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Deconstructing Ras Signaling in the Thymus

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Thymocytes must transit at least two distinct developmental checkpoints, governed by signals that emanate from either the pre-T cell receptor (pre-TCR) or the TCR to the small G protein Ras before emerging as functional T lymphocytes. Recent studies have shown a role for the Ras guanine exchange factor (RasGEF) Sos1 at the pre-TCR checkpoint. At the second checkpoint, the quality of signaling through the TCR is interrogated to ensure the production of an appropriate T cell repertoire. Although RasGRP1 is the only confirmed RasGEF required at the TCR checkpoint, current models suggest that the intensity and character of Ras activation, facilitated by both Sos and RasGRP1, will govern the boundary between survival (positive selection) and death (negative selection) at this stage. Using mouse models, we have assessed the independent and combined roles for the RasGEFs Sos1, Sos2, and RasGRP1 during thymocyte development. Although Sos1 was the dominant RasGEF at the pre-TCR checkpoint, combined Sos1/RasGRP1 deletion was required to effectively block development at this stage. Conversely, while RasGRP1 deletion efficiently blocked positive selection, combined RasGRP1/Sos1 deletion was required to block negative selection. This functional redundancy in RasGEFs during negative selection may act as a failsafe mechanism ensuring appropriate central tolerance.

T cell development is initiated when immature precursor cells emigrate from the fetal liver or adult bone marrow to the thymus. In the thymus, these cells undergo a receptor-driven differentiation program, passing through at least two distinct developmental checkpoints before emerging as functional T lymphocytes (2). At the first checkpoint, a properly rearranged T cell receptor β (TCRβ) chain pairs with a pre-TCRα chain to form a pre-T cell receptor (pre-TCR). The pre-TCR signals in a ligand-independent manner to promote proliferation and drive differentiation from the $CD4^-$ $CD8^-$ double-negative (DN) to the $CD4^+$ $CD8^+$ double-positive (DP) stage of thymocyte development. At the second checkpoint, the strength and quality of signaling through the mature TCR is interrogated. Cells that fail to signal through the TCR die by neglect, cells expressing a TCR that binds self-antigen in the context of the major histocompatibility complex (MHC) with strong affinity generate strong TCR signals and die via TCR-dependent apoptotic pathways (negative selection), whereas cells expressing a TCR that has weak affinity for self antigen-MHC complexes signal weakly and selectively survive (positive selection) (8). Genetic studies have shown that signaling from either the pre-TCR or the TCR, through the adaptors LAT and Slp-76, to the small G protein Ras and the downstream extracellular signal-regulated kinase (ERK) cascade is required for thymocyte development at both checkpoints (1, 7, 14, 17, 22, 24–26). However, an understanding of how Ras signals are generated to drive thymocyte development has remained enigmatic, despite intense study over the past 20 years.

Ras is thought to be activated in thymocytes by two families of RasGEFs: RasGRP1 and Son of Sevenless (Sos1 and Sos2). Recent studies have described two competing models describing the role of these RasGEFs in Ras/ERK activation during thymocyte development. The first model is based upon the signaling properties of each RasGEF and their pattern of activation at the TCR checkpoint. Studies using OT-I TCR transgenic thymocytes showed a correlation between weak, RasGRP1-dependent Ras/ERK activation during positive selection and strong, Sos-dependent Ras/ERK activation during negative selection (3). Furthermore, biochemical studies probing Ras activation in Jurkat cells have described a positive-feedback loop between RasGRP1 and Sos that can be engaged by strong, but not weak, TCR stimulation, leading to digital activation of the Ras/ERK pathway (4, 21). These studies, together with modeling data, have suggested an hypothesis in which in the developing thymus, weak TCR stimulation via low-potency ligands signals through RasGRP1 alone to support positive selection, while stronger ligands engage both Sos and RasGRP1, causing a marked increase in the amplitude of Ras/ERK activation to trigger negative selection (18). While this model does not make predictions about which RasGEFs are required for pre-TCR-mediated development, it provides a testable hypothesis for the role of Ras/ERK signaling during positive and negative selection.

