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Tracing the Pre-B to Immature B Cell Transition in Human Leukemia Cells Reveals a Coordinated Sequence of Primary and Secondary *IGK* Gene Rearrangement, *IGK* Deletion, and *IGL* Gene Rearrangement[1]

Florian Klein,* Niklas Feldhahn,* Jana L. Mooster,* Mieke Sprangers,* Wolf-Karsten Hofmann,‡ Peter Wernet,* Maria Wartenberg,† and Markus Müschen[2]*

The BCR-ABL1 kinase expressed in acute lymphoblastic leukemia (ALL) drives malignant transformation of pre-B cells and prevents further development. We studied whether inhibition of BCR-ABL1 kinase activity using STI571 can relieve this differentiation block. STI571 treatment of leukemia patients induced expression of the Ig L chain-associated transcription factors *IRF4* and *SPIB*, up-regulation of RAG1 and RAG2, Cκ and Cλ germline transcription, and rearrangement of Ig κ L chain (*IGK*) and Ig λ L chain (*IGL*) genes. However, STI571-treated pre-B ALL cells expressed λ L, but almost no κ L chains. This could be explained by STI571-induced rearrangement of the κ-deleting element (KDE), which can delete productively rearranged Vκ-Jκ joints. Amplifying double-strand breaks at recombination signal sequences within the *IGK*, *KDE*, and *IGL* loci revealed a coordinated sequence of rearrangement events induced by STI571: recombination of *IGK* gene segments was already initiated within 1 h after STI571 treatment, followed by KDE-mediated deletion of Vκ-Jκ joints 6 h later and, ultimately, *IGL* gene rearrangement after 12 h. Consistently, up-regulation of Cκ and Cλ germline transcripts, indicating opening of *IGK* and *IGL* loci, was detected after 1 and 6 h for *IGK* and *IGL*, respectively. Continued activity of the recombination machinery induced secondary *IGK* gene rearrangements, which shifted preferential usage of upstream located Jκ- to downstream Jκ-gene segments. Thus, inhibition of BCR-ABL1 in pre-B ALL cells 1) recapitulates early B cell development, 2) directly shows that *IGK*, *KDE*, and *IGL* genes are rearranged in sequential order, and 3) provides a model for Ig L chain gene regulation in the human. *The Journal of Immunology,* **2005, 174: 367–375.**

B cell precursors within the bone marrow undergo a sequence of Ig gene rearrangements defining distinct stages of early B cell differentiation (1). During their early development, B cell precursors have to pass checkpoints at which only cells carrying functional Ig gene rearrangements are selected for further development along the B cell lineage (2). For instance, the presence of a productive Ig μ H chain (*IGH*)[3] gene rearrangement is a prerequisite for the expression of the Ig μ H chain as a component of the pre-B cell receptor on the surface of pre-B cells (PBC). As soon as a B cell precursor successfully rearranges V_H, D_H, and J_H gene segments on one allele, the recombination machinery is halted. This prevents further rearrangement of the second allele, which constitutes allelic exclusion at the *IGH* locus (3). Termination of the rearrangement process at the *IGH* locus is followed by the initiation of Ig L chain gene recombination at the Ig κ L chain (*IGK*) and Ig λ L chain (*IGL*) loci, which defines the pre-B to immature B cell transition. Human B cells express more frequently κ L than λ L chains, at a κ/λ ratio of ~1.4 (4). To explain the relative overrepresentation of *IGK* gene rearrangements, which is even more striking in mice, two models have been proposed: the "stochastic model" (5, 6) postulates that *IGK* and *IGL* loci are independently accessible to recombinase activity and that *cis*-acting regulatory elements (e.g., the 3′ κ enhancer; Refs. 7 and 8) determine the ratio of κ L and λ L chain-expressing cells. This model was supported by the finding that λ-expressing B cells could arise from mice carrying inactivated *IGK* loci on both alleles (9, 10). On the contrary, the "ordered model" proposes that *IGK* genes, as a rule, rearrange before *IGL* gene recombination (11, 12). Consistent with an ordered model of *IGK* and *IGL* gene rearrangement, recent work showed that single human λ-expressing B cells (13) or B cell precursor leukemia clones (14) harbored inactivated Vκ-Jκ gene rearrangements almost in all instances in addition to productive Vλ-Jλ joints but not vice versa: only few κ-expressing B cells or leukemic B cell clones also carried inactive Vλ-Jλ joints

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[3] Abbreviations used in this paper: IGH, Ig μ H chain; PBC, pre-B cell; IGK, Ig κ L chain; IGL, Ig λ L chain; KDE, κ-deleting element; RSS, recombination signal sequence; ALL, acute lymphoblastic leukemia; SAGE, serial analysis of gene expression; HSC, hemopoietic progenitor cell; CMP, common myeloid progenitor cell; NBC, naive B cell; GCB, germinal center B cell; MBC, memory B cell; PC, plasma cell; TLP, T lymphoid progenitor; LM-PCR, ligation-mediated PCR.

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in addition to productive Vκ-Jκ gene rearrangements. These findings argue for sequential rearrangement of *IGK* and *IGL* genes. However, these studies do not formally demonstrate that rearrangement of *IGK* genes in λ-expressing B cells do necessarily precede successful Vλ-Jλ gene recombination. In λ-expressing B cells, for instance, *IGL* genes may well be rearranged first, followed by needless Vκ-Jκ gene rearrangements, which then would subsequently be inactivated by rearrangement of the κ-deleting element (KDE). The KDE may rearrange to recombination signal sequences (RSS) flanking germline Vκ gene segments or an RSS within the intron between the Jκ cluster and the Cκ gene, which leads to inactivation of a pre-existing Vκ-Jκ joint in either case (15). Also the existence of B cells expressing κ L together with λ L chains (16) suggests that recombination events in the *IGK* and *IGL* loci are not mutually exclusive in all instances. Instead of κ L or λ L chains, human PBC express surrogate L chains composed of VpreB and λ5.

In the vast majority of acute lymphoblastic leukemia (ALL), the malignant clone represents the outgrowth of a transformed PBC. In many cases, pre-B ALL clones carry specific oncogenic gene rearrangements defining both biological and clinical subentities (17). Among these translocation events, the t(9;22)(q34;q11) results in a fusion of the *BCR* and *ABL1* genes, which codes for a constitutive active variant of the ABL1 tyrosine kinase (18) and represents the most frequent recurrent aberration leading to ALL in adults (19). *ABL1* represents the human homologue of the transforming gene of the Abelson murine leukemia virus, v-abl. Although v-abl has been used for many years to transform murine PBC, recent work showed that v-abl also confers a differentiation block at the PBC stage of development and, hence, prevents rearrangement of *IGK* or *IGL* L chain genes (20, 21). Recently, a specifically designed inhibitor of BCR-ABL1, termed STI571, has become available and is now widely used as an anti-leukemia drug for BCR-ABL1$^+$ leukemias (22). Therefore, we investigated whether and in which way inhibition of BCR-ABL1 kinase activity by STI571 might induce differentiation including *IGK* and *IGL* gene rearrangement in pre-B ALL cells.

