Permissive roles of hematopoietin and cytokine tyrosine kinase receptors in early T-cell development

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Although several cytokines have been demonstrated to be critical regulators of development of multiple blood cell lineages, it remains disputed to what degree they act through instructive or permissive mechanisms. Signaling through the FMS-like tyrosine kinase 3 (FLT3) receptor and the hematopoietin IL-7 receptor α (IL-7Rα) has been demonstrated to be of critical importance for sustained thymopoiesis. Signaling triggered by IL-7 and thymic stromal lymphopoietin (TSLP) is dependent on IL-7Rα, and both ligands have been implicated in T-cell development. However, we demonstrate that, whereas thymopoiesis is abolished in adult mice doubly deficient in IL-7 and FLT3 ligand (FLT3L), TSLP does not play a key role in IL-7–independent or FLT3L-independent T lymphopoiesis. Furthermore, whereas previous studies implicated that the role of other cytokine tyrosine kinase receptors in T lymphopoiesis might not involve permissive actions, we demonstrate that ectopic expression of *BCL2* is sufficient not only to partially correct the T-cell phenotype of *Flt3l*$^{-/-}$ mice but also to rescue the virtually complete loss of all discernable stages of early T lymphopoiesis in *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice. These findings implicate a permissive role of cytokine receptors of the hematopoietin and tyrosine kinase families in early T lymphopoiesis. (Blood. 2008;111:2083-2090)

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Introduction

Growth factor dependent hematopoietic cell lines can on overexpression of the antiapoptotic regulator B-cell lymphoma-2 (*BCL2*) become independent of cytokines of the hematopoietin family.[1] In vivo studies demonstrating that defective thymopoiesis in interleukin-7 (IL-7)– and IL-7Rα–deficient (*Il7*$^{-/-}$ and *Il7r*$^{-/-}$) mice can be rescued on overexpression of *BCL2*[2-4] or by deleting the proapoptotic regulator *Bax*,[5] further support the hypothesis that hematopoietin family cytokine receptors play a permissive role in thymopoiesis. Hematopoiesis is also regulated by a distinct family of cytokines acting through tyrosine kinase receptors, such as KIT oncogene (KIT) and FMS-like tyrosine kinase 3 (FLT3).[6] Notably, although KIT, like IL-7Rα, is critically involved in the regulation of thymopoiesis, *BCL2* overexpression failed to rescue the impaired T lymphopoiesis in KIT-deficient mice.[7] This implies that at least some members of this family of cytokine receptors and ligands, unlike the hematopoietin family, might not promote hematopoiesis through permissive actions.

More recently, FLT3 ligand (FLT3L) has emerged as a key regulator of T lymphopoiesis, including of IL-7Rα–independent thymopoiesis,[8-12] but it has not been investigated to what degree FLT3 might act through permissive actions. Furthermore, as both IL-7 and thymic stromal lymphopoietin (TSLP) share the IL-7Rα, it is unclear to what degree the almost completely arrested T lymphopoiesis in adult *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice[8] reflects a role of FLT3L in conjunction with IL-7 and/or TSLP. TSLP also depends on and acts through a second IL-2R gamma chain (IL-2Rγ)-like TSLP receptor (TSLPR) subunit[13] and has been shown to modulate immunologic responses by activating T cells through regulation of dendritic cells.[14] TSLP has also been implicated to play an important role in IL-7–independent B lymphopoiesis, as *Il7r*$^{-/-}$ mice have a more severe reduction in B lymphopoiesis than *Il7*$^{-/-}$ mice.[15,16] Similarly although TSLP- and TSLPR-deficient (*Tpte2*$^{-/-}$) mice appear to have normal T lymphopoiesis,[17,18] there are indications that T lymphopoiesis is more severely affected in *Il7r*$^{-/-}$ than *Il7*$^{-/-}$ mice, compatible with a role of TSLP in IL-7–independent T lymphopoiesis.[19-22] This finding is supported by studies of TSLPR and IL-2Rγ deficient (*Tpte2*$^{-/-}$*Il2rg*$^{-/-}$) mice, in which a further reduction of total thymic cellularity compared with single *Il2rg*$^{-/-}$ mice has been reported.[18]

In the present studies, we first sought to establish the relative roles of IL-7, TSLP, and FLT3L in T lymphopoiesis. Notably, although *Il7r*$^{-/-}$ mice had a more severe block in thymopoiesis than *Il7*$^{-/-}$ mice, studies of *Tpte2*$^{-/-}$, *Il7*$^{-/-}$*Tpte2*$^{-/-}$, and *Flt3l*$^{-/-}$*Tpte2*$^{-/-}$ mice established that IL-7 and FLT3L are key regulators of normal thymopoiesis, with no discernable key role of TSLP. We also investigated to what degree the role of FLT3L in thymopoiesis is mediated through permissive actions. Through correction of the T-cell phenotype of *Flt3l*$^{-/-}$ mice, most notably through partial rescue of the almost complete loss of thymocytes in adult *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice by *BCL2* overexpression, we provide evidence for the critical role and interaction of IL-7 and FLT3L in T lymphopoiesis, being mediated at least in part through permissive actions.

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Methods

Mice

Flt3l$^{-/-}$ mice[9] were on a pure C57BL/6 background. *Il7r*$^{-/-}$[21] (Jackson Laboratory, Bar Harbor, ME), *Il7*$^{-/-}$[23] and *BCL2* transgenic mice[24] were backcrossed for a total of 10 generations to C57BL/6. *Tpte2*$^{-/-}$ mice[17] were kept on the original mixed 129/SvJ/C57BL/6 background. Double-deficient mice were generated through intercrossing the above mentioned strains except for the mice double-deficient in FLT3L and IL-7Rα, which were obtained by cross breeding of *Flt3l*$^{-/-}$ (pure C57BL/6) mice with *Il7r*$^{-/-}$ mice backcrossed 5 generations to C57BL/6.[8] All experiments were approved by the Ethical Committee at Lund University.

