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Highly Restricted Usage of Ig H Chain V<sub>H</sub>14 Family Gene Segments in Slp65-Deficient Pre-B Cell Leukemia in Mice

Van B. T. Ta,* Marjolein J. W. de Bruijn,* Louise Matheson,† Markus Zoller,‡ Martina P. Bach,§ Hedda Wardemann,† Hassan Jumaa,§ Anne Corcoran,† and Rudi W. Hendriks*

Mice deficient for the adapter protein Slp65 (also known as Blnk), a key component in precursor-BCR (pre-BCR) signaling, spontaneously develop pre-B cell leukemia. In these leukemias, proliferation is thought to be driven by constitutive Jak3/Stat5 signaling, mostly due to autocrine production of IL-7, together with high surface expression of the pre-BCR. In this study, we investigated whether particular IgH specificities would predispose Slp65-deficient pre-B cells to malignant transformation. Whereas V<sub>H</sub>-D-J<sub>H</sub> junctions were diverse, we found highly restricted Ig V<sub>H</sub> gene usage: 55 out of 60 (∼92%) leukemias used a V<sub>H</sub>14/SM7-family gene, mainly V<sub>H</sub>14-1 and V<sub>H</sub>14-2. When combined with surrogate or conventional L chains, these V<sub>H</sub>14 IgH chains did not provide increased proliferative signals or exhibit enhanced poly- or autoreactivity. We therefore conclude that pre-BCR specificity per se did not contribute to oncogenic transformation. Remarkably, in a high proportion of Slp65-deficient leukemias, the nonexpressed IgH allele also harbored a V<sub>H</sub>14-family rearrangement (10 out of 50) or was in the germline configuration (10 out of 50). V<sub>H</sub>14-1 and V<sub>H</sub>14-2 gene regions differed from their neighboring V<sub>H</sub> genes in that they showed active H3K4me3 histone modification marks and germline transcription at the pre-B cell stage in Rag1-deficient mice. Taken together, these findings demonstrate that in Slp65-deficient malignancy, the IgH region V<sub>H</sub>14 family is largely limited to particular pre-B cells that originate from pro-B cells that had restricted IgH V<sub>H</sub> chain accessibility at the time of V<sub>H</sub>-to-D-J<sub>H</sub> recombination.

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requires an N-linked glycosylation site in the IgH μ chain (N46), whereby binding of A5 to N46 mediates autonomous cross-linking (13). However, it cannot be excluded that pre-B cell proliferation can also be initiated or enhanced by binding of particular self-Ags. This is conceivable because pre-BCR signaling shapes the V<sub>H</sub> repertoire at the pro-B to pre-B transition (7). Furthermore, findings of interaction between the pre-BCR and galec tin-1 (14) and binding of the non-Ig tail of A5 to stromal cell-associated heparan sulfate (15) would support this notion. The pre-BCR is poly- reactive and capable of recognizing multiple (self-)Ags including DNA, LPS, and insulin, via the non-Ig tail of A5 (16). Thus, pre-BCR autoreactivity may serve to clonally expand those cells that produce a functional IgH μ chain and ensures that this selection can occur in the absence of foreign Ags. In support of this hypothesis, in SLC-deficient mice, mainly autoreactive pre-B cells are selected, resulting in the accumulation of autoreactive Abs (17). But also in the presence of SLC, more than half of the Abs in early B cell compartments of healthy individuals are polyreactive (18). It is therefore conceivable that expression of particular VH genes together with SLC results in the formation of autoreactive or polyreactive pre-BCRs that provide signals promoting tumor generation in Slp65<sup>−/−</sup> mice. This would parallel the proposed role of BCR signaling in the pathogenesis of neoplasms of mature B cells: because stereotyped clusters of particular IgH μ chains were identified in chronic lymphocytic leukemia (CLL), mantle cell lymphoma, and marginal zone B cell lymphoma, malignant transformation of B cells is thought to be driven by antigenic stimulation (19–21) or in CLL by cell-autonomous Ag-independent signaling involving an internal BCR epitope (22).

The molecular mechanisms involved in the formation of pre-B cell leukemias in Slp65<sup>−/−</sup> mice are unclear. It has been shown that Slp65 downregulates IL-7–mediated proliferation and survival through direct inhibition of Jak3, which is an essential IL-7R signaling component (23). Moreover, in leukemic pre-B cells from Slp65<sup>−/−</sup> mice, the Jak3/Stat5 signaling pathway is constitutively activated, mostly due to autocrine IL-7 production. Interestingly, haploinsufficiency of the transcription factors Pax5 or Ebf1 synergizes with constitutive Stat5 activation to rapidly induce acute lymphocytic leukemia in mice (24). Both Pax5 and Ebf1, which bind to various crucial cis-regulatory promoter and enhancer elements in the IgH locus, have been implicated in IgH locus accessibility (4, 25). Thus, mutations that contribute to oncogenic transformation of Slp65<sup>−/−</sup> pre-B cells may also effect V<sub>H</sub>-to-DJ<sub>H</sub> recombination and V gene usage.

