A small molecule Abl kinase inhibitor induces differentiation of Abelson virus–transformed pre-B cell lines

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Abelson murine leukemia virus–transformed cell lines have provided a critical model system for studying the regulation of B cell development. However, transformation by v-Abl blocks B cell development, resulting in the arrest of these transformants in an early pre-B cell–like state. We report here that treatment of Abelson virus–transformed pre-B cell lines with the small molecule Abl kinase inhibitor (STI571) results in their differentiation to a late pre-B cell–like state characterized by induction of immunoglobulin (Ig) light chain gene rearrangement. DNA microarray analyses enabled us to identify two genes inhibited by v-Abl that encode the *Igk* 3′ enhancer–binding transcription factors Spi-B and IRF-4. We show that enforced expression of these two factors is sufficient to induce germline *Igk* transcription in Abelson-transformed pro-B cell lines. This suggests a key role for these factors, and perhaps for c-Abl itself, in the regulated activation of Ig light chain gene rearrangement.

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Lymphocyte development requires the somatic assembly of antigen receptor genes from their component germline gene segments by a process known as variable-diversity-joining, or V(D)J, recombination[1,2]. In pro-B cells, the immunoglobulin (Ig) heavy chain locus (*Igh*) frequently rearranges before the Ig light chain loci, with D to J_H rearrangement preceding V to DJ_H rearrangement. The result of productive *Igh* rearrangement, the μ heavy chain, is incorporated into a cell-surface signaling complex known as the pre-B cell receptor (pre-BCR). The pre-BCR signal is required for pro-B cells to mature into pre-B cells. Early large pre-B cells shut-off expression of the recombination-activating genes 1 and 2 (*Rag1* and *Rag2*), undergo several rounds of cell division and then exit the cell cycle[1,3]. These late small pre-B cells reactivate RAG expression and complete rearrangement of an Ig light chain gene, becoming surface IgM-expressing immature B cells. At the same time, pre-B cells preserve heavy chain gene allelic exclusion by ensuring no further rearrangement at the *Igh* locus. So-called germline transcription of the various Ig gene segments at specific stages of development correlates with their activation for recombination[4,5].

Fundamental insights into the sequence of events in B cell development have been provided by the study of Abelson virus–transformed pre-B cell lines[6,7]. The Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus whose oncogene encodes a fusion between retroviral gag and an N-terminally truncated form of the nonreceptor protein tyrosine kinase c-Abl. This truncation removes the SH3 domain and N-terminal cap of c-Abl, conferring constitutive tyrosine kinase activity on v-Abl[8,9]. When adult murine bone marrow or fetal liver cells are infected with A-MuLV, pro-B cells undergo transformation that enables them to proliferate in culture in the absence of their growth factor, interleukin 7 (IL-7), but fail to undergo maturation past the pro- to pre-B cell transition. Abl transformants typically display cell surface markers characteristic of the pro-B or early pre-B stage of development ($B220^+CD43^+CD25^-$). Although certain Abl transformants, such as PD31, contain a productive heavy chain gene rearrangement, they fail to express a late pre-B cell surface marker phenotype ($B220^+CD43^-CD25^+$). In addition, they infrequently rearrange their Ig light chain genes and do not go on to express surface IgM[10]. Indeed, little if any germline transcription of either the *Igk* (encoding Igκ) or *Igl* (encoding Igλ) loci can be observed in Abelson transformants[5,11]. Further evidence that such cells have not received the pre-BCR signal is their failure to maintain *Igh* allelic exclusion. Abl-transformed pre-B cells harboring a productive VDJ_H rearrangement attempt further V to DJ_H rearrangements[12]. Thus, it is possible that v-Abl expression somehow interferes with pre-BCR signaling, resulting in defective pre-B cell maturation.

Studies on a pre-B cell line, 103-bcl2, that was transformed with a temperature-sensitive A-MuLV mutant showed that v-Abl expression inhibits Ig light chain gene rearrangement *via* at least two mechanisms: down-regulation of V(D)J recombinase expression and inhibition of nuclear NF-κB DNA-binding activity[13,14]. Here, we used DNA microarrays and a newly available pharmacologic inhibitor of Abl kinase activity, STI571, to determine how v-Abl mediates a developmental block at the pro- to pre-B cell transition.

Results

Inhibition of v-Abl promotes differentiation

Although they frequently lack cytoplasmic Ig heavy chain protein, murine progenitor B cells transformed with A-MuLV resemble early large pre-B cells according to a variety of criteria, including rapid proliferation, low

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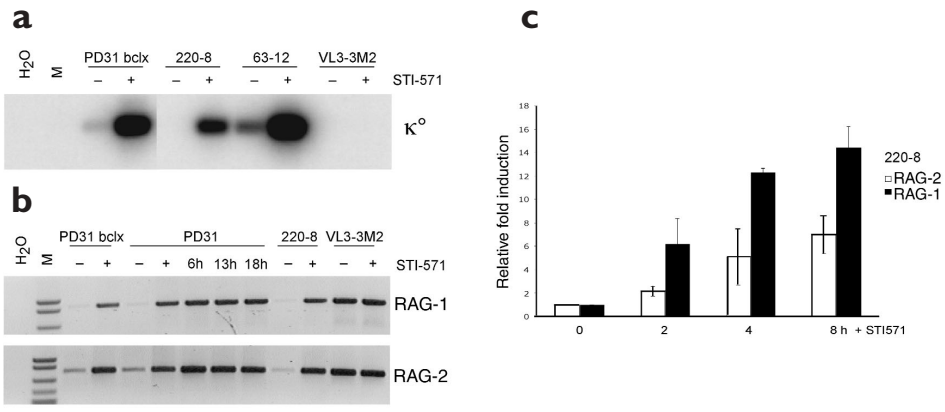

Figure 1. STI571 induces germline *Igk* and *Rag1* and *Rag2* transcription in pro-B cell lines. Equivalent amounts of total RNA from untreated (–) and STI571-treated cells, for either 12 h (+) or the indicated times, were reverse-transcribed and used as a template for PCR to detect the relative abundance of the indicated transcripts. VL3-3M2 is a T cell thymoma line; 220-8, 63-12, PD31 and PD31 bclx are all Abelson-transformed pro-B cell lines (see Methods). (**a**) Phosphorimage of a Southern blot of the RT-PCR assay for Igκ germline transcripts (κ°) probed with a transcript-specific oligonucleotide probe. (**b**) Digital photograph (negative image) of agarose gel analyses of the RT-PCR assays for RAG-1 and RAG-2 transcripts. (**c**) Real-time quantitative RT-PCR results showing RAG-1 and RAG-2 transcript induction in 220-8 cells treated with STI571 for 2, 4 and 8 h compared to untreated (0 h). RAG-1 and RAG-2 levels are normalized to HPRT transcripts.

