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Cutting Edge: Cell-Autonomous Control of IL-7 Response Revealed in a Novel Stage of Precursor B Cells

Gabriel J. Sandoval,* Daniel B. Graham,* Deepta Bhattacharya,* Barry P. Sleckman,**† Ramnik J. Xavier,**‡ and Wojciech Swat*‡

During early stages of B-lineage differentiation in bone marrow, signals emanating from IL-7R and pre-BCR are thought to synergistically induce proliferative expansion of progenitor cells. Paradoxically, loss of pre-BCR–signaling components is associated with leukemia in both mice and humans. Exactly how progenitor B cells perform the task of balancing proliferative burst dependent on IL-7 with the termination of IL-7 signals and the initiation of L chain gene rearrangement remains to be elucidated. In this article, we provide genetic and functional evidence that the cessation of the IL-7 response of pre-B cells is controlled via a cell-autonomous mechanism that operates at a discrete developmental transition inside Fraction C′ (large pre-BII) marked by transient expression of c-Myc. Our data indicate that pre-BCR cooperates with IL-7R in expanding the pre-B cell pool, but it is also critical to control the differentiation program shutting off the c-Myc gene in large pre-B cells.

B lymphopoiesis progresses through a developmental program that links the ordered rearrangement of Ig gene segments with cellular expansion and differentiation events (1–6). The nomenclature of developing B cell subsets is based on the expression of cell surface markers and the rearrangement status of the IgH and IgL loci (7). The earliest B-lineage progenitors are contained within Fraction (Fr.) A of bone marrow (also termed “pro-B”). These cells begin the rearrangement of IgH chain genes and differentiate into Fr. B and C, which are distinguished by a pattern of expression of CD24 and BP.1 (Fr. B and C are collectively termed “pre-BI”). The completion of IgH rearrangement and the expression of μHC protein on the cell surface with the surrogate L chain (LC) proteins (VpreB and L5) to form the pre-BCR mark the transition to Fr. C′ (also termed “large pre-BII”), which is characterized by high levels of CD24 and CD25. Fr. C′ cells are large and undergo rapid proliferative expansion that is critically dependent on IL-7 and the pre-BCR. Subsequently, however, the pre-BCR induces differentiation of C′ cells into Fr. D (also termed “small pre-BII”), which cease to proliferate, upregulate RAG-1/-2 genes, and begin the rearrangement of LC gene loci (3, 4).

The exact mechanism that controls these transitions remains incompletely understood. Intriguingly, the loss of pre-BCR–signaling components results not only in a developmental arrest of pre-B cells, but in both mice and humans, it also leads to the development of spontaneous pre-B cell leukemias (8–11). In this context, the pre-BCR signaling is initiated by tyrosine phosphorylation of ITAM sequences in Igα and Igβ (CD79α/CD79β) subunits, followed by recruitment and activation of Syk tyrosine kinase and the assembly of the SLP65/BLNK signalosome (4, 12, 13). In contrast, IL-7 initiates signaling events by heterodimerization of the IL-7Rα/IL-7Rβ subunits, followed by recruitment and activation of STAT5A and STAT5B (14). This permits STATs to dimerize and translocate to the nucleus, where they act as transcription factors for a number of target genes. The IL-7Rα-chain also serves for direct recruitment and activation of the p85 subunit of PI3K that is responsible for many downstream survival and proliferation–related events (15).

Thus, although signals emanating from both IL-7R and the pre-BCR synergistically regulate proliferative expansion of early stage B-lineage cells by promoting c-Myc expression and their survival (16), paradoxically, the pre-BCR complex is also critical for cell cycle exit of large pre-B cells and their differentiation into small pre-B cells, because the loss of pre-BCR signaling results in an arrest in differentiation and leads to pre-B cell lymphoblastic leukemia characterized by expression of c-Myc (17, 18). In this study, we used a fluorescently tagged c-Myc gene knock-in approach to track transient expression of c-Myc protein in developing B cells. Strikingly, using this approach we discovered a previously unrecognized developmental stage of large pre-B cells. We present functional and biochemical evidence that during large...
pre-B cell differentiation, the ability of cells to respond to IL-7R stimulation is controlled in a cell-autonomous manner at a new developmental transition that we term C′-1 to C′-2.