The second model is based upon genetic studies and expression data of the RasGEFs at each developmental checkpoint. Single knockout studies have shown a requirement for Sos1 (but not RasGRP1) in pre-TCR-dependent proliferation and gene expression, while RasGRP1 (and not Sos1) is required for TCR-dependent positive selection (5, 12). These data, in conjunction with the marked downregulation of Sos1 and upregulation of RasGRP1 protein levels observed between DN and DP thymocytes, have suggested an alternative model in which the RasGEF expression profile is the major determinant of Ras activation at these critical intrathymic checkpoints (12). A caveat to this second model is that genetic studies to date have not described a role for either Sos1 or RasGRP1 in negative selection (12, 20). Therefore, whether Ras is involved in negative selection at the TCR checkpoint remains a critical, unanswered question.

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Here, we have assessed the independent and combined roles of the RasGEFs Sos1, Sos2, and RasGRP1 during thymocyte development. We show that there is an inverse relationship in the importance of Sos1 and RasGRP1 at the pre-TCR and TCR checkpoints that correlates with their previously described expression patterns. Downstream of the pre-TCR, Sos1 is the predominant RasGEF for development beyond the DN stage, yet combined Sos1/RasGRP1 deletion leads to a more severe DN thymocyte block than is seen upon Sos1 deletion alone. Downstream of the TCR, RasGRP1 alone is necessary and sufficient for positive selection, whereas combined RasGRP1/Sos1 deletion is required to block negative selection. Sos2 is not required for thymocyte development downstream of either the pre-TCR or TCR as its deletion does not alter thymocyte development regardless of the Sos1 or RasGRP1 genotype. These data require modification of the existing models describing Ras activation by RasGEFs during thymocyte development. These data further suggest some functional redundancy between Sos proteins and RasGRP1 that acts as a failsafe mechanism to ensure (i) the production of a sufficient repertoire of immature T cells at the pre-TCR checkpoint and (ii) appropriate negative selection when the TCR is stimulated with high-potency ligands.

MATERIALS AND METHODS

Mice. RasGRP1$^{-/-}$ mice were a gift from James Stone (5). Sos2$^{-/-}$ mice were generated at LCMB by Eugene Santos (6). AND mice were a gift from B. J. Fowlkes (10). OT-I and OT-II mice were purchased from The Jackson Laboratory (9). HY (11), CD4-Cre (13), and Lck-Cre (13) mice were purchased from Taconic. Genotyping for OT-I (9), OT-II (9), AND (10), HY (11), RasGRP1$^{-/-}$ (5), Sos1(T)$^{-/-}$ (12), Sos2$^{-/-}$ (6), and Cre (13) mice was carried out as detailed in the original publications. All mice were housed at the National Institutes of Health (NIH) following guidelines set forth by the National Cancer Institute (NCI)-Bethesda Animal Care and Use Committee.

***In vivo* SEB injections.** Mice were injected intraperitoneally (i.p.) with 10 μg of staphylococcal enterotoxin B (SEB [Sigma catalog no. S4481], diluted to 0.1 mg/ml in sterile phosphate-buffered saline [PBS]) on days 0, 2, and 4. On day 7, total thymocytes were isolated and subjected to flow cytometry.

***In vivo* 2C11 stimulations.** Mice were injected intraperitoneally (IP) with 50 μg of anti-CD3ε (145-2C11 NA/LE [BD Biosciences], diluted to 0.5 mg/ml in sterile PBS). At the indicated time postinjection, the total thymocytes were isolated and subjected to flow cytometry.

Negative selection of OT-I thymocytes *ex vivo*. A total of 10^5 EL4 cells (a gift from Pamela Schwartzberg) were preincubated with increasing concentrations of SIINFEKL peptide or altered peptide ligands with decreasing affinities for the OT-I TCR (Q4, T4, Q7, G4, scrambled [3]) for 1 h in 100 μl of RPMI 1640 in a 96-well plate to allow binding of peptides to MHC complexes. These were then overlaid with 5×10^5 isolated OT-I thymocytes, followed by incubation at 37°C in RPMI 1640 containing 1% fetal bovine serum and penicillin-streptomycin. After 20 h, the total thymocytes were isolated and subjected to flow cytometry. The percentage of live, DP thymocytes were then normalized to non-peptide-pulsed controls for each genotype (1).