Antibodies

Antibodies used for cell-surface staining were 2.4G2 (CD16/32), RA3-6B2 (B220), RB6-8C5 (Gr1), M1/70 (CD11b, Mac1), H129.19 (CD4), 53-7 (CD5), 53-6.7 (CD8α), LY-76 (Ter-119), 2B8 (CD117, c-kit), A20 (CD45.1), 104 (CD45.2), 3C7 (CD25), IM7 (CD44; all from BD PharMingen, San Diego, CA) and polyclonal goat-antirat Tricolor (Caltag, Invitrogen, Carlsbad, CA).

Fluorescence-activated cell sorting

For thymic SP and DP cell staging and splenic T-cell analysis, cells were incubated with anti–mouse-CD8 FITC and -CD4 APC. For DN1-4 staging, thymocytes were stained with a cocktail of purified rat antibodies against lineage markers B220, CD4, CD5, CD8α, CD11b, Gr1, and Ter119. Lineage-positive cells were visualized with a goat-antirat Tricolor staining. Thereafter, cells were incubated with anti–mouse-CD44 FITC, -CD25 PE, and -KIT APC. 7 Amino-Actinomycin (Sigma-Aldrich, St Louis, MO) staining was used to exclude dead cells. Analyses were performed on a FACSCalibur (BD). Data analysis was carried out using FlowJo software (Tree Star, Ashland, OR).

Polymerase chain reaction analysis

Genomic DNA and RNA were prepared using Trizol (Invitrogen) according to the manufacturer's instructions. Tcrb D-J, VDJ rearrangements were assayed by polymerase chain reaction (PCR), as previously described,[25,26] using 38 cycles (94°C 1 minute, 61°C 1.5 minutes, and 72°C 2 minutes) of PCR.

The PCR products were blotted onto Hybond N$^+$ nylon membranes (GE Healthcare, Little Chalfont, United Kingdom) using capillary blotting. Membranes were prehybridized in 5× Denhardts (Ficoll 10 g/L, polyvinylpyrrolidone 10 g/L, bovine serum albumin 10 g/L), 6× SSPE, 0.1% SDS, and 100 μg/mL Salmon Sperm DNA, at 45°C for 60 minutes and hybridized with a specific γ[^{32}P] labeled oligonucleotide for 12 hours at 45°C in the same solution. Membranes were washed at room temperature in 2× SSC (0.015 M Na citrate, pH 7.0, containing 0.15 M NaCL) supplemented with 0.1% SDS for 15 minutes and 0.1× SSC with 0.1% SDS for 5 to 10 minutes. The hybridized membrane was then subject to auto radiography.

The following oligonucleotides were used for PCR: Tcrb-J2: 5′-TCTCCTACTATCGATTTCCCTCCCG-3′; Tcrb-D1: 5′-GGTAGAC-CTATG-3; Tcrb-V5.2: 5′-GTGGAGAGAGACAAAGGATT-3′; Tcrb-V8.3: 5′-TTGGCTTCTCCCTCTCAGACATCTT-3′. The following oligonucleotide was used for hybridization: Jβ2: 5′-TTTCCCTC-CCGGAGATTCCCTAA-3′; Gata-1 PCR: Gata1 sense: 5′-CCACTAGT-CCTGGCCTAGTACC-3′. Gata1 anti sense: 5′-CTGGCCTTGGCTC-TGAGTCTCC-3′.

RNA was prepared from cells using RNeasy Micro Kit (QIAGEN, VWR International, Stockholm, Sweden), and cDNA was generated by annealing 1 μg total RNA to 0.5 μg of random hexamers in 10 μL diethyl pyrocarbonate-treated water. Reverse transcriptase (RT) reactions were performed with 200 U SuperScriptII (Invitrogen) in accordance with the manufacturer's recommendations. One-twentieth of the RT reaction was used in the PCR assays. PCR was performed with 1 U *Taq* polymerase (Invitrogen) in the manufacture's buffer supplemented with 0.2 mM dNTP, in a total volume 25 μL. β-actin (actb) was amplified by 25 cycles, whereas 35 cycles were used to amplify Lck, Ptcra, Il7r, and Rag2 cDNA (94°C, 30 s, 60°C, 30 s, and 72°C, 30 s). The following primers were added to a final concentration of 1 mM: Actb sense: 5′-GTTTGAGACCTTCAACACC-3′; Actb anti sense: 5′-GTGGCCATCCCTGCTCGAAGTC-3′; Lck sense: 5′-GGTCAGAGACTTCGACCAGAAC-3′; Lck anti sense: 5′-CCACTG-CATAAAGCCGGACTAG-3′; Ptcra sense: 5′-CACACGCTGGTAGATG-GAAGGC-3′; Ptcra anti sense: 5′-GTCAGGAGCACATCGAGCAGAAG-3′; Il7r sense: 5′-AGCTGTTTCTGGAGAAAGTGG-3′; Il7r anti sense: 5′-AACGATTTCAGGTCAGAGGG-3′; Rag2 sense: 5′-GGTGTGCT-ACATCATATATTCTCCCAG-3′ Rag2 anti sense: 5′-TTCAATCGTGT-TGTCCCCTAGAG-3′.

In vivo reconstitution experiments

A total of 5×10^6 unfractionated BM cells from *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ or *Flt3l*$^{-/-}$*Il7r*$^{-/-}$*BCL2* (CD45.2) littermates were transplanted into lethally irradiated *Flt3l*$^{-/-}$ (CD45.1) recipients. As positive controls, 5×10^6 littermate WT or *BCL2* unfractionated BM cells were transplanted into WT (C57, CD45.1) recipients. Recipient mice were analyzed for the presence of donor-derived (CD45.2) T (CD4 or CD8) cell reconstitution at 6 weeks after transplantation. Briefly, thymocytes were stained with antibodies against CD45.1 and CD45.2 to determine the level of donor cell reconstitution and CD4 and CD8 to determine T-cell reconstitution.