Materials and Methods

**Mice**

c-Myc<sup>eGFP</sup> mice were described previously (19). Rag-2<sup>−/−</sup> and Rag-1<sup>−/−</sup> mice were a gift from M. White (Washington University, St. Louis, MO). Mice were maintained in the specific pathogen-free facility in accordance with institutional policies of Washington University (St. Louis, MO).

**Flow cytometry**

Single-cell suspensions were stained with Abs to AA4.1, B220, CD43, CD127, CD132, c-kit, CXCR4, and surrogate L chain/pre-BCR (all from BD Phar-mingen), CD24 and CD25 (eBioscience), BP.1 (BioLegend), p-STAT5 and p-FoxO1/3a (Cell Signaling Technology), and IgM (Southern Biotech), according to standard protocols. Cell sorts were performed on a FACSARia II (Becton Dickinson). Intracellular stains were performed by fixing the cells in 2% PFA for 15 min, followed by washing with permeabilization buffer (PBS+2% FBS and 0.1% saponin).

**OP-9 cell cultures**

Sorted B cell subsets were cultured in the presence of 10 ng/ml IL-7 in DMEM-10 media in 96-well flat-bottom plates with a layer of 10<sup>4</sup> OP-9 cells and analyzed as indicated.

**Quantitative real-time-PCR analysis**

Sorted cell subsets were harvested in TRIzol (Invitrogen), RNA was extracted, and cDNA was generated using the SuperScript First-Strand RT system (Invitrogen), according to the manufacturer’s instructions. RT-PCR PCRs were performed in Supplemental Table I. Quantitative real-time PCR primers were performed with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and a Stratagene Mx3000P.

**PCR analysis of Igκ rearrangements**

C′-1 and C′-2 cells were sorted via FACS, and DNA was extracted. PCR reactions were performed for Vκ/Jκ and IL-2 as a loading control (Supplemental Table I), and PCR products were separated and analyzed on a PhosphorImager after Southern blotting using a Jκ probe and standard protocols.

**Autoconstitution assays**

c-Myc<sup>eGFP</sup> mice were sublethally irradiated (500 rad) and sacrificed at the time points indicated, bone marrow was harvested, and B cell populations were analyzed by flow cytometry.

**Statistical analysis**

Data are expressed throughout as mean ± SD. Datasets were compared using the two-tailed unpaired Student *t* test. Differences were considered statistically significant when *p* < 0.05.

Results and Discussion

c-Myc marks a novel stage in the Fr. C′ (large pre-BII) subset of precursor B cells

Precursor B cells have to coordinate a significant proliferative burst that is dependent on IL-7, with the termination of IL-7 signals to permit LC gene rearrangement (1–6, 20). However, IL-7R is still present on small pre-B cells (14); despite some evidence that IL-7R<sup>+</sup> large pre-B cells may migrate away from IL-7–producing stroma (6), the mechanism by which IL-7R signaling is terminated remains unclear. To precisely determine the stage of pre-B cell development at which the large Fr. C′ cells cease to proliferate, we decided to directly visualize c-Myc protein abundance in live cells ex vivo. To this end, we used a mouse model in which eGFP reporter is engineered to replace the start codon of Myc in the endogenous locus (e-Myc<sup>eGFP</sup>) in which c-Myc is still functionally expressed (19). This model is uniquely suited for these analyses because c-Myc expression in early lymphoid progenitors is thought to be primarily regulated by a posttranslational mechanism (21).

