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Distinct roles for Syk and ZAP-70 during early thymocyte development

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The spleen tyrosine kinase (Syk) and ζ-associated protein of 70 kD (ZAP-70) tyrosine kinases are both expressed during early thymocyte development, but their unique thymic functions have remained obscure. No specific role for Syk during β-selection has been established, and no role has been described for ZAP-70 before positive selection. We show that Syk and ZAP-70 provide thymocytes with unique and separable fitness advantages during early development. Syk-deficient, but not ZAP-70–deficient, thymocytes are specifically impaired in initial pre-TCR signaling at the double-negative (DN) 3 β selection stage and show reduced cell-cycle entry. Surprisingly, and despite overlapping expression of both kinases, only ZAP-70 appears to promote sustained pre-TCR/TCR signaling during the DN4, immature single-positive, and double-positive stages of development before thymic selection occurs. ZAP-70 promotes survival and cell-cycle progression of developing thymocytes before positive selection, as also shown by in vivo anti-CD3 treatment of recombinase-activating gene 1–deficient mice. Our results establish a temporal separation of Syk family kinase function during early thymocyte development and a novel role for ZAP-70. We propose that pre-TCR signaling continues during DN4 and later stages, with ZAP-70 dynamically replacing Syk for continued pre-TCR signaling.

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Abbreviations used: dKO, double KO; DN, double negative; DP, double positive; FL, fetal liver; ISP, immature SP; LAT, linker for activation of T cells; Rag, recombinase-activating gene; Slp-76, Src homology 2 domain–containing leukocyte phosphoprotein of 76 kD; SP, single positive; Syk, spleen tyrosine kinase; ZAP-70, ζ-associated protein of 70 kD.

ζ-Associated protein of 70 kD (ZAP-70) and spleen tyrosine kinase (Syk) are cytoplasmic protein tyrosine kinases involved in immunoreceptor signaling. Both have been implicated in TCR signaling during thymocyte development. The ZAP-70–deficient mouse displays an absolute block in thymocyte development at the positive selection stage (i.e., the transition from the $CD4^+CD8^+$ double-positive [DP] to the $CD4^-CD8^+$ or $CD4^+CD8^-$ single-positive [SP] stages), whereas the Syk-deficient thymus shows no obvious block (1, 2). Strikingly, the combined loss of both kinases results in an absolute block at the earlier TCRβ selection stage, a stage where thymocytes are selected for rearrangements of in-frame TCRβ chains (3). This finding, combined with an absolute requirement for Src family kinase signaling (4, 5), is consistent with the canonical two-step immunoreceptor pathway originally described in T cell lines that also apply to this early step of thymocyte development (6–8). That is, sequential Src family kinase–dependent immunoreceptor tyrosine-based activation motif phosphorylation, followed by Syk family kinase signaling, is required for normal progression through β selection.

Successful rearrangement and expression of an in-frame TCRβ chain creates a protein that pairs with the invariant pre-Tα chain to form a pre-TCR. The pre-TCR engages the already expressed signaling machinery in the immature thymocyte in an apparently ligand-independent manner and generates a signal (9). It is widely assumed that simple expression of a functional pre-TCR at the β selection stage generates a signal that, combined with other signals such as TNF and Notch signaling, is sufficient to generate normal DP cells that are ready to undergo thymic selection (10–13). The pre-TCR signal has many purposes, including rescue from apoptosis, proliferation, and differentiation among others (10, 12). Mice unable to rearrange TCR genes or signal through the pre-TCR illustrate the critical importance of β selection. For example, $rag^{-/-}$ (recombinase-activating gene [Rag] KO) thymocytes cannot rearrange TCRβ or TCRγδ loci and are completely arrested at this stage with thymic cellularity reaching only 2% that of the WT.

The β selection stage is readily identified in the mouse. Thymocytes from mice deficient in

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components of pre-TCR signaling are blocked at the $CD3^-CD4^-CD8^-CD11b^-CD19^-DX5^-Gr1^-TCR\gamma\delta^-CD44^-CD25^+$ (Lin^-) double-negative (DN) 3 stage. Thus, $rag^{-/-}$ thymocytes, or those lacking the nonredundant adapters Src homology 2 domain–containing leukocyte phosphoprotein of 76 kD (Slp-76) or linker for activation of T cells (LAT), or enzymes such as Src or Syk family kinase members, are completely blocked at DN3 (3–5, 14–16). Moreover, because no signal is generated, the DN3 cells remain small and quiescent, unable to enter the cell cycle and achieve a blast-phase morphology, as occurs in normal development. Although the double KO (dKO) of ZAP-70 and Syk suggests a degree of redundancy between these two kinases, it is not known whether ZAP-70 or Syk is preferentially used during β selection.

After TCRβ selection and pre-TCR signaling, thymocytes proceed through a sequence of proliferation and differentiation before reaching a second critical selection checkpoint at the $CD4^+CD8^+$ DP stage. Similar to the DN3 pre-TCR signaling stage, thymic selection also critically depends on the TCR signaling machinery. Positive (and negative) selection depends on ZAP-70, TCRα, and MHC molecules, as shown by the respective KOs (17). However, absolute blocks at the DN3 stage may preclude assessing a role at later stages. For example, it had been assumed that Slp-76 is required for positive/negative selection and peripheral T cell activation, yet direct evidence was lacking for thymic selection until recently (18). Similarly, the pre-TCR may be necessary and functioning at later stages, such as between DN3 and DP, and this may be masked by absolute blocks at DN3 (Slp-76 and LAT KOs) or progression to the DP stage because of kinase family redundancy (Fyn and Lck, as well as ZAP-70 and Syk, KOs). The transcription factors basic helix-loop-helix, TCF/LEF, and Egr families and the nuclear hormone receptor RORγ are implicated (for review see reference 19) during these stages. These factors are thought to act at the immature SP (ISP) $CD8^+CD4^{-/lo}TCR\beta^{lo}$ stage immediately preceding the DP stage or at the DP stage, before selection. Aside from these factors, there are few reports of mutations that interfere with development between the DN3 and DP stages. It is possible that proper pre-TCR signaling using either Syk or ZAP-70 protein is necessary for normal transcription factor induction and progression during the stages between DN3 and DP.

To investigate the individual roles of Syk and ZAP-70 during early thymocyte development, we competed thymocyte progenitors from $syk^{-/-}$ and $zap70^{-/-}$ donors against each other as they repopulated a normal thymus after depleting the endogenous thymocytes. We found that only $syk^{-/-}$ thymocytes are specifically impaired in progressing past the DN3 β selection stage of development despite up-regulation of ZAP-70 within TCRβ-selected DN3. Unexpectedly, only $zap70^{-/-}$ thymocytes are uniquely impaired after the DN3 and before the DP stages, despite continued Syk expression within the DN4 and ISP stages. Our experiments indicate unexpected and distinct functions for Syk and ZAP-70 during early development and suggest that pre-TCR/TCR signaling continues during the DN4, ISP, and DP stages to generate normal numbers of DP cells before they undergo thymic selection.

RESULTS

Syk and ZAP-70 proteins provide thymocytes with differential fitness during development

We used competitive repopulation of the thymus in irradiated hosts as a sensitive and discriminating assay for in vivo thymocyte development. Such a competitive repopulation strategy might, for example, reveal subtle differences in proliferative capacities or usage of limiting growth factors. We used stem cell progenitors from $zap70^{-/-}$ and $syk^{-/-}$ embryos or animals and distinguished the two donor populations based on the congenic markers CD45.1 and CD45.2 (20). This strategy is outlined in Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20070405/DC1). Lethal irradiation of WT hosts and reconstitution with donor stem cells resulted in ∼98–99% replacement of all thymocytes within 4 wk by all genotypes tested (unpublished data). The 1–2% residual host cells detected were always within the mature SP subsets. All other subsets were routinely replaced >99% by donor-derived cells.

