Highly Restricted Usage of Ig H Chain V\textsubscript{H}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

*J Immunol* published online 12 October 2012
http://www.jimmunol.org/content/early/2012/10/12/jimmunol.1201440

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2012/10/12/jimmunol.1201440.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Highly Restricted Usage of Ig H Chain V_{H}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_{H}14-D-J_{H} junctions were diverse, we found highly restricted Ig V_{H} gene usage: 55 out of 60 (~92%) leukemias used a V_{H}14-SM7-family gene, mainly V_{H}14-1 and V_{H}14-2. When combined with surrogate or conventional L chains, these V_{H}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_{H}14-family rearrangement (10 out of 50) or was in the germline configuration (10 out of 50). V_{H}14-1 and V_{H}14-2 gene regions differed from their neighboring V_{H} genes in that they showed active H3K4me3 histone modification marks and germline transcription at the pro-B cell stage in Rag1-deficient mice. Taken together, these findings demonstrate that in Slp65-deficient mice, malignant transformation is largely limited to particular pre-B cells that originate from pro-B cells that had restricted IgH V_{H} region accessibility at the time of V_{H} to D-J_{H} recombination.

Diversity of the Ab repertoire of B lymphocytes is generated by gene recombination events at the IgH and IgL chain loci that are initiated by the lymphocyte-specific Rag1 and Rag2 proteins (1, 2). V(D)J recombination starts in prepro-B cells with D-to-J_{H} rearrangement on both IgH alleles and is followed by V_{H} to D-J_{H} rearrangement in committed pro-B cells. Productive V_{H} to D-J_{H} recombination in pro-B cells results in surface deposition of the precursor-BCR (pre-BCR) comprised of IgH \mu chain and the surrogate L chain (SLC) components A5 and VprEB (2, 3). Regulation of these events relies on developmental stage-specific epigenetic changes affecting the accessibility of V, D, and J segments in the IgH locus to the Rag proteins. They include histone modifications, DNA demethylation, germline transcription, antisense intergenic transcription, IgH locus contraction, and looping of chromatin domains (4). Although proximal V gene segments are more frequently rearranged particularly during fetal ontogeny (5, 6), random use of VH gene segments is ensured by cellular selection processes, partly based on the capacity of IgH \mu chain to pair with the SLC (7). The pre-BCR serves as an important checkpoint to monitor proper expression of functional IgH chains and triggers clonal expansion, whereby pre-B cells acquire the capacity to respond to low concentrations of IL-7. After a limited number of cell divisions, large pre-B cells stop cycling and differentiate into small, resting pre-B cells in which IgL chain rearrangement occurs (2, 3).

The adaptor protein Slp65 (also known as Blnk or Bash) and the tyrosine kinase Bruton’s tyrosine kinase (Btk) are key components in the signaling pathway downstream of the (pre)BCR. In human, mutations in SLP65 or BTK result in defective pre-B cell proliferation and an almost complete arrest of B cell development at the pro-B to pre-B cell transition, associated with the immunodeficiency disorder agammaglobulinemia (3). In contrast, mice deficient for Slp65 or Btk show only a partial arrest at the large cycling pre-B cell stage, whereas a nearly complete block is present in Btk/Slp65 double-deficient mice (8–10). The lack of Slp65 results in defective pre-BCR internalization, and at the age of 6 mo, ∼5–10% of Slp65^{−/−} mice develop pre-B cell leukemia, expressing high levels of pre-BCR on their surface (8). Btk^{−/−} mice do not develop pre-B cell tumors, but Btk cooperates with Slp65 as a tumor suppressor, independently of its kinase activity (10, 11). Although high-level pre-BCR expression is thought to contribute to their strong proliferative capacity, we previously showed that transgenic (Tg) overexpression of SLC components is not sufficient to induce leukemia (12).