Statistics

All results were expressed as mean (SD). The statistical significance between 2 groups of mice with deletion of single versus multiple signaling pathways was determined using a 1-tailed Student *t* test as removal of additional signaling pathways was hypothesized to result in the same or a more severe T-cell phenotype. Aspin-Welch correction was used in cases of unequal variances between groups.

For the *BCL2*-mediated rescue studies we tested, as an approximation to investigating if $\mu_1/\mu_2 > \mu_3/\mu_4$, the null hypothesis H'_0: $\mu'_1 - \mu'_2 \leq \mu'_3 - \mu'_4$ versus the alternative H'_1: $\mu'_1 - \mu'_2 > \mu'_3 - \mu'_4$. μ_1, μ_2, μ_3 and μ_4 denote population means of the WT mice, cytokine knockout mice on WT background, *BCL2* mice and cytokine knockout mice on *BCL2* background, respectively, and μ'_1, μ'_2, μ'_3 and μ'_4 denote population means of the corresponding logarithmically transformed quantities. To test H'_0 we used a t-type statistic with an Aspin-Welch approximation to the number of degrees of freedom. (For further details see Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article.)

The number of mice included in each statistical analysis is specified in the figure legends.

Results

Critical roles of IL-7 and FLT3L but not TSLP in thymopoiesis

In accordance with previous studies[17,18] we found no changes at any stages of thymocyte development in adult *Tpte2*$^{-/-}$ mice (Figure 1A,B). TSLP has been suggested to be important for IL-7-independent lymphopoiesis, largely based on a more severe lymphoid phenotype of IL-7Rα than IL-7 signaling deficient mice.[15,16,20-22] To further explore whether TSLP might have a role in IL-7-independent thymopoiesis, we first compared thymopoiesis in age-matched *Il7r*$^{-/-}$ and *Il7*$^{-/-}$ mice (both on a C57BL/6 background) because *Il7r*$^{-/-}$ mice are deficient in IL-7 and TSLP-mediated signaling. In comparison to *Il7*$^{-/-}$ mice, *Il7r*$^{-/-}$ mice had further reductions in total (Figure 1C), double negative (DN; CD4$^-$, CD8$^-$) (Figure 1D), double positive (DP; CD4$^+$, CD8$^+$) and single positive (SP; CD4$^+$ or CD8$^+$; Figure 1E) thymocytes, indirectly supporting the idea that TSLP might play a role in

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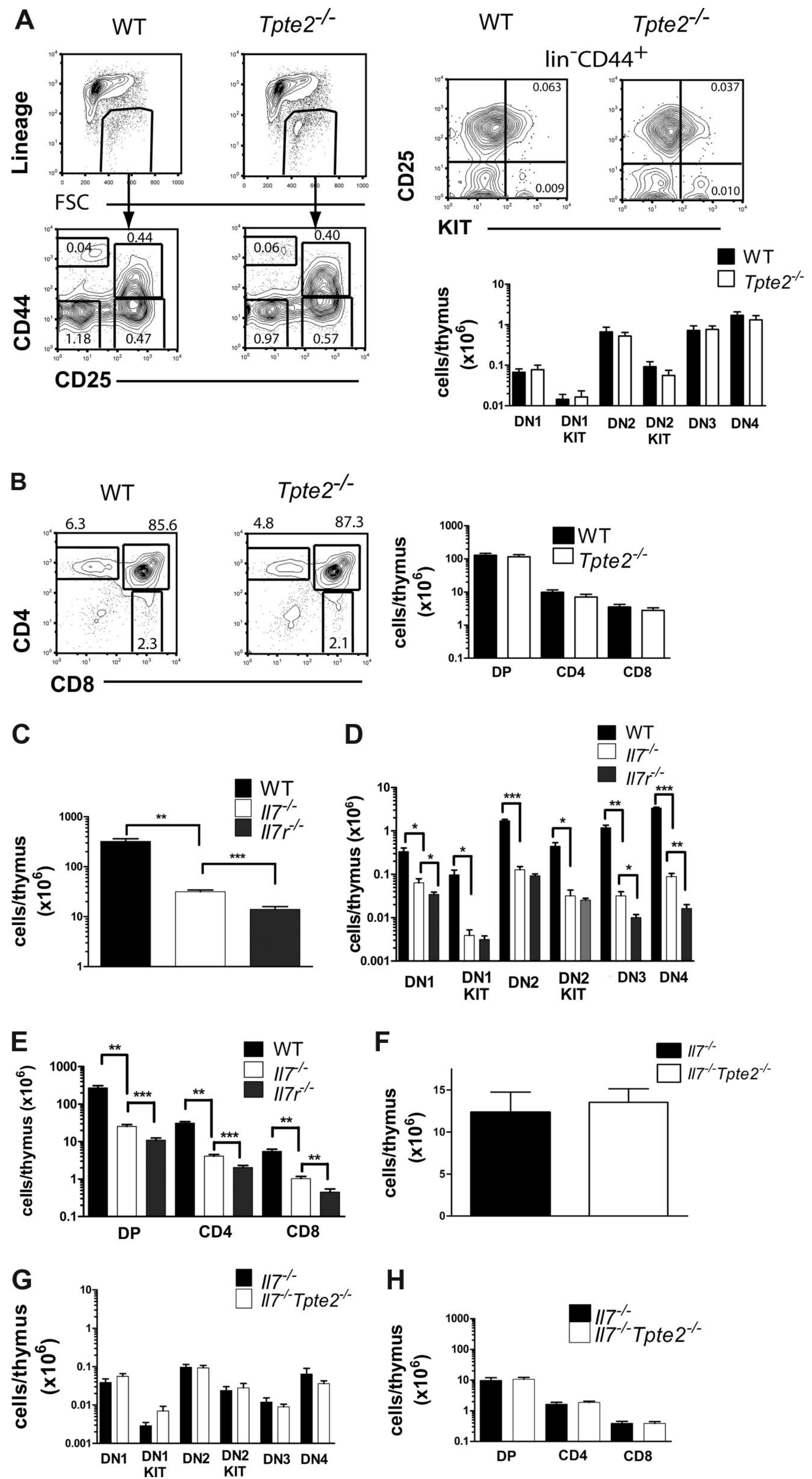

