A hypomorphic allele of ZAP-70 reveals a distinct thymic threshold for autoimmune disease versus autoimmune reactivity

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ZAP-70 is critical for T cell receptor (TCR) signaling. Tyrosine to phenylalanine mutations of Y315 and Y319 in ZAP-70 suggest these residues function to recruit downstream effector molecules, but mutagenesis and crystallization studies reveal that these residues also play an important role in autoinhibition ZAP-70. To address the importance of the scaffolding function, we generated a *zap70* mutant mouse (YYAA mouse) with Y315 and Y319 both mutated to alanines. These YYAA mice reveal that the scaffolding function is important for normal development and function. Moreover, the YYAA mice have many similarities to a previously identified ZAP-70 mutant mouse, SKG, which harbors a distinct hypomorphic mutation. Both YYAA and SKG mice have impaired T cell development and hyporesponsiveness to TCR stimulation, markedly reduced numbers of thymic T regulatory cells and defective positive and negative selection. YYAA mice, like SKG mice, develop rheumatoid factor antibodies, but fail to develop autoimmune arthritis. Signaling differences that result from ZAP-70 mutations appear to skew the TCR repertoire in ways that differentially influence propensity to autoimmunity versus autoimmune disease susceptibility. By uncoupling the relative contribution from T regulatory cells and TCR repertoire during thymic selection, our data help to identify events that may be important, but alone are insufficient, for the development of autoimmune disease.

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Abbreviations used: DP, double-positive; dsDNA, double-stranded DNA; ES, embryonic stem; ITAM, immunoreceptor tyrosine-based activation motif; SP, single-positive.

Signal transduction by the TCR plays a critical role in T cell development and in the protective and pathological responses mediated by mature T cells. The repertoire of mature T cells and the discrimination of self from nonself are largely determined within the thymus through TCR-dependent processes known as positive and negative selection. It is generally thought that quantitative or qualitative differences in TCR signaling determine the binary decision between positive or negative selection (Starr et al., 2003). Likewise, whether a productive mature T cell response will be made is dictated by signaling events induced by the TCR.

ZAP-70, a Syk family tyrosine kinase that associates with the TCR CD3 and ζ subunits, plays a critical role in TCR signaling in immature thymocyte selection and in mature T cell responses (Chan et al., 1992). ZAP-70 contains two N-terminal SH2 domains that mediate its association with doubly phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) after their phosphorylation by Lck. The ZAP-70 C-terminal catalytic domain phosphorylates the downstream adaptor molecules LAT and SLP-76 that play critical roles in T cell development and in T cell responses (Horejsí et al., 2004). Interdomain B bridges the C-terminal SH2 and the kinase domains and contains three tyrosine residues (Y292, Y315, and Y319) that are inducibly phosphorylated. Based on tyrosine to phenylalanine mutations, we and others have previously shown that phosphorylation of Y292 may exert a

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negative regulatory effect on ZAP-70, perhaps by functioning as a docking site for c-Cbl (Lupher et al., 1997; Meng et al., 1999; Rao et al., 2000). In contrast, phenylalanine mutations of Y315 and Y319 suggested that these sites positively regulate ZAP-70 function by recruiting Vav1 and Lck, respectively (Straus et al., 1996; Wu et al., 1997; Pelosi et al., 1999). These sites have also been reported to bind c-Crk (Gelkop and Isakov, 1999) and phospholipase Cγ1 (Williams et al., 1999). Thus, in addition to its catalytic activity, ZAP-70 may have an important scaffolding role in recruiting downstream effector molecules.

Surprisingly, in addition to this scaffolding role, we recently discovered that Y315 and Y319 play an important role in regulating ZAP-70 kinase activity. Whereas mutation of both residues to phenylalanine potently inhibits ZAP-70 function, deletion of interdomain B or mutation of these sites to alanine relieves an autoinhibitory conformation and renders the kinase relatively Lck-independent (Brdicka et al., 2005). Stabilizing the autoinhibited ZAP-70 conformation with the YYFF mutations (Y315F and Y319F) allowed the crystallization of full-length ZAP-70. The crystal structure, together with targeted mutagenesis studies, suggest that the Y315 and Y319 stabilize the autoinhibited conformation of the ZAP-70 kinase through hydrophobic interactions involving the aromatic rings of the Y315 and Y319 residues with residues in the inter-SH2 domain (Interdomain A) and the C-terminal lobe of the catalytic domain (Deindl et al., 2007). Comparison of the alignment of the SH2 domains in the autoinhibited conformation to the ITAM-bound SH2 domains (Hatada et al., 1995) further suggests that ZAP-70 undergoes a conformational change upon binding to a doubly phosphorylated ITAM, which allows Y315 and Y319 to be more accessible for phosphorylation. Y315 and Y319 appear to be Lck phosphorylation sites and their phosphorylation stabilizes the active "open" ZAP-70 conformation and prevents the kinase from returning to the autoinhibited conformation (Brdicka et al., 2005; Deindl et al., 2007; Levin et al., 2008). What remains unclear is the relative importance of their scaffolding function in effector molecule recruitment.

Two independent groups have generated knockin mice (Magnan et al., 2001) and transgenic mice (Gong et al., 2001) to study the in vivo individual contributions of Y315 and Y319 of ZAP-70 to signal transduction and to T cell development. However, those studies involved mutation of either Y315 or Y319 to phenylalanine, which would have helped stabilize the autoinhibited conformation and could have resulted in attenuated T cell signaling and consequent alteration of positive and negative selection. TCR-mediated calcium increase is greatly diminished in ZAP-70$^{-/-}$ mice expressing the Y319F transgenic mice whereas the Y315F mutation has a modest effect on calcium responses. Consistent with results from transgenic mice, phosphorylation of phospholipase C γ1, but not Vav1, is reduced in the Y315F knockin mice. These results suggest that Y315 and Y319 contribute to efficient generation of second messengers involved in thymic selection. However, these studies did not take into account the notion that the tyrosine to phenylalanine mutations might also stabilize the autoinhibitory conformation of ZAP-70 rather than only affecting the recruitment of downstream effectors.

To address the relative importance of the scaffolding function of these residues, we generated a knockin ZAP-70 mutant strain with Y315 and Y319 simultaneously mutated to alanine residues. By creating a YYAA ZAP-70 knockin mouse we were able to study the contribution of the scaffolding function of these sites without the confounding issue of autoinhibition in the context of phenylalanine mutants. These YYAA mice showed many of the features of a previously identified spontaneous mutant mouse called SKG. The SKG mouse has a syndrome that shares features with human rheumatoid arthritis, including autoimmune arthritis and rheumatoid factor production (Sakaguchi et al., 2003). SKG mice have a single point mutation in the C-terminal SH2 domain of ZAP-70 that results in attenuated TCR signaling and aberrant positive and negative selection. Results suggested that the autoimmune arthritis could be caused by expansion of autoreactive CD4$^+$ T cells that have escaped negative selection. Interestingly, unlike SKG mice, although the YYAA mice have similar defects in positive and negative selection in TCR transgenic systems and can be induced to develop rheumatoid factor antibodies, they do not develop autoimmune arthritis. Quantitative assessment of negative selection in response to endogenous superantigens suggests that YYAA and SKG mice have distinct TCR repertoires that could account for the increased susceptibility of both lines of mice to develop rheumatoid factor antibodies, but interestingly, only SKG mice develop arthritis. Our findings demonstrate the importance of phosphorylation of Y315 and Y319 in ZAP-70 as binding sites for key effector molecules. As well, these findings stress the importance of these sites for proper regulation of TCR signaling and for normal T cell repertoire selection and peripheral T cell function in resistance to autoimmune disease susceptibility.

RESULTS

Generation of ZAP-70 YYAA knockin mice

We generated a knockin mouse (YYAA mouse) that expresses a mutant form of ZAP-70 with Y315 and Y319 mutated to alanine residues (Fig. S1). Homozygous ZAP-70 YYAA mice were born at Mendelian ratios and were fertile and healthy and were bred onto C57BL/6 and BALB/c genetic backgrounds. Flow cytometry in both genetic backgrounds (Fig. S2) revealed lower ZAP-70 expression in double-positive (DP) thymocytes, CD8 single-positive (SP) thymocytes, and peripheral T cells in the YYAA mice compared with wild-type controls. However, CD4 SP thymocytes from YYAA mice express ZAP-70 at levels equivalent to wild-type controls. We also examined the ZAP-70 expression levels in the SKG mice on the BALB/c background. Interestingly, the SKG mutation results in even lower ZAP-70 expression in all T cell subsets examined. Targeted mutations of tyrosine 315 and 319 in YYAA mice and the W163C

SH2 domain mutation in SKG mice reduces ZAP-70 protein to ~60 and ~25% of wild-type BALB/c levels in DP thymocytes, respectively.

Impaired T cell development in ZAP-70 YYAA knockin mice

Based on overexpression and crystallization studies, we thought it possible that the ZAP-70 YYAA mutation might represent a hypermorphic signaling allele because it disrupted an autoinhibitory mechanism. However, analyses of thymus and secondary lymphoid organs of YYAA mutant mice on the C57BL/6 background revealed a substantial impairment in T cell development (Fig. 1 A). Although total thymic cellularity was only slightly reduced, the relative percentages and absolute numbers of both the CD4 and CD8 SP subsets in the thymus as well as in the periphery were substantially decreased. Further analysis of the CD8 SP thymocytes revealed that the majority of CD8 SP thymocytes represented the immature intermediate SP population with low levels of TCRβ (unpublished data).