Indeed, our analyses of the distribution of differentiation markers, including AA4.1, B220, c-kit, CD43, CD24, CD25, BP.1, and c-Myc<sup>eGFP</sup>, in bone marrow cells from c-Myc<sup>eGFP/eGFP</sup> and control mice showed that expression of c-Myc<sup>eGFP</sup> marked a distinct stage of developing pre-B cells within Fr. C′ (large pre-BI) (Fig. 1A, Supplemental Fig. 1A, 1B). Importantly, analyses of the expression of μHC genes showed that virtually all c-Myc<sup>eGFP</sup>+ cells express intracellular μHC protein (Fig. 1B) and surrogate L chain components (Supplemental Fig. 1B), which are the defining traits of Fr. C′ cells. Consistent with a view that c-Myc<sup>eGFP</sup>+ cells make up Fr. C′, they appear large (Fig. 1A) and express c-kit (Supplemental Fig. 1C). Strikingly, however, c-Myc<sup>eGFP</sup> cells completely lack expression of CD25, a conventional marker of C′ cells. Therefore, expression of c-Myc protein appears to mark a previously unrecognized stage within the Fr. C′ large pre-B cells, which we refer to as C′-1. In contrast, our data clearly indicate that large pre-B cells marked by expression of CD25 no longer contain c-Myc protein (Fig. 1A). We hypothesized that this latter subset, which we refer to as C′-2, may encompass a stage that acquires the ability to rearrange LC genes.

In agreement with this view, our analyses show that C′-1 cells express high levels of C<sub>cd43</sub>5 gene transcripts encoding cyclin D3, which are virtually absent in C′-2 cells (Fig. 2A). In sharp contrast, C′-2 cells have abundant expression of Rag2 and dsDNA break marker, Pim2, and appear to actively rearrange their Ig LC genes, as indicated by the presence of recombined V-Jκ–chain gene segments (Fig. 2). Moreover,
our intracellular staining analyses with phosphospecific Abs revealed significantly higher levels of p-FoxO1/3a and p-STAT5 in C9-1 cells, consistent with a scenario that these cells are responding to IL-7 in vivo (Supplemental Fig. 1D). Taken together, we interpret these data as indicating that the assembly and expression of the pre-BCR complex promote a discrete stage of pre-B cell development that is marked by the expression of c-Myc protein (C9-1), which is distinct from the large, but nonproliferative CD25+ stage (C9-2). Therefore, it appears that pre-BCR initially contributes to C’ cell proliferation during a discrete C9-1 stage but subsequently may induce differentiation to a nonproliferative C9-2 stage during which rearrangements of Ig LC genes are initiated.

Cell-autonomous control of IL-7–driven proliferation in novel subsets of large pre-B cells

Given these results, we hypothesized that proproliferative signals emanating from IL-7R in large pre-B cells may be suppressed at the C9-1/C9-2 transition in a cell-autonomous manner. To test this hypothesis, we analyzed the ability of isolated C9-1 and C9-2 cells to respond with proliferation to IL-7 treatment in vitro. Despite similar levels of surface expression of IL-7Rα-chain and IL-7Rγ-chain, C9-1 cells proliferate in response to IL-7 in OP9 cultures in vitro, whereas C9-2 cells fail to proliferate (Fig. 3A–C). Remarkably, C9-1 cells appear to spontaneously differentiate into C9-2 cells in vitro, with the accompanying loss of their ability to proliferate in response to IL-7. Moreover, blocking pre-BCR signaling by treatment with a specific Syk kinase inhibitor leads to an arrest at the C9-1 to C9-2 transition in vitro, with an accompanying increase in cell proliferation (Fig. 3D).

FIGURE 2. Cell cycle and LC gene rearrangement analysis of C9-1 and C9-2 stage cells. (A) Quantitative real-time PCR analysis of FACS-sorted cells was performed to detect RAG2 (RAG2), PIM2 (PIM2), and cyclin D3 (Cyclin D3) transcripts. Data are a composite of three independent experiments (n = 6). (B) Detection of LC gene (Vκ-Jκ) rearrangements in genomic DNA isolated from FACS-sorted cells. IL-2 gene was used as a loading control, WT splenic DNA was used as a positive control, and Rag-1–deficient pro-B cell DNA was used as a negative control. Data shown are representative of four independent experiments (n = 4). *p < 0.05, **p < 0.005.