Materials and Methods

Patient samples, primary cells, and cell lines

BCR-ABL1$^+$ pre-B ALL cells from five patients were analyzed. From three of these patients, leukemia cells were available before and during treatment with STI571 (cases III, IV, and V, Refs. 23 and 24). Samples of two other cases (cases I and II, Ref. 24) were used for serial analysis of gene expression (SAGE) analysis. Normal human CD10$^+$VpreB$^+$ PBC, CD34$^+$CD38^{low}CD133$^+$ hemopoietic progenitor cells (HSC), CD15$^+$CD34$^-$ myeloid progenitor cells, CD7$^+$CD10$^+$ T lymphoid progenitor cells (TLP) and mature B cell subsets including CD19$^+$CD27$^-$ naive B cells (NBC), CD20$^+$CD77$^+$ germinal center B cells (GCB), CD19$^+$CD27$^+$ memory B cells (MBC), and CD19^{low}CD138$^+$ plasma cells (PC) were isolated by MACS and FACS from bone marrow, umbilical cord blood, peripheral blood, or tonsils from healthy donors as previously described (25–28). For cell culture experiments, the *BCR-ABL1*$^+$ pre-B ALL cell lines BV173, NALM1, and SUP-B15 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) were used. Cell lines were treated with 10 μmol/L STI571 (Novartis) for the times indicated.

SAGE analysis

To identify differentially expressed genes between *BCR-ABL1*$^+$ pre-B ALL cells and normal PBC that play a role in Ig L chain gene regulation, we analyzed mRNA expression profiles generated by the SAGE method. A total of 592,000 SAGE tags were collected for 10 SAGE profiles. A total of 106,000 tags were analyzed from the CD34$^+$ HSC library, 99,000 for CD15$^+$ common myeloid progenitor cells (CMP), 110,000 for CD10$^+$CD19$^+$ PBC, 96,500 for CD7$^+$CD10$^+$ TLP, and each ~30,000 tags for two cases of bone marrow-derived pre-B ALL carrying a *BCR-ABL1* gene rearrangement (cases I and II), CD19$^+$CD27$^-$ NBC, CD20$^+$CD77$^+$ GCB, CD19$^+$CD27$^+$ MBC, and CD19$^+$CD138$^+$ PC (see Fig. 1*A*). All SAGE libraries were normalized to 100,000 tags. SAGE data were graphically visualized using the Cluster and Treeview software (http://rana.lbl.gov/) and sorted according to the ratio between SAGE-tag counts in PBC and in *BCR-ABL1*$^+$ ALL cases (26–28). In these SAGE profiles, 30 genes were identified which have been implicated in the regulation of Ig L chain expression. These genes were searched in the literature, as well as in UniGene (<www.ncbi.nlm.nih.gov/UniGene>) and OMIM (<www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM>) databases.

RT-PCR analysis of IRF4, SPIB, and RAG1 and RAG2 expression and germline Cκ and Cλ transcripts

mRNA levels of the Ig L chain-associated transcription factors *IRF4* and *SPIB* were measured in *BCR-ABL1*$^+$ leukemia samples from three patients (cases III, IV, and V, Ref. 24) before and during treatment with STI571 (see Fig. 1). From patient samples, total RNA was isolated and transcribed into cDNA as previously described (26–28). Amounts of cDNA were normalized by OD measurement and semiquantitative RT-PCR using 5′-TTAGCACCCCTGGCCAAG-3′ and 5′-CTTACTCCTTGGAGGCCATG-3′ for amplification of a cDNA fragment of *GAPDH*. For semiquantitative RT-PCR analysis of *IRF4* the oligonucleotides 5′-CAAGAGCAATGACTTTGAGG-3′ and 5′-TGGGACATTGGTACGGGAT-3′ were used and for *SPIB* 5′-AAGACTTACCGTTGGACAGC-3′ and 5′-CTTGGAGGAGAACTGGAAGA-3′.

To determine L chain locus germline transcription by RT-PCR, RNA was isolated from BV173, NALM1, and SUP-B15 cell lines with or without STI571 incubation, reverse-transcribed into cDNA, and normalized as described above. For amplification of Cκ germline transcripts, 5′-TTCAACAGGGGAGAGTGTTAGAG-3′ and 5′-ATGCGCCTTAGGATGACTACATA-3′ primers were used, and 5′-TGTCTGATCAGTGACTTCTACCC-3′ and 5′-CTGTAGCTTCTGTGGGACTTC-3′ primers were used for Cλ germline transcripts. RT-PCR for RAG1 and RAG2 was conducted using the oligonucleotides 5′-ATAGAAGAAAGCAACACAAAAGC-3′ and 5′-ATACTGAGTTCAATCCCTGAAGA-3′ for RAG1 and 5′-ATAGCAAGAGCTCTACACACTCC-3′ and 5′-AAAAATCAGATCAGAAATCCTCA-3′ for RAG2 (see Fig. 2*A*).

Analysis of nuclear RAG1 expression

BCR-ABL1$^+$ pre-B ALL cells (BV173 and SUP-B15) were cultured in the presence or absence of STI571 for 1, 2, or 4 h and subsequently stained with a Cy5-labeled Ab against CD19. After treatment with methanol and 4% paraformaldehyde, cells were stained with a mouse anti-human RAG1 IgG1 (BD Biosciences) together with a Cy2-labeled goat anti-mouse IgG Ab. Expression of CD19 and RAG1 was visualized by immunofluorescence (see Fig. 2*B*).

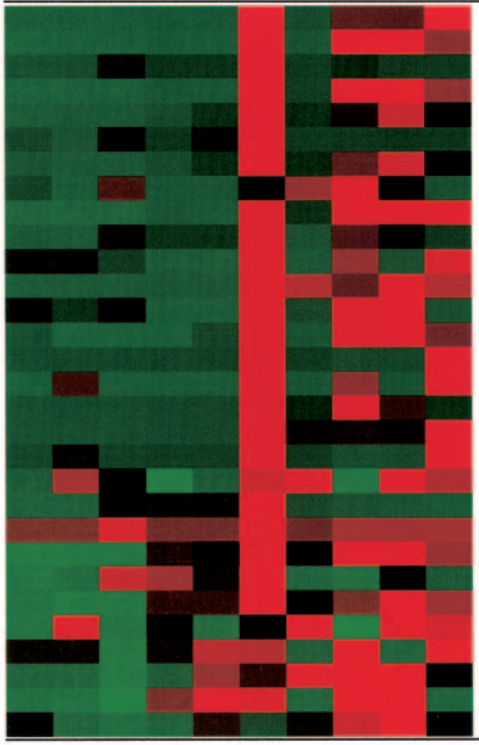
Flow cytometry

Surface expression of IGK or IGL on BV173, NALM1, and SUP-B15 pre-B ALL cells in the presence or absence of STI571 was monitored using Abs against IGK or IGL (BD Biosciences) after the incubation times indicated (see Fig. 3). Apoptotic or dead cells were identified by staining with FITC-labeled annexin V and propidium iodide (BD Biosciences) and excluded from analysis. Normal B cells were enriched from umbilical cord blood by MACS using immunomagnetic beads against CD19 (Miltenyi Biotec) and were used as a positive control for κ and λ staining (see Fig. 3).

