript; and all authors commented on the manuscript.

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Conflicts of interest

All authors declare that no competing interests exist.

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