Because thymocyte development occurs as a well-characterized series of stages, the percentage represented by each genotype at a given stage is indicative of the developmental fitness of the thymocyte up until that stage. Any impairment or enhancement in fitness would result in a decrease or increase, respectively, at the next stage of development. The competitive repopulation of the thymus using $syk^{-/-}$ versus $zap70^{-/-}$ progenitors is shown in Fig. 1. Injection of equal amounts of BM or fetal liver (FL) resulted in roughly equal representation at the earliest DN2 and DN3 stages identified. Equal representation of $syk^{-/-}$ and $zap70^{-/-}$ thymocytes within these earliest subsets suggests that neither kinase has an important unique function in development until at least the DN3 stage. In striking contrast, the two populations are differentially represented at multiple stages immediately after DN3. At least two changes seemed to occur: $Syk^{-/-}$ thymocytes became underrepresented at the DN4 stage when compared with DN3, and after that, $zap70^{-/-}$ thymocytes became underrepresented while progressing from the DN4 to the DP stage. These could be explained in multiple ways. One possibility is that Syk provides a positive regulatory role at the DN3 stage of development (thus, $syk^{-/-}$ accumulates at DN3), whereas ZAP-70 provides a different positive regulatory role at the DN4 and ISP stages. Another possibility is that ZAP-70 provides a negative regulatory role at the DN3 stage, allowing $zap70^{-/-}$ thymocytes a greater fitness advantage in transitioning from DN3 to DN4. Similarly, $syk^{-/-}$ thymocytes may have an advantage in progression from DN4 to DP. Our results differ from previous work that had reported that ZAP-70's only unique function in thymocyte development was during thymic selection, at the DP stage (21). We also saw this effect in our assays, as ZAP-70 deficiency resulted in a complete block in positive selection as shown by

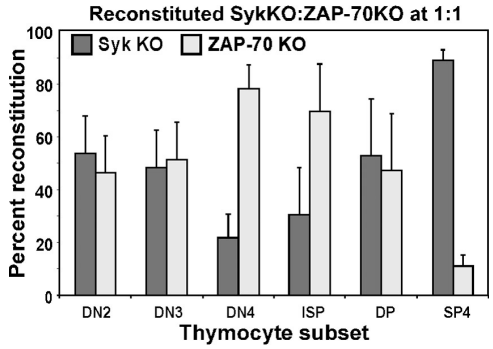

Figure 1. Syk-deficient and ZAP-70–deficient thymocytes show differential fitness during development. Competitive repopulation assay in which *syk*$^{-/-}$ and *zap70*$^{-/-}$ BM cells were injected at a 1:1 ratio, and the reconstituted chimeric thymus was analyzed 6 wk later. Syk-deficient BM was created by reconstituting lethally irradiated WT mice with *syk*$^{-/-}$ FL and harvested after 8 wk. Data are representative of four mice. Bars depict means ± SD. The experiment was repeated using FL from both genotypes with similar results.

the decrease in representation of *zap70*$^{-/-}$ thymocytes between the DP and SP4 stages. These data reveal that Syk and ZAP-70 uniquely contribute to the developmental fitness of early thymocytes at distinct stages well before positive selection occurs.

Syk And ZAP-70 proteins are inversely expressed during early thymocyte development

To understand the basis of differential fitness between *syk*$^{-/-}$ and *zap70*$^{-/-}$ developing thymocytes, we sought to determine the expression patterns of both kinases during all stages of thymocyte development. To investigate the expression patterns of Syk and ZAP-70 kinases, we quantitated the relative levels of mRNA in all thymocyte subsets. We sorted cells and used real-time fluorigenic RT-PCR to quantitate mRNA expression. The DN subsets were isolated based on standard phenotyping: CD25$^+$CD44$^+$ (DN2), CD25$^+$CD44$^-$ (DN3), and CD25$^-$CD44$^-$ (DN4). We found that *Syk* mRNA was robustly expressed at DN2, whereas *Zap70* was hardly detectable, almost 100-fold less than in peripheral T cells (Fig. 2 A). *Zap70* is first up-regulated within the DN3 population and increases thereafter. Interestingly, *Syk* mRNA expression decreases from DN3 to DN4 and spikes at the DP stage, before decreasing again at the SP stage. This pattern of Syk family kinase mRNA expression is consistent with another recent study (22).

After characterizing mRNA expression, we investigated ZAP-70 and Syk protein expression. We previously characterized Syk (23), but not ZAP-70, protein expression during thymocyte development. To do so, we conjugated an mAb previously made in this laboratory against ZAP-70 (24) to Alexa Fluor dyes. This gave a 25-fold increased signal in T versus B cells. This was specific, as NK cells and the 1–2% remaining T cells from *zap70*$^{-/-}$ mice provided a negative control (unpublished data).

Expression of ZAP-70 and Syk in all stages of thymocyte development is shown in Fig. 2 B. The overall pattern of expression of Syk agrees with our previous findings (23). We found ZAP-70 expressed at low levels during the earliest stages of development. In fact, robust ZAP-70 expression is not seen until DN4, which exhibits a four- to fivefold increase in ZAP-70 levels compared with DN3. ZAP-70 protein continues to increase thereafter (Fig. 2, B and C). CD8$^+$TCRβ^{hi} SP thymocytes had slightly more ZAP-70 than CD4$^+$ SP thymocytes, owing to their slightly larger size (unpublished data).

We sorted CD25$^+$ DN cells (pooled DN2/3) and performed immunoblot analysis on lysed cells (Fig. 2 D) to confirm low ZAP-70 protein expression in DN3. We also sorted CD25$^-$ DN cells and compared these cells with various lymphoid cell lines. This analysis confirmed that Syk is easily detectable within DN2/3 cells, whereas ZAP-70 is expressed at low levels. Overall, Syk and ZAP-70 are reciprocally expressed across thymocyte development, where Syk is highly expressed in early development and ZAP-70 is highly expressed in later development. However, there is considerable overlap where both kinases are detectable, including the DN3, DN4, and ISP stages.

Syk is necessary for normal progression past DN3 only

Intracellular flow cytometry, combined with our initial competitive repopulation assays, suggested that Syk may be serving a positive regulatory role at DN3 and serves a reduced role from the DN4 through the DP stages as its protein expression disappears. To test this possibility, we competed Syk-deficient progenitors against the WT in a repopulating thymus. This ruled out confounding effects brought on by competing against *zap70*$^{-/-}$ thymocytes. Because Syk is highly expressed in DN1 and possibly earlier, we assayed representation of DN1 after varying the ratios of FL injected into the host as a measure of the fitness of colonizing the thymus. Analysis of DN1 representation revealed no substantial changes in donor ratios (Fig. 3 A) compared with what was injected. Therefore, colonization is not substantially affected in *syk*$^{-/-}$ thymocyte progenitors after lethal irradiation of the host, consistent with our results in Fig. 1.

We then assessed donor representation within subsequent stages of thymocyte development. Fig. 3 B shows that *syk*$^{-/-}$ thymocytes were consistently underrepresented only after DN3. This was true at all starting ratios. Interestingly, *syk*$^{-/-}$ thymocytes were overrepresented within the CD4$^+$ SP and CD8$^+$ SP TCR^{hi} populations (not depicted). This was true whether the irradiated host was CD45.1$^+$ or CD45.2$^+$, suggesting that *syk*$^{-/-}$ thymocytes may be more fit than the WT at progressing from the DP to the SP stage.

We focused our experiments on whether Syk deficiency impairs TCRβ rearrangement and protein expression. DN3 cells from WT and *syk*$^{-/-}$ thymocytes had equal percentages of intracellular TCRβ (TCRβic$^+$) cells (Fig. 3 C). However, cell-cycle progression was impaired 40–50% in *syk*$^{-/-}$ DN3 (Fig. 3 D), as shown by decreased BrdU incorporation.