Figure 1. Lack of role of TSLP in IL-7-dependent and independent T lymphopoiesis. (A,B) fluorescence-acivated cell sorting (FACS) profiles and mean (SD) numbers of (A) DN subsets and (B) CD4 and CD8 SP and DP cells in thymus of 9- to 11-week-old littermate WT and *Tpte2*$^{-/-}$ mice. Data are from 5 littermate WT and 7 *Tpte2*$^{-/-}$ mice from 3 separate litters. Numbers in FACS profiles show the mean percentages of cells within the indicated gates of total thymocytes. (C-E) Mean (SD) numbers of (C) total thymocytes, (D) DN cells, and (E) CD4 and CD8 SP and DP thymocytes of 3-week-old WT, *Il7*$^{-/-}$ and *Il7r*$^{-/-}$ mice. Data are from 4 WT, 7 *Il7*$^{-/-}$, and 8 *Il7r*$^{-/-}$ mice from at least 2 separate litters. (F-H) Mean (SD) numbers of (F) total thymocytes, (G) DN cells, and (H) CD4^{+} and CD8^{+} thymocytes of 3-week-old littermate *Il7*$^{-/-}$ and *Il7*$^{-/-}$*Tpte2*$^{-/-}$ mice. Data are from 7 littermate *Il7*$^{-/-}$ and 11 *Il7*$^{-/-}$*Tpte2*$^{-/-}$ mice from 4 separate litters. All groups were investigated for statistical differences by Student *t* test (*$P < .05$, **$P < .01$, ***$P < .001$). Unless otherwise indicated differences were nonsignificant.

IL-7–independent T lymphopoiesis. In an attempt to get direct evidence for a role of TSLP in IL-7–independent thymopoiesis, we next investigated *Il7*$^{-/-}$ and *Il7*$^{-/-}$*Tpte2*$^{-/-}$ mice. Notably, no differences were observed in cellularity or at any stage of thymocyte development (Figure 1F-H), providing evidence for TSLP playing no key role in steady state adult IL-7–independent T lymphopoiesis.

Compared with mice deficient in FLT3L or IL-7Rα, thymopoiesis in mice double-deficient in FLT3L and IL-7Rα (*Flt3l*$^{-/-}$*Il7r*$^{-/-}$) is severely impaired and almost completely arrested already in young adult mice.[8] To establish the relative role of IL-7 and TSLP in FLT3L-independent thymopoiesis, we compared thymopoiesis in *Flt3l*$^{-/-}$*Il7*$^{-/-}$ and *Flt3l*$^{-/-}$*Tpte2*$^{-/-}$ mice. Notably, whereas *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ and *Flt3l*$^{-/-}$*Il7*$^{-/-}$ mice

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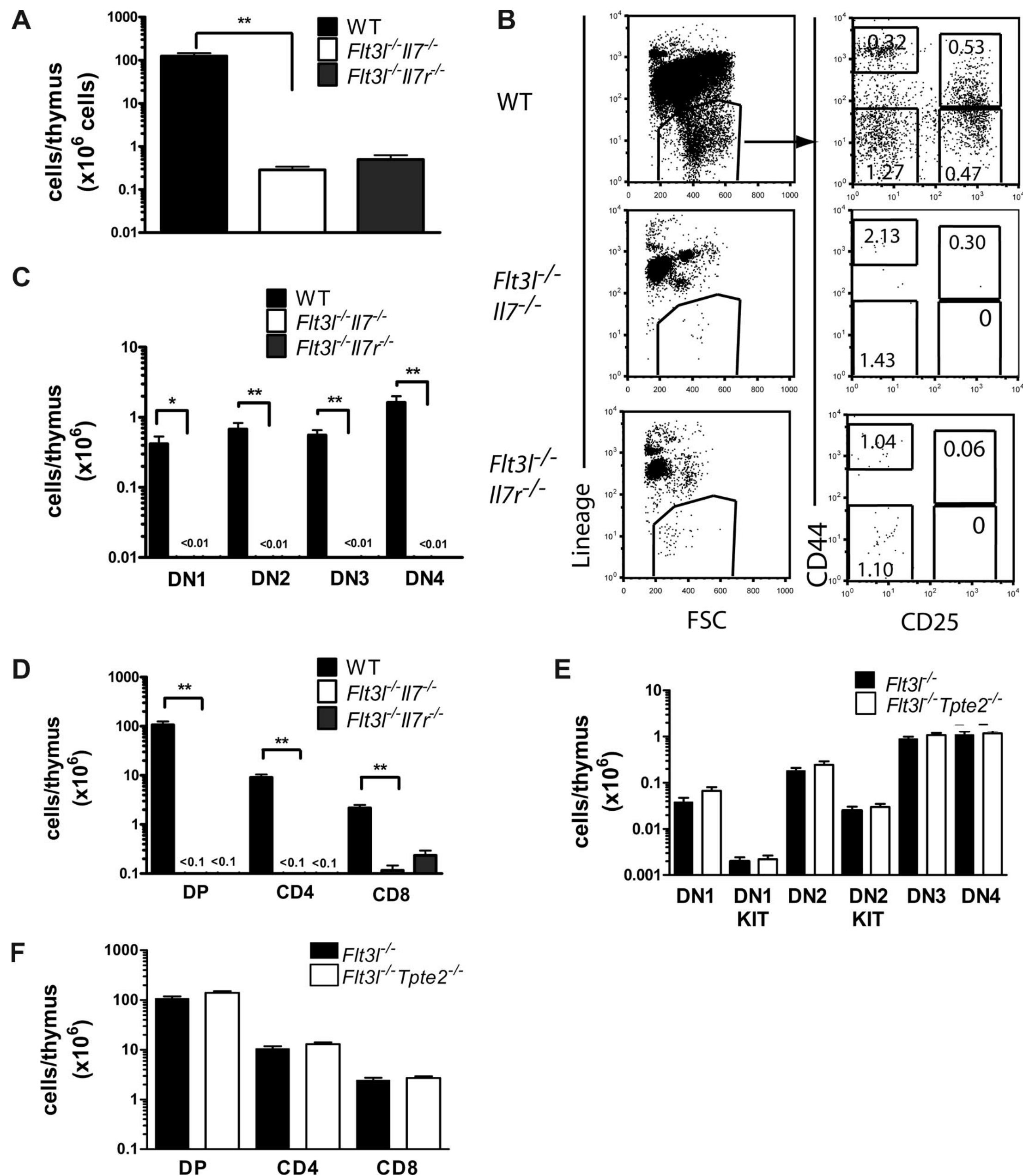