Because pre-BCR signals in Slp65<sup>−/−</sup> leukemia cells may contribute to their strong proliferative capacity, we investigated whether particular pre-BCR specificities may predispose these cells to malignant transformation. We performed DNA sequence analysis of IgH chain V regions of 60 Slp65<sup>−/−</sup> leukemias and remarkably found highly restricted usage of the V<sub>H</sub>14/SM7 gene family.

**Materials and Methods**

**Pre-B cell leukemias from Slp65<sup>−/−</sup> mice**

Pre-B cell leukemias from Slp65<sup>−/−</sup> (8), Slp65<sup>−/−</sup>Btk<sup>−/−</sup> (10), or Slp65<sup>−/−</sup>p53<sup>−/−</sup> mice (26) were identified as previously described (8, 10). Mice were bred and maintained in the Erasmus Medical Center animal care facility under pathogen-free conditions and killed after signs of leukemia. Experimental procedures were reviewed and approved by the Erasmus Medical Center committee of animal experiments.

**PCR analysis**

The following primers were used in PCR amplifications: D-I H forward primer (27) and a reverse primer located in Igα (5′-TGAGGAGCGGTGCAGCTGAGG-3′); germline configuration was analyzed using the D<sub>H</sub>Q52 primer (28) and a J<sub>H</sub>1 primer (5′-TGGAGGACCGTGACCAGTG-3′); and PCR for V<sub>H</sub>14 rearrangements was performed with a V<sub>H</sub>14 forward primer (5′-CAGTCCTGCTCAGGGACTAA-3′) and the Igα reverse primer. Primers to amplify methylthioadenosine phosphorylase were located in exons 8 (forward: 5′-CAGGGCTAAGGGAGCAAACTC-3′; reverse: 5′-CGCCTCGACATTAACACTGGA-3′).

**DNA sequencing**

For DNA sequence analyses, DNA and cDNA samples were amplified using two high-degeneracy primers located in the framework region 1 (29) in combination with a primer located in the framework region 3 (30) together amplified using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase on an ABI PRISM 377 automated sequencer (Applied Biosysytems). All IgH chain regions were sequenced in two directions from at least two independent PCR products and analyzed by the international ImMunoGeneTics information system IMGT/V-Quest (http://www.IMGT.org).

**ELISA studies and indirect immunofluorescence assays**

Ab production and ELISA studies were essentially performed as described (18). Briefly, 293T human embryonic kidney cells (American Type Culture Collection, CRL-11268) were cultured in DMEM supplemented with 10% FCS (Invitrogen) and cotransfected with 10–15 μg IgH and IgL chain encoding plasmid DNA using polyethylenimine (Sigma-Aldrich). Cells were cultured for 4 d in DMEM supplemented with 1% Nutridoma SP (Roche). Supernatants were collected and purified on protein G Sepharose (GE Healthcare). For reactivity with specific Ags, microtiter plates (Costar Easywash Polystyrene Plates; Corning) were coated with 10 μg/ml ssDNA, dsDNA, or LPS (Sigma-Aldrich) or 5 mg/ml recombinant human insulin (Fischer). Tissue culture supernatants were used at 1 mg/ml Ab concentrations and three 1:4 dilutions in PBS. All ELISAs were developed with HRP-labeled goat anti-human IgG Fc Ab (Jackson ImmunoResearch Laboratories) and HRP Substrate (Bio-Rad). OD<sub>405</sub> was measured using a microplate reader (Molecular Devices). For indirect immunofluorescence assays, human epithelial Hep-2 cell coated slides (Bion Enterprises, CA) were incubated with purified Abs at 50–100 μg/ml at room temperature for 30 min, washed in PBS, and visualized with FITC anti-human Ig by fluorescence microscopy. Control stainings with the highly polyreactive control Ab ED38 were included in all experiments (18).

**Retroviral constructs and transductions**

Esrrogen receptor ligand-binding domain 2 (ERT2)-SLP65 (31) and SLp65 were expressed from retroviral vectors encompassing tdTomato (32) (kind gift from R.Y. Tsien, University of California, San Diego, La Jolla, CA) as an internal ribosome entry site cassette. IgH chains were expressed from retroviral vectors encompassing a fusion of the yeast GCN4 leucine zipper and a C-terminal fragment of CFP, whereas expression vectors for SLC included the leucine zipper and an N-terminal fragment of YFP. IgH chains were generated by PCR amplification of Slp65<sup>−/−</sup> tumor-derived IgV<sub>H</sub>14, D<sub>H</sub>3 regions and ligation to the murine m558L HC C region. Retroviral transductions were essentially performed as described (33).