Because T cell development in the YYAA mice is substantially compromised, we reasoned that ZAP-70 YYAA allele is, instead, a hypomorphic allele of ZAP-70. We examined signaling by the TCR directly by treating thymocytes from wild-type and YYAA mice with anti-CD3 and -CD4 antibodies and analyzed protein tyrosine phosphorylation. The response was globally diminished in thymocytes from YYAA mice (Fig. 1 B). We recently confirmed these observations in the ZAP-70 deficient Jurkat line p116 that was stably reconstituted with the YYAA mutant at wild-type levels of expression (Kadlecek, T.; personal communication; unpublished data). Therefore, our results suggest that the YYAA mutation has a negative impact on ZAP-70 function in TCR-mediated signaling.

Comparison of thymic development in ZAP-70 YYAA mice and SKG mice

We compared the YYAA mice, backcrossed 7 generations onto the BALB/c background, with the SKG mice that also have compromised TCR signaling and similarly defective thymocyte development. Thymocyte development in YYAA mice and SKG mice were very similarly affected, in that fewer thymocytes from YYAA and SKG mice matured into either CD4 SP or CD8 SP stages (Fig. 2 A). There were mild decreases in total thymic cellularity when YYAA mice and SKG mice were compared with wild-type mice, but the absolute numbers of CD4 SP and CD8 SP thymocytes were profoundly reduced, and fewer mature $CD4^+$ and $CD8^+$ T cells were present in the periphery (Fig. 2, A and B). Therefore, despite differences in the nature of the ZAP-70 mutations, similar effects on thymic development and peripheral T cell subsets were observed.

Differential impact of ZAP-70 YYAA and SKG alleles on TCR signaling

SKG T cells are hyporesponsive to in vitro TCR stimulation. To address how similar the impact of the YYAA mutations are on TCR signaling, we analyzed the TCR-induced increase in cytoplasmic free calcium in thymocytes and peripheral T cells. Compared with immature DP, CD4 SP thymocytes and mature peripheral $CD4^+$ and $CD8^+$ T cells from wild-type controls, thymocytes, and T cells isolated from YYAA mice or SKG mice manifested attenuated TCR-induced increases in cytoplasmic free calcium (Fig. 3 A and not depicted). SKG T cells had a more profound impairment in the calcium responses compared with YYAA T cells, suggesting that these two hypomorphic alleles have different impacts on downstream TCR signaling strength.

To further compare the signaling differences between YYAA and SKG T cells, we analyzed the surface expression of CD5 and CD69 on thymocyte subsets. The level of CD5 expression on DP thymocytes correlates with basal TCR signaling strength. During positive selection, TCR signals result in up-regulation of CD5 and CD69 (Azzam et al., 1998; Puls et al., 2002). The levels of CD5 and CD69 expression on DP thymocytes were markedly reduced in SKG mice, but less so on YYAA DP thymocytes (Fig. 3 B). The graded signaling strength of different ZAP-70 alleles was also observed in CD4 SP thymocytes, but to a lesser extent (Fig. 3 B). Similarly, analysis of Erk phosphorylation revealed that the SKG allele had a greater negative impact than the YYAA allele on the induction of Erk phosphorylation in thymocytes. This result was not caused by the different levels of TCRβ expression in the three different ZAP-70 genotypes (Fig. 3 D) because we compared Erk phosphorylation from thymocytes expressing the same levels of TCRβ. These results confirmed that both SKG and YYAA alleles compromise TCR signaling, but that the SKG allele does so to a greater extent.

Responses of SKG and YYAA peripheral T cells to TCR stimulation

Given the TCR signaling defects in SKG and YYAA thymocytes, we addressed whether the impairments of peripheral T cells were similar in response to TCR stimulation. Whereas wild-type T cells underwent several rounds of cell division after 72 h, YYAA and SKG T cells showed poor proliferative responses to TCR and CD28 stimulation (Fig. 4 A). The CFSE patterns of SKG T cells stimulated with medium alone or with a low dose of anti-CD3 plus anti-CD28 were virtually identical (Fig. 4 A). Interestingly, although the proliferative response of YYAA T cells was defective to a lower dose of anti-CD3, the YYAA T cells partially overcame the proliferative defect in response to higher doses (Fig. 4 A). We also measured IL-2 production in response to low and high doses of anti-CD3 stimulation. Similarly, IL-2 production after CD3 stimulation was greatly reduced in YYAA and SKG mice (Fig. 4 B). Collectively, our results indicate that proliferative responses and IL-2 production in response to TCR stimulation correlates well with TCR signaling strength in YYAA and SKG T cells. An increased dose of anti-CD3 was able to partially, but not fully, compensate for the impaired TCR signaling in YYAA T cells, but not in SKG T cells.

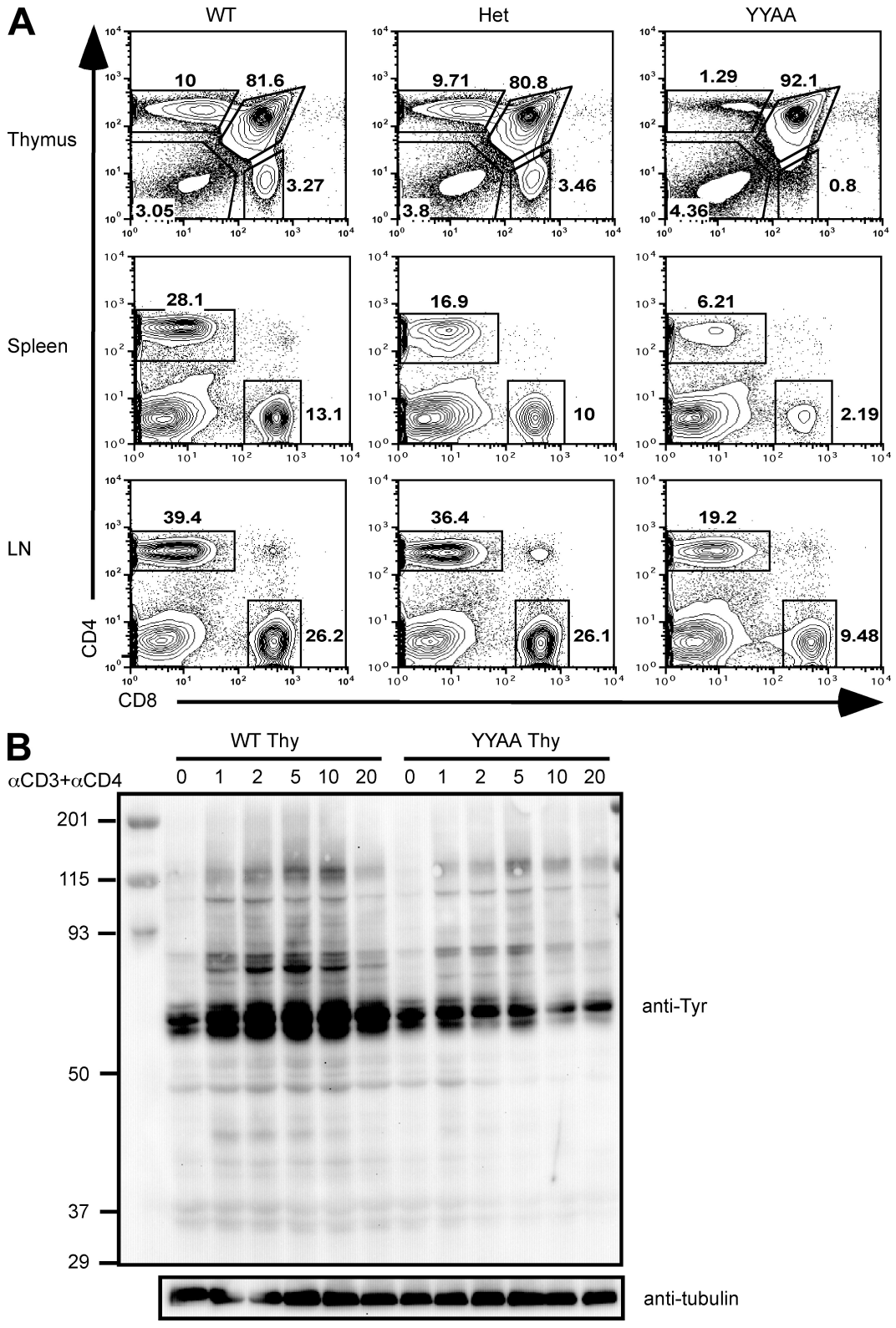

Figure 1. T cell development is impaired in the ZAP-70 YYAA mice on the C57BL/6 background. (A) Representative FACS plots of CD4 versus CD8 expression of total thymocytes (top), splenocytes (middle), and LN cells (bottom) from individual 2-mo-old mice. (B) Reduced total tyrosine phosphorylation in ZAP-70 YYAA mice. Thymocytes were stimulated with anti-CD3 and -CD4 for the times indicated, whole-cell lysates were prepared, and total tyrosine phosphorylation (top) and α-tubulin were assessed by immunoblotting. Data are representative of at least three independent experiments.