Figure 2. Critical roles of FLT3L and IL-7 but not TSLP in adult thymopoiesis. (A) Mean (SD) thymic cellularity, (B) FACS profiles of DN development, and mean (SD) numbers of (C) DN cells and (D) CD4 and CD8 SP and DP thymocytes of 9- to 11-week-old WT, *Flt3l*$^{-/-}$ *Il7*$^{-/-}$ and *Flt3l*$^{-/-}$ *Il7r*$^{-/-}$ mice. Numbers in FACS profiles show the mean percentages (of total thymocytes) of cells within the indicated gates. Data are from 5 WT, 6 to 13 *Flt3l*$^{-/-}$ *Il7*$^{-/-}$ and 4-10 *Flt3l*$^{-/-}$ *Il7r*$^{-/-}$ mice from at least 2 separate litters. (E,F) Mean (SD) numbers of (E) DN cells and (F) CD4 and CD8 SP and DP thymocytes of 9- to 11 week-old littermate *Flt3l*$^{-/-}$ and *Flt3l*$^{-/-}$ *Tpte2*$^{-/-}$ mice. Data are from 8 littermate *Flt3l*$^{-/-}$ and 10 *Flt3l*$^{-/-}$ *Tpte2*$^{-/-}$ mice from 2 separate litters. All groups were investigated for statistical differences by Student *t* test (*$P < .05$, **$P < .01$). Unless otherwise indicated, differences were nonsignificant.

had indistinguishable thymocyte deficiencies with a virtually complete loss of all thymocyte subsets (Figure 2A-D), *Flt3l*$^{-/-}$*Tpte2*$^{-/-}$ mice had a thymocyte phenotype indistinguishable from *Flt3l*$^{-/-}$ mice (Figure 2E,F). Thus, whereas FLT3L and IL-7 play critical and complementary roles in T lymphopoiesis, TSLP appears to have no important role in IL-7–independent or FLT3L-independent thymopoiesis.

Overexpression of *BCL2* partially rescues all stages of T-cell development in *Flt3l*$^{-/-}$ *Il7r*$^{-/-}$ mice

We next investigated to what degree the role of FLT3L in thymopoiesis is mediated through permissive actions. Although somewhat contentious,[7] IL-2Rγ and IL-7Rα have, through overexpression of *BCL2,* been suggested to largely act in a permissive manner in αβ T lymphopoiesis.[2-4] In contrast, and through a similar strategy, it was not possible to obtain support for a permissive role of the cytokine tyrosine kinase receptor KIT in thymopoiesis.[7] To investigate to what degree the critical importance of FLT3 signaling in early T-cell development might involve permissive actions, we examined thymopoiesis in *Flt3l*$^{-/-}$, *Il7r*$^{-/-}$ and *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice on a *BCL2* transgenic (*BCL2*) background, obtained by intercrossing each of the strains with transgenic mice with high pan-hematopoietic expression of *BCL2*.[24] Because the loss of thymocytes in all these strains increases with age, we performed this analysis in 7- to 9-week-old mice at which time only minute numbers of DN cells are sustained in *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice (Figure

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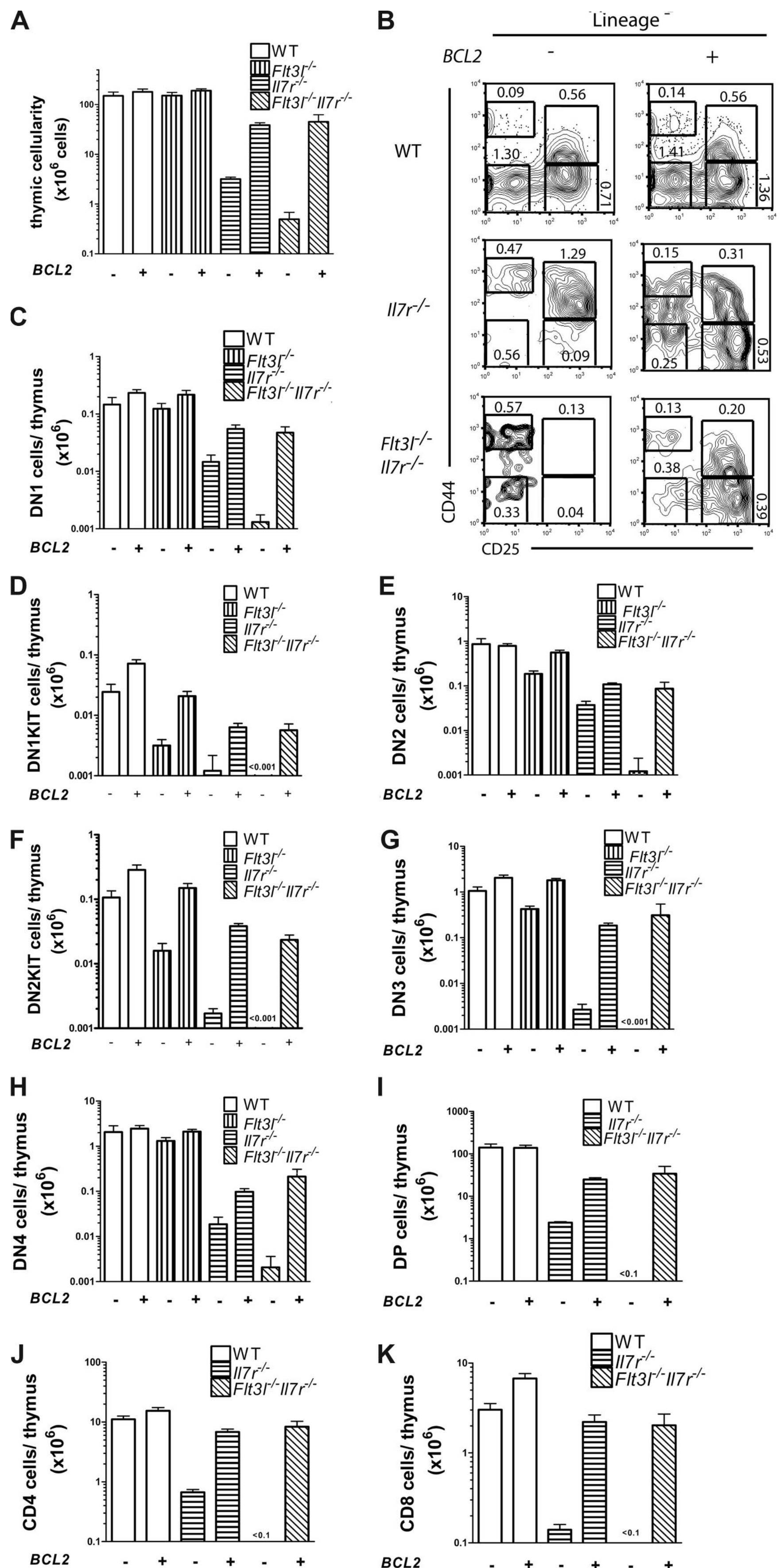