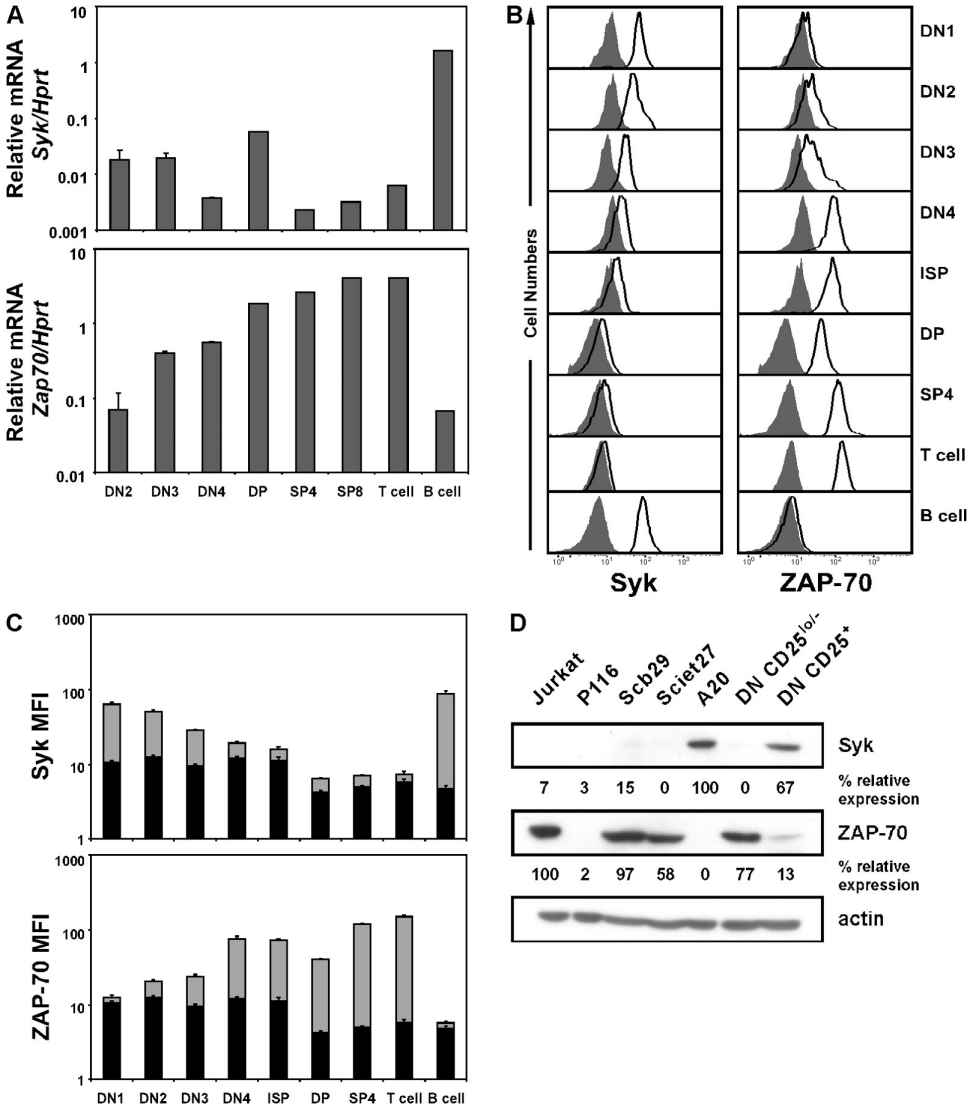

Figure 2. ZAP-70 and Syk protein are differentially expressed during thymocyte development. (A) The bar graphs indicate means ± SD of real-time quantitation of *Zap70* and *Syk* mRNA from the sorted cells (>98% purity) relative to a control gene (*Hprt*). The entire sort and RNA quantitation was performed twice using two groups of 20 mice with similar results. (B) Flow cytometric histograms showing ZAP-70 and Syk protein expression in all major thymocytes subsets. Shaded histograms show an isotype-matched mouse IgG1 conjugated to the same fluorescent dye. (C) Bar graphs quantitatively present the means ± SD of ZAP-70 and Syk mean fluorescence intensity (MFI) protein expression, as shown in B, from three adult animals. This was repeated at least 16 times with similar results. Black bars indicate background IgG1 mean fluorescence intensity. (D) Immunoblot of ZAP-70 and Syk protein expression in sorted $CD25^{+}$ (DN2/3) and $CD25^{lo/-}$ (DN1/4) DN thymocytes at >98% purity (not depicted) and various lymphoid cell lines. Densitometry was used to quantitate the relative expression levels of the kinases in each lane.

In contrast, DN4 cell cycling was comparable in $syk^{-/-}$ and WT cells, indicating less of a role for Syk at this stage. Because apoptosis is difficult to detect within a steady-state thymus, we could not detect reproducible differences in apoptosis between genotypes within the mixed chimeras. Therefore, our competitive repopulation experiments indicated that Syk-deficient thymocytes have a fitness defect compared with WT thymocytes at the DN3 stage, and loss of Syk is only partially compensated for by ZAP-70. Furthermore, although TCRβ rearrangement seems to occur normally, Syk deficiency impairs cell-cycle progression at the DN3 stage.

Syk is preferentially required for the initial DN3-E pre-TCR signal that leads to DN3-L blast phase

DN3 cells are a mixture of at least two populations: small cells actively rearranging TCRβ loci (DN3-E or DN3a) and large cells that have successfully expressed a TCRβ chain, initiated pre-TCR signaling, and entered a cell cycle (DN3-L or DN3b) (25, 26). Our repopulation assays suggested that Syk is required for normal progression through one or both of these stages. To investigate which stage is affected, we analyzed $TCR\beta ic^{+}$ expression and compared it to cell size to discriminate DN3-E and DN3-L cells within WT and $syk^{-/-}$

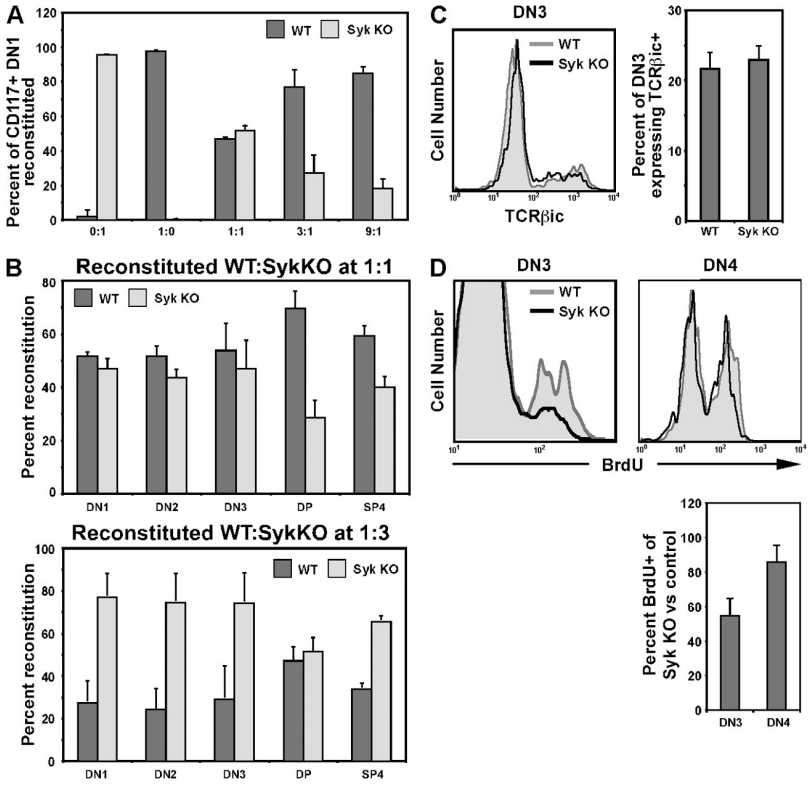

Figure 3. Syk is uniquely required for optimal progression past DN3. (A) WT and *syk*$^{-/-}$ FL cells from E15.5 embryos were injected at various ratios into lethally irradiated B6 hosts, and the reconstituted chimeric thymus was analyzed 5–7 wk later. DN1 was characterized by further excluding CD25^{+} events while gating on CD44^{+}CD117^{+}. Data indicate three to five mice per group in all experiments. Bars depict means ± SD. (B) The contribution of each genotype to major subsets after competing at 1:3 and 1:1 ratios. (C) Histograms showing representative intracellular TCRβ within DN3 for WT and *syk*$^{-/-}$ within the same mouse. Bar graph depicts means ± SD of percent TCRβ^{+}. (D) Histograms of representative BrdU incorporation within DN3 and DN4 of WT and *syk*$^{-/-}$ within the same mouse 1.5 h after injecting 2 mg BrdU. Bar graph indicates means ± SD of BrdU^{+} *syk*$^{-/-}$ thymocytes as a percentage of the mean of BrdU^{+} WT thymocytes within the same mice.

thymocytes. For a given mouse, TCRβic^{+} correlated well with increased cell size in WT thymocytes. However, in *syk*$^{-/-}$ thymocytes within the same mouse, TCRβic^{+} did not correlate with increased cell size, where the DN3-L population was greatly reduced despite normal levels of TCRβic^{+} expression (Fig. 3 C; and Fig. 4, A and B). We performed a similar analysis of DN3 from WT and *zap70*$^{-/-}$ mixed chimeric mice (further discussed in the following section). *Zap70*$^{-/-}$ thymocytes had normal numbers of DN3-L cell formation after TCRβic^{+} expression. Similar results were seen in mice reconstituted with varying ratios, or that were noncompetitively reconstituted, and in nonreconstituted WT and *zap70*$^{-/-}$ mice. Collectively, these results indicate that Syk has a nonredundant function in early pre-TCR signaling and is needed for normal blast formation and cell-cycle progression after TCRβ selection and protein expression. Additionally, our mixed chimeric studies indicate that this defect is thymocyte intrinsic.