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expression of *Rag1* and *Rag2*, minimal germline *Igk* and *Igl* locus transcription and infrequent light chain gene rearrangement. To determine whether inhibition of Abl activity could promote further differentiation of these cells, we treated a series of Abelson-transformed cell lines with the Abl kinase inhibitor STI571 and analyzed RNA and DNA from these cells for changes in gene expression and V(D)J recombination.

Treatment of the Abelson-transformed pro-B cell lines PD31, 220-8 and 63-12 with STI571 resulted in increased germline *Igk* transcription (**Fig. 1a**). Identical treatment of a transformed thymocyte cell line, VL3-3M2, did not induce this transcript, which indicated that the effect of STI571 on *Igk* transcription was limited to B-lineage cells. Similarly, treatment of these pro-B cell lines with STI571 activated transcription of both *Rag1* and *Rag2*, although there was no effect on *Rag1* and *Rag2* transcription in the T cell line (**Fig. 1b**). Using a quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) assay, we determined that in 220-8 cells, STI571 induced *Rag1* and *Rag2* transcription within 2 h and, after 8 h of drug treatment, *Rag1* and *Rag2* transcripts were up-regulated nearly 15-fold and 7-fold, respectively (**Fig. 1c**).

Given the induction of RAG-1, RAG-2 and germline κ transcripts (which correlate with recombinase accessibility) by STI571, we examined next genomic DNA purified from these cells for signs of V to J_κ gene rearrangement. The initial stages of V(D)J recombination consist of recognition and double-stranded DNA (dsDNA) cleavage at the recombination signal sequences (RSSs) flanking rearranging gene segments. We used a ligation-mediated PCR (LM-PCR) assay[15] to test DNA purified from PD31 pre-B cells cultured in the presence of STI571 for dsDNA breaks (DSBs) at the J_κ5 RSS. We observed the time-dependent accumulation of RSS breaks that correlated with the time course of induction of *Rag1* and *Rag2* transcription in these same cells (**Fig. 2a**). We also found that concentrations of STI571 between 1.25 and 10 μM were equally efficient in inducing recombinase activity at the J_κ1, J_κ5 and J_λ1-3 RSSs in 220-8 pro-B cells (**Fig. 2b**).

The pro- to pre-B cell transition is also associated with a change in the structure of chromatin at the light chain loci that makes them accessible to recognition and cleavage by the V(D)J recombinase *in vitro*[16]. To determine whether STI571 treatment can promote such changes in chromatin structure, we purified nuclei from the RAG-2$^{-/-}$ Abl-transformed cell line 63-12 cultured in the absence or presence of STI571 for 16 h. These purified nuclei were incubated *in vitro* with purified recombinant RAG-1 and RAG-2 core proteins and a fetal cow thymus nuclear extract, then genomic DNA was purified and analyzed by LM-PCR for DSBs associated with J_κ and J_λ RSSs. Nuclei purified from STI571-treated cells were cleaved by the recombinase at these RSSs, whereas the same loci remained inaccessible in untreated 63-12 cell nuclei (**Fig. 3**).

Thus, by the criteria of *Rag1*, *Rag2* and germline *Igk* loci transcription, light chain gene rearrangement and chromatin accessibility, the inhibition of Abl activity by STI571 promotes differentiation of these transformed cells to a late pre-B cell–like state.

v-Abl inhibition causes global gene expression changes

To identify the Abl-regulated genes that may have played a role in B cell development and transformation, we used Affymetrix murine oligonucleotide microarrays, which contain ~12,000 probe sets. Our experimental approach involved the comparison of RNA purified from untreated and STI571-treated (for 5 or 16 h) 220-8 and PD31 pre-B cell lines. In addition, we studied RNA from 103bcl-x7, a pro-B cell line transformed with

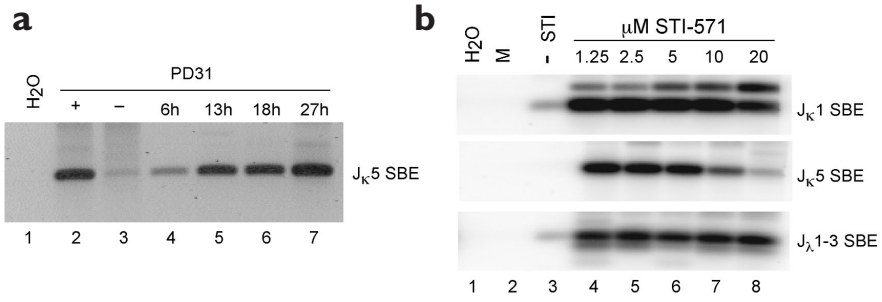

Figure 2. STI571 stimulates Ig light chain gene rearrangement. (**a**) LM-PCR assay to detect DSBs at the J_κ5 RSS. Purified genomic DNA from untreated PD31 (lane 3) and PD31 cells treated with 10 μM STI571 for the indicated times was reacted with a blunt-ended DNA linker, which ligates to broken RSS ends. The linker-ligated DNAs were then analyzed for specific RSS breaks by PCR[15]. Lane 1 is a 'no template' control reaction. The LM-PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide (EtBr). (**b**) LM-PCR assays to detect J_κ1, J_κ5 and J_λ1, 2 and 3 RSS breaks. 220-8 pro-B cells were cultured in the absence (– STI) or presence of the indicated concentrations of STI571 for 16 h. DNA was purified and analyzed for RSS breaks at the indicated light chain gene segments by LM-PCR. The phosphorimage of a Southern blot hybridized with ^{32}P-labeled locus-specific oligonucleotide probes is shown.