Defective positive and negative selection in YYAA mice

Because YYAA DP thymocytes failed to substantially up-regulate CD5 and CD69 markers, the YYAA allele might affect positive selection. To determine whether positive selection is defective in YYAA mice, we examined the expression levels of CD24 (HSA), which is down-regulated after positive selection (Lucas et al., 1993). The numbers of the $HSA^{lo}TCR\beta^{hi}$ cells were substantially decreased in YYAA and SKG mice as compared with thymocytes from wild-type mice (Fig. S3). These data suggest that positive

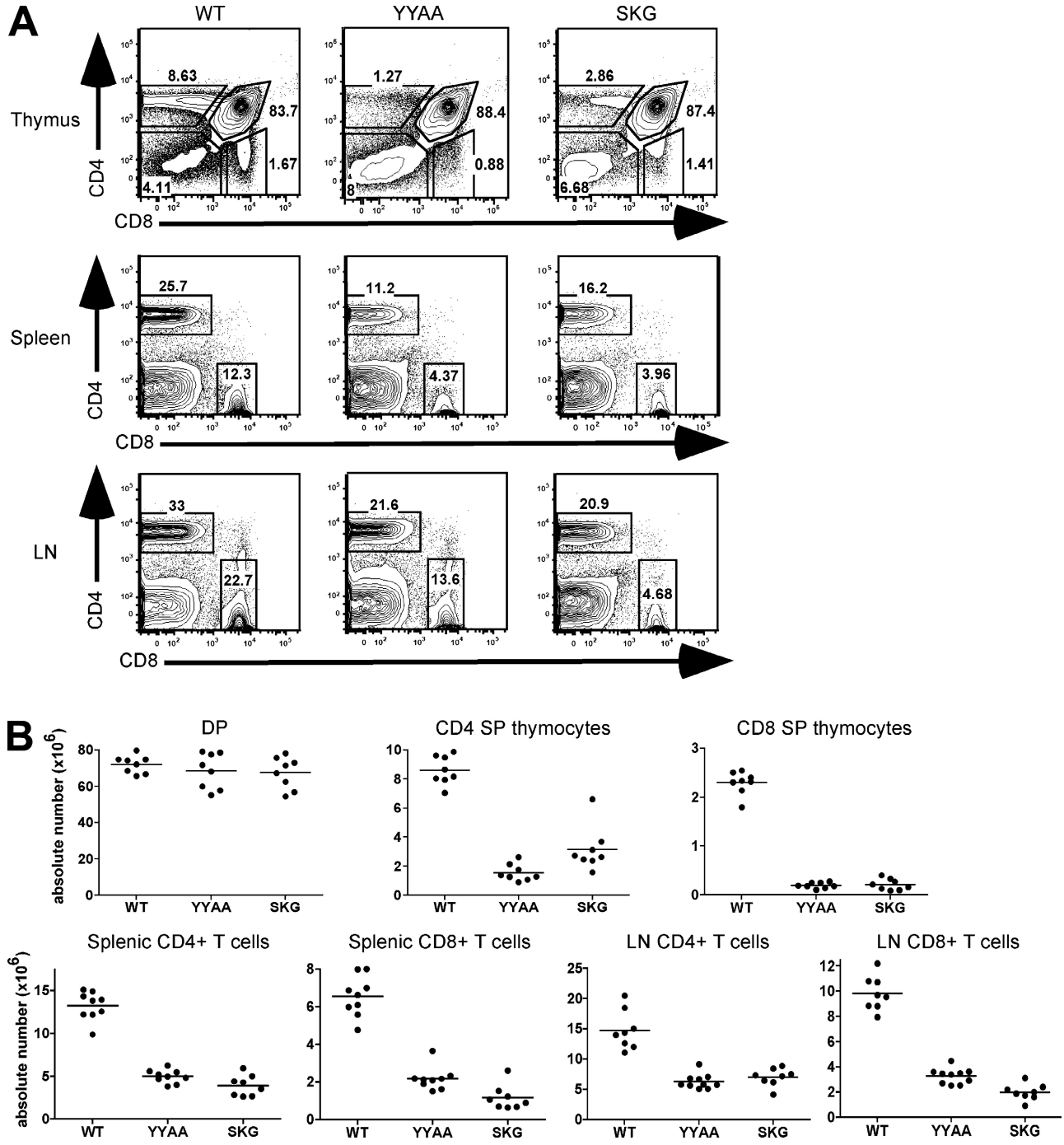

Figure 2. Similar impairments in thymic development in ZAP-70 YYAA and SKG mice on the BALB/c background. (A) Thymocytes (top), splenocytes (middle), and LN cells (bottom) from 2-mo-old wild-type, ZAP-70 YYAA, and SKG mice were stained with anti-CD4 and -CD8. Percentages of DN, DP, and SP populations are displayed. (B) The absolute numbers of DP, and SP thymocytes (top) and $CD4^+$ and $CD8^+$ lymphocytes from spleen and LNs (bottom) of wild-type, YYAA, and SKG mice were calculated based on the relative percentages determined by FACS analysis in A and total numbers from thymus, spleen, and LNs. $n = 8$ mice for each genotype. Pooled results from three independent experiments are shown.

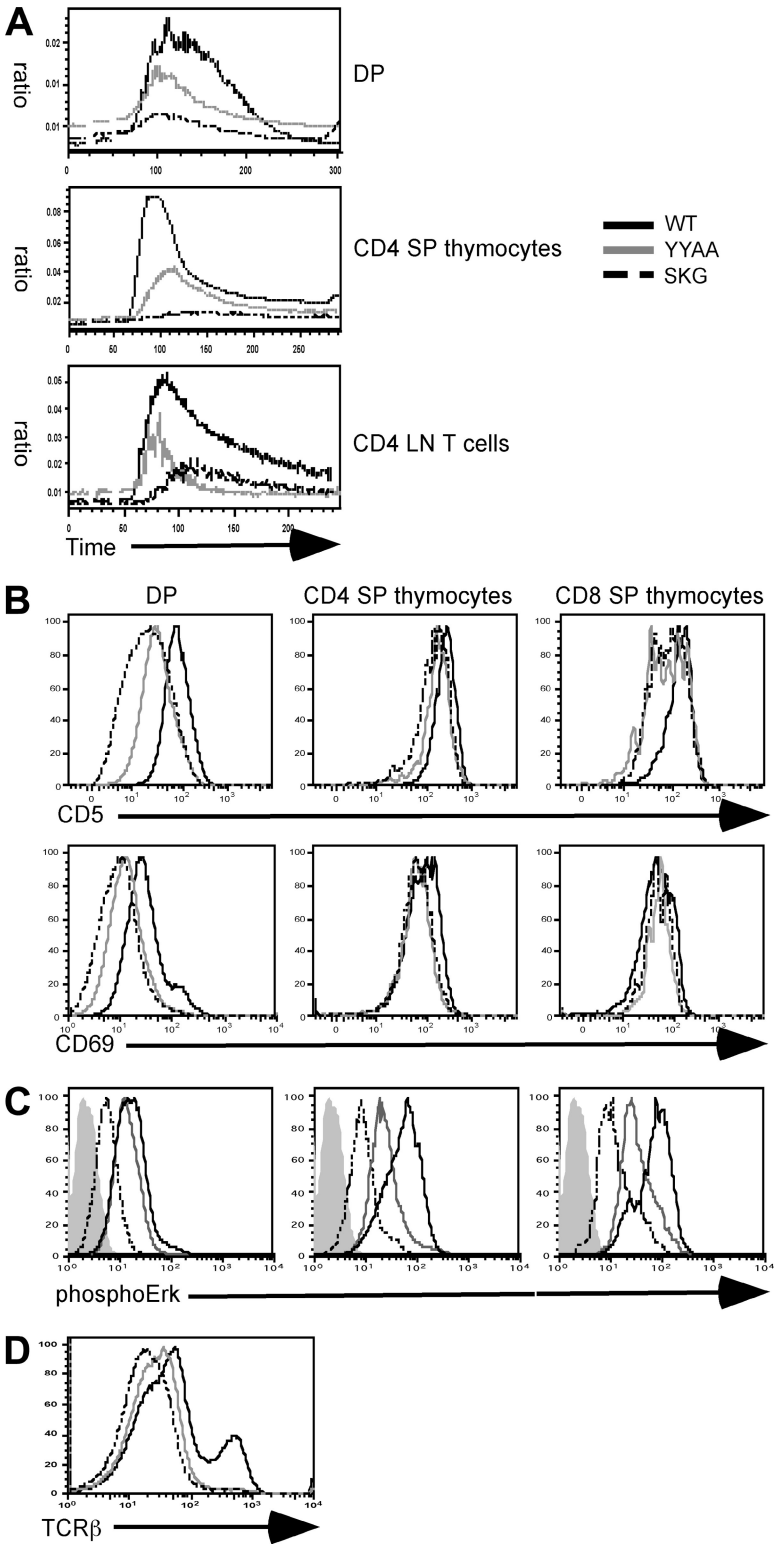

Figure 3. ZAP-70 YYAA and SKG alleles have different impacts on TCR signaling. (A) TCR-induced calcium increased in DP thymocytes (top), CD4 SP thymocytes (middle), and CD4+ LN T cells (bottom) from wild-type, ZAP-70 YYAA, and SKG mice. (B) CD5 and CD69 expression on DP, CD4 SP, and CD8 SP thymocytes from wild-type, ZAP-70 YYAA, and SKG mice. (C) DP, CD4 SP, and CD8 SP thymocytes from wild-type, ZAP-70 YYAA, and SKG mice were analyzed for levels of phospho-Erk by flow cytometry. Unstimulated T cells (filled gray) and stimulated T cells from wild-type mice (black line), YYAA mice (gray line), and SKG mice (dashed line) are shown. (D) Histograms show TCRβ surface expression levels on total thymocytes from wild-type, ZAP-70 YYAA, and SKG mice. Data are representative of at least three independent experiments.

selection might be impaired in YYAA mice expressing a diverse TCR repertoire.