We next wished to directly characterize the biochemical nature of the initial pre-TCR signal in WT and *syk*$^{-/-}$ thymocytes. However, traditional biochemical methods were prohibitive because of the paucity of DN subsets and the limitations of making sufficient chimeric mice. As an alternative to traditional biochemistry, we used flow cytometry to detect phosphorylation site–specific antibody staining in TCRβic^{-} DN3-E cells before and after CD3 cross-linking as a correlate of kinase activation. The phospho–ZAP-70 (Tyr319)/Syk (Tyr352) antibody used detects activated forms of both ZAP-70 and Syk. Small amounts of CD3 reach the surface of Rag1-deficient DN3 cells (27), and we speculated that this would be true of normal TCRβic^{-} DN3-E cells. Anti-CD3 stimulation of WT DN3-E thymocytes resulted in a twofold increase in phosphorylated Syk/ZAP proteins (Fig. 4 C, top left). Preincubating with PP2, an Src-kinase specific inhibitor, decreased the phospho-ZAP/Syk signal below background (not depicted), confirming that the signal emanates from a CD3-Src kinase–dependent pathway. Similar stimulations of *syk*$^{-/-}$ DN3-E thymocytes revealed that any phosphorylation of ZAP-70 is undetectable (Fig. 4 C, top right). As a control, CD4^{+} SP thymocytes from WT and *syk*$^{-/-}$ thymocytes (which express equal levels of ZAP-70 and no Syk) were equally phosphorylated on Syk family kinases after CD3 cross-linking. These data suggest that Syk, not ZAP-70, is preferentially activated downstream of

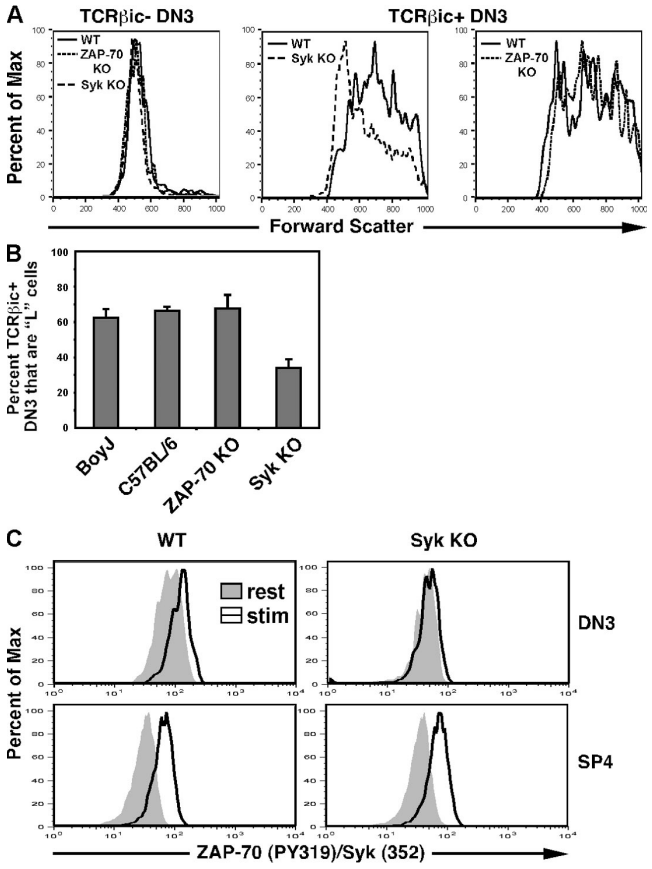

Figure 4. Syk deficiency impairs initial DN3 pre-TCR signaling and blast formation in a cell-autonomous manner. (A, left) Histogram depicts cell size of DN3 cells before TCRβic expression. (middle) Histogram compares the cell size of *syk*$^{-/-}$ with WT TCRβic^{+} DN3 within one mixed chimeric mouse. (right) Histogram compares the cell size of *zap70*$^{-/-}$ with WT TCRβic^{+} DN3 within one mixed chimeric mouse. (B) Quantitation of data in A, where each genotype is represented by at least nine chimeric mice. Bars depict percent means ± SD of TCRβic^{+} that are DN3-L using an arbitrary gate equally applied to all samples. (C) Activation state phosphorylation of Syk family kinases is measured in small CD25^{+}CD4^{-}CD8^{-} DN cells and CD4^{+}CD8^{-} SP after in vitro stimulation of unfractionated thymocytes with anti-CD3. Shaded histograms represent basal levels, whereas open histograms are after 2 min of stimulation. The experiment was repeated three times with similar results.

the CD3 complex within WT DN3-E cells and that this may account for the blast defect seen in *syk*$^{-/-}$ TCRβic^{+} DN3 thymocytes.

ZAP-70 has an unexpected and unique function in generating DP thymocytes before positive selection occurs

We next wished to further test whether ZAP-70 serves a positive regulatory role before positive selection. Our initial competitive repopulation experiments suggested that ZAP-70 might provide two novel functions: a negative regulatory function at DN3 as well as a positive function immediately after DN3 and before positive selection. Unlike *syk*$^{-/-}$ thymocytes, *zap70*$^{-/-}$ thymocytes had no discernable fitness impairment or enhancement at the DN3 stage, as measured by competitive repopulation versus the WT (Fig. 5 A). Blast size, TCRβ rearrangement, and protein expression were similar to the WT, as was cell-cycle status in DN3 and DN4 cells (Fig. 5, B and C). These data suggest that ZAP-70 has no notable role (positive or negative) at the DN3 stage that is not compensated for by Syk in the *zap70*$^{-/-}$ thymocyte during competitive repopulation. Competitive repopulation revealed that SP thymocytes are lacking in *zap70*$^{-/-}$ thymocyte subsets, consistent with the reported phenotype (21).

Surprisingly, *zap70*$^{-/-}$ thymocytes were also substantially impaired before the DP stage. *Zap70*$^{-/-}$ thymocytes were largely impaired even before the CD8^{+}CD4$^{-/lo}$TCRβ^{lo} ISP stage (Fig. 5 A). Thus, competition of *zap70*$^{-/-}$ versus the WT and *syk*$^{-/-}$ versus the WT is entirely complementary with our results from directly competing *syk*$^{-/-}$ against *zap70*$^{-/-}$ (Fig. 1, Fig. 3, and Fig. 5). Collectively, these data suggest a novel positive regulatory function for ZAP-70 during the DN4, ISP, and DP stages and before positive selection, as well as a distinct positive role for Syk at DN3-E. The individual competitions against the WT argue against any enhanced fitness upon deleting the kinases and any obvious negative regulatory functions.

ZAP-70 is necessary for normal generation of DP thymocytes after cross-linking CD3 on Rag DN3 thymocytes

It is possible that ZAP-70 is not involved in pre-TCR signaling but has some other function that is important and merely correlates with the pre-TCR^{+} cellular stage. For example, the pre-TCR may generate a single Syk-dependent signal at the DN3-E stage that leads to the expected 6–10 cell divisions and characteristic gene expression changes, whereas ZAP-70 functions in a parallel but different pathway. Alternatively, initial Syk-dependent pre-TCR signaling may give way to continuous ZAP-70–dependent pre-TCR and/or TCR signaling that is MHC independent between the DN3 and DP stages.