FIGURE 3. Functional and precursor–product relationship analysis of C9-1 and C9-2 cells. (A) C9-1 cells were sorted via FACS and then cocultured with OP-9 stromal cells supplemented with IL-7. After 3 d in culture, cells were analyzed by flow cytometry to assess differentiation into C9-2 cells. Data shown are representative of five independent experiments (n = 8). (B) Freshly isolated C9-1 and C9-2 cells were directly FACS sorted at 10 cells/well into microtiter plates and cocultured with OP-9 stromal cells in the presence of 10 ng/ml IL-7. Wells were scored daily to determine IL-7–induced proliferation by microscopic observation. Data are representative of two independent experiments (n = 4). (C) Flow cytometry analysis of IL-7Rα and γ in C9-1 and C9-2 cells. Mean fluorescence intensity (MFI) is shown; data are a composite of three independent experiments (n = 6). (D) Analysis of pre-B cell proliferation in the presence of Syk kinase inhibitor (BAY61-3606). B220+/CD43+/CD25- cells were FACS sorted and cultured in the presence of 10 ng/ml IL-7, with or without BAY61-3606 (10 nM). Cells were counted every 2 d and analyzed for CD25 expression on day 10. Data are the composite of four independent experiments (n = 6). (E) Autoreconstitution analysis of pre-B cell repopulation kinetics. c-Myc eGFP/eGFP mice were sublethally irradiated (500 rad), and pro/pre-B cell populations were analyzed, as indicated. (F) Percentages of C9-1 and C9-2 cells were measured. Data are representative of four independent experiments; n > 10 for (E) and n = 5 for (F). *p < 0.05, **p < 0.005.
Thus, although the generation of C′-2 stage cells is virtually blocked by Syk inhibitor in this system, developmentally arrested C′-1 cells remain viable and retain their proliferative properties (Fig. 3D). We interpret these results as suggesting that expression of c-Myc marks C′-1 stage cells that respond to IL-7R signals with proliferation but later differentiate into C′-2 stage cells that no longer proliferate in response to IL-7.

In addition to IL-7 and pre-BCR, environmental cues, such as cytokines induced by stress or tissue injury, can exert progenitor cell proliferation, differentiation, and modulate c-Myc protein abundance in progenitor cells (22–24). To further address the precursor/product relationship between the C′-1 and the C′-2 subsets, we treated c-MycGFP/c-MyceGFP mice with low doses of gamma radiation, inducing ablation of B-lineage cells and activation of quiescent progenitor cells. Analyses of bone marrow at 24 h postirradiation showed an absence of virtually all B-lineage cells (Fig. 3E). Strikingly, analyses of the repopulation kinetics revealed that development of C′-1 cells preceded C′-2 cells by 3–5 d (Fig. 3F), consistent with the view that the former subset represents an earlier stage compared with the latter.

Taken together, these observations indicate that expression of c-Myc protein in C′-1 large pre-B cells is subject to pre-BCR-dependent regulation. Thus, the pre-BCR may function to suppress signals emanating from IL-7R and/or, conceivably, is involved in the recruitment of downstream effectors necessary for the suppression of c-Myc expression and the induction of C′-1 cell differentiation into the nonproliferative C′-2 stage. In this context, our analyses revealed virtually identical levels of IL-7Rα–chain and IL-7Rγ–chain on the surface of C′-1 and C′-2 cells (Fig. 3C). Therefore, the more mature C′-2 cells maintain IL-7R expression but are unresponsive to IL-7.