**Cell culture and enrichment studies**

Triple-deficient (TKO) cells (31) were cultured in Iscove’s medium containing 10% heat-inactivated FCS (Vitromex). 100 U/ml penicillin, 100 U/ml streptomycin (Life Technologies), and 50 mM 2-ME. Supernatant of J558L mouse plasmacytoma cells stably transfected with a vector for murine IL-7 was added. Calcium measurements were done as described before (33). Cells were treated with 2 μM 4-hydroxymethylxanthin (OHT) and 10 μg/ml anti-IGM (Southern Biotechnology Associates). Calcium flux was measured with the LSRII (BD Biosciences).

For enrichment studies, transduced cells were cultured in Iscove’s medium supplemented with IL-7, and the proportions of CYFP-positive cells were determined by flow cytometry at days 1 and 10 after transduction. To measure cell proliferation, transduced cells (1 × 10<sup>5</sup>) were labeled with Cell Proliferation Dye eFluo670 (Biocisence) for 10 min at 37°C in the dark, and eFluo670 labeling was analyzed directly by flow cytometry. Cells were cultured in Iscove’s medium supplemented with IL-7, and eFluo670 labeling was analyzed after 4 d.

**Chromatin immunoprecipitation and sequencing and total nuclear RNA sequencing**

Nuclei were obtained from 2.5 × 10<sup>5</sup> FACS-sorted Rag2<sup>−/−</sup> pro-B cells (B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>) or C57BL/6 Hardy fraction B (CD19<sup>−</sup>CD43<sup>−</sup>BP1<sup>−</sup>) and fraction C (CD19<sup>−</sup>CD43<sup>−</sup>BP1<sup>−</sup>) lacking pro-B cells by incubation in 5 ml
buffer RIN (50 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1.5 mM MgCl2, 1 mM DTT, and 0.4% Nonidet P-40) for 5 min on ice. RNA was isolated using the Qiagen RNeasy kit and treated with Turbo DNase (Ambion). Paired-end, strand-specific RNA sequencing (RNA-seq) libraries for Illumina sequencing were generated essentially as described (34), except that polya’ RNA selection was not performed, first-strand cDNA synthesis was performed using solely random hexamer primers, and double-stranded cDNA was fragmented using a Diagenode Bioruptor (Diagenode).

Chromatin immunoprecipitation (ChIP) was performed as described (35) using an Ab against histone H3K4me3 (Ab8580; Abcam). Cross-links were reversed by incubation in 200 mM NaCl, 12.5 mM EDTA, 50 mM NaHCO3, 0.5% SDS, and 100 μg/ml proteinase K overnight at 65°C, and ChIP DNA was purified by phenol-chloroform extraction and isopropanol precipitation. Paired-end ChIP and sequencing (ChIP-seq) libraries were prepared according to standard Illumina ChIP-seq library generation protocols. Cluster generation and 36-bp paired-end sequencing of libraries were performed on an Illumina Genome Analyzer IIx (Illumina), according to the manufacturer’s instructions. Reads were mapped to the C57BL/6 mouse genome (National Center for Biotechnology Information m37) and analyzed using Seqmonk software (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/).

Results

Restricted V<sub>H</sub> repertoire of expressed IgH μ chains in Slp65<sup>−/−</sup> pre-B cell leukemias

We sequenced the expressed IgH chain V regions in a panel of pre-B cell leukemias from Slp65<sup>−/−</sup> (n = 17), Slp65<sup>−/−</sup>Btk<sup>−/−</sup> (n = 26), or Slp65<sup>−/−</sup>p53<sup>−/−</sup> (n = 17) mice. Remarkably, we found that 55 out of 60 (~ 92%) used a V<sub>H</sub>4/SM7 gene segment (Supplemental Table I, Table I), despite the small size of this V<sub>H</sub>4 family with only four members. In particular, V<sub>H</sub>14-1 and V<sub>H</sub>14-2 were used in 24 (~40%) and 19 (~32%) of the cases, respectively. This restricted V<sub>H</sub>14 usage is in stark contrast with the diverse repertoire found in normal pre-B cells expressing D<sub>i</sub> (aspartic acid and glutamate) were included.