To assess in greater detail whether positive and negative selection are defective in the YYAA mice, we bred the YYAA mice to the MHC class I–restricted HY TCR transgenic mice (B6 background) and the class II MHC–restricted DO 11.10 TCR transgenic mice (BALB/c background). Defects in positive and negative selection were well documented using these same TCR transgenes in SKG mice (Sakaguchi et al., 2003). In wild-type HY TCR transgenic mice, thymocytes in female mice expressing the TCR specific for the male HY antigen are positively selected as shown by an increase of CD8 SP (Fig. 5 A). In contrast, there were substantially fewer $CD8^+$ HY-TCR^+ thymocytes in YYAA female mice (HY-TCR YYAA). Consequently, many fewer $CD8^+$ HY-TCR^+ T cells were found in the lymph nodes of HY-TCR YYAA female mice.

To address the ability of thymocytes from YYAA mice to undergo negative selection, we compared the thymocyte development in HY-TCR male mice. Because of the premature expression of the HY-TCR in DN subsets, HY-TCR male mice exhibit a marked reduction in thymic cellularity and nearly complete deletion of DP and SP thymocytes (Takahama et al., 1992). Despite strong deletion seen in the wild-type mice, a substantial number of DP and CD8 SP thymocytes developed in HY-TCR YYAA male mice. There was an approximately threefold increase in total numbers of thymocytes in HY-TCR YYAA male mice, with a nearly 40-fold increase in DP thymocytes. This result suggests a requirement for tyrosines 315 and 319 in negative selection.

To assess the requirements of these two tyrosine residues in positive selection of MHC class II-restricted TCRs, we introduced the DO11.10 TCR, a transgenic TCR specific for chicken ovalbumin (OVA) peptide, onto the YYAA mutant background.

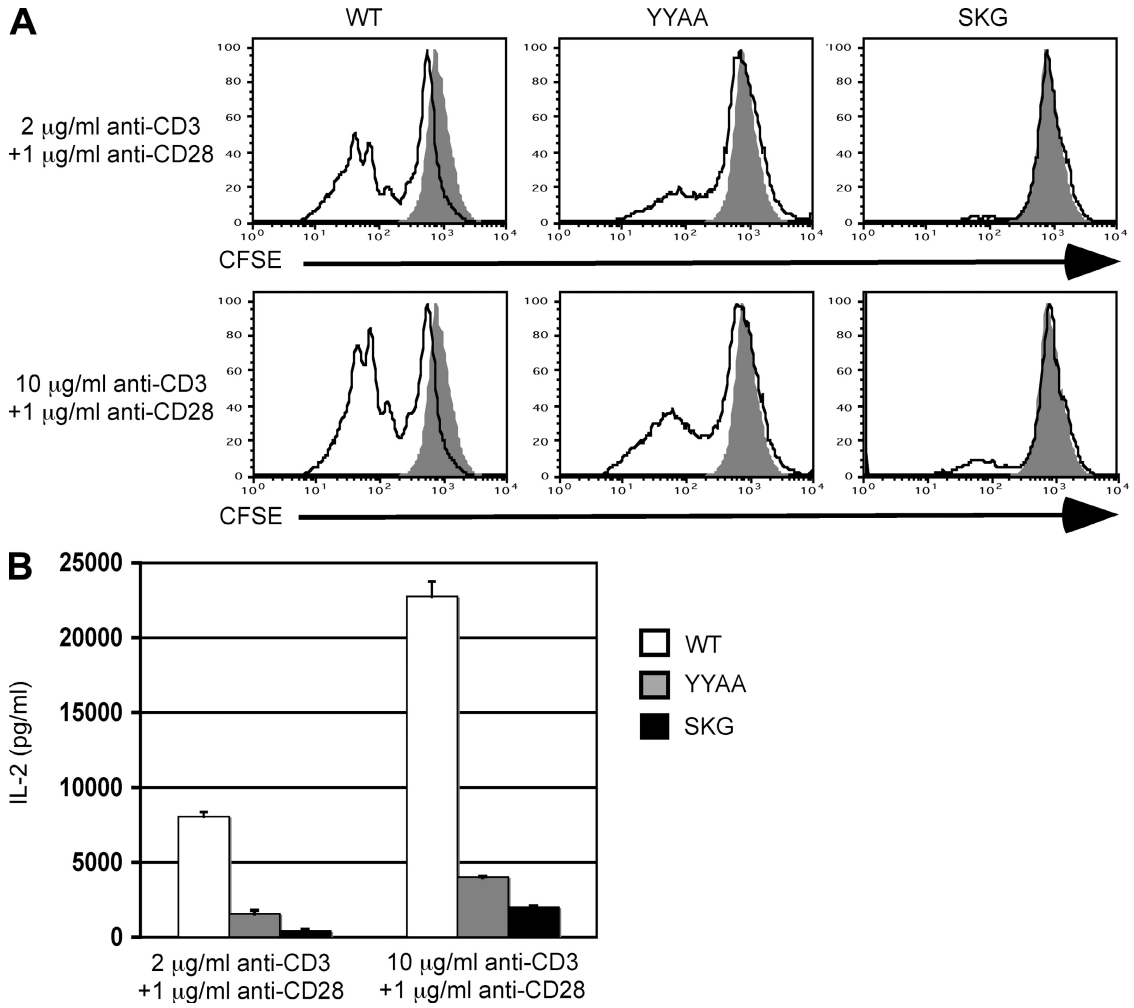

Figure 4. Proliferative responses and IL-2 production by ZAP-70 YYAA and SKG T cells to in vitro TCR stimulation. $CD4^+$ T cells purified from wild-type, ZAP-70 YYAA, and SKG mice were stimulated with plate-bound anti-CD3 (2 or 10 μg/ml) and soluble anti-CD28 (1 μg/ml) for 72 h (A) or 24 h (B). Proliferation of the $CD4^+$ cells was measured by dilution of CFSE on day 3 (A). The shaded line graph is the CFSE fluorescence from cells stimulated with medium. Supernatants were harvested and assayed for IL-2 production by ELISA (B). Results are presented as mean ± SEM from three independent experiments. Error bars indicate SEM.

Positive selection of DO11.10$^+$ T cells on an I-A^d background results in accumulation of immature SP thymocytes that are nearly all CD4 SP thymocytes and express high amounts of the DO11.10$^+$ TCR. Analysis of YYAA mice expressing the DO11.10 TCR (designated DO11$^{YYAA/YYAA}$) revealed that there was a marked reduction in the proportion and absolute numbers of CD4 SP thymocytes (Fig. 5 B). The very small numbers of thymocytes that were able to mature to the CD4 SP stage in DO11$^{YYAA/YYAA}$ mice appear to express an endogenously rearranged TCR, in contrast to thymocytes from DO11.10$^+$ wild-type mice (Fig. 5 B). As a consequence, this resulted in lower percentages and lower absolute numbers of DO11.10$^+$CD4$^+$ T cells in the spleens and lymph nodes of DO11$^{YYAA/YYAA}$ mice (Fig. 5 B and not depicted). Collectively, our results from two different TCR transgenic models suggest that Y315 and Y319 of ZAP-70 are required for proper positive and negative selection. These results mirror the defects in positive and negative selection previously reported for the SKG mice in these two TCR transgenic systems.