Amplification of double-strand RSS breaks by ligation-mediated PCR

From ~2.5×10^6 untreated BV173, NALM1, and SUP-B15 cells or after treatment with 10 μmol/L STI571 after the times indicated, genomic DNA was isolated and ligated to a blunt-end linker using T4 DNA ligase (Invitrogen Life Technologies) at 14°C overnight. The linker was constructed by annealing the oligonucleotides 5′-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG-3′ and 3′ amino (C7)-GACGAGCTTAAGTTCGAAGATTGCTACATGCCCCT-5′ and protruding 3′ overhangs were removed by 3′→5′ exonuclease activity of the Klenow fragment of *Escherichia coli* DNA polymerase I (Invitrogen Life Technologies). Ligation-mediated PCR (LM-PCR; Ref. 29) was conducted with modifications as previously described (28). In two seminested rounds of amplification (35 and 45 PCR cycles at an annealing temperature of 59°C), RSS intermediates with a DNA double-strand break at the 5′ heptamer of Jκ gene segments were amplified (see Figs. 4 and 5*A*) using 5′-GTAATTAACATTCAGTCTACTTTC-3′ as external forward and 5′-TAACATTCAGTCTACTTTCTAAAA-3′ as internal forward primers together with 5′-TCCCCGTACATCGTTAGAAG-3′ as reverse primer specific for DNA-ligated linker molecules. To amplify RSS intermediates with a DNA double-strand break at the 5′ heptamer of Jλ gene segments, 5′-TTCTCAC

A



Gene	Proposed function in light chain regulation	Reference
IRF4	required for *IGK* and *IGL* gene rearrangement;	Eisenbeis *et al.*, 1995
SPIB	activates *IGK* and *IGL* transcription;	Su *et al.*, 1996
RAG2	recombination activating gene;	Schatz *et al.*, 1989
OBF1	required for recombination of some *IGK* genes;	Casellas *et al.*, 2002
BLNK	increases IGK light chain production;	Xu and Lam, 2002
RAG1	recombination activating gene;	Schatz *et al.*, 1989
BTK	increases *IGL* gene rearrangement;	Dingjan *et al.*, 2001
IRF8	required for *IGK* and *IGL* gene rearrangement;	Lu *et al.*, 2003
Igα	opens *IGK* and *IGL* loci for rearrangement;	Maki *et al.*, 2000
E2A	activates *IGK* and *IGL* gene transcription;	Kee and Murre, *1998*
RASA1	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
AP4	activates *IGK* gene transcription;	Aranburu *et al.*, 2001
KRAS2	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
PU.1	activates *IGK* and *IGL* gene transcription;	Schwarzenbach *et al.*, 1995
MEF2B	activates *IGK* and *IGL* gene transcription;	Satyaraj and Storb, 1998
CREM	activates *IGK* and *IGL* gene transcription;	Pongubala and Atchison, 1995
HRAS	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
MEF2A	activates *IGK* and *IGL* gene transcription;	Satyaraj and Storb, 1998
ATF1	activates *IGK* and *IGL* gene transcription;	Pongubala and Atchison, 1995
JUN	activates *IGK* and *IGL* gene transcription;	Schanke *et al.*,1994
TdT	N-nucleotide addition	Landau *et al.*, 1987
NRAS	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
Igβ	opens *IGK* and *IGL* loci for rearrangement;	Maki *et al.*, 2000
NF-κB2	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
REL-B	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
FOS	activates *IGK* and *IGL* gene transcription;	Schanke *et al.*,1994
REL-A	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
POLμ	required for *IGK* and *IGL* gene rearrangement;	Satyaraj and Storb, 1998
NF-κB1	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
YY1	represses *IGK* gene transcription;	Park and Atchinson, 1991

B

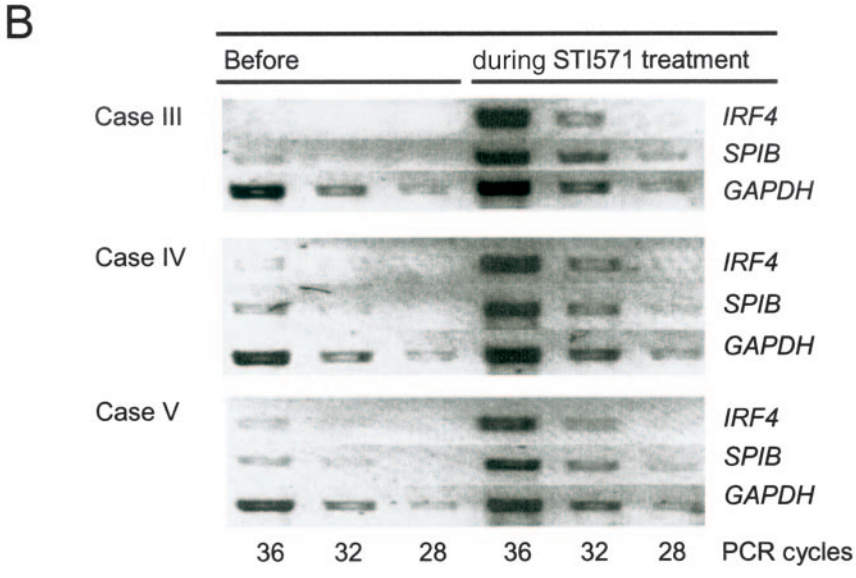


FIGURE 1. mRNA levels of Ig L chain-associated genes in *BCR-ABL1*$^+$ pre-B ALL cells. *A*, Focusing on 30 genes implicated in the regulation of Ig L chain expression, genome-wide SAGE profiles of two *BCR-ABL1*$^+$ pre-B ALL cases (ALL1 and ALL2) were compared with SAGE profiles of normal CD34$^+$CD38^{low} HSC, CD15$^+$ CMP, CD7$^+$CD10$^+$ TLP, CD10$^+$CD19$^+$ PBC, CD19$^+$CD27$^-$ NBC, CD20$^+$CD77$^+$ GCB, CD19$^+$CD27$^+$ MBC, and CD19^{low}CD138$^+$ PC. SAGE-tag counts are visualized by colors, red indicating high and green depicting low levels or no expression, respectively. SAGE data were sorted according to the ratio of SAGE-tag counts in normal B cell subsets to leukemia samples. *B*, From three patients with *BCR-ABL1*$^+$ pre-B ALL, leukemia samples were available before and during treatment with the BCR-ABL1 kinase inhibitor STI571. In these matched leukemia sample pairs, mRNA levels of *IRF4* and *SPIB* were compared by semiquantitative RT-PCR. cDNA amounts were normalized by OD measurements and amplification of a specific fragment of the housekeeping gene *GAPDH*.