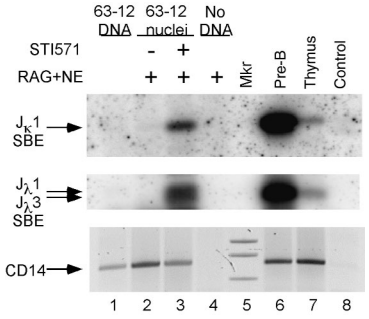

Figure 3. STI571 treatment promotes the accessibility of light chain genes to RAG-1 and RAG-2–mediated RSS cleavage *in vitro*. Nuclei were purified from the RAG-2$^{-/-}$ pro-B cell line 63-12 cultured either in the absence (lane 2) or presence (lane 3) of 10 μM STI571 for 16 h. These nuclei were then reacted with purified recombinant RAG-1 and RAG-2 and a fetal bovine thymus nuclear extract (RAG + NE)[16]. Lane 4 is a similar reaction, which lacked any DNA template. Genomic DNA was purified from these reactions and analyzed for the indicated signal broken ends (SBE) by LM-PCR[15]. LM-PCR controls included genomic DNA from 63-12 cells (lane 1), sorted primary pre-B cells or thymus and water. LM-PCR products were analyzed by electrophoresis and Southern blot hybridization to specific radiolabeled oligonucleotide probes and visualized with a PhosphorImager. (Upper) LM-PCR assay to detect Jκ1 SBE; (middle) LM-PCR assay that simultaneously detected Jκ1 and Jλ3 SBE; (lower) direct PCR amplification of *Cdl4*, a nonrearranging locus which serves as a control for DNA quality and quantity. The digital image of an EtBr-stained agarose gel is shown. Lane 4 contains DNA size markers.

a temperature-sensitive mutant of v-Abl[14]. In this cell line v-Abl kinase activity can be inhibited either with STI571 or by growth at the restrictive temperature (39 °C). Previous work suggests that the 103 cell line undergoes changes upon temperature shift, which resemble those we observed upon STI571 treatment of other Abl transformants[13,14].

In analyzing these microarray data, we focused on genes whose expression changed at least twofold as a consequence of STI571 treatment in all three cell lines as well as by the temperature shift of 103bcl-x7 (**Fig. 4** and **Web Tables 1** and **2** online). As expected, microarray analyses identified *Rag1*, *Rag2* and *Igk-C* (germline κ transcript, Ig-Cκ) among the genes induced in each cell line when Abl is inhibited. Some of the genes we identified as being induced by Abl inhibition were independently identified as also being pre-B cell–specific transcripts[17]. These genes might encode factors important in pre-B cell differentiation. However, the previous analysis failed to identify certain genes. For example, CD25 (also known as IL-2Rα) has been described as a pre-B cell–specific cell surface marker[18,19], but was not identified in the previous study[17]. Our analysis showed that Abl-transformed pro-B cells are CD25$^-$ but become CD25$^+$ upon STI571 treatment (**Fig. 4** and **Web Fig. 1** online). CD62 ligand (CD62L, also known as L-selectin), a cell surface marker not typically used to phenotype bone marrow B lymphocytes, is also induced upon STI571 treatment (**Web Fig. 1** online). *Spib* and *Irf4* encode transcription factors that can partner each other to bind to the *Igk* locus 3′ enhancer (Eκ3′) and the *Igl* locus enhancers (Eλ1-3 and Eλ2-4). We investigated these genes further (see below).