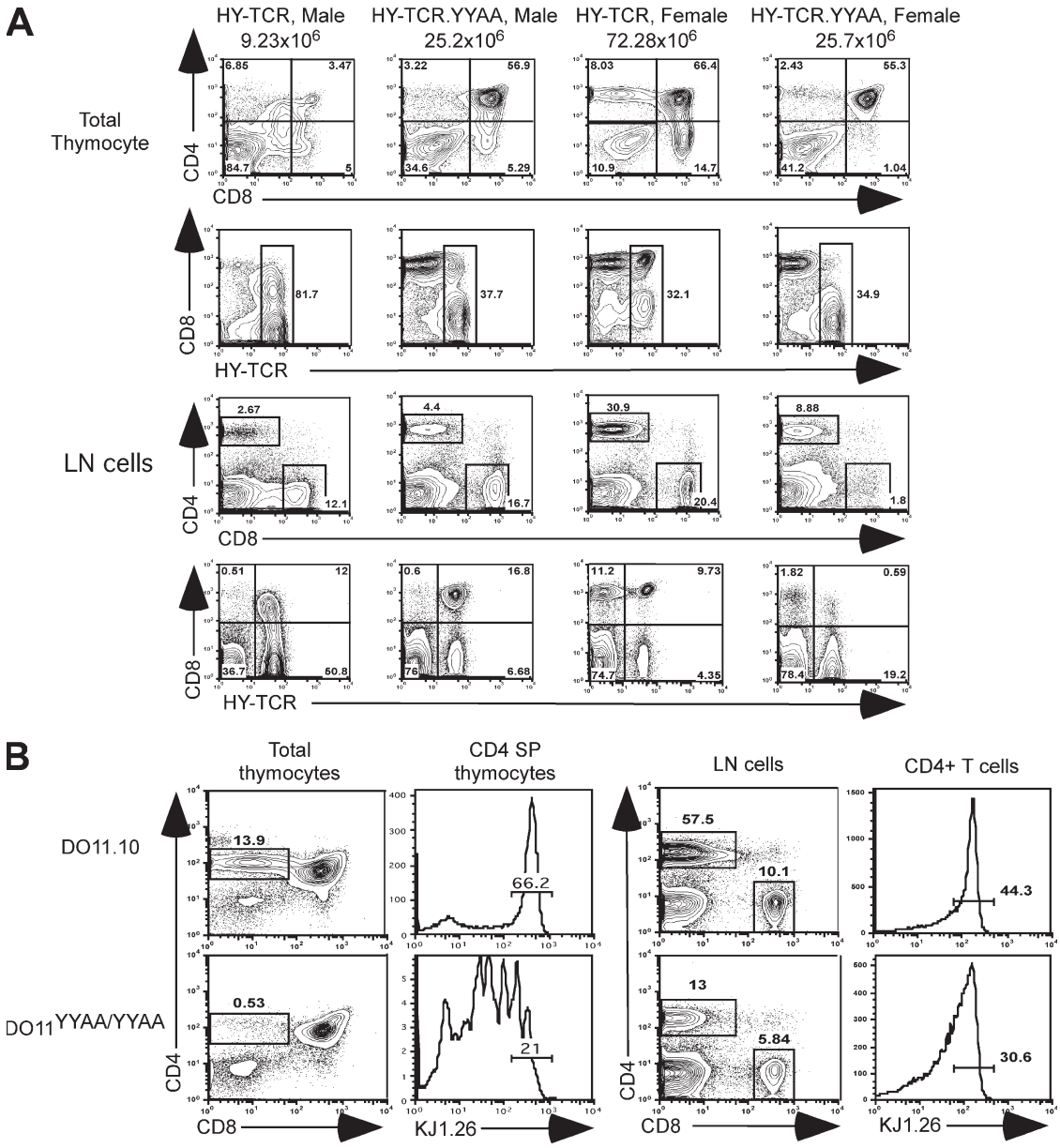

Figure 5. Defective positive and negative selection occurs in the ZAP-70 YYAA mice. (A) Total thymocytes and LN cells from wild-type ZAP-70 YYAA male and female mice expressing the HY-TCR transgene were analyzed for the expression of CD4, CD8, and the transgenic TCR chain used by the HY-TCR (T3.70). Numbers above the plots indicate the total number of thymocytes. n = 4–6 mice per group. (B) Thymocytes and LN cells from wild-type and ZAP-70 YYAA mice bearing DO11.10 TCR were analyzed for the expression of CD4 and CD8. Representative histograms show the levels of DO11.10 TCR (clonotypic antibody KJ1.26) on either CD4 SP thymocytes or LN CD4$^+$ T cells. The data are representative of three independent experiments.

Effect of different ZAP-70 mutants on susceptibility to autoimmunity

Thus, both YYAA and SKG mutants exhibit similar defects in positive and negative selection, but exhibit some differences in TCR signaling strength. In light of the susceptibility of SKG mice to develop autoimmune arthritis, we investigated whether the quantitative or qualitative changes in signaling strength observed in YYAA and SKG mice influence the susceptibility of YYAA to develop autoimmunity. First, we failed to detect any manifestations of systemic autoimmunity in YYAA mice histologically (unpublished data). In addition, neither YYAA nor SKG mice developed substantial amounts of anti–double-stranded DNA (dsDNA) autoantibodies after zymosan stimulation (Fig. 6 A).

Previous studies have demonstrated that SKG homozygous mice housed under specific pathogen-free conditions develop arthritis after zymosan challenge. Zymosan challenge activates the innate immune system. Both SKG and YYAA mice developed RF antibodies, which are anti-IgG antibodies, after zymosan challenge (Fig. 6 A). We examined whether development of RF antibodies in YYAA mice would lead to the development of autoimmune arthritis in YYAA mice or compound F1 heterozygotes of YYAA and SKG after the injection of zymosan. Although SKG homozygous mice developed severe arthritis triggered by zymosan challenge, only 1 out of 12 YYAA mice and 1 out of 6 SKG/YYAA mice developed mild arthritis during the 12-wk observation (Fig. 6 B).

In light of the impaired negative selection in the YYAA mice, together with the rare development of arthritis in YYAA and SKG/YYAA mice, we examined these mice for other evidence of T cell dysregulation or autoimmune responses. First, the frequency of activated/memory $CD44^{hi}CD62L^{lo}$ T cells was more than doubled and tripled in the YYAA and SKG mice with diverse TCR repertoires, respectively (Fig. S4 A). Consistently, the percentages of $CD45RB^{lo}$ $CD4^{+}$ T cells were increased in both YYAA and SKG mice; however, they increased to a greater extent in SKG mice (Fig. S4 B). Interestingly, CD69, an early activation marker, was found more frequently on peripheral $CD4^{+}$ T cells from SKG mice, whereas CD69 expression on YYAA $CD4^{+}$ T cells was nearly the same as in the wild-type $CD4^{+}$ T cells (Fig. S4 B). Increased levels of CD69 were detected in the one YYAA mouse that developed mild arthritis after zymosan injection. From these data, we conclude that $CD4^{+}$ T cells from both YYAA and SKG mice have a striking increase in $CD44^{hi}CD62L^{lo}$ $CD45RB^{lo}$ population, suggestive of T cells that might be responding to endogenous autoantigens or are responding to a lymphopenic environment.

IL-17 production is increased in both YYAA and SKG mice

Numerous studies have suggested that cytokines are directly involved in the pathogenesis of arthritis. A recent study of SKG mice reported that the arthritis seen in the SKG mouse critically depends on IL-17–producing T cells (Th17). Using antibody blockade and a genetic approach, IL-6–deficient SKG mice and IL-17–deficient SKG mice failed to develop arthritis, presumably because IL-6 not only promotes Th17 differentiation, but inhibits the differentiation/expansion of IFN-γ–producing cells (Hirota et al., 2007). In contrast, lack of IFN-γ in SKG mice exacerbates disease because of enhanced Th17 differentiation. Collectively, these data suggest that cytokines play important roles in the development and progression of arthritis.

To test whether T cell cytokine production is different between SKG and YYAA mice, we measured cytokine production in $CD4^{+}$ T cells from SKG and YYAA mice treated with zymosan. As shown in Fig. S5, the percentages of IL-17–producing cells were 6- and 15-fold higher in the YYAA and SKG mice than in the wild-type controls, respectively. When we compared mice stimulated with zymosan or PBS, the increases in the number of IL-17–producing cells were similar in SKG and YYAA mice. Therefore, we conclude that the expansion or differentiation of T_H-17 cells occurs in both YYAA and SKG mice, albeit to a lesser degree in YYAA mice, after activation of innate immunity by zymosan injection.

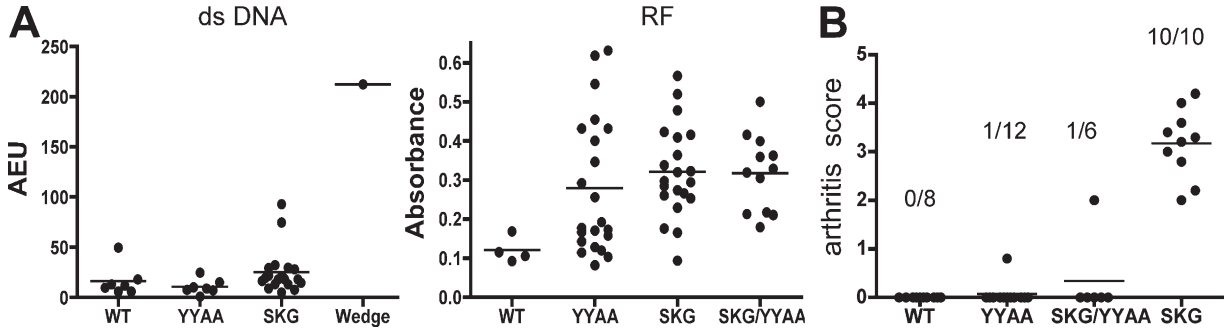

Figure 6. Comparison of autoimmune phenotypes cells in ZAP-70 YYAA and SKG mice. (A) Autoantibody production in the indicated ZAP-70 genotypes 8 wk after zymosan injection. Double-stranded DNA antibodies were detected by ELISA in sera from the indicated genotypes (left). Results are represented as arbitrary ELISA units (AEU). A positive control was provided by serum from CD45E613R wedge mutant mice. IgM rheumatoid factor was measured by ELISA (right). Absorbance >0.2 is considered definitely positive. (B) Arthritis scores of four different genotypes. 8–10-wk-old female mice from each genotype received a single i.p. injection of 2 mg zymosan. Arthritis score was determined 12 wk after injection. Pooled results from three independent experiments are shown.

Both YYAA and SKG mutations impair the development of regulatory T cells

Given the important role of regulatory T (T reg) cells in maintaining self-tolerance and preventing autoimmunity, we first investigated whether ZAP-70 YYAA and SKG mutations affect T reg cell development. There was a marked reduction in both thymic T reg cell frequency as well as absolute T reg cell numbers in both YYAA and SKG mice (Fig. 7, A and D). T reg cell markers, such as cytotoxic T lymphocyte antigen 4 and glucocorticoid-induced TNF receptor were reduced, supporting the finding that thymic T reg cell development was defective in both YYAA and SKG mice (Fig. 7 B). Notably, as compared with wild-type mice, the percentage of peripheral T reg cells was nearly double in the SKG mice whereas the percentage in the YYAA mice was slightly lower (Fig. 7 C). The absolute numbers of peripheral T reg cell in the SKG mice was not significantly decreased compared with wild-type mice.