To test this, we used an in vivo model of pre-TCR signaling. *Rag1*$^{-/-}$ (Rag) thymocytes can be induced to differentiate into DP thymocytes by in vivo stimulation with anti-CD3 mAbs (27, 28). This process yields normal numbers of DP thymocytes and is dependent on kinases and the nonredundant T cell adapters Slp-76 and LAT (14, 15). We injected increasing amounts of anti-CD3 into *rag1*$^{-/-}$ mice to determine whether DP development was titratable. Increasing amounts of anti-CD3 resulted in a dose-dependent increase in total thymocyte production after 5 d and was primarily representative of DP production (Fig. 6 A, bar graphs). Absolute DN4 production also increased with increasing antibody, whereas DN3 cells decreased (not depicted), probably owing to the slow rate of replacement from BM stem cells and increased differentiation to DN4. This assay allowed us to ask whether ZAP-70 is necessary for DP generation when we control the strength and specificity of a pre-TCR–like signal. Anti-CD3 stimulation resulted in 7–10-fold less DP generation at all antibody concentrations in *rag1*$^{-/-}$*zap70*$^{-/-}$

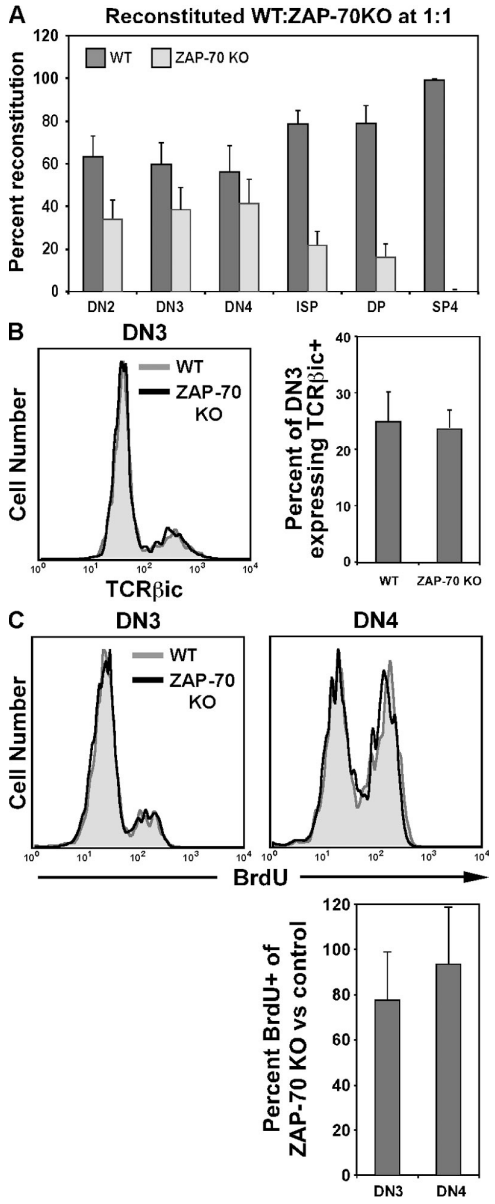

Figure 5. ZAP-70 is unexpectedly and uniquely required before positive selection for normal generation of DP thymocytes in a cell-autonomous manner. (A) Competitive repopulation assay in which 5×10^6 WT and *zap70*$^{-/-}$ BM cells were injected at a 1:1 ratio, and the reconstituted chimeric thymus was analyzed 7 wk later. The experiment represents seven mice and was repeated three times with similar results. Bars depict means ± SD. (B and C) Intracellular TCRβ and BrdU incorporation were analyzed as in Fig. 3 (C and D).

(RagZAP dKO) compared with Rag controls. The difference slightly decreased with increasing antibody concentrations, suggesting that increased signaling can lead to a normalization of cell numbers. Nevertheless, within the time frame studied, RagZAP dKO DN3 required 10-fold more antibody injected to achieve the same overall cellularity (compare RagZAP dKO at 100 μg with Rag at 10 μg). We believe this result represents a ZAP-70–dependent cell intrinsic defect downstream of the CD3 pathway before the positive selection stage.

Representative phenotypic profiles of Rag and RagZAP dKO thymocytes are shown in Fig. 6 A (right). No overall increase in cell numbers was seen in RagZAP dKO stimulated with 10 μg anti-CD3, yet 68% of cells differentiated to the DP stage, reflecting decreased absolute DN3s. At 30 μg, the frequency of DP is similar with or without ZAP-70, yet the overall and DP cellularity is greater than sevenfold less without ZAP-70. The absolute counts of DN3 and DN4 are also decreased two to fivefold in RagZAP dKO compared with Rag mice (not depicted).

To determine how ZAP-70 affects generation of DP cells in this assay, we measured levels of BrdU incorporation and Annexin V binding as markers of cell-cycle status and apoptosis, respectively. For both genotypes, the fraction of cells in cell cycle in all subsets increased with increasing antibody. Examples of BrdU incorporation at 100 μg are shown in Fig. 6 B. RagZAP dKO DN3 and DN4 cells incorporated ∼80% BrdU compared with the respective Rag KO cells, and RagZAP dKO DP cells incorporated only ∼35% (Fig. 6 B). Interestingly, the relative decrease in cell cycle in RagZAP dKO compared with Rag subsets was constant at various antibody concentrations. These data suggest that the fraction of cells in cell cycle in all subsets is proportional to the CD3-mediated (i.e., pre-TCR) signal and that this signal depends on ZAP-70.

Because the initiating CD3-based signal was largely synchronized in these experiments, assessment of apoptosis was possible. For both genotypes, apoptosis was highest after minimal stimulation with 10 μg anti-CD3 and is shown in Fig. 6 C. DN3, DN4, and DP subsets in RagZAP dKO were 5–20-fold more apoptotic than Rag after 10 μg anti-CD3 (Fig. 6 C). This difference decreased to approximately twofold in all subsets at greater antibody concentrations. Thus, unlike cell cycle, the effect of ZAP-70 on survival was proportional to the pre-TCR signal strength but appeared to be threshold dependent. The cell-cycle and apoptosis results suggest that ZAP-70 affects sustained pre- TCR signaling at all stages between DN3 and DP. We attempted similar experiments using *rag1*$^{-/-}$ and *rag1*$^{-/-}$*syk*$^{-/-}$ reconstituted animals (into lethally irradiated *rag1*$^{-/-}$ hosts) but could not achieve reproducible levels of proliferation even within *rag1*$^{-/-}$ reconstituted mice. This may be because of radiation damage to the vascular system of the host mouse (29) and inefficient absorption of the anti-CD3 antibody.

ZAP-70 protein is robustly up-regulated only after DN3 cells have signaled through the pre-TCR

Because ZAP-70 is quickly up-regulated within DN4 cells, we speculated that ZAP-70 might be a direct target or indicator of pre-TCR signaling. As noted before, DN3 thymocytes are a mixture of two populations: small, quiescent TCRβ-unrearranged DN3-E (DN3a) cells and large, cycling TCRβ-rearranged DN3-L (DN3b) cells that are beginning the progression toward DP. Our RNA and initial flow cytometry