The non-Ig tail of the A5 SLC component induces ligand-independent pre-BCR cross-linking, which signals for pre-B cell expansion (see Refs. 2, 3 for review). Recently, we demonstrated that functional pre-BCR formation and autonomous signaling...
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_{H} repertoire at the pro-B to pre-B transition (7). Furthermore, findings of interaction between the pre-BCR and galecin-I (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 polyreactive 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-D<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 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-Ig<sub>H</sub> forward primer (27) and a reverse primer located in J<sub>IgH</sub>4 (5′-TGGAGGACGCGTGACGACG-3′); germine configuration was analyzed using the J<sub>IgH</sub> Q52 primer (28) and a J<sub>IgH</sub> primer (5′-TGGAGGACGCGTGACGACG-3′); and PCR for V<sub>H</sub>14 rearrangements was performed with a V<sub>H</sub>14 forward primer (5′-CA-CAGCTCTGGCCTCAACA-3′) and the J<sub>IgH</sub>4 reverse primer. Primers to amplify methylthioadenosine phosphorylase were located in exon 8 (forward: 5′-CAGCCGCTAAAGGACCAATAC-3′ and reverse: 5′-CGCTCG-ACATTAACCCTGGGA-3′).

DNA sequencing

For DNA sequence analysis, DNA and cDNA samples were amplified using two high-degeneracy primers located in the framework 1 region (29) in combination with a primer located in the V<sub>H</sub> 5′ region (30). PCR products were directly sequenced using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase on an ABI PRISM 377 automated sequencer (Applied Biosystems). 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). Cultures were fed 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 μg/ml recombinant human insulin (Fitzgerald). 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>450</sub> was measured using a microplate reader (Molecular Devices). For indirect immunofluorescence assays, human epithelial Hep-2 cell coated slides (Bion Enterprises) 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

Estrogen receptor ligand-binding domain 2 (ERT2)-SLP65 (31) and SLP65 were expressed from retroviral vectors encompassing tdTomato (32) (kind gift from R. 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 CPP, 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 IgH<sub>14</sub> D-J<sub>558</sub> 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. Supernatants of J558L mouse plasmacytoma cells stably transfected with a vector for murine IL-7 were added. Calcium measurements were done as described before (33). Cells were treated with 2 μM 4-hydroxytamoxifen (OHT) and 10 μg/ml anti-IgM (Southern Biotechnology Associates). Calcium flux was measured by the LSRII (BD Biosciences).

For enrichment studies, transduced cells were cultured in Iscove’s medium supplemented with IL-7, supernatant, 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>6</sup>) were labeled with the Cell Proliferation Dye eFluor670 (eBioscience) for 10 min at 37˚C in the dark, and eFluor670 labeling was analyzed directly by flow cytometry. Cells were cultured in Iscove’s medium supplemented with IL-7 supernatant, and eFluor670 labeling was analyzed after 4 d.

Chromatin immunoprecipitation and sequencing and total nuclear RNA sequencing

Nuclei were obtained from 2.5 × 10<sup>6</sup> FACS-sorted Rag<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>TSC<sup>-</sup>) pro-B cells by incubation in 5 ml
buffer RLN (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). Cross-links were reversed by incubation in 200 mM NaCl, 12.5 mM EDTA, 50 mM NaHCO3, 0.5% SDS, and 100 μg/ml protease 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 was 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 with Seqmonk software (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/).

Results

Restricted VH repertoire of expressed IgH μ chains in Slp65−/− pre-B cell leukemias

We sequenced the expressed IgH chain V regions in a panel of pre-B cell leukemias from Slp65−/− (n = 17), Slp65−/−/Btk−/− (n = 26), or Slp65−/−/p53−/− (n = 17) mice. Remarkably, we found that 55 out of 60 (∼92%) used a VH14/SM7 gene segment (Supplemental Table I, Table I), despite the small size of this VH family with only four members. In particular, VH14-1 and VH14-2 were used in 24 (∼40%) and 19 (∼32) of the cases, respectively. This restricted VH14 usage is in stark contrast with the diverse repertoire found in large pre-B cells of normal mice, in which VH genes belonging to the VH1 (VHJ558), VH2 (VHQ52), and VH3 (VHI183) family are represented in >80% of pre-B cells (7). However, <8% of Slp65−/− leukemias used a member of the VH1, VH2, or VH3 family.