We compared the regulatory potential of peripheral T reg cells from either wild-type, YYAA, and SKG mice to suppress in vitro the proliferation of CD3 antibody-stimulated naive T cells. Interestingly, our result demonstrated that SKG T reg cells showed little or no impairment in suppressive activity. In contrast, YYAA T reg cells were much less efficient at suppressing proliferation of wild-type naive T cells (Fig. 7 E). We conclude that the YYAA mutation impairs T reg cell development as well as their function.

Apparent differences in deletion to endogenous superantigens in YYAA and SKG mice

We next sought to determine whether the difference between YYAA and SKG mice in the development of arthritis might be influenced by an alteration in their TCR repertoires. On the BALB/c ($H\text{-}2^d$, $I\text{-}E^+$) background, viral superantigens encoded by Mtv-8 and Mtv-9 induce clonal deletion in thymocyte cells bearing Vβ5 and Vβ11 (Matsutani et al., 2006). We confirmed efficient deletion of $V\beta5^+$ and $V\beta11^+$ cells in both $CD4^+$ and $CD8^+$ populations in wild-type mice (Fig. 8). Interestingly, we found graded increases in the frequencies of $V\beta5^+$ and $V\beta11^+$ T cells in YYAA, SKG/YYAA, and SKG mice, with SKG mice having the greatest defect in deletion of $V\beta5^+$ and $V\beta11^+$ T cells (Fig. 8). In contrast, T cells expressing the TCR Vβ8 chain, which cannot interact with Mtv-8 and Mtv-9, were present at similar frequencies among mice with different ZAP-70 alleles. These findings reveal a graded increase in $V\beta5^+$ and $V\beta11^+$ T cells that correlates with loss of TCR signal strength among different ZAP-70 mutants. These results suggest TCR signaling differences lead to different TCR repertoires as a consequence of differential sensitivity to negative selection in YYAA and SKG mice, and these differences could contribute to their different susceptibility to induction of autoimmune arthritis.

DISCUSSION

The present study shows that replacement of ZAP-70 tyrosines 315 and 319 with alanines results in attenuated TCR signaling, leading to compromised development in $CD4^+$ and $CD8^+$ thymocytes, as well as defective positive and negative selection in the context of certain fixed transgenic TCR systems. This results in impaired development of normal numbers of T cells and in an apparent shift in the TCR repertoire. Because of the apparent similarities between YYAA and SKG mice, with regard to T cell development, in this study we compared these two mice and explored the mechanisms that contribute to or protect from the development of autoimmune arthritis in the YYAA mice.

The YYAA mice have diminished TCR signaling, and consequently, perturbed thymocyte development. One explanation for the reduced level of TCR signaling may be the decrease in YYAA ZAP-70 protein expression levels. The most severe reduction is seen in DP thymocytes. However, in the CD4 SP thymocytes, the level of the YYAA protein is comparable to the level of the wild-type protein. Yet, signaling is still substantially reduced in the CD4 SP population as indicated by diminished calcium flux and Erk phosphorylation in response to TCR stimulation. Although in the periphery the level of the YYAA ZAP-70 is reduced to ∼50% wild-type levels, we and others have not seen a substantial reduction in signaling in heterozygous ZAP-70 KO mice that express half the normal level of protein. The basis for the reduction of the YYAA protein is not clear, but could reflect a less stable protein or the influence of compromised ZAP-70–dependent TCR signaling. We favor the latter hypothesis, as we have not noted difficulties in expressing the YYAA mutant in cell lines. Moreover, at similar levels of wild-type or YYAA ZAP-70 protein in reconstitution studies of a deficient Jurkat line, we still note decreased TCR signaling as indicated by diminished total tyrosine phosphorylation as well as that of specific substrates (T. Kadlecek, personal communication; unpublished data). The reduction of ZAP-70 protein in the SKG mice is much more profound in all subsets than the YYAA and, yet, these mice have sufficient T cell functional activity in the periphery to mediate T cell-dependent responses that result in autoimmune arthritis. Thus, it appears that the scaffolding function mediated by the phospho-Y315 and -Y319 to recruit critical effector molecules is, indeed, important.

The YYAA and SKG mice have very different ZAP-70 mutations; compromised scaffolding function and decreased binding to phosphorylated ITAMs, respectively. Because of the similar developmental defects in YYAA and SKG mice, we compared TCR signaling events in wild-type, YYAA, and SKG mice and found quantitative decreases in TCR signaling, as indicated by calcium responses, phosphorylation of Erk, and surface expression of CD5 and CD69 at different stages of T cell development. Consistent with the TCR signaling strength, the patterns of CFSE dilution and IL-2 production in response to TCR stimulation in vitro were greatly reduced in SKG T cells and more moderately decreased in YYAA T cells. Thus, the SKG mutation has a greater negative impact on TCR signaling than the YYAA mutation. Despite the greater impact on TCR signaling in the SKG mice, the absolute numbers of CD4 SP and CD8 SP thymocytes are very similar in SKG and YYAA mice. Our results

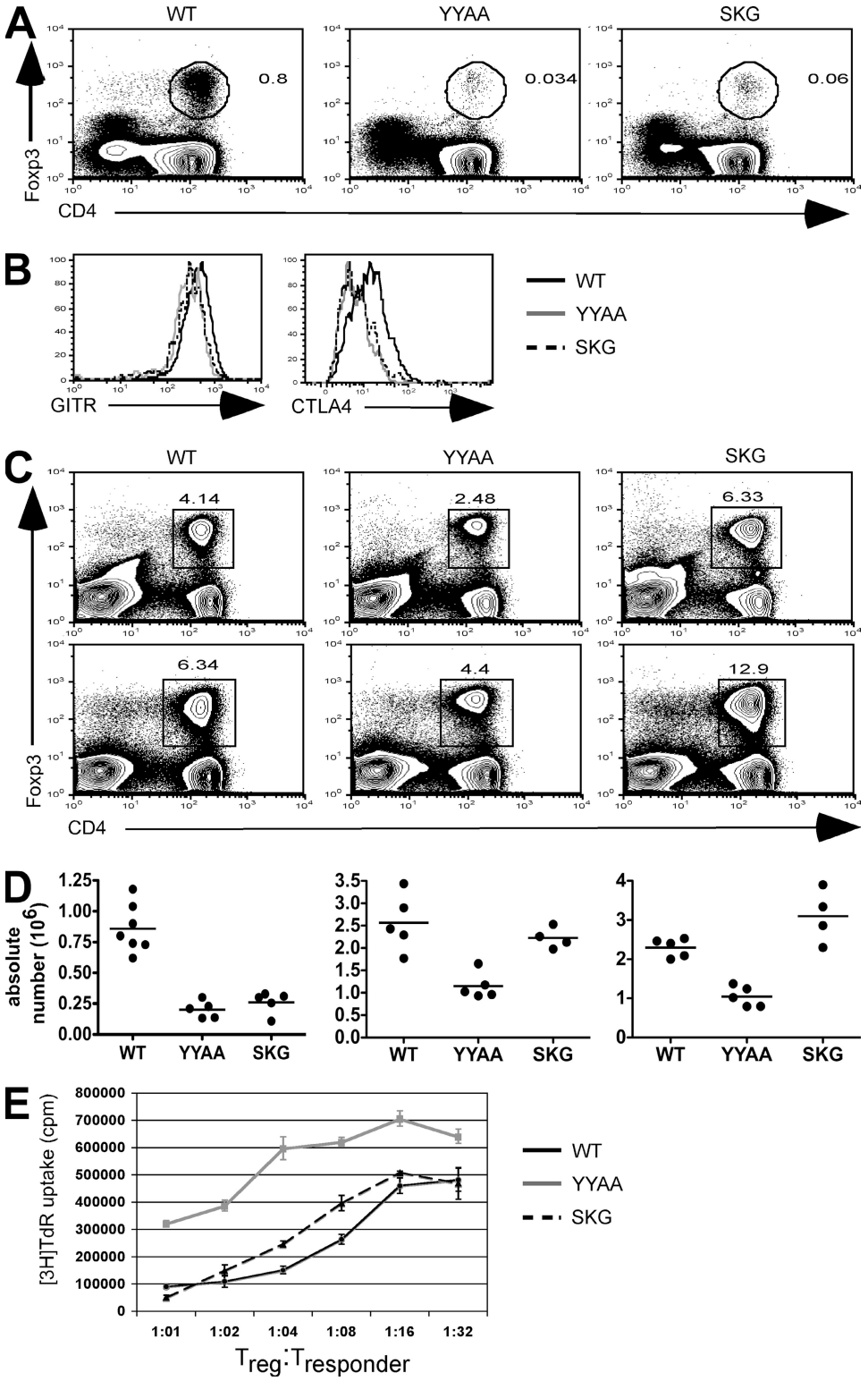

Figure 7. Phenotypic and functional characterization of thymic and peripheral T reg cells in ZAP-70 YYAA and SKG mice. (A) Significant reduction of thymic T reg cells in both ZAP-70 YYAA and SKG mice. (B) Expression of various T reg cell markers on $CD4^+Foxp3^+$ thymocytes from wild-type, YYAA, and SKG mice. $CD4^+Foxp3^+$ thymocytes were further analyzed by other T reg cell markers such as CTLA4 and GITR. (C) Increased percentage of peripheral T reg cell population in ZAP-70 SKG, but not YYAA, mice. FACS analysis of CD4 and Foxp3 expression on splenocytes (top) and lymph node cells (bottom) from 8-wk-old mice. (D) The absolute numbers of T reg cells from thymus (left), spleen (middle), and LNs (right) of wild-type, YYAA, and SKG mice were shown. $n = 5$ mice for each genotype. (E) In vitro T reg cell–mediated suppression assay with purified $CD4^+CD25^+$ T reg cells from either wild-type, YYAA or SKG mice. Proliferation is assessed as uptake of $[^3H]$thymidine. The data are representative of three or more independent experiments.