Figure 3. BCL2-mediated rescue of defective thymopoiesis in adult *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice. (A) Mean (SD) thymic cellularities, (B) FACS profiles of DN staging, and mean (SD) numbers of (C) DN1, (D) DN1KIT^{+}, (E) DN2, (F) DN2KIT^{+}, (G) DN3, (H) DN4, (I) DP, (J) CD4 SP, and (K) CD8 SP cells in the thymus of 7- to 9-week-old WT, *Flt3l*$^{-/-}$, *Il7r*$^{-/-}$ and *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ mice on WT or *BCL2* transgenic background. Data are from 3 to 4 WT, 12 *Flt3l*$^{-/-}$, 8 to 12 *Il7r*$^{-/-}$, 4 to 10 *Flt3l*$^{-/-}$*Il7r*$^{-/-}$, 7 to 9 *BCL2*, 14 *Flt3l*$^{-/-}$*BCL2*, 12 *Il7r*$^{-/-}$*BCL2*, and 6 to 7 *Flt3l*$^{-/-}$*Il7r*$^{-/-}$*BCL2* mice, in all cases from at least 2 separate litters. Numbers in FACS profiles show the mean percentage (of total thymocytes) of cells within the indicated gates. For each strain and cell type, cutoff numbers in graphs are indicated. However, for one mouse in the *Flt3l*$^{-/-}$*Il7r*$^{-/-}$ group, in which no DN1KIT^{+} and DN2KIT^{+} cells were detected, too few events were acquired to be applicable to the cutoff value.

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3A-H). Notably, not only could we demonstrate an extensive rescue (although not completely to the levels in *BCL2* mice) of the earliest as well as later stages of T lymphopoiesis in the thymus of $Il7r^{-/-}$ mice, but also the reduction in DN progenitors in $Flt3l^{-/-}$ mice were largely rescued on *BCL2* overexpression, including rescue of KIT-positive DN1 and DN2 immature T-cell progenitors[27-29] (Figure 3A-H). Most notably, the virtually complete loss of all stages of DN as well as DP and SP thymocytes in $Flt3l^{-/-}Il7r^{-/-}$ mice was substantially rescued by *BCL2*, resulting in the recovery of all stages of thymocyte progenitors to the same levels observed in $Il7r^{-/-}$ mice in which *BCL2* had been overexpressed (Figure 3A-K). For all populations, statistical analyses were performed to test whether the reduction of lymphocyte populations in the cytokine deficient mice on a WT background was more severe than on a *BCL2* background, using data transformed to a logarithmic scale (see "Methods" and Document S1 for details). Based on these calculations, the reductions of DN1KIT$^+$, DN2-DN4, DP and SP CD4/8 thymocyte populations were all significantly ($P < .01$) reduced by *BCL2* in IL-7Rα-deficient mice (Figure 3A-K). Similarly, reductions of DN2, DN2KIT$^+$, and DN3, were significantly ($P < .05$) reduced by *BCL2* in FLT3L-deficient mice (Figure 3E-G). Comparing the double deficiency of FLT3L and IL-7Rα on a WT and *BCL2* background, the reductions of thymocytes on the *BCL2* background was much less severe for all thymocyte populations (Figure 3A-K, $P < .01$). Moreover, in $Flt3l^{-/-}Il7r^{-/-}$ mice, numbers of CD4$^+$ and CD8$^+$ T cells in spleen were increased 99- and 15-fold, respectively, in mice on a *BCL2* compared with WT background (Figure S1). Notably, numbers of splenic T cells in $Flt3l^{-/-}Il7r^{-/-}BCL2$ mice were not significantly different from T cells in $Il7r^{-/-}BCL2$ mice (Figure S1).