Large CDR3 diversity and increased JH4 usage in Slp65−/− pre-B cell leukemias

Next, we analyzed the CDR3 diversity of IgH μ chains expressed in 60 Slp65−/− 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 pre-B cells expressing Δμ protein was abolished, resulting in similar usage of the three D gene segment reading frames (RF). However, our Slp65−/− leukemias analyzed still displayed RF2 counterselection (Fig. 1C). They also showed preferential usage of JH4 (Fig. 1D), in contrast to equal JH usage normally found (36).

Table I. VH representation in Slp65−/− pre-B cell leukemias

<table>
<thead>
<tr>
<th>VH Gene</th>
<th>VH Representation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH14-1</td>
<td>24 (40.0)</td>
</tr>
<tr>
<td>VH14-2</td>
<td>19 (31.7)</td>
</tr>
<tr>
<td>VH14-3</td>
<td>3 (5.0)</td>
</tr>
<tr>
<td>VH14-4</td>
<td>9 (15.0)</td>
</tr>
<tr>
<td>VH1-family (1-52, 1-64, 1-75)</td>
<td>3 (5.0)</td>
</tr>
<tr>
<td>VH2-2</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>VH4-1</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
</tr>
</tbody>
</table>

Data are number (%).

FIGURE 1. Characteristics of CDR3 regions of IgH μ chains of Slp65−/− leukemias. DNA sequences from expressed IgH μ chains from Slp65−/− leukemias (n = 60) were analyzed. (A) Distribution of VHβDH 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) DH RF usage. (D) Observed relative frequency of JH 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 shared characteristics of IgH chains of 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−/− leukemias are very diverse and may have characteristics of polyreactive or autoreactive Abs.

IgH μ chains from Slp65−/− 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 λ5 to N-linked sugar groups (13) and may be enhanced by binding self-Ags (14, 15). Although to date the VH14 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−/− leukemic cells have increased pre-BCR polyreactivity or autoreactivity due to their unique CDR3 characteristics.
We cloned 10 different V_{H}14 and 5 non-V_{H}14 IgH \( \mu \) chains from Slp65\textsuperscript{−/−} leukemias and expressed these together with SLC, nonpolyreactive (murine B1-8 and human mGO53) and polyreactive (human BC62) IgL chains (40). We used ELISA and Hep-2 indirect immunofluorescence assay techniques (41) to determine polyreactivity, 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_{H}14 IgH chains (PS14 and S13) 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).

Next, to investigate whether pre-BCR expression conferred a growth advantage, the frequencies of cells with detectable green fluorescence signals at day 1 after retroviral transduction and after 10 d of culture were compared. These analyses demonstrated an increase of ~8.3 times in the control B1-8 or BC62 IgH \( \mu \) chain-expressing cell cultures and average values of ~4.6 times in the V_{H}14 or non-V_{H}14 IgH \( \mu \) chains derived from Slp65\textsuperscript{−/−} pre-B cell tumors (Fig. 2C). Thus, when combined with SLC components to form a pre-BCR, V_{H}14 or non-V_{H}14 IgH \( \mu \) chains derived from Slp65\textsuperscript{−/−} pre-B cell leukemias did not induce enhanced proliferative signals when compared with control B1-8 or BC62 IgH \( \mu \) chains. Also, when cell division was monitored by labeling cells with the cell proliferation dye eFluor670, which is equally distributed between daughter cells, we found at day 4 similar mean fluorescence intensities between IgH \( \mu \) chains derived from Slp65\textsuperscript{−/−} pre-B cell tumors and control \( \mu \) chains (Fig. 2D).

Finally, we combined the IgH \( \mu \) chains from Slp65\textsuperscript{−/−} leukemias with the non–self-reactive murine B1-8 Ig L 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 polyreactive 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 Slp65\textsuperscript{−/−} pre-B cell leukemias did not induce enhanced proliferation compared with the nonautoreactive B1-8 IgH \( \mu \) chain.