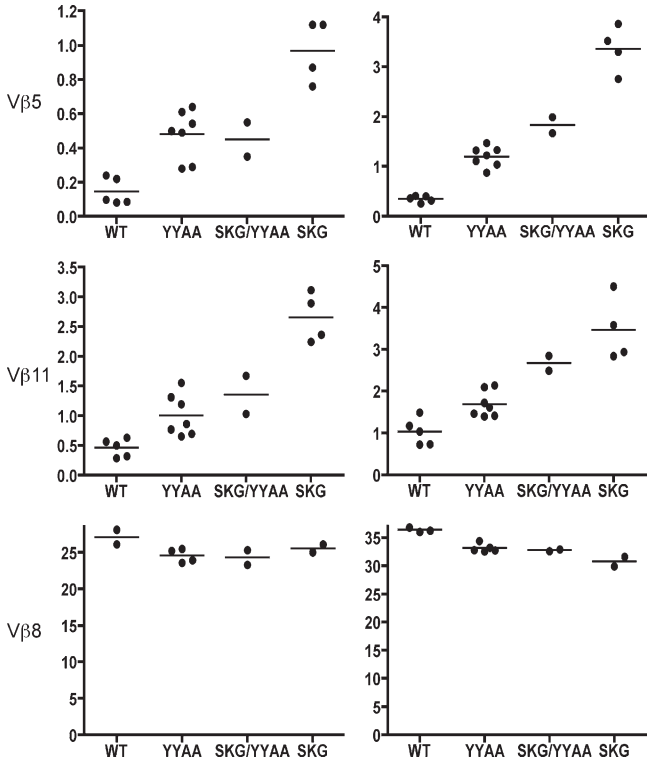

Figure 8. Graded changes in the efficiency of negative selection from different ZAP-70 variants. CD4+ (left) and CD8+ (right) T cells from wild-type, YYAA, SKG/YYAA, and SKG mice were evaluated for expression of Vβ5, Vβ11, and Vβ8. The percentage of these Vβ chains is shown. The data are representative of two independent experiments.

with the YYAA and SKG mice contrast with another more severe hypomorphic allelic series of ZAP-70 in which the absolute numbers of CD4 SP and CD8 SP thymocytes correlate with the signaling strength of different ZAP-70 alleles (Siggs et al., 2007). The discrepancy might be caused by different efficiencies in positive and negative selection in various ZAP-70 alleles, or in quantitatively or qualitatively different signals based on the specific functions of ZAP-70 impacted by the individual mutations.

The reduced number of single positive thymocytes in the YYAA mice suggested that the impairment in TCR signaling results in defective positive selection. This is supported by the reduced numbers of TCR^{hi} HSA^{low} DP thymocytes, indicative of cells that have recently been positively selected. By crossing the YYAA mutation with two TCR transgenic mice, we have demonstrated that, like the SKG mice, the efficiency of positive selection in YYAA mice is reduced in both transgenic systems. As compared with the reported positive selection defects in SKG mice in both HY TCR and DO 11.10 TCR systems (Sakaguchi et al., 2003), the efficiency of positive selection in YYAA mice appears to be very similar.

Based on analyses of male HY TCR transgenic mice, negative selection in YYAA mice bearing the HY-TCR is similarly defective as in the SKG mice bearing this transgene (Sakaguchi et al., 2003). Given the defects in both positive and negative selection in YYAA mice, we anticipated a breakdown of normal self-tolerance and the appearance of autoimmunity in the YYAA mice. Surprisingly, YYAA mice failed to develop spontaneous autoimmunity. Nor did they develop the autoimmune arthritis seen in the SKG mice after zymosan challenge. However, the YYAA mice, unlike wild-type mice, did develop serum rheumatoid factor. Thus, activation of the innate immune system in YYAA mice, as in SKG mice, led to some evidence of autoimmunity but, unlike SKG, did not lead to autoimmune disease. Further studies of autoantibodies and histological examination failed to reveal any evidence of other autoimmune manifestations in YYAA mice. However, we did find evidence for peripheral T cell activation and this correlated with the signaling strength of both ZAP-70 alleles. But only SKG mice, which showed the highest increase in $CD44^{hi}CD62L^{low}$, $CD45RB^{low}$, and $CD69^{+}$ cells in the periphery, develop autoimmune arthritis. These findings raise three possibilities. First, differences in peripheral TCR signaling caused by the YYAA and SKG mutations may determine whether autoimmune arthritis develops because of differences in the quality of the immune response generated. Second, peripheral tolerance is relatively intact in YYAA but defective in SKG mice. Third, the skewed repertoires selected in the thymus of the SKG and the YYAA mice substantially differ, leading to induction of autoimmune arthritis only in the SKG mice after zymosan challenge.

Because $CD4^{+}$ T cells are responsible for autoimmune arthritis in the SKG mouse model, we reasoned that altered cytokine production would likely be important in the evolution of T cell–mediated disease. We specifically examined IL-17 production because it has been implicated in the pathogenesis of autoimmune arthritis (Hirota et al., 2007). However, when challenged with zymosan, an elevation of IL-17 production was seen in both YYAA and SKG T cells. The number of IL-17–producing cells increased somewhat in both YYAA and SKG mice after treatment with zymosan, although the induced increase in IL-17–producing cells in the YYAA mouse was somewhat lower than that seen in T cells from the SKG mouse. One interpretation of these data are that YYAA $CD4^{+}$ T cells are less able to differentiate into Th17 cells, although the change compared with the SKG T cells is rather modest. Another alternative possibility is that some cytokines such as IFN-γ in the YYAA mice suppress Th17 development. We found that IFN-γ–deficient YYAA mice did not show enhanced development of Th17 T cells (unpublished data). Collectively, these data suggest that the failure to develop arthritis in the YYAA mice is not likely to be caused by a selective difference in the ability of the YYAA and SKG mice to generate increased numbers of IL-17–secreting effector cells. However, it is possible that other differences in cytokine production or local production might influence responses leading to arthritis.

Phenotypic and functional characterization of T reg cells, which are essential for peripheral self-tolerance, indicated that much lower percentages and absolute numbers of thymic T reg cell were found in the YYAA and SKG mice. This

marked decrease is probably the result of their severely impaired abilities to produce IL-2, partial TCR signaling defect, or both. Interestingly, unlike another partial T cell deficiency mouse mutant, LAT^{Y136F} in which T reg cells are nearly absent in the periphery (Koonpaew et al., 2006), the percentages of $Foxp3^+$ T reg cell in the YYAA and SKG mice were essentially normal or increased, respectively. Whereas YYAA T reg cells can develop in the periphery despite fewer numbers, we demonstrated that the suppressive activity of YYAA T reg cells consistently was not as efficient as WT controls. Given that SKG T reg cells have relatively normal suppressive function in vitro, the lower suppressive activity of YYAA T reg cell, which was consistently observed, is unexpected. Whether YYAA T reg cells are less functional in vivo remains to be determined. However, significant decreases in both thymic and peripheral T reg cell numbers in the YYAA mice suggest that YYAA T reg cells are impaired to some extent. Despite the observation that T reg cell mediated–function appears to be defective in the YYAA mice in contrast to the SKG mice, very limited autoimmunity was observed in the YYAA mice. Thus, the development of arthritis in SKG mice does not reflect a greater impairment in T reg cell numbers or function than in the YYAA mice.

To explore the third possibility that TCR repertoire skewing may differ in the YYAA and SKG mice, we examined how signaling strength affects TCR repertoire usage. The YYAA mice exhibited defects in positive selection in the HY-TCR or DO11.10-TCR systems that were similar to those reported for the SKG mice (Sakaguchi et al., 2003). However, the difference in TCR signaling could lead to differences in negative selection in a polyclonal repertoire that were not revealed by the model HY TCR transgenic systems. Therefore, we used a more direct and quantitative assessment of polyclonal TCR Vβ deletion in response to endogenous MMTV superantigens. Indeed, the magnitude of bias in Vβ deletion to endogenous superantigens correlates with the signaling strength in these different ZAP-70 allelic variants. The skewing in repertoires is not associated with a monoclonal or pauciclonal repertoire in either YYAA or SKG mice, as assessed by immunoscope analysis (Fig. S6). These findings suggest that the window of thymic selection has been shifted to different levels in these ZAP-70 mutants. This could well result in differences in the diverse T cell repertoires that result in distinct predispositions for the induction of autoimmune disease such as arthritis. These findings support the idea that development of autoimmune disease requires breakdowns of multiple tolerance checkpoints (Goodnow, 2007).