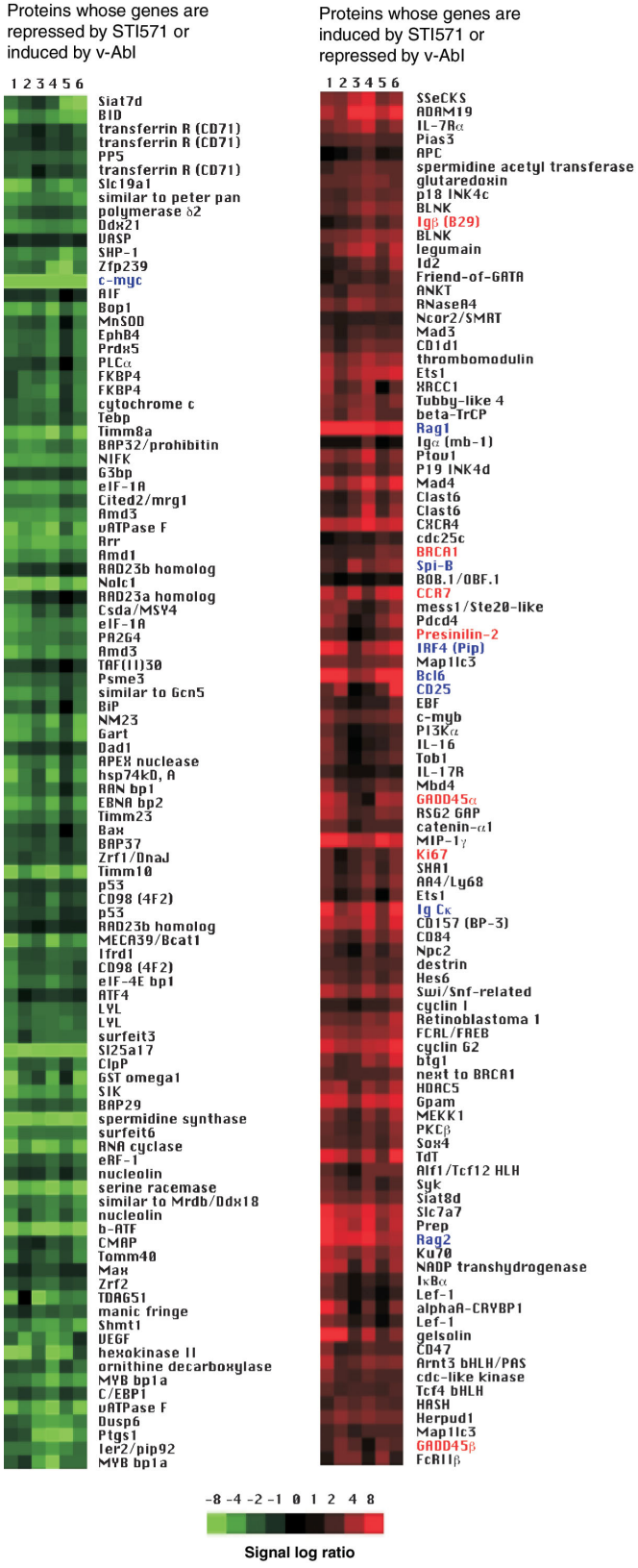

Figure 4. Results of an Affymetrix microarray screen for genes regulated by STI571. Each column is a single Affymetrix GeneChip experiment compared to the respective baseline control (that is, untreated cells of each type). Each row represents data (signal log ratio values) for a given probe set. Intensity of squares reflects the fold-repression (green) or fold-induction (red) after STI571 treatment or a temperature shift to 39 °C (lane 1), according to the ratio color scale at the bottom (where black indicates no significant change in gene expression). Data from 100 representative probe sets from each category of candidate target genes (repressed or induced) were compiled and clustered hierarchically with Cluster, and the resulting figure was generated with TreeView. Lanes 1 and 2, 103bcl-x7 grown at 39 °C for 12 h or 33 °C in the presence of 10 μM STI571 respectively compared to untreated 103bcl-x7 grown at 33 °C without drug; lanes 3 and 4, PD31 treated with 10 μM STI571 for 5 or 16 h, respectively, compared to untreated PD31; lanes 5 and 6, 220-8 treated with 10 μM STI571 for 5 or 16 h, respectively, compared to untreated 220-8. Gene names printed in blue are discussed in the text. Gene names in red were identified in an Affymetrix microarray analysis of developmental fractions of bone marrow B cells as being transcriptionally activated in pre-B cells[17].

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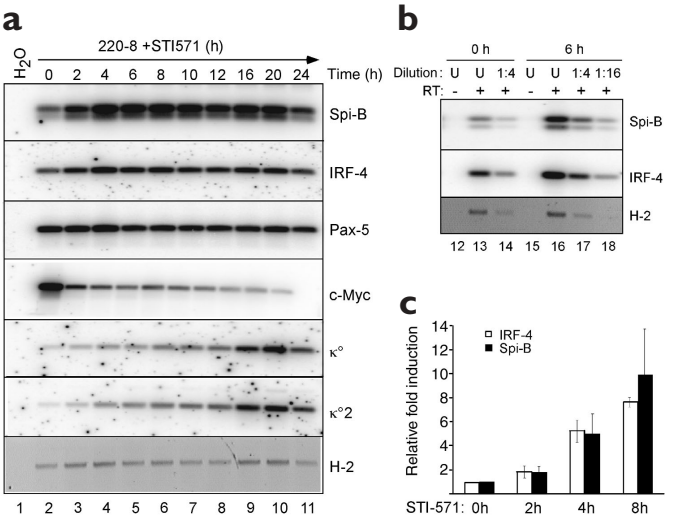

Figure 5. Analysis of transcripts in 220-8 pro-B cells during a time-course of STI571 treatment. (**a**,**b**) RT-PCR analyses of the indicated transcripts. Equivalent amounts of total RNA from untreated and STI571-treated 220-8 cells were reverse-transcribed and used as template for PCR to detect the abundance of the following transcripts: Spi-B, IRF-4, Pax-5, c-Myc, κ° and κ°2 Igκ germline transcripts (κ° denotes short and κ°2 denotes long germline κ transcripts) and H-2 (an MHC class I transcript). The PCR products were analyzed by agarose gel electrophoresis and visualized by EtBr staining in the case of H-2 and by Southern blot hybridization with specific oligonucleotide probes in the case of the others. Lane 1 is a 'no template' PCR control; lanes 2 and 13 are products of cDNA prepared from untreated 220-8; lanes 3–11 and 16 are samples treated with 10 μM STI571 for the indicated number of hours. (**b**) Template dilution assays demonstrate the semi-quantitative nature of the assays for Spi-B, IRF-4 and H-2. Lane 14 used a 1:4 dilution of the cDNA from lane 13 (same as lane 2). Similarly, lanes 17 and 18 used a 1:4 and 1:16 dilution, respectively, of the cDNA from lane 16 (same as lane 5). In addition, minus-RT (RT step was omitted) controls were provided to demonstrate that the respective assays are specific for the indicated transcript (lanes 12 and 15 and data not shown). U, undiluted. (**c**) Graphical representation of real-time quantitative RT-PCR (Taqman) results showing Spi-B and IRF-4 transcript induction in 220-8 cells treated with STI571 for 2, 4 and 8 h compared to untreated cells (0 h). Spi-B and IRF-4 levels are normalized to HPRT transcripts.

Other genes that we found are induced by STI571 treatment (or repressed by v-Abl expression) included *Mad3*, *Mad4*, *Bcl6*, *Sox4*, *Lef1* *Gadd45a* and *Gadd45b* (**Web Table 2** online). Syk, Igα, Igβ and BLNK are crucial components of the pre-BCR signaling pathway[2] and their down-regulation by v-Abl may explain how v-Abl antagonizes pre-BCR signaling. Inhibition of pre-BCR signaling may also explain why Abl-transformed pre-B cells show no allelic exclusion of heavy chain gene rearrangement[12]. Finally, tumor suppressor genes encoding Ku70, BRCA1 and Rb may be transcriptionally repressed and contribute to transformation by the oncogenic v-Abl.

Conversely, among the genes that are repressed by STI571 treatment (induced by v-Abl) are the proto-oncogenes *Myc*[20], *Nmyc1* and *Lyl1* (**Web Table 1** online). The genes in this category may be factors that contribute to cellular transformation and inhibition of differentiation. *Nmyc1* and *Myc* are also regulated by the critical lymphoid progenitor growth factor IL-7 in cultured primary pro-B cells[21], and thus similarities can be found between Abl and IL-7 signaling.

Understanding *Igk* activation through Abl inhibition

As noted above, our microarray studies showed that Spi-B and IRF-4 (also called Pip or LSIRF)—transcription factors of the Ets and IRF families, respectively—were induced by the inhibition of Abl activity. Although these factors can bind DNA independently of one another, they can bind synergistically to an Ets-IRF composite binding site (EICE), a well studied example of which exists in Eκ3′, the *Igk* locus 3′ enhancer[22]. In addition, Spi-B and IRF-4 bind to sites in the two *Igl* enhancers[22]. *In vivo* footprinting studies have shown that the Eκ3′ EICE becomes occupied as bone marrow B cell progenitors progress from the pro-B to the pre-B stage of development[23]. Thus, we tested here the hypothesis that induction of Spi-B and IRF-4 activates the germline κ transcription and recombinase accessibility observed in STI571-treated pre-B cell lines.