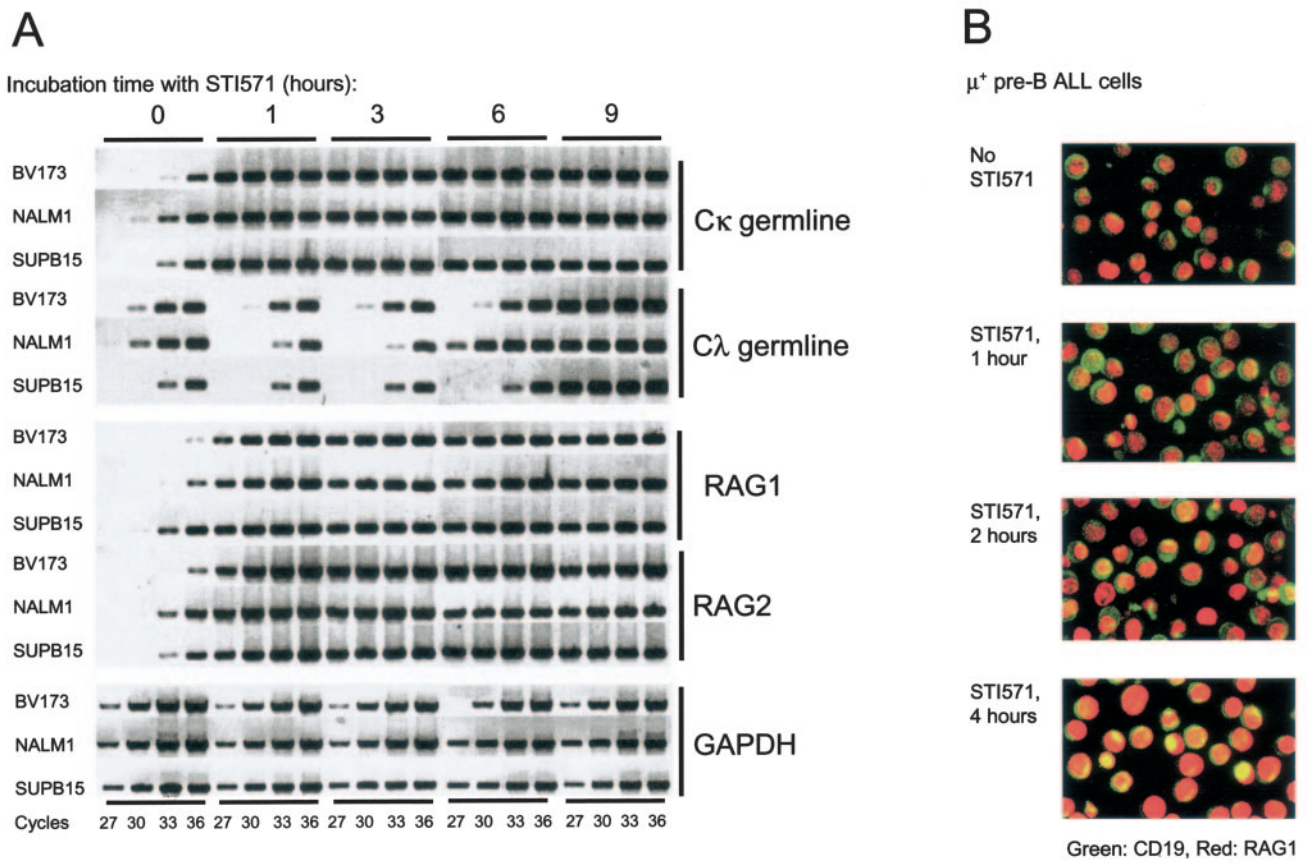


FIGURE 2. Inhibition of BCR-ABL1 by STI571 results in Cκ and Cλ germline transcription and rapid up-regulation of RAG1 and RAG2. Three *BCR-ABL1*$^+$ pre-B ALL cell lines were incubated in the presence or absence of 10 μmol/L STI571 for the times indicated and analyzed for the expression of Cκ and Cλ germline transcription, *RAG1*, *RAG2*, and *GAPDH* (control) by RT-PCR (*A*). Nuclear RAG1 expression in individual leukemia cells was measured by staining for CD19 (green) and intracellular RAG1 (red; *B*).

TTCTTCCATGGTGAC-3′ and 5′-ACTTCTTCCATGGTGACAGTCT-3′ were used in two rounds of PCR amplification as described above (see Fig. 6). Accordingly, 5′-TCCTCCTCACTGAGCCTCCCTTGAAT-3′ and 5′-CTCACTGAGCCTCCCTTGAATAGTCC-3′ were used to amplify RSS intermediates with a DNA double-strand break at the 5′ heptamer of the KDE. LM-PCR products were cloned and five randomly picked clones were sequenced showing that indeed Jκ1 RSS-linker ligation products were amplified.

Amplification of Vκ-Jκ and Vλ-Jλ gene rearrangements

Genomic DNA from 5 × 10^6 STI571-treated (24 h) or untreated BV173, NALM1, and SUP-B15 cells was isolated and subjected to two rounds of seminested PCR (35 and 45 cycles) using six Vκ and nine Vλ family-specific primers together with Jκ- and Jλ-specific primers, respectively. Primers and PCR conditions were used as previously described (30).

Analysis of the genomic configuration of the KDE

Three primers (KDE germline: 5′-CTCACTGAGCCTCCCTTGAAT AGTCC-3′; JκCκ intron: 5′-CCGCGGTTCTTTCTCGATTGAGTGG-3′; KDE external reverse: 5′-CTTCATAGACCCTTCAGGCACATGC-3′) were added to Vκ family-specific primers for amplification of Vκ-KDE rearrangements (Vκ primers and KDE external reverse primer), JκCκ intron RSS-KDE rearrangements (JκCκ intron and KDE external reverse primer), and KDE in germline configuration (KDE germline and KDE external reverse primer). One-microliter aliquots of the first rounds were used in separate second rounds for the six family-specific Vκ primers together with 5′ Jκ primers and the internal reverse KDE primer (5′-AGACAGGTCCTCA GAGGTCAGAGC-3′), and one second round with JκCκ intron and KDE internal reverse and another with KDE germline and internal reverse KDE (see Fig. 5). Vκ family-specific primers used are 5′-GACATCCRGWTGAC CCAGTCTCCWTC-3′ for Vκ1, 5′-CAGWCTCCACTCTCCCTGYC CGTCA-3′ for Vκ2, 5′-TTGTGWTGACRCAGTCTCCAGSCACC-3′ for Vκ3, 5′-AGACTCCCTGGCTGTGTCTCTGGGC-3′ for Vκ4, 5′-CAGTCT CCAGCATTCATGTCAGCGA-3′ for Vκ5, and 5′-TTTCAGTCTGTGACT CCAAAGGAGAA-3′ for Vκ6. Jκ-specific primers used are 5′-TTGATYTC CASCTTGGTCCCYTGGC-3′ for Jκ1 and Jκ2, 5′-TTGATATCCACTTT GGTCCCAGGGC-3′ for Jκ3, 5′-TTGATCTCCACCTTGGTCCCTC CGC-3′ for Jκ4, and 5′-TTAATCTCCAGTCGTGTCCCTTGGC-3′ for Jκ5.

Analysis of secondary IGK gene rearrangement

To analyze potential secondary *IGK* recombination events, we tested whether rearrangement of individual Jκ-gene segments occurs sequentially. Genomic DNA were isolated from untreated *BCR-ABL1*$^+$ pre-B ALL cells or treated with STI571 for the times indicated and subjected to two rounds of seminested PCR. 5′-GCTCAGCTCCTGGGGCTCCTGC-3′ and 5′-GACATCCRGWTGACCCAGTCTCCWTC-3′ were used as primers specific for the leader and framework region I, respectively, of Vκ1 gene segments. 5′-TTGATYTCCASCTTGGTCCCYTGGC-3′ was used for amplification of Jκ1 and Jκ2 gene rearrangements and 5′-TTAATCTC CAGTCGTGTCCCTTGGC-3′ for Jκ5.