Large CDR3 diversity and increased J<sub>H</sub>4 usage in Slp65<sup>−/−</sup> pre-B cell leukemias

Next, we analyzed the CDR3 diversity of IgH μ chains in 60 Slp65<sup>−/−</sup> tumors. The CDR3s were heterogeneous in sequence and length (Supplemental Table I). Their average length was 14.8 ± 2.1 aa (Fig. 1A), reflecting an increase compared with the reported average CDR3 length of ~12 aa in normal pre-B cells (36). The average number of N nucleotide additions was 10.2 ± 4.0 (Fig. 1B), which is higher than ~7 nt in wild-type pre-B cells (36). Hayashi et al. (37) reported that in Slp65-mutant mice, counterselection of pro-B cells expressing D<sub>i</sub> protein was abolished, resulting in similar usage of the three D gene segment reading frames (RF). However, our Slp65<sup>−/−</sup> leukemias analyzed still showed RF2 counterselection (Fig. 1C). They also showed preferential usage of J<sub>H</sub>4 (Fig. 1D), in contrast to equal J<sub>H</sub> usage normally found (36).

Table I. V<sub>H</sub> representation in Slp65<sup>−/−</sup> pre-B cell leukemias

<table>
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</table>

Data are number (%).

FIGURE 1. Characteristics of CDR3 regions of IgH μ chains in Slp65<sup>−/−</sup> leukemias. DNA sequences from expressed IgH μ chains from Slp65<sup>−/−</sup> leukemias (n = 60) were analyzed. (A) Distribution of V<sub>μ</sub>H<sub>μ</sub> CDR3 lengths. CDR3 length is in amino acids. The average CDR3 length was 14.8 aa. (B) Distribution of N nucleotide additions. For determination of N nucleotide addition, P nucleotides were not included. The average value was n = 10.2 nt. (C) D<sub>i</sub> RF usage. (D) Observed relative frequency of J<sub>H</sub> usage. (E) Proportions of IgH CDR3s with charged amino acids. Positive (arginine, lysine, and histidine) and negatively charged amino acids (aspartic acid and glutamate) were included.

The CDR3 regions showed characteristics of IgH chains of autoimmune Abs, including long CDR3 regions that were enriched in aromatic and positively charged amino acids (Fig. 1E). In most cases, there was at least one arginine or aspartate acid present (Supplemental Table I). Although arginines are abundant in CDR3 regions from autoreactive Abs, including long CDR3 regions that were enriched in aromatic and positively charged amino acids (Fig. 1E). In most cases, there was at least one arginine or aspartate acid present (Supplemental Table I). Although arginines are abundant in CDR3 regions from autoreactive Abs, in healthy mice, only ~25% of pre-B cells contain arginines in their CDR3 regions (17, 38).

Taken together, these findings indicate that CDR3s of Slp65<sup>−/−</sup> leukemias are very diverse and may have characteristics of polyreactive or autoreactive Abs.

IgH μ chains from Slp65<sup>−/−</sup> pre-B cell leukemias are not highly poly- or autoreactive

Pre-B cell proliferation is thought to be induced by binding of the SLC component A5 to N-linked sugar groups (13) and may be enhanced by binding self-Ags (14, 15). Although to date the V<sub>H</sub>14 family has not been associated with autoreactivity in rheumatoid arthritis or systemic lupus erythematosus (38, 39), it is conceivable that the IgH μ chains of Slp65<sup>−/−</sup> leukemic cells have increased pre-BCR polyreactivity or autoreactivity due to their unique CDR3 characteristics.
We cloned 10 different VH14 and 5 non-VH14 IgH \( \mu \) chains from \( \text{Slp65}^{-/-} \) leukemias and expressed these together with SLC, nonpolypotentive (murine B1-8 and human mGO53) and polypotentive (human BC62) IgL chains (40). We used ELISA and Hep-2 indirect immunofluorescence assay techniques (41) to determine polycytactivity, autoreactivity, and subcellular staining patterns of the IgH \( \mu \) chains (Supplemental Fig. 1). When combined with SLC, most IgH \( \mu \) chains showed Hep-2 reactivity and efficient binding to all Ags tested by ELISA, including ssDNA, dsDNA, LPS, and insulin (Supplemental Fig. 1, Table II), as previously found for other pre-BCRs (16). Only two \( V_{\text{H}14} \) IgH chains (PS14 and SI3) did not show Hep-2 reactivity, but we could not confirm by Western blotting whether the two pre-BCRs were completely assembled (which we could for the other 13 pre-BCRs). When combined with the B1-8, mGO53, or BC62 IgL \( \lambda \) chains, we found that the individual IgH \( \mu \) chains of \( \text{Slp65}^{-/-} \) leukemic cells behaved quite differently (Table II). Out of the 10 \( V_{\text{H}14} \) IgH \( \mu \) chains tested, 2–5 (~20–50%) were poly- or autoreactive, and out of the 5 non-VH14 IgH \( \mu \) chains, this was the case for 2–4 (40–80%) (Supplemental Fig. 1C, 1D). When combined with the nonpolypotent B1-8 and mGO53 IgL \( \lambda \) chains, the polycytactivity of the \( V_{\text{H}14} \) IgH \( \mu \) chains detected in the ELISA was limited compared with the \( V_{\text{H}14} \)-64 and \( V_{\text{H}14} \)-75 IgH \( \mu \) chains (Table II).