We used RT-PCR to compare the time course of activation of *Spib*, *Irf4* and two distinct germline κ transcripts in STI571-treated pre-B cells (**Fig. 5a,b**). We found that *Spib* and *Irf4* were coordinately regulated and their activation preceded that of the germline κ locus. These data were confirmed by real-time PCR analyses, which showed an eight- to tenfold induction of these transcripts within 8 h of STI571 treatment (**Fig. 5c**). To test whether this correlation might indicate causality, we stably transfected 220-8 tTA10—a derivative of the 220-8 pre-B cell line, which expresses the tetracycline-repressible transcriptional activator[24]—with a tetracycline-regulated expression vector containing Spi-B and IRF-4 cDNAs separated by an IRES sequence to allow their translation from a single bi-cistronic mRNA. Removal of tetracycline from the growth media resulted in the subsequent activation of endogenous germline κ transcription after 36 h, but had no effect on *Myc* transcription (**Fig. 6a**). Germline κ transcription coincided with accumulation of Spi-B and IRF-4, which also occurred by 36 h of tetracyline withdrawal (**Fig. 6b**). Thus, we concluded that the repression of Spi-B and IRF-4 by v-Abl may be responsible for the lack of κ locus transcription and recombinase accessibility in transformed pre-B cell lines and perhaps during B cell development as well.

To test whether the induction of Spi-B and IRF-4 may be responsible for developmentally regulated activation of the *Igk* locus during primary B cell

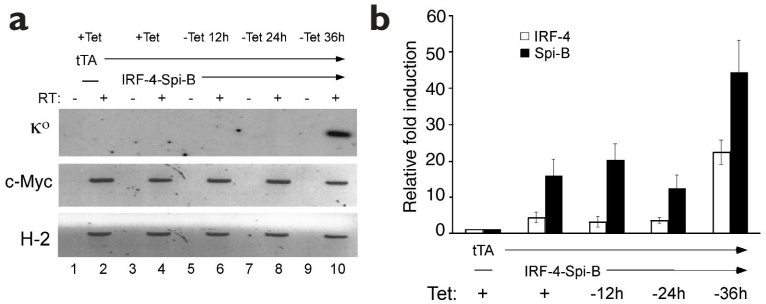

Figure 6. Overexpression of *Spib* and *Irf4* activates *Igk* germline transcription. (**a**) RT-PCR analysis of Igκ germline (κ°), c-Myc and H-2 transcripts in 220-8 cells transfected with a tetracycline-regulated IRF-4-IRES-Spi-B expression vector. Odd numbered lanes contain 'minus-RT' control reactions. Lane 2, the parental 220-8 tTA.10 cell line expressing the tetracycline-regulated transactivator (Tet-off); lanes 4, 6, 8 and 10, 220-8 tTA.10 cell line stably transfected with the tetracycline-regulated IRF-4-IRES-Spi-B construct cultured in the presence (+) or absence (–) of tetracycline for 12, 24 or 36 h. (**b**) Graphical representation of real-time quantitative RT-PCR results showing Spi-B and IRF-4 transcript levels from the 'Tet-off' IRF-4-IRES-Spi-B construct in the 220-8 tTA.10 cell line at various times after tetracycline withdrawal. Samples of cDNA reactions are as in **a**. Spi-B and IRF-4 levels from minus-RT control reactions were subtracted from reactions in which RT was included to account for genomic DNA contamination. These adjusted Spi-B and IRF-4 levels were then normalized to HPRT transcripts. Normalized Spi-B and IRF-4 levels in the parental 220-8 tTA.10 cell line cultured in tetracycline were arbitrarily set to 1.

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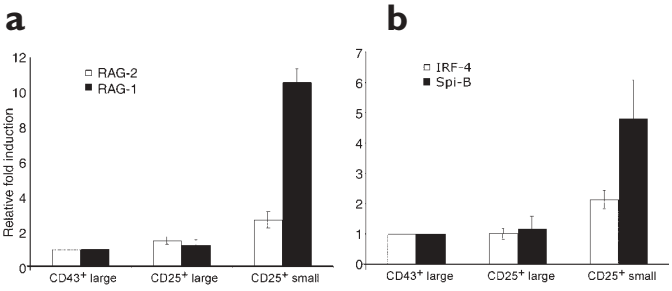

Figure 7. Quantitative analysis of RAG-1 and RAG-2, IRF-4 and Spi-B transcripts in various sorted primary pre-B cell fractions. (**a**) Graphical representation of real-time quantitative RT-PCR results showing RAG-1 and RAG-2 transcript levels in CD25$^+$ small pre-B cells (B220$^+$IgM$^-$) compared to fraction C′ large pre-B cells (either B220$^+$IgM$^-$CD43$^+$ or B220$^+$IgM$^-$CD25$^+$). RAG-1 and RAG-2 levels were normalized to HPRT transcripts and the level of transcripts in B220$^+$IgM$^-$CD43$^+$ large pre-B cells was arbitrarily set to 1. (**b**) Graphical representation of real-time quantitative RT-PCR results showing Spi-B and IRF-4 transcript induction in the same developing B cell subpopulations as described above. Spi-B and IRF-4 levels were normalized to HPRT transcripts and the levels of Spi-B and IRF-4 in B220$^+$IgM$^-$CD43$^+$ large pre-B cells were arbitrarily set to 1.

development, we proceeded to quantify transcripts in RNA purified from sorted large and small bone marrow pre-B cells. Using multi-parameter fluorescence-activated cell sorting (FACS) we isolated large pre-B cells according to two criteria: cell size by forward scatter and B220$^+$IgM$^-$CD43$^+$ or B220$^+$IgM$^-$CD25$^+$ cell-surface marker expression. We sorted small pre-B cells based on cell size by forward scatter and B220$^+$IgM$^-$CD25$^+$ cell surface marker expression. Consistent with published results, this analysis showed that RAG-1 and RAG-2 transcripts were low in large pre-B cells sorted by two different criteria and then became re-expressed in small pre-B cells (**Fig. 7a**)[1,3]. Spi-B and IRF-4 transcript levels were likewise low in large pre-B cells and increased as developing B cells progressed to the B220$^+$IgM$^-$CD25$^+$ small pre-B cell stage (**Fig. 7b**). This finding was consistent with our model, in which Abl-transformed pre-B cells represent large cycling pre-B cells. In both these cell lines and their primary cell counterparts, Spi-B and IRF-4 transcripts were transcriptionally repressed, perhaps downstream of Abl kinase activation.