One model proposes that pre-BCR signaling upregulates the transcription factor IRF4, which, in turn, promotes higher expression of the chemokine receptor CXCR4 on pre-B cells and their movement, directed by the chemokine CXCL12, away from stromal cells expressing IL-7, thereby attenuating IL-7 signaling (6). Notably, IRF4 also induces germine transcription of Ig LC loci and, consequently, their accessibility for recombination (6, 25, 26). However, in our study, both C′-1 and C′-2 stage cells show similar levels of expression of surface CXCR4 (Supplemental Fig. 1E), even though the developmental transition from C′-1 to C′-2 is accompanied by a dramatic increase in IRF4 production (Supplemental Fig. 1F). Moreover, our analyses of expression of critical transcription factors involved in pre-B cell differentiation indicate that C′-2 cells contain significantly higher levels of Irf4 and Isef1 gene (encoding Ikaros) compared with C′-1 cells (Supplemental Fig. 1F), consistent with the view that these factors negatively regulate pre-B cell proliferation by directly suppressing c-Myc expression (3). Notably, our data also reveal a significant increase in Socs-I gene expression at the transition from C′-1 to C′-2 (Supplemental Fig. 1F). These results indicate that the increase in Socs-I expression that we find in C′-2 cells could conceivably contribute to downregulation of STAT5 signaling in this population, as suggested by previously published studies (27). However, we note that Socs-I expression levels continue to increase dramatically in Fr. D cells (Supplemental Fig. 1F).

Thus, whatever the mechanism, to our knowledge, we show the first evidence of the existence of two distinct subsets of Fr. C′ cells distinguished by their ability to respond to IL-7. Our data clearly indicate that termination of IL-7 signaling is a cell-autonomous process critically dependent on signals emanating from the pre-BCR. Strikingly, pharmacological inhibition of pre-BCR signaling leads to a dramatic enhancement of proliferation in response to IL-7 that coincides with a developmental arrest at the C′-1 stage expressing c-Myc.

We propose that these results help to explain observations of spontaneous leukemia upon loss of pre-BCR in mice and humans (8–10).

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1.

A

Fr.B  Fr.C  Fr.C’-1  Fr.C’-2  Fr.D

c-Myc

B

control  C’-1  C’-2

Pre-BCR  AA4.1

C

C-Kit

D

pFoxO1/3a  pSTAT5

E

CXCR4

MFI

Arbitrary Units

CXCR4  IRF4  BLNK  Ikaros  Socs-1

F

C’-1  C’-2  C’-1  C’-2  C’-1  C’-2  C’-1  C’-2  C’-1  C’-2  D

MFI

Arbitrary Units
Supplemental Figure 1. Characterization of C’-1 and C’-2 cells.

(A) c-Myc levels in developing B cells. Data shown is representative of over 10 experiments.
(B) Flow cytometry analysis of intracellular SLC/pre-BCR and surface AA4.1 expression in C’-1 and C’-2 cells. Data shown is representative of 4 separate experiments (N=4).
(C) Flow cytometry analysis of c-kit (CD117) expression in C’-1 and C’-2 cells. MFI +/- SD is shown, data is composite of 3 independent experiments (n=4).
(D) Flow cytometry analysis of pFoxO1/3a and pSTAT5 expression in C’-1 and C’-2 cells. MFI +/- SD is shown, data is composite of 3 independent experiments (n=5).
(E) Flow cytometry analysis of CXCR4 in C’-1 and C’-2 cells. MFI +/- SD is shown, data is composite of 3 independent experiments (n=4).
(F) qRT-PCR analysis of Irf4 (IRF4), Blnk (BLNK), Izkf1 (Ikaros), and Socs-1 (Socs-1) gene expression in C’-1 or C’-2 cells. Data shown is representative of 3 independent experiments (n=4).
**Supplemental Table 1.**

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Supplemental Table 1. Primers used for QRT-PCR and PCR.
Shown in the top table are the forward and reverse primers used for QRT-PCR analysis as well as control primers (GAPDH). The bottom table shows the primers used to observe the LC kappa region in B cells as well as the primers for the loading control (IL-2).