In summary, the IgH \( \mu \) chains of \( \text{Slp65}^{-/-} \) leukemias tested were not intrinsically strongly polycytactive or autoreactive. Similar to regular IgH \( \mu \) chains, their IgH \( \mu \) chain reactivity was influenced by the specificity of SLC or IgL \( \lambda \) chain pairing.

IgH \( \mu \) chains from \( \text{Slp65}^{-/-} \) pre-B cell leukemias do not induce enhanced proliferation

Although we did not find evidence for increased poly- or autoreactivity, the unique structural properties of \( V_{\text{H}14} \) gene segments might confer a proliferative advantage to pre-B cells expressing this particular \( V_{\text{H}4} \) domain.

To measure the signaling capacity of IgH \( \mu \) chains, we used bone marrow (BM)-derived cells from mice deficient for Rag2, A.S, and Slp65 (TKO cells) as a reconstitution system to introduce combinations of various IgH chains with SLC (16, 31). As previously described (16), we used bimolecular fluorescence-complementation expression vectors that provide detectable green fluorescence signals only in those cells that contain both constructs (Supplemental Fig. 2). Using this TKO reconstitution system, we found that levels of pre-BCR expression were comparable between IgH \( \mu \) chains using Slp65\(^{-/-}\)-leukemia-derived \( V_{\text{H14}} \) segments (S25, BS40, PS19, and PS27) or non-VH14 segments (BS72, PS11) and control non-self-reactive B1-8 or polycytactive BC62 IgH \( \mu \) chains (Fig. 2A). Notably, compared with the large amounts of BCR expressed when cells are transduced with plasmids encoding the mouse BCR (Supplemental Fig. 2B), surface pre-BCR expression is low, as a consequence of constitutive, ligand-independent pre-BCR signaling and internalization that is characteristic for pre-B cells (42). In the TKO cells, Slp65 protein with ERT2 fused to the N terminus was induced using retroviral transduction and addition of the synthetic ligand OHT, which is equally distributed between daughter cells, we found at day 4 similar mean fluorescence intensities between IgH \( \mu \) chains derived from Slp65\(^{-/-}\)-pre-B cell tumors and control \( \mu \) chains (Fig. 2D).

Finally, we combined the IgH \( \mu \) chains from \( \text{Slp65}^{-/-} \) leukemias with the non-self-reactive murine B1-8 Ig \( \lambda \) \( \lambda \) chain. We found high levels of surface BCR expression, whereby robust calcium mobilization was only detected upon OHT treatment in combination with anti-IgM stimulation. The leukemia-derived IgH \( \mu \) chains showed similar calcium flux profiles as the nonautoreactive B1-8 IgH \( \mu \) chain, and only the polycytactive BC62 control IgH \( \mu \) chain manifested ligand-independent calcium mobilization (Supplemental Fig. 2).

Therefore, in combination with either SLC or conventional IgL chain, the IgH \( \mu \) chains from \( \text{Slp65}^{-/-} \) pre-B cell leukemias did not induce enhanced proliferation compared with the nonautoreactive B1-8 IgH \( \mu \) chain.

Slp65\(^{-/-}\) pre-B cell leukemias have increased \( V_{\text{H14}} \) family gene recombination on the nonproductive IgH chain allele

Restricted \( V_{\text{H14}} \) family usage in Slp65\(^{-/-}\) pre-B cell leukemias may alternatively be explained by preferential malignant transformation of those pre-B cells that had limited IgH V region accessibility at their pro-B cell stage at the time of VH to D-JH recombination.

The \( V_{\text{H14}} \) family usage observed in Slp65\(^{-/-}\) leukemias could not be explained solely on the basis of \( V_{\text{H14}} \) localization, because \( V_{\text{H14}} \) gene segments belonging to various other families (e.g., \( V_{\text{H}3}, V_{\text{H}4}, \) and \( V_{\text{H}11} \)), which are interspersed between and adjacent to the \( V_{\text{H14}} \) gene members, were not used in Slp65\(^{-/-}\) tumors. To investigate whether Slp65\(^{-/-}\) pre-B cell leukemias had increased \( V_{\text{H14}} \) gene accessibility in pro-B cells, we determined the \( V_{\text{H}14}/D_{\text{J}H} \) gene configuration of the nonproductively rearranged IgH chain alleles. As these nonexpressed alleles do not confer a selective advantage or disadvantage to pre-B cells, their \( V_{\text{H}14}/D_{\text{J}H} \) gene configuration should reflect the accessibility of individual \( V_{\text{H14}} \) gene segments in pro-B cells.