Discussion

A-MuLV, like many transforming viruses, arrests development upon transformation. In the case of A-MuLV–transformed murine progenitor B cell lines, developing B cells arrest in an early pre-B cell–like state characterized by rapid proliferation, but minimal expression of recombinase activity. In addition, the Ig light chain loci remain inaccessible to the recombinase. Unlike primary pre-B cells, however, they do not necessarily express μ heavy chain protein or the pre-BCR and are therefore not genuine pre-B cells. We found that treatment of many different A-MuLV–transformed B cell lines with the Abl inhibitor STI571 caused the cells to resume their differentiation program and advance to a small pre-B cell–like state. These results were similar to those in a report on a single pro-B cell line transformed with a temperature-sensitive A-MuLV mutant[13,14]. These temperature-sensitive Abl cells adopt a pre-B cell–like phenotype upon shift from the permissive (33 °C) to the nonpermissive (39 °C) temperature. The system we present here is much more flexible, however, as STI571 can apparently induce the differentiation of any v-Abl–transformed cell line—including those generated from mutant mouse bone marrow—which makes this approach potentially useful in analysis of the myriad of induced mutations with B cell developmental phenotypes. In addition, with the development of techniques that improve the utility of RNAi in mammalian cells[25], the role of a gene in the pro- to pre-B cell transition can be readily tested in this STI571-inducible system. One limitation of this system, however, is the fact that these cells (as well as the temperature-sensitive Abl-transformed 103 cell line) stop dividing and undergo apoptosis within 24–36 h (data not shown).

A large number of genes underwent changes in expression in response to STI571 treatment. A subset of these changes were predictable based on previous observations with the temperature-sensitive Abl cell line described above and on the known patterns of gene activity during B cell development[17]. Additionally, we found many genes, some of which have unknown function, that were differentially regulated by STI571. These genes are attractive candidates for factors that are either involved in cell transformation, regulated by Abl *in vivo* and/or play a role in facilitating the pro- to pre-B cell transition. For example, an expressed sequence tag (Mm.44219) induced by v-Abl (repressed by STI571) that bears similarity to a human DEAD-box RNA helicase regulated by c-Myc has been uncovered[26]; it may play a role in ribosome biogenesis, RNA splicing or folding or gene silencing. This system, along with the data we present here, should be useful for building provocative testable models of regulation of the pro- to pre-B cell transition and of the mechanism of v-Abl–mediated cell transformation.

Pro-B cells are dependent on IL-7 for their survival and the appropriate activation of V_H to DJ_H rearrangement[27]. Cultured pro-B cells are driven to proliferate by this cytokine but typically do not mature into IgM$^+$ mature B cells. Among the changes that occur upon the pro- to pre-B cell transition is the loss of IL-7 responsiveness[28]. These IL-7–unresponsive cells exit the cell cycle and activate light chain gene rearrangement. This transition can be mimicked *ex vivo* by withdrawal of IL-7 from primary pro-B cell cultures[29]. Transformation with v-Abl relieves pro-B cells of this dependence on IL-7, allowing them to survive and continuously proliferate in culture. v-Abl may mimic the IL-7 signal that promotes cell survival and division up to the early pre-B cell stage[30]. STI571, then, would block Abl activity and release these cells to undergo the pre-B cell transition. Because cell cycle arrest is insufficient to induce the pre-B cell transition in A-MuLV–transformed pro-B cell lines (data not shown), v-Abl must arrest development through other regulatory changes. We suggest that upon inhibition of v-Abl, the activation of BLNK and Syk as downstream components of the pre-BCR signaling pathway and perhaps other target genes also contribute to this change in differentiated state.

Much effort has been spent trying to uncover c-Abl's physiologic role beyond its involvement in leukemic transformation. It has been suggested that V(D)J recombinase-mediated DSBs lead to recruitment and activation of ATM kinase[31], which, in turn, can phosphorylate and activate c-Abl kinase[32]. Because c-Abl is activated by DSB, it is an attractive candidate for the molecular sensor of ongoing V(D)J rearrangement in developing lymphocytes. For instance, in early pre-B cells, inactivation of c-Abl kinase activity may signal to the cell that V(D)J rearrangement has ceased at the *Igh* locus and that it is safe to proceed to the next step in development. A precedent for such a model exists in thymocyte development. Thymocyte development is arrested at the double negative stage in RAG-1 and RAG-2–deficient mice, but can be rescued so that development to the double-positive stage occurs through inactivation of p53, another well recognized molecular sensor of DNA damage[33]. It would be interesting to determine whether inactivation of c-Abl may effect a similar rescue in RAG-1 and RAG-2–deficient mice.

The ability of v-Abl to block development and for STI571 to release that block raises the question of whether regulated c-Abl activity plays a similar role in the normal pro- to pre-B cell transition *in vivo*. Perhaps regulated activation and inactivation of c-Abl contributes to the pre-BCR–mediated stimulation of early pre-B cell division or the subsequent cessation of cell cycle activity. c-Abl mutant mice have been generated by

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gene targeting, but detailed analyses of B cell development in these mice did not reveal a stage-specific block[34–36]. C-Abl$^{-/-}$ mutant hematopoiesis is characterized by pan-cytopenia and reduced total B cell numbers at each developmental stage. Complicating interpretation of the c-Abl$^{-/-}$ phenotype, however, is expression of the homologous gene *Abl2* (which encodes Arg)[37]. c-Abl$^{-/-}$ Arg$^{-/-}$ double-deficient mice are embryonic-lethal, thus precluding an analysis of lymphocyte development[38]. Conditional deletion of *Abl1* (which encodes c-Abl) on an Arg$^{-/-}$ background might reveal a critical function for c-Abl in pro- or pre-B cells. Alternatively, B cell development in mice treated with pharmacologic doses of STI571 might reveal stage-specific Abl-related phenotypes. One must keep in mind, however, that this drug may affect other related tyrosine kinases such as PDGF-R and c-Kit, the receptor for stem cell factor[39], complicating the interpretation of such *in vivo* studies.