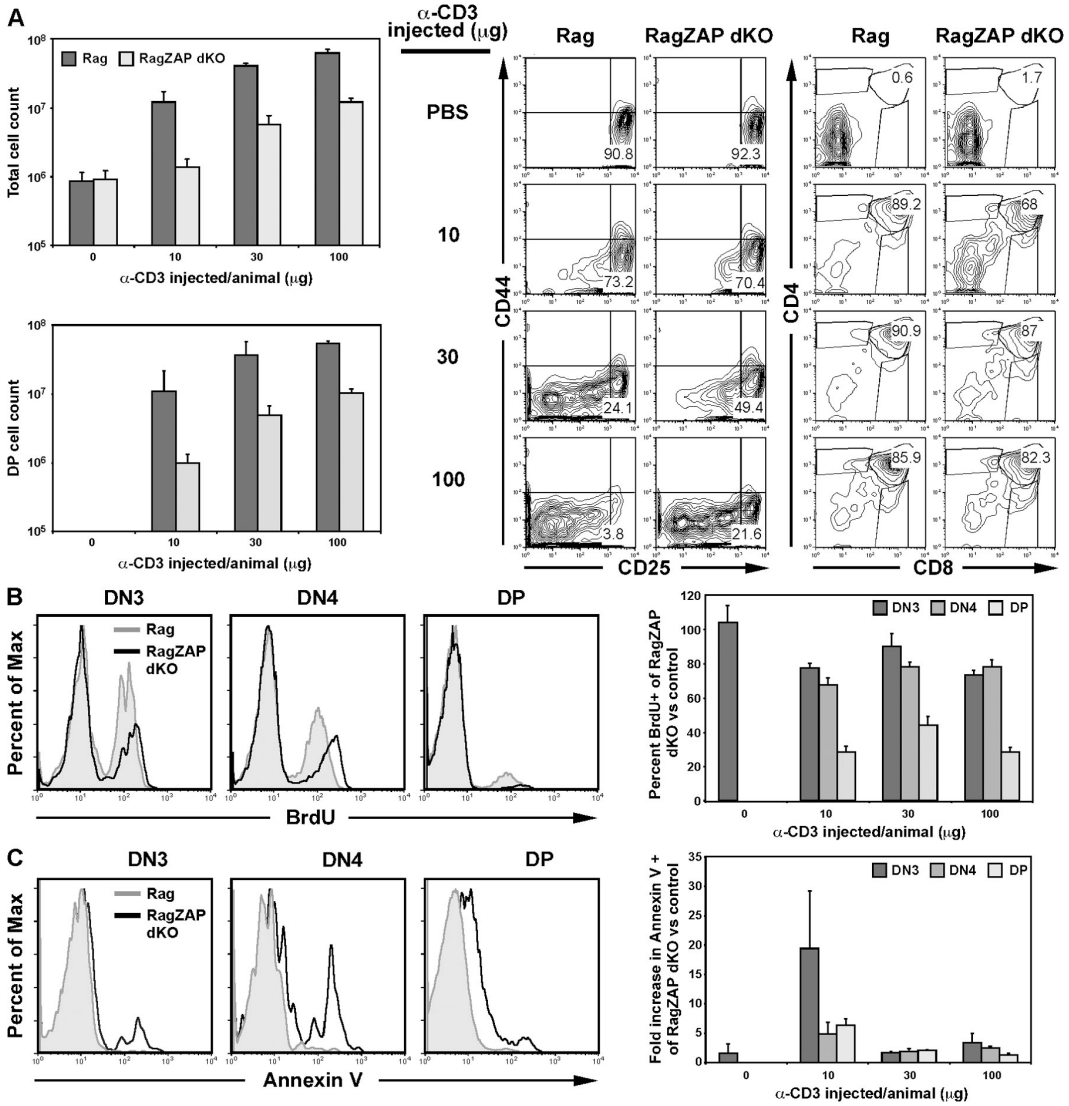

Figure 6. ZAP-70 is required for efficient generation and survival of DP cells in Rag mice after in vivo anti-CD3 stimulation. (A, top) Graph shows total cellularity from *rag1*$^{-/-}$ and *rag1*$^{-/-}$*zap70*$^{-/-}$ thymocytes after 5 d of in vivo treatment with increasing anti-CD3 mAb. (bottom) Graph shows DP cellularity. Each group represents three mice. Representative plots illustrate the CD25/CD44 profile of DN subsets and the CD4/CD8 profile for DP subset. Numbers on DN plots indicate percent DN3. Numbers on DP plots indicate percent DP. (B) Representative histograms of incorporated BrdU after a 100-μg anti-CD3 treatment. (right) Bar graph shows the mean percentage of BrdU^{+} cells in *rag1*$^{-/-}$*zap70*$^{-/-}$ subsets divided by the mean of *rag1*$^{-/-}$ control for 0, 10, 30, and 100 μg mAb injected. (C) Histograms of Annexin V^{+} staining in *rag1*$^{-/-}$*zap70*$^{-/-}$ dKO and *rag1*$^{-/-}$ thymocytes after anti-CD3 treatment as depicted in B. Representative example shown is after 10 μg anti-CD3. (right) Bar graph is similarly quantitated as in B.

studies conflicted on whether ZAP-70 was first expressed within the DN3 stage. We noticed that DN3 cells had a reproducible positive shoulder of ZAP-70 protein but not Syk (Fig. 7 A), suggesting that subpopulations have differential ZAP-70 expression.

We focused on CD25^{+} DN2/3 thymocytes and assayed for simultaneous intracellular expression of TCRβ and either ZAP-70 or Syk protein to investigate the earliest detectable TCRβ-selected cell. TCRβic^{+} cleanly separated a ZAP-70$^{lo/-}$ cell from a ZAP-70^{+} population. Thus, TCRβ selection correlates with ZAP-70 protein up-regulation. The increase in expression was about threefold. The antibody specificity was confirmed by staining equivalent *zap70*$^{-/-}$ CD25^{+} DN cells (unpublished data). ZAP-70 correlated with cell size, which also correlated to TCRβic^{+} expression (Fig. 7 A; and unpublished data). We also found that ZAP-70^{+} cells corresponded to CD27^{+} DN3b cells (unpublished data) (26).

These results suggested that ZAP-70 is up-regulated after TCRβ is rearranged and expressed. Therefore, the up-regulation of ZAP-70 may be a consequence of initial pre-TCR signaling itself. To test this, we assayed ZAP-70 expression in Rag KO mice, as they are completely blocked in pre-TCR

signaling and arrested at DN3. We injected increasing amounts of anti-CD3 mAb into Rag KO mice and examined ZAP-70 expression in DN3, DN4, and DP cells. ZAP-70 protein increased in a dose-dependent manner after anti-CD3 treatment, as shown for DN4 in Fig. 7 B. Treated RagZAP dKO mice confirmed the specificity of the signal (Fig. 7, B and C). ZAP-70 protein is almost completely absent in resting Rag KO DN3, consistent with a requirement of an initial pre-TCR signal for robust ZAP-70 up-regulation. ZAP-70 expression increased in DN3, DN4, and DP cells (Fig. 7 C; and unpublished data) after anti-CD3 injection in a dose-dependent manner. In addition, we assayed mice in which TCRβ rearrangement still occurs yet no signal is generated (*lat*$^{-/-}$ and *lck*$^{-/-}$*fyn*$^{-/-}$) and found no up-regulation of ZAP-70 or CD27 and, therefore, no correlation with intracellular TCRβ (unpublished data). Collectively, our data suggest that ZAP-70 is expressed at low levels in DN3-E thymocytes and is up-regulated only after initial pre-TCR signaling in DN3-L. Thus, ZAP-70, along with CD27, is among the earliest targets or indicators of pre-TCR signaling. Developing thymocytes have the unique feature by which initial Syk-dependent pre-TCR signaling at DN3-E quickly up-regulates ZAP-70, which then replaces Syk during sustained pre-TCR/TCR signaling during the DN3-L, DN4, ISP, and DP stages.

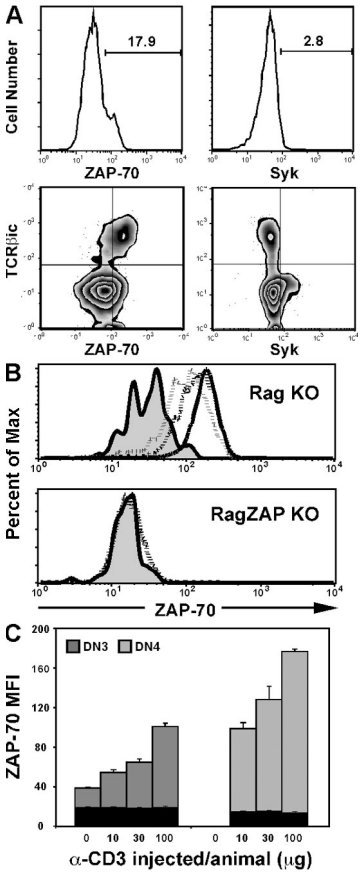

Figure 7. ZAP-70 protein is up-regulated after pre-TCR signaling. (A, top) ZAP-70 and Syk expression in all DN3 cells. (bottom) ZAP-70 protein compared with intracellular TCRβ chains within DN3s. (B, top) Dose-dependent increased ZAP-70 expression in DN4 from *rag1*$^{-/-}$ mice 5 d after in vivo anti-CD3 injection. Shaded histogram is background DN4 from PBS-treated mice. Other histograms show 10 μg (dotted line), 30 μg (dashed line), and 100 μg (open) of anti-CD3 treatment. (bottom) ZAP-70 staining in control-treated *rag1*$^{-/-}$*zap70*$^{-/-}$ dKO. (C) Bars depict quantitation of means ± SD of ZAP-70 expression as shown in B and from the DN3 and DN4 subsets 5 d after in vivo anti-CD3 treatment in *rag1*$^{-/-}$ mice. Three mice were included for each condition. Overlaid black bars show means ± SD of ZAP-70 expression in similarly treated *rag1*$^{-/-}$ *zap70*$^{-/-}$ dKO. This experiment was repeated twice with similar results.