By analysis of genomic DNA from our Slp65\(^{-/-}\) pre-B cell leukemia panel by a set of four PCR reactions, we were able to identify the configuration of the nonproductively rearranged allele in 50 of 60 tumor samples. We performed genomic PCR reactions: 1) with primers localized near DSQ2 and J\( \text{H}2 \), to identify alleles with a complete germline configuration; and 2) with primers for a D-J\( \text{H}4 \) PCR to detect alleles with D-J\( \text{H}4 \) rearrangements only in the absence of \( V_{\text{H14}} \) gene segment recombination. These analyses showed that out of 50 nonproductive alleles, 10 were in the germline configuration and 18 contained a D-J\( \text{H}4 \) rearrangement (Fig. 3A, Table III).

Next, using a \( V_{\text{H}14}/I_{\text{A}4} \) PCR to detect recombination of \( V_{\text{H}14} \) gene segments on both alleles, we observed that 8 out of 50 Slp65\(^{-/-}\) tumors contained a rearrangement with \( V_{\text{H}14} \) on both alleles (Fig. 3B, Table III). Remarkably, two pre-B cell leukemias (PS08 and PS16) expressing non-VH14 IgH chains (VH1-64 and VH4-1, respectively) had a \( V_{\text{H}14} \) segment recombination on the nonproductively rearranged allele (Fig. 3B). Using a PCR with two highly degenerate primers located in the framework 1 region (29) together with a primer located in the IgH 5' \( C_{\mu} \) region (30),
<table>
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*N Nuclear (N) or cytoplasmic (C) staining as determined in Hep2 immunofluorescence assay (see Supplemental Fig. 1A).

*Reactivity to dsDNA, ssDNA, LPS, or insulin, as determined by ELISA. In these assays, highly and low polyreactive Abs (ED38 and JB40, respectively) as well as the nonpolyreactive Ab mG053 were used as positive and negative controls (18) (see Supplemental Fig. 1B).

*Leukemias from Slp65-deficient mice (S) or from mice that were additionally deficient for Btk (BS) or P53 (PS), as detailed in Supplemental Table I.

*Not determined (Nd) (because IgH + SLC protein expression was not detectable by Western blot).

-, Negative; +, positive; ++, strongly positive.
we identified 12 pre-B cell leukemias with a rearrangement with a non-VH14 segment on the nonproductive allele (Table III).

To confirm the clonality of the amplified PCR products, we used heteroduplex analysis (43). In these assays, homoduplexes and heteroduplexes resulting from denaturation and renaturation of IgH V region PCR products were separated in nondenaturing polyacrylamide gels based on their conformation (data not shown). In 10 samples, the configuration could not unambiguously be determined (e.g., because of the presence of identical rearrangements on both alleles, chromosome loss, or limited tumor load).

In summary, the finding of increased VH14 family recombination, both at the productive (55 out of 60 VδJH genes) and the nonproductive (10 out of 22 VδDJH genes) IgH chain allele in Slp65−/− pre-B cell leukemias, indicated that these cells had a limited Vδ gene choice at their pro-B cell stage.

VH14 family usage is not increased in Btk−/− Slp65−/− or IL-7 Tg pre-B cells mice

Although Slp65 functions downstream of the pre-BCR and BCR, it is already expressed early in B cell development and may therefore be involved in regulating IgH accessibility. To investigate whether the absence of Slp65 would lead to increased VH14 family usage in nontransformed B-lineage cells, we analyzed VH family expression by quantitative RT-PCR in fractions of purified CD19+Igα Igλ pro/pre-B cells from BM from wild-type and Btk−/− Slp65−/− mice, which did not yet develop leukemia. In these experiments, we
observed comparable VH14 family usage in wild-type and Btk−/−/Slp65−/− pre-B cells (Supplemental Fig. 3A).

Because Slp65−/− pre-B cell leukemias manifest autocrine IL-7 production (23), it is conceivable that in Slp65−/− mice, only those pre-B cells are transformed that have gained autocrine IL-7 production and thus constitutive Jak/Stat5 signaling as a first event. Autocrine IL-7 production would result in increased pro-B cell proliferation and may thereby hamper V(D)J recombination, because the Rag2 protein is unstable in cycling cells (44, 45). Moreover, IL-7R signaling is also coupled to phosphatidylinositol-3-OH kinase and Akt activation, which suppresses the expression of Rag proteins as well as FoxO1, Pax5, and Ikaros (46, 47).