The developmentally regulated activation of light chain gene rearrangement depends upon a pre-BCR signal that activates germline κ locus transcription and recombinase accessibility[2]. The *Igk* locus contains at least two developmentally regulated transcriptional enhancers termed Eκi and Eκ3′. NF-κB is bound to Eκi in both primary pro-B and pre-B cells, whereas the pattern of transcription factor binding at Eκ3′ changes across this developmental transition[23]. *In vivo* footprinting analyses showed that the Pax-5 (also known as BSAP) binding site in Eκ3′ is occupied in pro-B cells, whereas the adjacent EICE is vacant. In pre-B cells, Pax-5 association can no longer be detected, but the EICE is occupied. Thus it was proposed that these changes, and not NF-κB or Eκi function, were associated with the developmentally regulated onset of *Igk* locus transcription and its accessibility to the V(D)J recombinase[23].

The results we present here are consistent with this model: a drug that induces *Igk* locus transcription, accessibility and rearrangement caused a temporally correlated increase of Spi-B and IRF-4 mRNA levels. We propose that Spi-B and IRF-4 binding to the EICE removes Pax-5 from Eκ3′ (which may serve as a transcriptional repressor at this site), allowing for transcriptional activation. Spi-B and IRF-4 also bind to the *Igl* locus enhancers, which perhaps accounts for their activation in STI571-treated Abelson transformants. We propose that additional factors may be required for *Igl* activation in primary pre-B cells, as *Igk* rearrangement appears to precede *Igl* rearrangement *in vivo*.

Spi-B is a lymphoid-specific Ets family transcription factor closely related to PU.1[40]. Both are expressed in developing B cells. B cell development in Spi-B$^{-/-}$ mice is relatively normal, perhaps due to genetic redundancy with PU.1[41]. However, Spi-B cannot substitute for PU.1 to fully support B cell development[42]. PU.1$^{-/-}$ mice are embryonic-lethal[43]. Spi-B$^{-/-}$ splenic B cells respond poorly to BCR stimulation and show defects in germinal center formation[41,44]. PU.1$^{+/-}$Spi-B$^{-/-}$ B cells show a more severe phenotype in both of these regards[44]. It remains possible that the induction of either PU.1 or Spi-B in pre-B cells would be sufficient to activate germline *Igk* locus transcription.

The transcription factor IRF-4 belongs to the family of interferon-regulatory factors closely related to IRF-8 (also called ICSBP)[22,45]. As noted above, Spi-B as well as PU.1 commonly partner IRF-4 to regulate the transcription of target genes by binding to EICE. The *Igk* locus contains a well described EICE within its 3′ enhancer[46]. The *Igl* locus contains an EICE within each of its two enhancers, Eλ3-1 and Eλ2-4[47,48]. A dominant-negative IRF-4–PU.1 forced-dimer capable of abrogating Igλ expression in a plasmacytoma cell line demonstrates the importance of the EICE in λ enhancer activity[48].

IRF-4–deficient mice show defective serum Ig production[49]. However, neither IRF-4 nor Spi-B seem to be required for Ig expression or rearrangement during early B cell development[44,49]. This is consistent with the finding that there are two redundant enhancers capable of regulating the *Igk* locus. Elimination of either *Igk* enhancer does not absolutely abrogate *Igk* rearrangement or expression[50,51]. Thus it seems that although IRF-4 can contribute to the regulation of *Igk* rearrangement, it is not required. IRF-4 may only be required in plasma cells for maximal activation of the 3′ κ enhancer when large amounts of antibody production are required. It also remains possible that there are other IRF family members such as IRF-8 that can substitute for IRF-4 function during B cell development in the bone marrow. Another possibility is that although the Ets factors Spi-B and PU.1 synergize with the IRF factors IRF-4 and IRF-8, this cooperation is not required for early B cell development. It will be useful to know whether an Ets factor can function in isolation without interacting with an IRF factor, and *vice versa*, to activate the germline κ locus.

Methods

Cell culture. The cell line 220-8 is an Abl-transformant with nonproductive VDJ_H rearrangements on both alleles derived from adult BALB/c bone marrow[10]; PD31 is a primary subclone of an Abl-transformant (PD, also called 300-18) derived from bone marrow cells of an NIH-Swiss mouse[52]; 103bcl-x7 is the temperature-sensitive Abl-transformed 103 line[14] stably transfected with a Bcl-x expression construct; and 63-12 is an Abl transformant derived from the fetal liver of RAG-2$^{-/-}$ mice[53]. VL3-3M2 is a mouse thymoma line. The 220-8 tTA10 (Tet-off) cell line was as described[24]. All of the Abelson-transformed pro-B cells and VL3-3M2 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml of penicillin, 100 μg/ml of streptomycin and 50 μM 2-mercaptoethanol. Except for 103bcl-x7, all cells were grown at 37 °C in a 5% CO_2 atmosphere. The 103bcl-x7 cells were grown at the permissive temperature of 33 °C or the restrictive temperature of 39 °C.

STI571. Fresh 1000x stock solution of 10 mM STI571 (Gleevec, Novartis, East Hanover, NJ) was made before each experiment by dissolving 5 mg of STI571 per ml of PBS and filtering through a 0.2 μm syringe filter for sterilization. The final concentration of STI571 used was 10 μM, unless indicated otherwise.