DISCUSSION

We have examined the expression patterns and in vivo functions of the Syk family kinases Syk and ZAP-70 during early stages of thymocyte development. *Syk*$^{-/-}$ thymocytes are impaired in transitioning from DN3 to DN4 during competitive repopulation of the thymus. Analyses of mRNA abundance and intracellular flow cytometry indicated that early T cell progenitors express high levels of Syk and little or no ZAP-70, and this remains true as cells commit to the T lineage pathway and become DN2 cells. After TCRβ selection has occurred, Syk is necessary in quiescent DN3-E (DN3a) cells for optimal initial pre-TCR signaling and entry into cell cycle and transition to DN3-L (DN3b) cells. ZAP-70 appears to have a preferred function during sustained pre-TCR signaling from the DN4 through the DP stages and aids in the normal expansion and differentiation of thymocytes despite concurrent Syk expression (through the ISP stage), after which positive selection occurs. In vivo stimulation with anti-CD3 mAbs in Rag mice confirms that ZAP-70 uniquely functions downstream of the pre-TCR/TCR after the DN3-E stage and before DP selection. Loss of ZAP-70 mostly increases apoptosis levels but also decreases cell-cycle progression, especially at the DP stage. ZAP-70 expression is induced by the pre-TCR and is quickly up-regulated, coincident with CD27 when newly formed pre–T cells transition from DN3-E to DN3-L. Our data suggest the pre-TCR is a dynamic signaling module in which the signaling machinery is fundamentally altered during development. We propose a model where Syk-dependent pre-TCR signaling is replaced with ZAP-70–dependent pre-TCR signaling.

Previous work on the role of Syk in T cell development has been conflicting. Although the original KO papers suggested that Syk had little role in development (1, 2), later reports argued for either a unique role in TCRγδ development (30) or a more quantitative role in general T cell development (31). Our work is consistent with and expands on the results of Colucci et al. (31). That study found that DN3 cells were found at higher frequencies in *syk*$^{-/-}$ reconstituted mice, but they never competed *syk*$^{-/-}$ thymocytes versus the WT within a given mouse. Our studies allow direct comparison of the in vivo fitness of *syk*$^{-/-}$ versus WT and *syk*$^{-/-}$ versus *zap70*$^{-/-}$ thymocytes within the same mouse, allowing us to assess a quantitative impairment of the block and to conclude that the impairment is cell intrinsic. Further still, we demonstrate that ZAP-70 protein is poorly expressed within

the DN3-E thymocytes, providing a logical explanation for *syk*$^{-/-}$ impairment.

Clearly, the pre-TCR has several well-defined functions (10). However, it is not established when these functions are used. Rescue from apoptosis may be necessary at the DN3-E stage but may also be necessary at every stage from DN3-L to DP for normal population expansion. One can imagine continuous pre-TCR signaling enabling maximal cell-cycle progression at all stages. In contrast to this, decreasing Rag activity and enforcing allelic exclusion of the TCRβ locus might only be necessary at the DN3-E stage. Some of these distinct functions have been shown to diverge downstream of the pre-TCR (19). We propose that they also temporally diverge during differentiation and expansion. ZAP-70 may provide continuous pre-TCR/TCR signaling during the DN3-L, DN4, ISP, and/or DP stages that is needed for optimal development. Indeed, our data from in vivo–stimulated Rag mice demonstrate a strong correlation with anti-CD3 titration and ZAP-70 protein expression in all subsequent stages and absolute DP generation. A similar function has recently been proposed for Slp-76 (18). Continuous or constitutive pre-TCR signaling has been previously suggested. Studies of *c-cbl*$^{-/-}$ thymocytes suggest a continuous pre-TCR signal, as these cells have pre-TCRs that fail to be continuously internalized (32). DN4 cells are shown to activate NF-κB and NFAT downstream of the pre-TCR (33). A recent study demonstrated temporal separation of Egr3 and RORγ induction after pre-TCR signaling, both of which are necessary for proper DP generation (34).

Other signaling components are also known to promote the proliferation and survival of DN and DP stages. Early stages of TCRβ selection require activation of Notch1 (11), p53 (35), FADD (36), and NF-κB (33, 37) pathways, among others, many of which are likely to be affected downstream of the pre-TCR. Because these molecules exert their effects at or immediately after the DN3 stage, they may be optimally activated by an Syk-dependent pre-TCR signal. However, Bcl-X, RORγ, and TCF/LEF all affect the survival of DP cells (38, 39), and they may be preferentially influenced downstream of a ZAP-70–dependent pre-TCR signal.

Notch and E2A activities precede/activate a host of T lineage genes such as CD3 and Lck as early as stage DN1 (40–42). Therefore, it is surprising that ZAP-70, a largely T lineage–specific protein, is not highly expressed until after the first T lineage commitment checkpoint. One teleological explanation may be that Syk is better able to transduce the initial pre-TCR signal. This could either be a quantitative or a qualitative difference. Quantitatively, Syk is a more active kinase in vitro, causes more basal activation in T cells, and is less Src kinase–dependent in T cell lines (for review see reference 43), which makes it less coreceptor dependent. In fact, the use of Syk for initial pre-TCR signaling may explain why the Lck KO is more leaky at pre-TCR signaling than at positive selection. Qualitatively, Syk may activate/inactivate specific genes. After initial pre-TCR signaling, ZAP-70 may be better able to transduce continuous pre-TCR signals that are necessary for DN to DP transitioning. A previous study addressed whether Syk can substitute for ZAP-70 during positive selection by transgenic expression of Syk in a ZAP-70–deficient mouse strain (44). These thymocytes overexpressed Syk after β selection and through the DP stage, suggesting that if Syk were overexpressed it could replace ZAP-70 during continuous pre-TCR signaling and DP expansion. However, the efficiency of DP generation was not determined, as these thymocytes were not tested by competitive repopulation, leaving open the question of whether Syk could comparably replace all ZAP-70 functions if its expression was maintained from the DN3 to the DP stage. A recent study showed that Syk-expressing B cells require the traditional mitogen-activated protein kinase kinase pathway for p38 activation, whereas ZAP-70–expressing T cells can also activate p38 in a novel mitogen-activated protein kinase kinase 3/6–independent manner (45). Thymocytes require that p38 not be activated during initial pre-TCR signaling (46), and Syk may suffice. However, p38 becomes necessary for normal transitioning from the DN4 to the DP stage (47) and, thus, may be largely induced by ZAP-70–based pre-TCR signaling. We are currently testing these possibilities.

It is certainly possible that the differential fitness of Syk and ZAP-70–deficient thymocytes may, at least in part, be reflective of the distinct expression levels of each kinase during these stages and may not be caused by a unique function of the given kinase at a certain stage. To formally test this would require a model system involving the competition of thymocytes that express comparable levels of either ZAP-70 or Syk throughout thymic development on a null background for both kinases. Additionally, as TCRγδ cells diverge from TCRαβ cells at this DN3 stage because of a purported difference in signal strength, the balance of γδ versus αβ T cells may be altered (48). A relative overexpression of ZAP-70, Syk, or both could alter this ratio by increasing the strength of the signal generated, thereby increase relative numbers of TCRγδ. Our comparative studies detail for the first time the extent to which thymocytes differentially express both Syk family kinases and show that such differential expression/and or function results in differential cellular fitness during early development, where a Syk family–driven pre-TCR signal is necessary for normal DP expansion.