To directly examine whether enhanced IL-7R signaling in pro-B cells is associated with limited IgH locus accessibility, we analyzed VH gene usage in Tg mice in which overexpression of IL-7

FIGURE 3. Analyses of nonproductive alleles in Slp65−/− pre-B cell leukemias. (A) Analysis of germline and D-JH configurations of the IgH chain loci in Slp65−/− pre-B cell leukemias. Using primers localized near DQ52 and JH1 (top panel), PCR products demonstrating the presence of IgH alleles with germline configuration were seen in leukemias S19, S75, and S98. Very faint bands in other lanes (e.g., S37, S96, and S99) represent signals derived from nonleukemic cells present in tumor samples (top panel). Likewise, D1H rearrangements were analyzed using a forward primer located in the leader of the Dα segments together with a J4-specific primer, whereby Dβ2 or Dα3 rearrangements were identified in BS45, BS54, BS70, and BS72 (bottom panel). (B) PCR analyses of VH14 usage (top panel) with amplification of methylthioadenosine phosphorylase performed as a control (bottom panel).

Discussion
In this report, we show that Slp65−/− pre-B cell leukemias had highly restricted usage of VH14-family genes and preferential usage of JH4, whereas their CDR3 regions were heterogeneous in sequence and length. To date, the VH14 gene family has not been associated with autoimmunity in mice or humans, and, accordingly, we found that VH14 IgH μ chains expressed by Slp65−/− pre-B cell leukemias did not have enhanced polyreactive or autoreactive specificities. When combined with SLC in a Rag2/Slp65 reconstruction system, these VH14 IgH μ chains did not exhibit increased calcium mobilization or proliferation. Importantly, nonproductively rearranged IgH alleles in Slp65−/− pre-B cell leukemias also exhibited preferential VH14 gene segment recombination, indicating increased accessibility of VH14-family genes at the time of VH to D-JH recombination in these cells. Furthermore, in a large proportion of Slp65−/− pre-B cell leukemias, the nonexpressed IgH chain locus had retained the germline configuration, which is unusual because D to JH recombination events are generally initiated on both IgH alleles. Finally, we show that VH14-1 and VH14-2 gene regions differed from their neighboring VH genes in that they showed active H3K4me3 histone modification marks and germline transcription, indicative for enhanced accessibility, at the pro-B cell stage in Rag1−/− mice.

RESTRICTED VH USAGE IN Slp65-DEFICIENT LEUKEMIA

As local differences in VH gene accessibility are reflected by germline transcription that precedes or accompanies VH-to-DJH recombination (25), we analyzed germline transcription by nuclear RNA sequencing. We looked in Rag1−/− pro-B cells, which are arrested at a stage in which IgH chain D-to-JH gene recombination is initiated. In general, VH gene transcription is low: in contrast to 300–800 reads for every JH gene, within an ~380-kb portion of the VH region containing 27 genes, we detected no reads for most VH genes, except for VH14-1 and VH14-2 (Fig. 4A). We did not find evidence for antisense transcription over VH genes, except for a region encompassing VH14-4, VH7-3, and VH9-3. When we also included VH flanking regions, RNA sequencing indicated that sense germline transcription was restricted to VH14 family members, whereby, in particular, VH14-2 showed abundant transcription (Fig. 4B). In sorted pre-B cells from wild-type BM (Hardy fractions B and C), many of which harbor n-to-JH gene rearrangements, transcription was still most abundant close to VH14 family genes (Fig. 4B). In addition, some of the neighboring VH genes were transcribed, which might well originate from rearranged VH genes.

Transcription of lymphocyte receptor loci facilitates rearrangement in part via trimethylation of histone H3 at lysine 4 (H3K4me3), which recruits Rag2 protein (49, 50). ChIP-seq showed increased H3K4me3 at the VH14-2 region in Rag1−/− pro-B cells (Fig. 4A). Analyses of the JH regions revealed numerous H3K4me3 marks, in agreement with a recent report (51), whereby these epigenetic marks were most abundant in the JH4 region (ranging from 256 reads for JH1 to 410 reads for JH4). In summary, the VH14-1 and VH14-2 gene regions differed from their neighboring V genes in that they showed active H3K4me3 histone modification marks and germline transcription, indicative for enhanced accessibility, at the pro-B cell stage in Rag1−/− mice.

Table III. VH Representation on nonproductive alleles of Slp65−/− pre-B cell leukemias

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bility. This might be supported by the finding that IgH enhancer-promoter communication is controlled by transcription factors (TFII-I, Bright, and Oca-B) that are connected to the signaling molecules Btk and Syk (52–54). However, in RT-PCR experiments, we did not detect increased VH14 family gene usage in untransformed Slp65<sup>−/−</sup> pre-B cells, excluding the possibility that Slp65 deficiency increases VH14 gene accessibility. Second, expression of a VH14 family pre-BCR may predispose Slp65<sup>−/−</sup> pre-B cells for oncogenic transformation. This is less likely, because pre-BCRs with a VH14 family IgH<sub>m</sub> chains did not show increased proliferative signals nor exhibited enhanced poly- or autoreactivity. Even if VH14<sub>m</sub> chain expression would somehow promote transformation, this model would not explain the observed increase in VH14-family rearrangements on the nonexpressed IgH alleles in Slp65<sup>−/−</sup> pre-B cell leukemias.