Slp65\textsuperscript{−/−} pre-B cell leukemias have increased V_{H}14 family gene recombination on the nonproductive IgH chain allele

Restricted V_{H}14 family usage in Slp65\textsuperscript{−/−} 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 V_{H} to D-IgJ recombination.

The V_{H}14 family usage observed in Slp65\textsuperscript{−/−} leukemias could not be explained solely on the basis of V_{H}14 localization, because V_{H}14 gene segments belonging to various other families (e.g., V_{H}3, V_{H}14, and V_{H}11), which are interspersed between and adjacent to the V_{H}14 gene members, were not used in Slp65\textsuperscript{−/−} tumors. To investigate whether Slp65\textsuperscript{−/−} pre-B cell leukemias had increased V_{H}14 gene accessibility in pro-B cells, we determined the V_{H}DJ_{H}4 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_{H}DJ_{H}4 gene configuration should reflect the accessibility of individual V_{H} gene segments in pro-B cells.

By analysis of genomic DNA from our Slp65\textsuperscript{−/−} 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 out of 60 tumor samples. We performed genomic PCR reactions: 1) with primers localized near DQ52 and IgJ1 to identify alleles with a complete germline configuration; and 2) with primers for a D-IgJ4 PCR to detect alleles with D-IgJ rearrangements only in the absence of V_{H}4 gene segment recombination. These analyses showed that out of 50 nonproductive alleles, 10 were in the germline configuration and 18 contained a D-IgJ rearrangement (Fig. 3A, Table III).

Next, using a V_{H}14-IgJ4 PCR to detect recombination of V_{H}14 gene segments on both alleles, we observed that 8 out of 50 Slp65\textsuperscript{−/−} tumors contained a rearrangement with V_{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_{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′ IgC region (30),
Table II. Overview of reactivity of VH14 and non-VH14 family IgH chains from Slp65-deficient leukemias

<table>
<thead>
<tr>
<th>No.</th>
<th>VH Gene</th>
<th>Leukemia</th>
<th>Hep2 C/N</th>
<th>dsDNA</th>
<th>ssDNA</th>
<th>LPS</th>
<th>Insulin</th>
<th>Hep2 C/N</th>
<th>dsDNA</th>
<th>ssDNA</th>
<th>LPS</th>
<th>Insulin</th>
<th>Hep2 C/N</th>
<th>dsDNA</th>
<th>ssDNA</th>
<th>LPS</th>
<th>Insulin</th>
<th>Hep2 C/N</th>
<th>dsDNA</th>
<th>ssDNA</th>
<th>LPS</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14-1</td>
<td>S13</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>14-1</td>
<td>BS73</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>14-1</td>
<td>BS93</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>14-1</td>
<td>PS19</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>14-1</td>
<td>PS14</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>14-2</td>
<td>S99</td>
<td>C+N</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>14-2</td>
<td>BS70</td>
<td>C+N</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>14-2</td>
<td>PS27</td>
<td>C+N</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>14-3</td>
<td>S25</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>14-3</td>
<td>BS40</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>1-52</td>
<td>BS72</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>1-64</td>
<td>PS08</td>
<td>C+N</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>1-75</td>
<td>S92</td>
<td>C+N</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>C+N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>2-2</td>
<td>PS11</td>
<td>C+N</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>4-1</td>
<td>PS16</td>
<td>C+N</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**a**Nuclear (N) or cytoplasmic (C) staining as determined in Hep2 immunofluorescence assay (see Supplemental Fig. 1A).

**b**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).

**c**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.

**d**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 VHDJH genes) and the nonproductive (10 out of 22 VHDJH genes) IgH chain allele in Slp65−/− pre-B cell leukemias, indicated that these cells had a limited VH 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+ Igk−Igl− pro/pre-B cells from BM from wild-type and Btk−/− Slp65−/− mice, which did not yet develop leukemia. In these experiments, we