Results and Discussion

Regulation of genes implicated in Ig L chain expression in BCR-ABL1$^+$ *leukemia cells*

Comparing genome-wide gene expression profiles from normal bone marrow PBC with two cases of *BCR-ABL1*$^+$ pre-B ALL generated by the SAGE technique, we identified 16,786 individual transcripts (unique tags) in PBC (26) and each ~9,200 in the pre-B ALL cases, respectively. To elucidate transcriptional regulation during the pre-B to immature B cell transition, we identified 182 genes for which a specific role in early B cell development is known (26). Among these genes, transcription factors such as *PAX5*, *E2A*, and *EBF* and genes coding for (pre-) B cell receptor signaling molecules (e.g., *LYN*, *BTK*, *SYK*, *FYN*) are silenced in

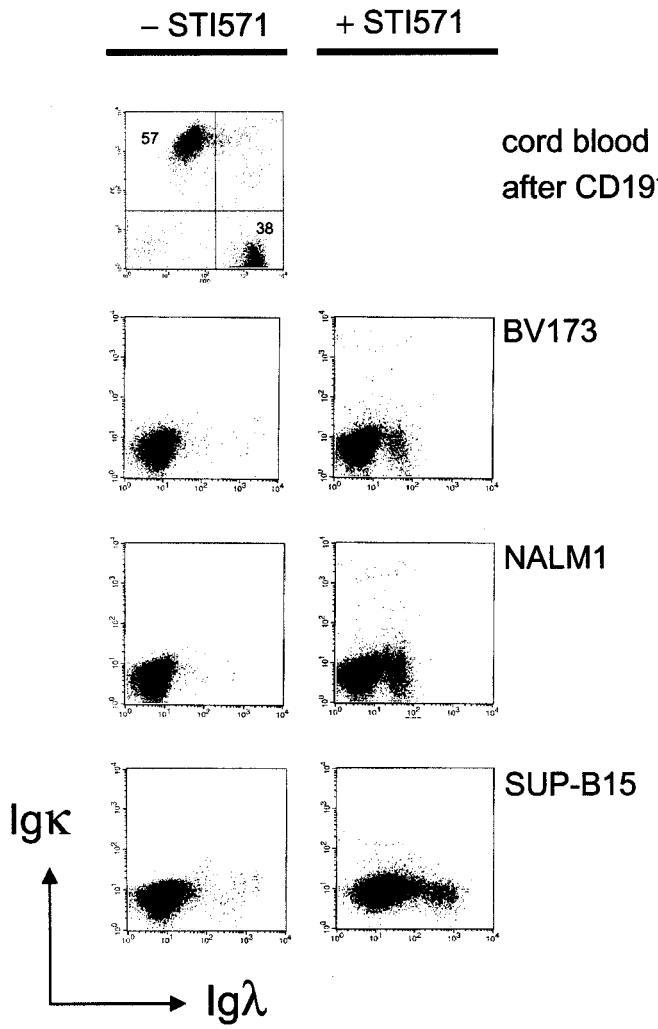


FIGURE 3. Ig L chain expression resulting from BCR-ABL1 inhibition is heavily biased for λ L chains. *BCR-ABL1*$^+$ BV173 cells, NALM1 cells, and SUP-B15 cells were cultured in the presence or absence of 10 μmol/L STI571 for 2 days and surviving cells were analyzed for surface expression of κ and λ L chains by flow cytometry. B lymphocytes from umbilical cord blood were enriched by MACS and used as positive control for κ and λ staining.

the leukemia cells (24). Furthermore, we focused on a subset of 30 genes which are specifically implicated in Ig L chain regulation (Fig. 1). Here, transcription factors that positively regulate Ig L chain gene transcription (*IRF4*, *SPIB*, *OBF1*, *IRF8*, *E2A*, *AP4*, *PU.1*, *CREM*, *MEF2*, *ATF1*, *JUN*) are down-regulated in the leukemia cells compared with normal PBC, which argues for a differentiation block at the PBC to immature B cell transition. However, besides genes implicated in Ig L chain gene rearrangement (pre-B to immature B cell transition), also genes coding for components of the pre-B cell receptor (μ-chain: 565 tags in PBC and 48 and 148 tags in leukemia cells (two cases in Fig. 1); VpreB: 278 tags in PBC and 4 and 12 tags in leukemia cells; λ5: 1,932 tags in PBC and 12 and 20 tags in leukemia cells) at the pro-B to pre-B cell transition are down-regulated in the leukemia cells. This may suggest that in the leukemia cells, expression and function of the pre-B cell receptor is impaired in general, including its specific function at the PBC to immature B cell checkpoint (2). We next investigated whether down-regulation of the Ig L chain-associated transcription factors *IRF4* and *SPIB* is indeed linked to BCR-ABL1 kinase activity. To this end, we analyzed primary *BCR-ABL1*$^+$ leukemia samples, which were derived from three patients before and during treatment with the BCR-ABL1-inhibitor STI571 (cases III, IV, V, Ref. 24, Fig. 1*B*). Comparing matched pairs of patient-derived leukemia cells, *IRF4* and *SPIB* mRNA levels were increased when BCR-ABL1 kinase activity was blocked by STI571.

Before rearrangement of Ig L chain genes can be initiated, *IGK* and *IGL* loci have to be opened to become accessible to the recombination machinery. Therefore, Cκ and Cλ germline transcription indicating transcriptional activation of Ig L chain loci was analyzed by RT-PCR in *BCR-ABL1*$^+$ cell lines treated with or without STI571 (Fig. 2*A*). Upon inhibition of BCR-ABL1, germline transcription of Cκ was up-regulated already after 1 h, followed by Cλ germline transcription after 6 h. However, low germline transcription activity at both loci could also be detected in the absence of STI571. These data suggest that *IGK* and *IGL* L chain loci become in sequential order accessible to Ig-specific transcription factors and the recombination machinery.

As constituents of the recombination machinery, the expression of recombination activation genes, *RAG1* and *RAG2*, was analyzed by RT-PCR (Fig. 2*A*). mRNA levels for both *RAG* genes were significantly up-regulated after 1 h of STI571 treatment. To investigate RAG1 expression in individual cells, RAG1 protein was stained by immunofluorescence in a leukemia cell line (SUP-B15), from which we previously amplified a productive $V_H3.53$-$D_H2.8$-J_H6 Ig H chain gene rearrangement (24), in the presence or absence of the BCR-ABL1-inhibitor STI571. Although some cells already exhibit nuclear RAG1 expression before STI571 treatment, RAG1 protein levels were visibly up-regulated after 2 h in some and after 4 h in almost all cells (Fig. 2*B*).