Affymetrix gene expression profiling. 220-8 and PD31 cell cultures were either left untreated or treated with STI571 for 5 or 16 h. 103bcl-x7 cultures were either untreated at 33 °C, heat-shifted to 39 °C without STI571 for 12 h or treated with STI571 at 33 °C for 14 h. Total RNA was extracted with an RNeasy kit (Qiagen, Valencia, CA), which was followed by mRNA purification with the Oligotex mRNA Kit (Qiagen). We synthesized double-stranded cDNA from 2 μg of mRNA with the SuperScript Choice System (Invitrogen, Carlsbad, CA) primed with the T7-(dT)24 primer (Genset Corp, La Jolla, CA). We checked the quality of the cDNA by agarose gel electrophoresis. This cDNA was then used to prepare biotin-labeled cRNA by an *in vitro* transcription reaction done by T7 RNA polymerase in the presence of biotinylated UTP and CTP, according to the manufacturer's protocol (Enzo Diagnostics, Farmingdale, NY). The cRNA product was purified with RNeasy columns. We checked the quality of cRNA by agarose gel electrophoresis. These cRNAs were fragmented and 40 μg of each hybridized to Affymetrix Murine Genome U74Av2 oligonucleotide microarrays according to the manufacturer's instructions along with controls provided in the GeneChip Eukaryotic Hybridization Control Kit (Affymetrix, Santa Clara, CA). The hybridized products were revealed with phycoerythrin (PE)–streptavidin (Molecular Probes, Eugene, OR) in conjuction with antibody amplification and scanned with the confocal HP GeneArray Scanner, as suggested by Affymetrix. Before scanning, the arrays were washed and stained on the GeneChip Fluidics Station 400. Comparisons were made between untreated samples and their respective Abl-inhibited samples. We analyzed the data using Gene Chip Comparison Analysis Algorithm with both Microarray Suite version 4.0 and 5.0 and Data Mining Tool version 2.0 and 3.0 (Affymetrix GeneChip Software, Affymetrix) to determine the relative fold induction of transcripts for each probe set in Abl-inhibited samples compared to untreated samples (where the untreated sample probe hybridization was defined as the baseline and the Abl-inhibited sample hybridizations were defined as the experimental data). In our analysis, we relied primarily on the metrics generated by the statistical algorithms in Microarray Suite version 5.0; however, data analyses with both versions of the software yielded essentially similar results. For Fig. 4, expressed sequence tags with no known homology or function were excluded and the selected data were processed further for presentation with Cluster and TreeView[54].

RT-PCR. The ThermoScript RT-PCR system, which used RNAse H-minus avian myeloblastosis virus reverse transcriptase (AMV-RT) and random hexamer oligonucleotide primers, was used to synthesize cDNA from purified total RNA according to the manufacturer's instructions (Invitrogen). In brief, RNA was denatured at 65 °C for 5 min before carrying out the cDNA synthesis reaction first at 25 °C for 10 min followed by 50 °C for 50 min. For a 20 μl reaction, ~3 μg of total RNA, 100 ng of random hexamers, 40 U of RNAse inhibitors and 15 U of AMV-RT were used. The cDNA was then treated with 2 U of RNase H for 20 min at 37 °C before use in PCR. Typically 1–2 μl of cDNA were used as template for PCR. Transcript-specific PCR primers were designed so that they spanned at least one intron to avoid amplification of contaminating genomic DNA.

Quantitative real-time RT-PCR. Real-time 5′-nuclease PCR reactions with TaqMan hydrolysis probes were run and analyzed with the DNA Engine Opticon System (MJ Research,

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Waltham, MA). All hydrolysis probes were dual-labeled 5′ with 6-carboxyfluorescein succinimidyl ester (FAM) reporter fluorophore and 3′ with 6-carboxytetramethyl rhodamine (TAMRA) quencher (MWG-Biotech, High Point, NC). JumpStart Taq polymerase (Sigma, St. Louis, MO) was used at 15 U per ml in the following reaction conditions: 20 mM Tris at pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 4% glycerol and 200 μM each dNTP. Cycling conditions were 10 min at 95 °C followed by 40 cycles of 94 °C for 15 s and 58.7 °C for 1 min. PCR primers and TaqMan probes spanned an intron and included *Spib* sense 5′-TCTCG GACAGTGAGTCAGACGA-3′, *Spib* probe FAM-5′-CTTCTTGCGTGCACCTGCCTCA GAT-3′-TAMRA, *Spib* antisense 5′-TAGGAGCAACCCCAGCAAGA-3′, *Irf4* sense 5′-GA AGCCTTGGCGCTCTCA-3′, *Irf4* probe FAM-5′-CTGCCGGCTGCATATCTGCCTGT-3′-TAMRA, *Irf4* antisense 5′-TCACGAGGATGTCCCGGTAA-3′. RAG-1 sense 5'-CATTCTAG-CACTCTGGCCGG-3'; RAG-1 probe FAM-5'-AAGGTAGCTTAGCCAACATG GCTGCCTC-3'-TAMRA; RAG-1 antisense 5'-TCATCGGGGCAGAACTGAA-3'; RAG-2 sense 5'-TTAATTCCTGGCTTGGCCG-3'; RAG-2 probe FAM-5'-AGGGATAAGCAGCCC-CTCTGGCC-3'-TAMRA; RAG-2 antisense 5'-TTCCTGCTTGTGGATGTGAAAT-3'. The assay for HPRT transcripts was as described[55]. All primers spanned an intron. RNA without previous reverse-transcription was used as negative control template to demonstrate the specificity of the assay for RNA transcripts. Analysis used version 1.4 Opticon Monitor software (MJ Research).

Analysis of DSBs associated with V(D)J gene rearrangement. Assays were done as described[15,16,56].

Flow cytometry. Single-cell suspensions were stained for FACS with the following antibodies (from Pharmingen, San Diego, CA). Biotin–anti-B220 (RA3-6B2), fluorescein isothiocyanate (FITC)–anti-IgM (II/41), PE–anti-CD25 (PC61), PE–anti-CD43 (S7), PE–anti-CD62L–L-selectin (MEL-14) and anti-CD16/32 (FcBlock). Quantum red–streptavidin- (Sigma) was used to reveal biotinylated antibodies. Stained cells were analyzed with a Beckman-Coulter EPICS XL flow cytometer and CellQuest software (Becton Dickinson, Palo Alto, CA). Dead cells were excluded from the analysis by gating on live lymphocytes with forward and side scatter. A Beckman-Coulter EPICS Elite cell sorter equipped with an argon ion 15 mW air-cooled laser was used for cell sorting.

Plasmids. Spi-B cDNA[57] was subcloned downstream of an IRES sequence (from encephalomyocarditis virus) in the pBluescript plasmid. The IRF-4 cDNA[22] was then subcloned upstream of this IRES-Spi-B sequence. This IRF-4-IRES-Spi-B bi-cistronic cassette was then subcloned into the pTetSpliceNeo plasmid[24].

Note: Supplementary information is available on the Nature Immunology *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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