We next investigated whether the *BCL2*-rescued thymocytes in $Flt3l^{-/-}Il7r^{-/-}$ mice expressed genes critical for T-cell development. RT-PCR analysis demonstrated that thymocytes from $Flt3l^{-/-}Il7r^{-/-}BCL2$ mice expressed recombination activating gene 2 (*Rag2*) and Pre-T-cell antigen receptor α (*Ptcra*), required for T-cell receptor (TCR) rearrangements and assembly of the pre-TCR. In contrast, expression of these genes was undetectable in residual thymocytes from $Flt3l^{-/-}Il7r^{-/-}$ mice (Figure 4A). Furthermore, *Tcrb-D-J* (Figure 4B) and *Tcrb-V-DJ* (Figure 4C) rearrangement diversity was observed in $Flt3l^{-/-}Il7r^{-/-}BCL2$ thymocytes in a pattern comparable with WT thymocytes, supporting the hypothesis that *BCL2* rescues polyclonal thymopoiesis in adult $Flt3l^{-/-}Il7r^{-/-}$ mice.

Multipotent progenitor cells in the BM are required to continuously replace short-lived thymocytes.[30] However, $Flt3l^{-/-}Il7r^{-/-}$ BM cells fail to reconstitute thymopoiesis.[8] Strikingly, whereas transplantation of $Flt3l^{-/-}Il7r^{-/-}$ BM cells into lethally irradiated $Flt3l^{-/-}$ recipients failed to reconstitute thymopoiesis, thymic reconstitution was observed with $Flt3l^{-/-}Il7r^{-/-}BCL2$ BM cells (Figure 5A,B). Specifically, total donor-derived thymic cellularity, DP, CD4 SP, and CD8 SP thymocytes were increased 37-fold, 98-fold, 163-fold, and 49-fold, respectively, in recipients of $Flt3l^{-/-}Il7r^{-/-}BCL2$ BM compared with $Flt3l^{-/-}Il7r^{-/-}$ BM cells (Figure 5A,B).

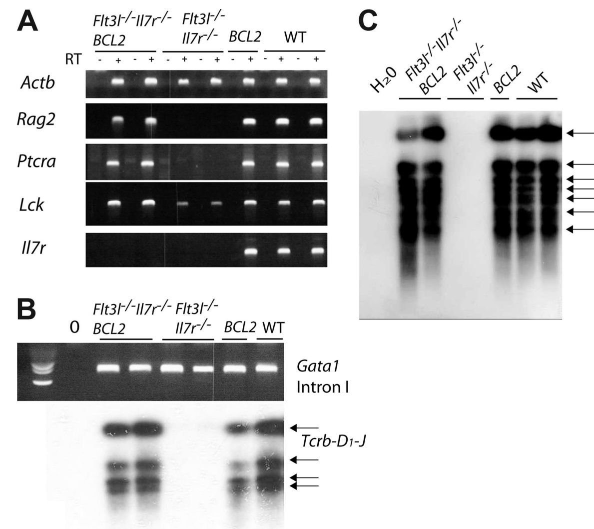

Figure 4. Molecular evidence for *BCL2*-induced rescue of FLT3L and IL-7Rα–dependent thymopoiesis in $Flt3l^{-/-}Il7r^{-/-}$ mice. (A) Ethidium bromide-stained agarose gels with resulting PCR products from RT-PCR analysis of *Rag2, Ptcra, Lck*, and *Il7r* message in thymus from mice of the indicated genotypes. Autoradiograms of (B) *Tcrb-D1-J* rearrangements and (C) *Tcrb-V-DJ* rearrangements in the thymus of WT, *BCL2*, $Flt3l^{-/-}Il7r^{-/-}$, and $Flt3l^{-/-}Il7r^{-/-}BCL2$ mice. Amplification of a region in the *Gata1* gene was performed to verify that the DNA preparations were of comparable quality. Results are from representative mice of each genotype. Arrows indicate rearrangement products. Please note that lines visible on reproduction of gels represent reproduction artifacts.

Discussion

Mice doubly deficient in FLT3L and IL-7Rα have virtually no existing adult thymopoiesis.[8] Because the IL-7Rα has 2 ligands, IL-7 and TSLP, $Flt3l^{-/-}Il7r^{-/-}$ mice lack FLT3L, IL-7, as well as TSLP-mediated signaling. In the present studies, we explored the relative importance of these key cytokines, all implicated in early T-cell development. TSLP has, by comparative studies in IL-7Rα and IL-7 signaling deficient mice, been suggested to have a major role in IL-7–independent B lymphopoiesis.[15,16] Furthermore, findings in support of a role for TSLP in IL-7–independent T-cell development have been reported, as mice double-deficient in IL-2Rγ and TSLPR show an additional thymic phenotype compared with single IL-2Rγ–deficient mice.[18] Our comparative studies of IL-7Rα– and IL-7–deficient mice confirmed a more severe T-cell phenotype in $Il7r^{-/-}$ than $Il7^{-/-}$ mice, further supporting the idea that TSLP might play a role in IL-7–independent thymopoiesis. However, when directly investigating the role for TSLP in IL-7–independent thymopoiesis, we found no clear differences at any stage of thymocyte development between $Il7^{-/-}$ and $Il7^{-/-}Tpte2^{-/-}$ mice, clearly indicating that TSLP does not play a key role in IL-7–independent T lymphopoiesis. In light of these findings, the more aggravated T-cell phenotype in $Il7r^{-/-}$ compared with $Il7^{-/-}$ mice may instead be explained by alternative, TSLP-independent, mechanisms. Such mechanisms may potentially include yet unidentified, IL-7Rα ligands or cross-activation of IL-7Rα in $Il7^{-/-}$ mice through other pathways, such as KIT, as recently suggested.[31] Although our findings do not support a key role of TSLP in IL-7–dependent or –independent steady state thymopoiesis, it remains possible that TSLP might be involved in T lymphopoiesis in conditions other than steady state.