Interestingly, one unusual feature that distinguishes TCRα selection from the analogous Ig light chain selection is that TCRα chains are not allelically excluded (49, 50). Although the mechanisms leading to allelic exclusion are not well understood, Syk may be uniquely suited to better transduce these signals in both lineages. Although ZAP-70 can perform some Ig heavy chain allelic exclusion (51), Syk is mostly responsible for this. It is unknown whether Syk or ZAP-70 differentially effect allelic exclusion of the TCRβ chain.

In summary, our studies identify a specific role for Syk during β selection and cell-cycle entry and uncover an unexpected relative requirement for ZAP-70 during early thymocyte development. This occurs despite overlapping expression of both kinases within DN3-L and DN4 and ISP cells. Our

data are also consistent with a model in which Syk-dependent pre-TCR signaling induces ZAP-70 expression, which then replaces Syk for ZAP-70–dependent pre-TCR signaling as thymocytes progress toward the DP stage.

MATERIALS AND METHODS

Mice. Zap70$^{-/-}$ mice were backcrossed at least seven times onto the B6 background. Syk$^{+/-}$ mice were carried as heterozygotes because of their homozygotic perinatality and were backcrossed at least 11 generations to B6. Syk$^{+/-}$ was later crossed to the BoyJ (B6 mice with the CD45.1^{+} congenic marker B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ) background for one generation. B6 and rag1$^{-/-}$ mice used for breeding were originally obtained from the Jackson Laboratory. B6 mice used as hosts for competitive repopulation were obtained from Charles River Laboratories. BoyJ mice were purchased from Taconic and the Jackson Laboratory. For BrdU studies, 1–2 mg BrdU (Sigma-Aldrich) at 20 mg/ml was injected i.p. 1–2 h before killing the animal. All animals were housed in specific pathogen-free facility at the University of California, San Francisco according to university and National Institutes of Health guidelines.

Real-time RNA quantitation. RNA was extracted using TRIzol (Invitrogen), according to the manufacturer's instructions. cDNA was created using the Sensiscript protocol (QIAGEN). Primers and probes used for quantitative PCR span introns and were as follows. For mouse *ZAP70*, 5′-GCATGCGCAAGAAGCAGATT-3′ (forward primer), 5′-GGGCCTCTCGCATCATCTC-3′ (reverse primer), and [6-FAM]CTTTGTCGGCCTTCTCTGTGCCCTG[BHQ1~Q] (probe) were used. For mouse *Syk*, 5′-CTGGTTCCATGGCAACATCTC-3′ (forward primer), 5′-TGGCCCTGATCAGGAATTTTC-3′ (reverse primer), and [6-FAM]TGACCCTATGAGGACCGTCTGCTCTGAT[BHQ1~Q] (probe) were used. *HPRT* primers and PCR conditions were previously described (52).

Immunoblotting. Cell lysates were prepared using a 1% NP-40 lysis buffer with inhibitors and transferred to membranes after SDS-PAGE, as previously described (53). The A20 B cell line, Jurkat and P116 (ZAP-70–deficient) cells, and the pre–T cell lines Sciet27 and SCB29 were used as ZAP-70– and Syk-expressing lymphoid cell lines. Lysates were corrected for protein content before loading. Antiactin mAb was obtained from Sigma-Aldrich. Lanes were quantitated using an Image Station (model 440DF; Kodak) and ID image analysis software (version 3.5; Kodak).

Competitive repopulations and chimeras. For competitive repopulation assays, FL versus FL or BM versus BM cells were injected i.v. into lethally irradiated B6 or BoyJ hosts, and reconstituted thymi were analyzed 4–8 wk later. FL was from timed pregnancies at E15.5–16.5, and BM was from 8–12-wk-old adults. *Syk*$^{-/-}$ embryos were visually identified and confirmed by either PCR or flow cytometry of reconstituted blood. For some experiments, *syk*$^{-/-}$ FL was used to generate chimeric mice, and the resulting BM was used for competing or reconstituting irradiated hosts. No differences were seen using these BM compared with FL. WT recipients were irradiated with two doses of 600 rads, 3–5 h apart. Mixed FL chimeras were injected i.v. at ratios of 1:0, 0:1, 1:3, 3:1, 1:9, or 9:1. Each FL was resuspended in 1 ml media, and 200 μl (mixed or not) was used per mouse. For BM, 5×10^6 total cells were injected i.v. into each mouse. Males were used as recipients for most experiments, and females were used for some with no qualitative differences. Within an experiment, all hosts were sex and age matched and were between 5–10 wk old.

Antibodies and flow cytometry. Thymi were separated to single-cell suspensions using glass slides. $1–3 \times 10^6$ cells were used for each stain. mAbs against CD3, CD4, CD8, CD11b, CD19, and Gr-1 were used to exclude lineage-committed cells and delineate DN thymocytes and were conjugated to either PE or allophycocyanin (APC; BD Biosciences and eBioscience, respectively). Biotin-DX5, -TCRγδ and -CD69 (BD Biosciences) with streptavidin-APC or -PE were used to amplify the signal and/or exclude these cells. For Syk and ZAP-70 quantification in thymocyte subsets, DN1 was defined as Lin^{-} CD44^{+}CD117^{+}CD25^{-}. T and B cells were from the LN and spleen and defined by CD3^{+}CD19^{-} for T cells and CD3^{-}CD19^{+} for B cells. CD25, CD44, CD117, CD45R, and TCRβ (BD Biosciences) were conjugated to PE, PE-Cy5.5, or PerCP-Cy5.5 and FITC and further characterized DN subsets. mAbs against CD45.1 and CD45.2 were conjugated to FITC, PE, or APC. mAbs against mouse/human ZAP-70 1E7 (24) and mouse Syk 5F5 (23) were conjugated to Alexa Fluor 488 or Alexa Fluor 647 at fluorophore/protein ratios of 6.5–7:1 or 5:1, respectively, using the manufacturer's instructions (Invitrogen). Anti-BrdU was purchased from Invitrogen. Annexin V–FITC was obtained from Caltag. Fc binding was blocked using anti-CD16/32 2.4G2 (Harlan). For intracellular staining, cells were surfaced stained; fixed in Fix and Perm Medium A (Caltag); stained for intracellular ZAP-70, Syk, TCRβ, BrdU, or Lck with Fc block for 30–60 min in medium B (Caltag); washed with Perm/Wash (BD Biosciences); and fixed. BrdU staining also included 4.1 mM $CaCl_2$ and 20 U DNaseI (Roche). For phospho-ZAP/Syk analysis, cells were stimulated with 20 μg/ml anti-CD3 (2C11), followed 20 s later by 50 μg/ml goat anti–hamster (Jackson ImmunoResearch Laboratories) secondary cross-linker and fixed to terminate stimulation. Cells were permeablized using 90% ice-cold methanol for 30 min, stained with 2 μl phospho–Zap-70 (Tyr319)/Syk (Tyr352) antibody (Cell Signaling) for 1 h, stained with surface markers and donkey anti–rabbit–PE (Jackson Immunoresearch Laboratories) for 1 h, and fixed. Data were collected on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc.). For RNA and immunoblot analysis, cellular subsets were sorted using a sorter (MoFlo; DakoCytomation).

Anti-CD3 injections. 8–12-wk-old *rag1*$^{-/-}$ and *rag1*$^{-/-}$*zap70*$^{-/-}$ mice were used for in vivo anti-CD3 treatment. Anti-CD3 mAb 2C11 was injected i.p. at 10, 30, or 100 μg/animal diluted to 500 μl in PBS, and mice were killed and analyzed 5 d later. All experiments were repeated at least twice with similar results, and data are represented as the mean ± SD. For *rag1*$^{-/-}$ and *rag1*$^{-/-}$*syk*$^{-/-}$ experiments, chimeric mice were made from FL and were used 5–6 wk after reconstitution.

Online supplemental material. After stem cell transfer and reconstitution, competitive repopulation was assessed as shown in Fig. S1. To ensure an unbiased assessment, all subpopulations were identified first, followed by interrogation of the CD45 allele marker. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070405/DC1.

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