To determine whether up-regulation of Ig-L chain-associated transcription factors *IRF4* and *SPIB* increased expression of RAG1

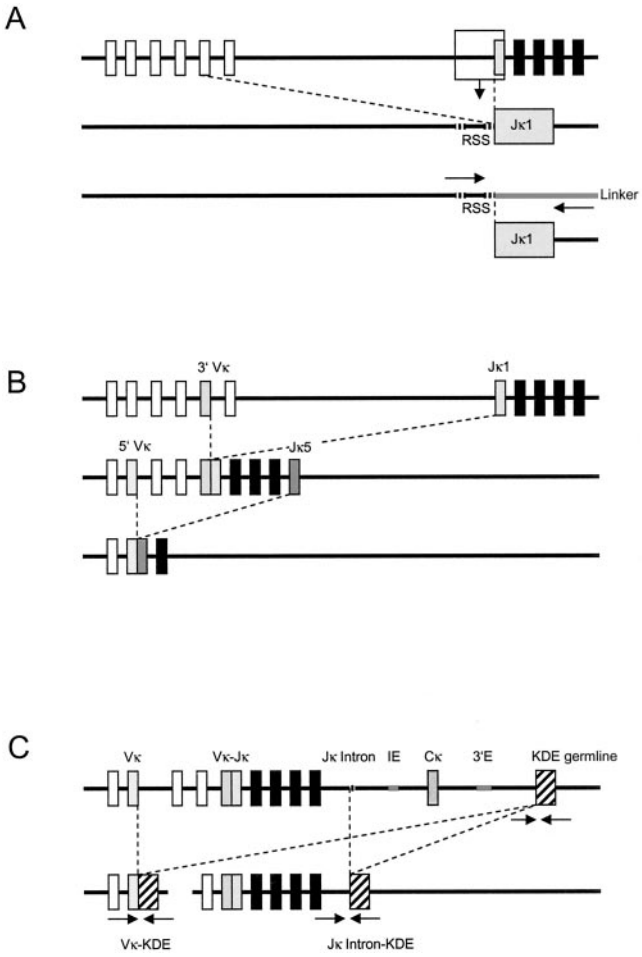


FIGURE 4. Molecular analysis of recombination events within the *IGK* locus. *A*, The rearrangement of Vκ to Jκ segments is initiated by DNA double-strand breaks at the heptamer RSS sequence (RSS) immediately flanking the Jκ segment. DNA double-strand breaks result in hairpin formation at the Jκ-break and 5′ phosphorylated blunt ends at the RSS break, which can be ligated to a blunt-ended linker molecule (Linker). Using PCR primers specific for sequences upstream of the RSS and the ligated linker sequence, broken-ended Jκ-RSS DNA intermediates can be amplified. *B*, During primary recombination events, a Vκ gene segment rearranges to a (mostly upstream located) Jκ gene segment. In secondary rearrangements, pre-existing Vκ-Jκ joints are deleted by juxtaposition of more upstream V to a more downstream J element. *C*, A preformed Vκ-Jκ joint (*top*) can be inactivated by two types of rearrangement of the KDE. Using an upstream Vκ-RSS of an unrearranged Vκ-gene segment, rearrangement of the KDE results in a large deletion within the κ locus including downstream unrearranged Vκ-gene segments, the pre-existing Vκ-Jκ joint, unrearranged Jκ-gene segments, and the Cκ gene (*bottom*, *left*). In alternative, the KDE can rearrange to an intronic RSS between the cluster of Jκ-gene segments and the Cκ gene. In this case, KDE rearrangement results in a deletion of the Cκ gene and both κ enhancers (*bottom*, *right*).

and RAG2 and whether Cκ and Cλ germline transcription is followed by the expression of conventional L chains, we analyzed three *BCR-ABL1*$^+$ pre-B ALL cell lines for κ and λ L chain expression in the presence or absence of STI571 by flow cytometry (Fig. 3). After 48 h of STI571 treatment, >50% of the cells had already undergone apoptosis and were excluded from the analysis. Apoptotic or dead cells were identified by annexin V membrane expression and uptake of propidium iodide. Among the surviving cells, ~10% exhibit de novo Ig L chain expression (Fig. 3). Unexpectedly, BCR-ABL1 inhibition resulted in a pattern of L chain expression heavily biased for λ L chains (Fig. 3). Only a few κ L chain-producing leukemia cells were detectable, while the anti-κ Ab used clearly identified normal κ-expressing B cells (Fig. 3). For control stainings, B cells from umbilical cord blood were used that were enriched by MACS for CD19 expression (Fig. 3).

Extensive KDE rearrangement results in preferential λ L chain expression on STI571-surviving leukemia cells

Using three different PCR strategies (Fig. 4), we investigated at which level the expression of κ L chains may have been impaired. By LM-PCR (Fig. 4*A*), we first showed that inhibition of BCR-ABL1 kinase activity induces DNA double-strand breaks at RSS within the *IGK* locus after 8 h (Fig. 5*A*). For validation of the LM-PCR assay, PCR products were cloned and five randomly chosen clones were sequenced. Sequence analysis confirmed Jκ1 RSS-linker ligation products (not shown). Such RSS-specific DNA strand breaks represent an initial step within the recombination process of *IGK* genes and demonstrate that the *IGK* locus is indeed targeted by the recombination machinery upon treatment with STI571. Because Jκ-RSS-specific DNA strand breaks represent a precondition for Vκ-Jκ gene rearrangements, we next amplified Vκ-Jκ rearrangements from genomic DNA of B cell precursor leukemia cells treated with or without STI571 (Fig. 4*B*). Although sporadic Vκ-Jκ and Vλ-Jλ joints were already detectable under control conditions, V-J rearrangements at both *IGK* and *IGL* loci were clearly inducible by STI571 (Fig. 5*B*). As a specificity control, genomic DNA of BCR-ABL1$^+$ chronic myeloid leukemia cells was also subjected to amplification of Vκ-Jκ and Vλ-Jλ rearrangements but no product was obtained. Integrity of DNA isolated from chronic myeloid leukemia cells was verified by amplification of a Cβ1 germline fragment of the TCRβ locus (Fig. 5*B*). In agreement with a previous study on the effect of STI571 on v-abl-transformed murine pre-B cell lines (21), we observed a polyclonal pattern of Vκ and Vλ gene rearrangement upon STI571 treatment. STI571-induced V-J gene rearrangements involved all six *IGK* and all nine *IGL* V gene families. Whether *IGK* and *IGL* genes were also rearranged at similar frequencies in response to STI571 treatment remains unclear, because the PCR approach used here was not quantitative.

Rearrangement of the KDE may lead to inactivation of productively recombined Vκ-Jκ joints and hence represents another level at which Ig L chain expression may be impaired (15). To assess whether pre-existing functional Vκ-Jκ joints may have been inactivated by rearrangement of the KDE, we analyzed both types of KDE recombination within the *IGK* locus (Fig. 4*C*). The KDE can rearrange either to an RSS site within the intron region between the cluster of Jκ gene segments and the κ intronic enhancer or to an RSS site immediately flanking a Vκ gene segment (Fig. 4*C*). KDE rearrangement leads to deletion of both κ enhancers and the Cκ gene in the former (Jκ intron RSS-KDE) or to deletion of a Vκ-Jκ joint in the latter case (Vκ RSS-KDE). To investigate whether KDE rearrangement may contribute to reduced κ L chain usage in STI571-treated leukemia cells, we amplified specific DNA fragments for KDE-germline configuration, Jκ intron RSS-KDE rearrangement, and Vκ RSS-KDE rearrangement from three *BCR-ABL1*$^+$ leukemia cell lines (BV173, NALM1, SUP-B15) in the absence or presence of STI571 (Fig. 5*C*). Although KDE was found in germline configuration in untreated leukemia cells, STI571-mediated inhibition of BCR-ABL1 induced deletion of *IGK* alleles by rearrangement of the *KDE* to Jκ intron RSS sites and to Vκ RSS sites. Targeting of the *IGK* locus by the recombination machinery and generation of Vκ-Jκ joints appears to be normal, but overrepresentation of λ L chains can be attributed to extensive KDE rearrangement observed here (Fig. 5*C*). In the