We attempted to determine whether decreasing the TCR signaling level from the YYAA T cell level to one closer to the SKG level might result in autoimmune arthritis. Therefore, we examined whether compound heterozygous mice sharing one SKG and one YYAA allele (SKG/YYAA) might develop arthritis. However, it appears that $CD4^+$ T cells developing in YYAA mice or SKG/YYAA mice never reach the selection threshold or altered peripheral immune responsiveness of those T cells in the SKG mice for development of autoimmune arthritis. This could reflect a dominant ability of the YYAA allele to interact with phospho-ITAMs over the SKG allele. It is interesting that in contrast to another hypomorphic allelic series recently reported that developed anti-DNA autoantibodies (Siggs et al., 2007), the YYAA, SKG, and the SKG/YYAA mice did not develop anti-DNA autoantibodies. Whereas genetic background can play a role in the development of autoimmunity, YYAA did not develop anti-dsDNA antibodies in either C57BL/6 or BALB/c backgrounds. By comparing the numbers of T cells developing on the B6 background in YYAA mice to those described in the studies by Siggs, et al., it is likely that neither the YYAA nor SKG mutations impair TCR signaling as severely. Therefore, we suspect it is likely that differences in TCR signaling that are responsible for differences in TCR repertoire explain the failure to develop arthritis or anti-dsDNA antibodies in the YYAA mice.

In conclusion, our studies demonstrate that in addition to the recently reported autoinhibitory function of Y315 and Y319 in ZAP-70, these phosphorylation sites play other important roles in TCR signaling, presumably by recruiting SH2 domain effector molecules to activated ZAP-70. These sites are important for the generation of a normal T cell repertoire and a normally functioning peripheral T cell compartment. Although reduced T reg cell development could also contribute to the development of arthritis in SKG mice, it is clearly insufficient as indicated by the failure of YYAA mice to develop frank arthritis. Our studies suggest that it is likely that distinct T cell repertoires containing potentially autoreactive cells are selected in YYAA and SKG mice based on differences of Vβ deletion to endogenous superantigens. Even in the presence of induced RF production, the YYAA mice fail to develop arthritis, whereas SKG mice do. These findings strongly suggest rather narrow windows of repertoire selection may dictate whether autoimmunity or autoimmune disease develops to the same challenge of the immune system.

MATERIALS AND METHODS

Generation of YYAA knockin mice. A 220-bp SmaI–HphI fragment covering exon 7 was subjected to site-directed mutagenesis changing tyrosines 315 and 319 to alanines and simultaneously introducing a diagnostic EcoRV site. The mutated fragment was used to replace the wild-type fragment and was then subcloned into the pAM20neo vector containing Neo cassette floxed by loxP sequence, 1.5-kb 5′ arm fragment, and 5-kb 3′ arm fragment (Fig. S1). Linearized DNA was electroporated into 129S4/Sv-derived PrmCre embryonic stem (ES) cells (O'Gorman et al., 1997). Homologous recombinants were selected in the presence of G418. DNA from each colony was digested with EcoRI and XbaI and analyzed by Southern blotting using the 5′ external and 3′ external probes shown in Fig. 1 C and described in Magnan et al. (2001). Two positive ES clones were injected into C57BL/6 blastocysts. Chimeric mice were generated and mated with C57BL/6 females for germline transmission. The *neo* gene was present in all the chimaeras, but efficiently excised in all offspring, in accordance with the Cre recombinase being expressed in the haploid germ cells of male chimaeras. Excision of the *neo* gene was further verified by PCR and Southern blotting on genomic DNA (Fig. S1). Heterozygous mutant mice were backcrossed to C57BL/6 or BALB/c background for at least 7 generations before intercrossing to obtain homozygous mutant mice of both C57BL/6 and BALB/c background. The primers used for screening are as follows: 5′ primer,

5′-GCTGATGTAGGCAATCCTCATTG-3′; 3′ primer, 5′-CATTTGGGCACTGTTTCCGC-3′. The size of the PCR product is 723 bp.

Mice. HY-TCR transgenic mice were obtained from Taconic. DO11.10 TCR transgenic mice and SKG mice were kindly provided by A. Abbas (University of California, San Francisco, San Francisco, CA) and S. Sakaguchi (Kyoto University, Kyoto, Japan), respectively. All mice were analyzed between 6 and 12 wk of age. All animal experiments were approved by the University of California San Francisco Institutional Animal Care and Use Committee.

Antibodies. The following monoclonal antibodies were purchased from BD: anti–CD3ε-PE, –PE-Cy7(145-2C11); anti–CD4-PE, –PerCP-Cy5.5, and -APC (RM4-5); anti–CD5-FITC (53–7.3); anti–CD8α-FITC, –PE-Cy7, and –APC (53–6.7); anti-CD24-FITC (M1/69); anti–CD44-FITC (IM7); anti-CD45RB-PE (16A); anti–CD62L-APC (MEL-14); anti–B220-PE-Cy7 (RA3-6B2); anti–DO11.10-PE (KJ1-26); anti–Vβ5-PE (MR9.4); anti–Vβ8-PE (F23.1); and anti–Vβ11 (RR3-15). The following antibodies were purchased from eBioscience: anti–TCRβ-FITC (H57-597); anti–CD4-Pacific blue (RM4-5); and anti–IL-17-PE (TC11-18H10). The following antibody was obtained from Invitrogen: anti-CD25-PE-Cy5.5 (CD35-3G10).

Calcium flux. Thymocytes or peripheral T cells were incubated with the calcium-sensitive dyes Fura-Red and Fluo-3 (Invitrogen) for 30 min at 37°C in RPMI 1640 and 5% fetal bovine serum, washed, and stained with anti–CD4-PE and –CD8-APC. After surface staining, cells were resuspended in RPMI medium with anti-CD3ε mAb (clone 2C11; 10 μg/ml) and warmed to 37°C for 5 min before stimulation. The baseline Ca^{2+} levels were measured for 25 s before addition of the cross-linking antibody goat anti–Armenian hamster IgG (50 μg/ml; Jackson Immunoresearch Laboratory). Ca^{2+} increase was measured as the ratio of Fluo-3 to Fura-Red fluorescence and was displayed as a function of time.

Intracellular staining. For the phospho-Erk flow assay, thymocytes or peripheral T cells were rested in serum-free medium for 30 min at 37°C, after which they were stimulated with 25 μg/ml of anti-CD3 (clone 2C11; PharMingen), followed by the addition of 50 μg/ml goat anti–Armenian hamster IgG (Jackson Immunoresearch Laboratory) at 37°C for 2 min. The addition of 4% paraformaldehyde stopped the reaction. Cells were pelleted, washed, and stained with anti–TCRβ–FITC for thymocytes and anti–CD44-FITC for peripheral T cells, respectively. Cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and washed with FACS buffer. Ice-cold methanol was added drop-wise, and cells were incubated on ice for 30 min followed by two washes in FACS buffer. Cells were stained with 3 μg/ml anti–phospho-p44/42 MAPK (Thr202/Tyr202; Cell Signaling Technology), followed by staining with donkey anti–rabbit Ig-APC, anti–CD4-Pacific blue, and anti–CD8-PE. Intracellular cytokine staining was assayed as previously described (Hirota et al., 2007).

Immunoblotting. Thymocytes were stimulated as in the phospho-Erk flow assay described in the previous section. The stimulation was stopped by the addition of ice-cold PBS. Cells were pelleted and lysed in buffer containing 1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, and protease and phosphatase inhibitors. Lysates were pelleted by centrifugation at 14,000 *g* for 10 min at 4°C and analyzed by immunoblotting.

Proliferation assay. $CD4^+$ T cells from lymph nodes were purified by negative magnetic bead separation using a CD4 T cell purification kit (Miltenyi Biotec). Purified cells were loaded with CFSE (Invitrogen), washed, and incubated with plate-bound anti-CD3 mAb (2 or 10 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) for 3 d. Cells were harvested for flow cytometry.

ELISA. Purified $CD4^+$ T cells from lymph nodes were stimulated as in the proliferation assay described above. 24 h after stimulation, supernatants were analyzed by ELISA for the presence of IL-2 using reagents and the protocol from BD. Anti-dsDNA autoantibody was assayed as previously described (Majeti et al., 2000). Rheumatoid factor antibody titers were determined with mouse RF ELISA kit (Alpha Diagnostic International).

Suppression assay. T reg cell and responsor $CD4^+$ T cells were isolated from the lymph nodes and spleen. Purified wild-type $CD4^+CD25^-$ responder cells (1×10^5) were co-cultured with $CD4^+CD25^-$ T reg cell from three genotypes, antigen-presenting cells from TCR $C\alpha^{-/-}$ mouse, and 1 μg/ml anti-CD3 mAb for 3 d. Various ratios of responder T cells to T reg cells were used in this assay. Proliferation was assessed as uptake of $[^3H]$thymidine.

Online supplemental material. Fig. S1 shows the targeting strategy for generation of ZAP-70 YYAA mice. Fig. S2 shows ZAP-70 expression levels in the wild-type, YYAA, SKG, and ZAP-$70^{-/-}$ mice. Fig. S3 shows defective positive selection in the YYAA and SKG mice. Fig. S4 shows increased percentages of memory or activated $CD4^+$ T cells in both YYAA and SKG mice. Fig. S5 shows production of IL-17^+ cells in wild-type, YYAA, and SKG mice. Fig. S6 shows immunoscope analysis in various T cell subsets from wild-type, YYAA, and SKG mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082902/DC1.

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