**FIGURE 2.** IgH μ chains from Slp65−/− pre-B cell leukemias do not induce enhanced proliferation. Flow cytometric analysis of Slp65, λ5, Rag2 TKO cells expressing ERT2-SLP65 reconstituted with control B1-8 or BC62 IgH μ chains or with the indicated leukemia-derived IgH μ chains, all in conjunction with the λ5 SLC component. In the bottom panel, control pre-BCR–negative TKO cells transduced with empty vector are shown. (A) Expression profiles of μ H and λ5 SLC are shown as dot plots. (B) Cells were subjected to Ca2+ measurements upon addition of 2 μM OHT (+OHT) or the addition of both 2 μM OHT and 10 μM anti-IgM, as indicated. (C) Enrichment of pre-BCR–expressing cells is measured by quantification of the proportions of cYFP-positive cells in the cultures at day 1 (initial) and 10 d after transduction. The empty vector pair served as a control (bottom panel). (D) For proliferation analysis, cells were labeled with the cell proliferation dye eFluor670 and analyzed after 4 d in culture. Data are shown as histograms of gated cYFP+ cells for the individual pre-BCR–positive TKO cells (black histograms), together with those of pre-BCR–negative TKO cells transduced with empty vector (gray histograms; mean fluorescence intensity [MFI] 147). MFI values are shown.
observed comparable V_{H}14 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 V_{H} gene usage in Tg mice in which overexpression of IL-7 is driven by the mouse MHC class II Ea promoter (48). These mice show an expansion of the number of pro/pre-B cells and immature B cells in the BM (48) (Supplemental Fig. 3B). When we evaluated BM fractions of purified CD19^{+}Ig^{-}Ig^{-} pro/pre-B cells from wild-type and IL-7 Tg mice for Ig_{H} family expression by quantitative RT-PCR, we did not find evidence for increased V_{H}14 family usage in IL-7 Tg mice (Supplemental Fig. 3C).

These findings indicate that Slp65 deficiency or IL-7 overexpression does not directly affect Ig_{H} usage.

### Table III. V_{H}4 Representation on nonproductive alleles of Slp65^{-/-} pre-B cell leukemias

<table>
<thead>
<tr>
<th>Productive Allele</th>
<th>Nonproductive Allele</th>
<th>V_{H}4 Usage</th>
<th>ND</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{H}14-1</td>
<td>Germine</td>
<td>3</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>V_{H}14-2</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>V_{H}14-3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>V_{H}14-4</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Non-V_{H}14</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>18</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

V_{H}14-1 and V_{H}14-2 gene regions show active epigenetic marks and germline transcription in pre-B cells

As local differences in V_{H} gene accessibility are reflected by germline transcription that precedes or accompanies V_{H} to DJ_{H} recombination (25), we analyzed germline transcription by nuclear RNA sequencing. We looked in Rag1^{-/-} pre-B cells, which are arrested at a stage in which IgH chain n-to-J_{H} gene recombination is initiated. In general, V_{H} gene transcription is low: in contrast to 300–800 reads for every J_{H}, gene, within an ~380-kb portion of the V_{H} region containing 27 genes, we detected no reads for most V_{H} genes, except for V_{H}14-1 and V_{H}14-2 (Fig. 4A). We did not find evidence for antisense transcription over V_{H} genes, except for a region encompassing V_{H}14-4, V_{H}14-3, and V_{H}9-3. When we also included V_{H} flanking regions, RNA sequencing indicated that sense germline transcription was restricted to V_{H}14 family members, whereby, in particular, V_{H}14-2 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-J_{H} gene rearrangements, transcription was still most abundant close to V_{H}14 family genes (Fig. 4B).

### Discussion

In this report, we show that Slp65^{-/-} pre-B cell leukemias had highly restricted usage of V_{H}14-family genes and preferential usage of J_{H}, whereas their CDR3 regions were heterogeneous in sequence and length. To date, the V_{H}14 gene family has not been associated with autoimmunity in mice or humans, and, accordingly, we found that V_{H}14 IgH μ chains expressed by Slp65^{-/-} pre-B cell leukemias did not have enhanced polyreactive or autoantibody specificities. When combined with SLC in a Rag2/Slp65 reconstitution system, these V_{H}14 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 V_{H}14 gene segment recombination, indicating increased accessibility of V_{H}14-family genes at the time of V_{H} to DJ_{H} 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 J_{H} recombination events are generally initiated on both IgH alleles. Finally, we show that V_{H}14-1 and V_{H}14-2 genes differed from their neighboring V_{H} 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-deficient mice.