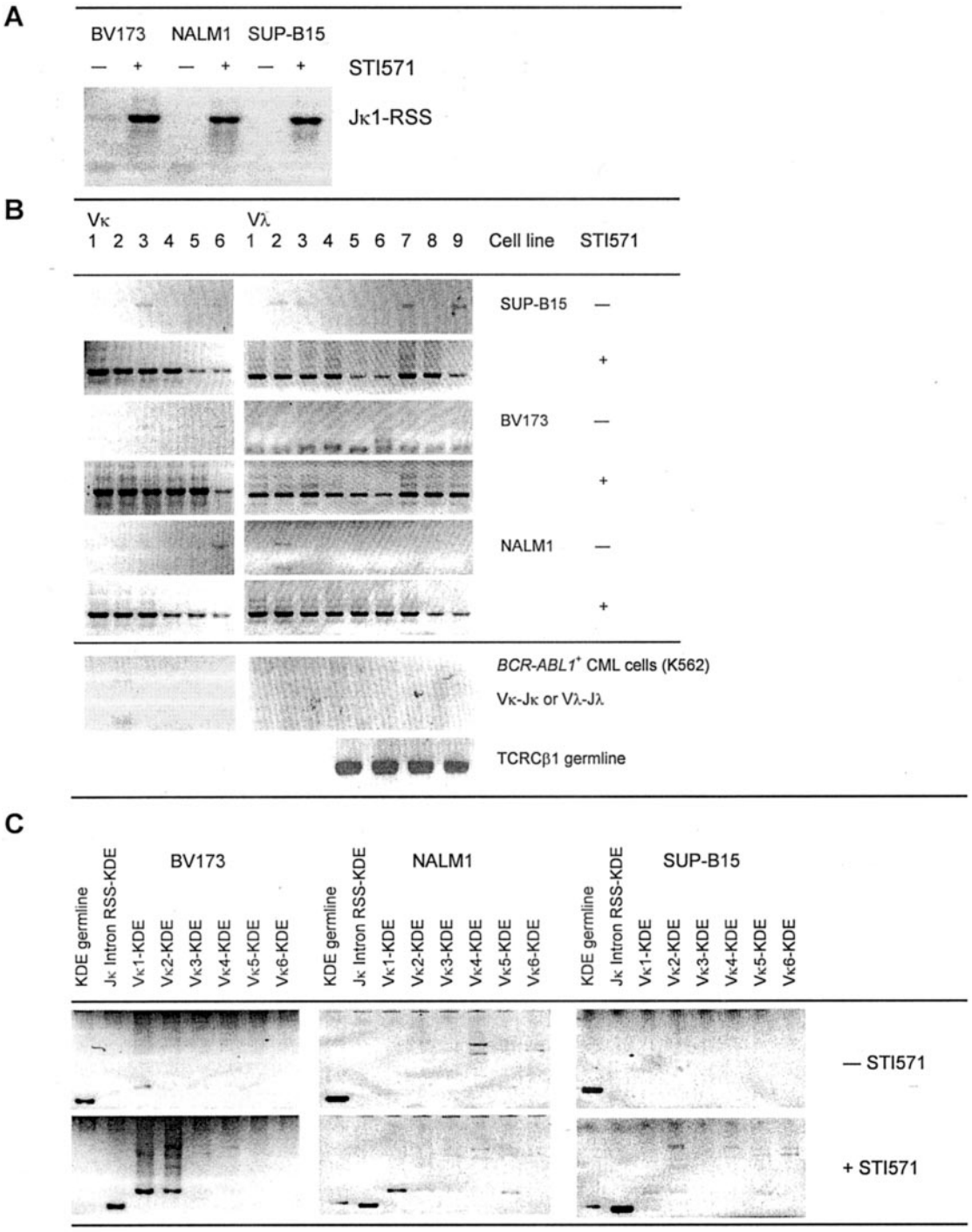


FIGURE 5. Inhibition of BCR-ABL1 causes extensive rearrangement of κ and λ L chain genes including the KDE. Using LM-PCR, we could amplify short-lived Jκ1-RSS DNA-double strand breaks predominantly in leukemia cells treated with STI571 (10 μmol/L) for 8 h, which demonstrates active recombinase activity within the *IGK* loci (*A*). We next investigated Vκ-Jκ and Vλ-Jλ gene rearrangements in STI571-treated and -untreated leukemia cells. Comparing *IGK* and *IGL* loci, there was no indication of preferential Vλ-Jλ gene rearrangement (*B*). As a specificity control, Vκ-Jκ and Vλ-Jλ joints were also amplified from *BCR-ABL1*$^+$ chronic myeloid leukemia cells (K562 cells). To control for amount and integrity of DNA used, a germline Cβ1 fragment of the TCRβ locus was amplified from K562 cells. Amplifying specific fragments of KDE germline configuration, KDE rearrangement to an RSS within the Jκ intron and KDE rearrangement to a Vκ-associated RSS, we observed that the KDE was mainly in germline configuration in untreated leukemia cells, whereas STI571 treatment induced KDE rearrangement predominantly to the Jκ intron RSS (*C*).

absence of BCR-ABL1 kinase activity, differentiating leukemia cells seem to undergo multiple rounds of rearrangement targeting both κ and λ L chain loci, which ultimately leads to preferential λ L chain expressiondue to KDE-mediated deletion of potentially productive Vκ-Jκ joints.

Rearrangements at the IGK and IGL loci occur in sequential order

For this reason, we considered STI571-induced Ig L chain gene rearrangement as a model for the pre-B to immature B cell transition in normal B cell development and investigated whether *IGK*, *KDE*, and *IGL* gene rearrangements follow a defined sequence of

events. Sequential rearrangement of *IGK*, *KDE*, and *IGL* genes would be predicted by the ordered model of L chain gene recombination. To the contrary, according to the stochastic model of L chain gene rearrangement, *IGK*, *KDE*, and *IGL* genes would be recombined independently at each locus. Therefore, we incubated the three *BCR-ABL1*$^+$ pre-B ALL cell lines in the presence or absence of STI571 and analyzed genomic DNA for RSS-specific double-strand breaks within the *IGK*, *KDE*, and *IGL* loci at different time points (Fig. 6). In the three cell lines, RSS-specific DNA double-strand breaks were already detectable at the *IGK* locus within 1 h after STI571 treatment. RSS breaks flanking the *KDE* followed after 6 h of STI571 treatment and RSS-DNA breaks were found at the *IGL* locus only after 12 h (Fig. 6). These findings are consistent with a sequential order of rearrangement events: ablation of BCR-ABL1 kinase activity almost immediately initiates *IGK* gene rearrangements, many of which are subsequently deleted by *KDE*- and ultimately replaced by *IGL*-gene rearrangements (Fig. 6). Of note, up-regulation of Cλ germline transcripts did not precede the onset of KDE RSS breaks (Figs. 2*A* and 6). Unlike normal B cell development, only very few STI571-treated leukemia cells seem to stay at a κ^+ stage, while the vast majority of cells continue to rearrange, first *KDE* and ultimately *IGL* genes. It should be noted that between 12 and 24 h of STI571 incubation, RSS breaks could be detected concomitantly in *IGK*, *KDE*, and *IGL* loci (Fig. 6). Therefore, we cannot exclude that during this period of time, *IGL* gene rearrangements may have occurred before recombination within the *IGK* locus. Stability of RSS-strand break intermediates is cell cycle-dependent and mainly found in G_1 and G_0 phases (29). Indeed, STI571 treatment induces cell cycle arrest in BCR-ABL1$^+$ B cell precursor leukemia cells (25). Thus, the detection of Jκ-RSS breaks for an extended period of time (1–24 h), partially overlapping with KDE-RSS and even Jλ-RSS breaks, can be explained by cell cycle arrest and increased stability of RSS breaks induced by STI571.