We favor a third model, whereby preferential VH14 family recombination on expressed and nonexpressed IgH alleles marks a subset of Slp65<sup>−/−</sup> pre-B cells that is predisposed to malignant transformation. Thus, although leukemic cells in Slp65<sup>−/−</sup> mice manifest a pre-B cell phenotype, rare early (epi-)genetic alterations already at the pro-B cell stage would then contribute to tumorigenesis and concomitantly alter VH gene choice. The model does not implicate Slp65 as a signal transducer in pro-B cells that influences VH accessibility. This is even unlikely, because we did...
not find increased VH14 family gene usage in untransformed Slp65−/−Btk−/− pre-B cells. Gain of autocrine IL-7 production might be involved, although this would not be supported by our finding that pre-B cells from IL-7 Tg mice have normal VH4 gene usage. Nevertheless, we cannot rule out the possibility that autocrine IL-7 production does affect VH14 usage in Slp65-deficient pre-B cells, which lack direct inhibition of Jak3 by Slp65 (23). It therefore remains possible that in Slp65−/− mice, only those pre-B cells are transformed that have gained autocrine IL-7 production and constitutive Jak/Stat5 signaling as a first event. Autocrine IL-7 production would result in increased proliferation and may hamper V(D)J recombination, because the Rag2 protein is unstable in cycling cells. Moreover, IL-7R signaling is also coupled to phosphoryldinositol-3-OH kinase and Akt activation, which suppresses the expression of Rag proteins (1, 2). A restricted usage of VH14-family genes is expected when Rag2 protein levels are low, because of their active 3K4me3 histone modification marks, which recruit Rag2 protein (49, 50). Also, when pre-B cells are stimulated by IL-7 in vitro, active chromatin marks were restricted to VH14 and VH43609 (55). Thus, even in the case of constitutive IL-7R signaling, VH14-1 and VH14-2 genes may still be the most accessible VH genes. It is unknown when and how Slp65−/− leukemic pre-B cells gained autocrine IL-7 production and/or altered IgH V region accessibility. This is difficult to address experimentally, because this should be analyzed in primary Slp65−/− early B-lineage cells, because Slp65−/− leukemic pre-B cells certainly have gained additional alterations. Although it is currently a matter of debate whether IL-7R signaling directly regulates IgH V gene accessibility (55, 56), it cannot be formally excluded that in preleukemic Slp65−/− pro-B cells, constitutive Jak/Stat5 signaling would indirectly alter V(D)J recombination events at the IgH locus (e.g., by affecting cellular proliferation and survival). Taken together, it is currently unclear whether autocrine IL-7 production is connected to the selective transformation of VH14-expressing pre-B cells. Because of the selective presence of active 3K4me3 histone modification marks and germline transcription at the VH14-1 and VH14.2 gene segments in early pro-B cells, VH14 family expression may mark a subset of cells in which the IgH V region was not fully accessible for recombination. Likewise, the preferential usage of JH4 would correlate with the finding that H3K4me3 marks in pro-B cells were most abundant in the JH4 region. In this context, it is interesting that haploinsufficiency of Pax5 or Ebf1, both of which have been implicated in IgH chain locus accessibility, synergizes with constitutive Stat5 activation to rapidly induce acute lymphocytic leukemia in mice (24). Therefore, it is attractive to speculate that transformation occurs almost exclusively in rare pre-B cells that had acquired, next to constitutive IL-7R signaling, a defect in the expression of one of the transcription factors involved in the regulation of IgH chain accessibility.

Preferential usage of particular IgH VH gene families is not unique to Slp65−/− pre-B cell leukemias in mice. It has been reported for several leukemias and lymphomas in human and mice including CLL, but is thought to reflect BCR signaling-dependent selection rather than preferential V gene segment recombination (19–22). Finally, we found that random combinations of VH14 IgH μ chains (Slp65−/− pre-B cell leukemia-derived IgH chains) and the nonpolyreactive IgL chains (mouse B1-8 and human mG053) generally did not lead to poly- or autoreactive specificities (Table II). This is intriguing, because we previously found predominant autoantibody production by early human B cell precursors in the BM (18). The high proportions of autoreactive B cells generated in the BM may therefore not originate from random recombination but rather from positive selection. In such a model, Slp65−/− pre-B cell leukemia-derived VH14 IgH μ chains would then not be subject to this selection mechanism.

To the best of our knowledge, we report for the first time in this study that a restricted VH repertoire would originate from altered VH4 to D-JH gene recombination instead of BCR-mediated antigenic selection. It is conceivable that in human acute lymphoblastic leukemia, restricted VH to D-JH gene recombination may also partly contribute to restricted VH gene usage.

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Disclosures
The authors have no financial conflicts of interest.

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