We also failed to obtain any evidence in support of TSLP playing a role in FLT3L-independent T lymphopoiesis as $Flt3l^{-/-}$ and $Flt3l^{-/-}Tpte2^{-/-}$ mice revealed indistinguishable T-cell phenotypes. Furthermore, the lack of active thymopoiesis in adult $Flt3l^{-/-}Il7^{-/-}$ as well as $Flt3l^{-/-}Il7r^{-/-}$ mice unequivocally demonstrated that intact TSLP function is insufficient for sustaining active thymopoiesis in the absence of FLT3L and IL-7–mediated signaling. Taken together, FLT3L and IL-7 are

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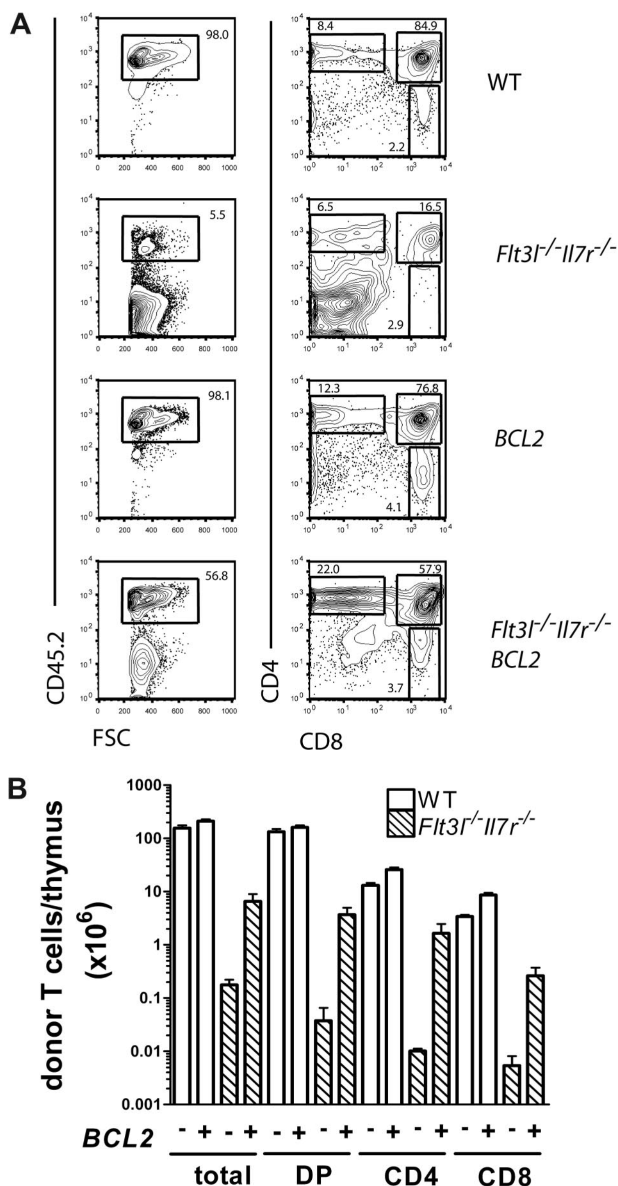

Figure 5. **Ectopic expression of BCL2 enhances the T-cell reconstitution potential of *Flt3l$^{-/-}$Il7r$^{-/-}$* BM cells**. 5×10^6 littermate *Flt3l$^{-/-}$Il7r$^{-/-}$* or *Flt3l$^{-/-}$Il7r$^{-/-}$BCL2* (CD45.2) unfractionated BM cells were transplanted into lethally irradiated *Flt3l$^{-/-}$* (CD45.1) recipients. As positive controls, 5×10^6 littermate WT or *BCL2* unfractionated BM cells were transplanted into WT (CD45.1) recipients. (A) FACS profiles showing donor (CD45.2)-derived CD4 and CD8 DP and SP cells in thymus 6 weeks after transplantation. Numbers in FACS profiles represent the mean percentage of total donor cells relative to total thymocytes and the contribution of CD4 and CD8 cells within donor cells. (B) Mean (SD) numbers of donor-derived total, DP, and SP CD4$^+$ and CD8$^+$ thymocytes per thymus, at 6 weeks after transplantation. Data are from 4 recipients of WT, *Flt3l$^{-/-}$Il7r$^{-/-}$*, *Flt3l$^{-/-}$Il7r$^{-/-}$BCL2* and 3 recipients of *BCL2* cells.

critical extrinsic regulators of thymopoiesis, whereas TSLP appears to plays no critical role.

Previous studies have questioned whether the important functions of cytokine tyrosine kinase receptors in T lymphopoiesis are mediated through permissive actions because overexpression of *BCL2* had no rescuing effect on defective T lymphopoiesis in KIT-deficient mice.[7] In contrast, we demonstrated here that the defect in early T-cell progenitors in *Flt3l$^{-/-}$* mice, and the virtually complete loss of all stages of thymocytes in adult *Flt3l$^{-/-}$Il7r$^{-/-}$* mice is largely rescued (although not fully to the levels of *BCL2* transgenic mice), and all developmental stages become clearly detectable, on enforced expression of *BCL2*. In further support of a *BCL2*-mediated rescue of normal polyclonal thymopoiesis in adult *Flt3l$^{-/-}$Il7r$^{-/-}$* mice, rearrangement diversity and expression of key genes required for normal T-cell development comparable with that of WT thymocytes were restored on overexpression of *BCL2* in *Flt3l$^{-/-}$Il7r$^{-/-}$* mice. Furthermore, the inability of adult *Flt3l$^{-/-}$Il7r$^{-/-}$* BM cells to reconstitute the thymus of FLT3L deficient mice was partially corrected on enforced expression of *BCL2*.

In conclusion, our studies demonstrate for the first time that the requirement for ligands of cytokine tyrosine kinase receptors (ie, FLT3), as ligands of the hematopoietin receptor family (ie, IL-7), can largely be substituted by the potent antiapoptotic regulator BCL2, supporting the hypothesis that both of these cytokine receptor pathways at least in part mediate critical functions in T-cell development through permissive actions.

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Authorship

Contribution: C.T.J., M.S., E.S., and S.E.W.J. designed and conceptualized the research, analyzed the data, and wrote the manuscript; C.T.J., S.K., C.B., M.S., and A.L. did the characterization of the different mouse strains; and T.R. analyzed the data and performed statistical analysis.

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