Indication for secondary IGK gene rearrangements

In contrast, the relatively wide time frame during which *IGK* gene rearrangements can occur might reflect that the leukemia cells are not all synchronized with respect to STI571-induced *IGK* gene rearrangement. This is consistent with a rather heterogenous pattern of RAG1 up-regulation following treatment with STI571 (Fig. 2). Moreover, RSS-specific DNA-strand breaks within the *IGK* locus do not necessarily reflect a single definitive Vκ-Jκ gene rearrangement and would also arise from any secondary recombination event during L chain revision (Fig. 4*B*). To determine whether ongoing κ L chain editing may extend the time window for RSS-specific DNA-strand breaks, we searched for traces of secondary rearrangements within the *IGK* locus. A pre-existing Vκ-Jκ joint can only be replaced by a rearrangement which uses an upstream-located Vκ- together with a downstream-located Jκ segment (Fig. 4*B*). Individual Vκ segments are not arranged in the order of Vκ gene families within the *IGK* locus. Therefore, we only compared the usage of upstream Jκ1 and Jκ2 segments to downstream Jκ5 elements (see Fig. 4*B*) at different time points after STI571-induced initiation of L chain gene rearrangement. Consistent with a shift from upstream to downstream Jκ elements, Jκ1 and Jκ2 gene rearrangements were detected slightly earlier than rearrangements involving the Jκ5 gene segment (Fig. 7). Given that the two round PCR approach used here for analysis of Vκ-Jκ gene rearrangements is not quantitative, the relative amount of PCR products does not necessarily reflect the usage of individual Jκ segments.

Concluding remarks

We conclude that inhibition of BCR-ABL1 initiates a coordinated sequence of Ig L chain gene rearrangement events: almost immediately after ablation of BCR-ABL1 kinase activity by STI571, the *IGK* locus opens and is targeted by the recombination machinery. The continuous generation of new Jκ RSS-specific DNA strand breaks even after 24 h together with a shift from upstream Jκ1 and Jκ2 elements to downstream Jκ5 segments suggests that initial Vκ-Jκ rearrangements are followed by at least one, perhaps multiple, round(s) of secondary rearrangements. A possible initiation

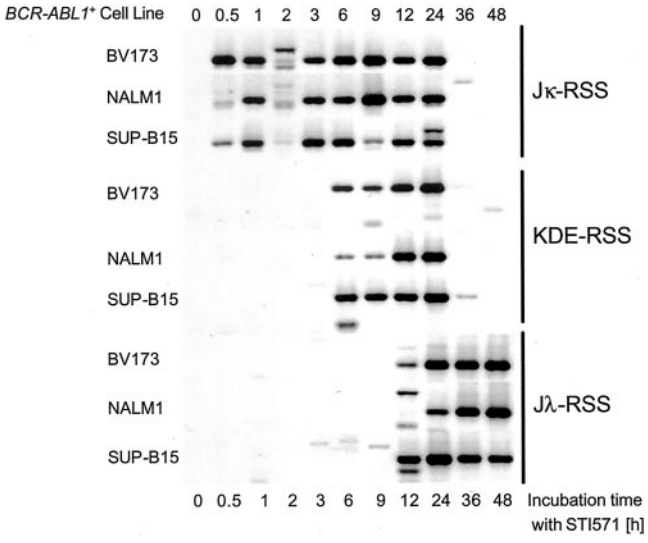


FIGURE 6. Sequentially ordered recombination events within the *IGK*, *KDE*, and *IGL* loci. *BCR-ABL1*$^+$ BV173, NALM1, and SUP-B15 cells were cultured in the presence or absence of 10 μmol/L STI571 for the times indicated. Genomic DNA was isolated from 5×10^6 cells. Broken-ended DNA strand breaks were ligated to linker molecules and subjected to two rounds of PCR amplification using primers specific for breaks at Jκ-RSS, KDE-RSS, and Jλ-RSS sites.

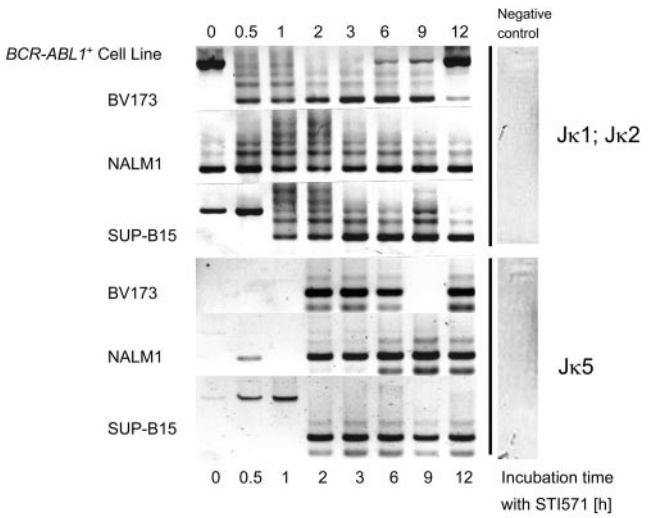


FIGURE 7. Shifting the preferential Jκ usage from upstream to downstream located Jκ gene segments indicates secondary *IGK* gene rearrangements. Vκ1 gene rearrangements were amplified from three *BCR-ABL1*$^+$ pre-B ALL cell lines using Vκ1 leader- and Vκ1 framework region I-specific primers together with either primers matching to Jκ1 and Jκ2 or primers matching to Jκ5. As controls, PCRs were performed to which water was added instead of DNA.

signal for subsequent *KDE* rearrangement could be exhaustion of the recombination potential of *IGK* alleles in multiple rounds of Vκ-Jκ rearrangement. Recombination of the *KDE* deletes Vκ-Jκ rearrangements from the chromosome and may initiate Vλ-Jλ gene rearrangements, which can first be detected after 12 h. According to these observations, it is not surprising that the vast majority of leukemia subclones that have been primed to differentiate, ultimately express λ L chains on their surface (Fig. 3). Obviously, the leukemia cells, unlike normal PBC, are lacking a feedback mechanism which prompts them to halt the recombination machinery in the presence of a productively rearranged *IGK* allele. Upon inhibition of BCR-ABL1, the leukemia cells seem to enter a pre-determined (ordered) sequence of rearrangement events, in which κ-expressing cells would only exist as a transition stage, inevitably leading to the expression of λ L chains.

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