Several explanations for how the restricted V_{H}14-family gene usage relates or predisposes for malignant transformation of Slp65^{-/-} pre-B cells should be considered. First, Slp65-dependent signaling could be directly involved in regulating IgH accessi-
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 \(Slp65^{−/−}\) pre-B cells, excluding the possibility that \(Slp65\) deficiency increases \(VH14\) gene accessibility.

Second, expression of a \(VH14\) family pre-BCR may predispose \(Slp65^{−/−}\) pre-B cells for oncogenic transformation. This is less likely, because pre-BCRs with a \(VH14\) family IgH \(\mu\) chains did not show increased proliferative signals nor exhibited enhanced poly- or autoreactivity. Even if \(VH14\) \(\mu\) chain expression would somehow promote transformation, this model would not explain the observed increase in \(VH14\)-family rearrangements on the nonexpressed IgH alleles in \(Slp65^{−/−}\) pre-B cell leukemias.

We favor a third model, whereby preferential \(VH14\) family recombination on expressed and nonexpressed IgH alleles marks a subset of \(Slp65^{−/−}\) pre-B cells that is predisposed to malignant transformation. Thus, although leukemic cells in \(Slp65^{−/−}\) 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

---

**FIGURE 4.** Active chromatin and early transcription of \(VH14\) genes. (A) Sense and antisense nuclear transcription and H3K4me3 and H3K36Me3 active chromatin marks in the IgH locus V region encompassing the VH14 family genes. Data are from nuclear RNA-seq and ChIP-seq experiments in sorted pro-B cells from \(Rag1^{−/−}\) mice and plotted as total numbers of reads over the indicated V genes, not including up- or downstream regions (sense and antisense RNA), including 400 bp upstream flanking regions (H3K4me3) or 1000 bp upstream flanking regions (H3K36Me3). (B) Sense nuclear transcription reads in an 
~260-kb IgH locus V region encompassing the VH14 family genes. Data are from nuclear RNA-seq in sorted pro-B cells from Rag1-deficient mice (top panel) or sorted Hardy B and C fractions from wild-type mice (bottom panel) and are plotted as total numbers of reads per 500-bp intervals.
not find increased VH14 family gene usage in untransformed Slp65<sup>-/-</sup>Btk<sup>-/-</sup> 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<sup>-/-</sup> mice, only those pre-B cells 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 phosphatidylinositol-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 pro-B cells are stimulated by IL-7 in vitro, active chromatin marks were restricted to V HI and VH3609 (55). Thus, even in the case of constitutive IL-7R signaling, VH14-1 and VH14-2 genes may still be the most accessible VH4 genes. It is unknown when and how Slp65<sup>-/-</sup>-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<sup>-/-</sup>-early B-lineage cells, because Slp65<sup>-/-</sup>-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 ruled out that this may mark a subset of cells in which the IgH V region was not fully accessible for recombination. Likewise, the preferential usage of J<sub>H4</sub> would correlate with the finding that H3K4me3 marks in pro-B cells were most abundant in the J<sub>H4</sub> 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 V gene families is not unique to Slp65<sup>-/-</sup>-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 V<sub>H14</sub> IgH μ chains (Slp65<sup>-/-</sup>-pre-B cell leukemia-derived Ig H chains) and the nonpolyreactive IgL chains (mouse B1-8 and human mGO53) 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<sup>-/-</sup>-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 VH 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 V gene usage.

Acknowledgments

We thank A. Langerak, S. Middendorp, R. Kersseboom, A. de Haan (Erasmus Medical Center), and the Erasmus Medical Center animal facility for assistance at various stages of the project and Daniela Finke (University Children’s Hospital, Basel, Switzerland) for providing IL-7 Tg BM cells.

Disclosures

The authors have no financial conflicts of interest.

References