Flow cytometry. Single cell suspensions from thymus were stained with the fluorochrome-conjugated monoclonal antibodies described in the text. For DN1 to DN4 profiling, thymocytes were gated as negative (not staining) for a mixture of CD4, CD8, CD11b, TCRβ, TCRγδ, Ter119, B220, and NK1.1 (lin$^-$). For the pERK studies, stimulated and fixed (BD Fix/Perm kit) thymocytes were stained with pERK (1:50, catalog no. 4370; Cell Signaling Technology), followed by fluorescein isothiocyanate (FITC)-conjugated TCRβ, phycoerythrin (PE)-conjugated CD4, and allophycocyanin (APC)-conjugated anti-rabbit secondary antibodies (Invitrogen). DP thymocytes were defined as CD4$^+$ TCRβ^{lo}. Flow cytometry was performed using a FACSCalibur and CellQuest software (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc.). All fluorochrome-conjugated antibodies were purchased from BD Biosciences except for anti-CD44 (eBioscience).

Cell purification. For preselection DP thymocytes, the cells were first stained with a cocktail containing anti-CD3ε–FITC, anti-CD25–FITC, and Fc block (anti-mouse CD16/CD32, BD Biosciences). The cells were then were isolated on MACS columns via depletion using anti-FITC microbeads (Miltenyi Biotech) according to the manufacturer's instructions. Cells were >90% DP following purification. Purified DP thymocytes were then resuspended in prewarmed RPMI at 10^6 cells per 10 μl and allowed to equilibrate to room temperature for 20 min (RasGEF blots) or rested for 4 h at 37°C in RPMI at 10^6 cells per ml (cell stimulations). Cells were never allowed to get below room temperature during the isolation procedure.

Cell stimulation and Western blotting. For the stimulation of purified preselection DP thymocytes, purified cells were resuspended in prewarmed RPMI at 10^6 cells per 10 μl. For each time point, 5×10^6 cells were preincubated with biotinylated anti-CD3ε (0.5 mg/ml; 145-2C11) with or without anti-CD4 (0.5 mg/ml; GK1.5) for 15 min at room temperature. The cells were then washed with RPMI and resuspended at 10^6 cells per 10 μl prior to the addition of 50 μl of streptavidin (20 μg/ml [final concentration]). Stimulation was terminated by the addition of 2× sodium dodecyl sulfate (SDS) sample buffer containing 100 mM dithiothreitol and boiling for 10 min.

Samples were loaded at 0.5×10^6 cells per lane (10^6 cells for RasGEF blotting) and separated by SDS–10% PAGE. Blots were incubated with primary antibodies (pERK, 1:2,000 [Cell Signaling Technology; catalog no. 4370]; ERK1/2, 1:2,000 [Cell Signaling Technology; catalog no. 4695]; β-actin, 1:5,000 [Sigma-Aldrich; catalog no. AC-40]; Sos1, 1:500 [Santa Cruz; sc-256]; Sos2, 1:200 [Santa Cruz; sc-15358]; RasGRP1, 1:500 [Santa Cruz; sc-8430]) at 4°C overnight and secondary horseradish peroxidase-conjugated antibodies (1:20,000; Millipore) at room temperature for 1 h. Enhanced chemiluminescence was used to visualize protein products (Super Signal West Pico and Super Signal West Femto [Pierce]). The protein bands were quantified using ImageJ.

pERK measurement via flow cytometry. Total thymocytes were resuspended in prewarmed RPMI at 10^6 cells per 10 μl. For each time point, 2×10^6 cells were preincubated with the indicated concentrations of biotinylated anti-CD3ε for 15 min at room temperature. The cells were then washed with RPMI and resuspended at 2×10^6 cells per 50 μl prior to the addition of 50 μl of streptavidin (20 μg/ml [final concentration]) for the indicated times (0 to 5 min). Stimulation was terminated by the addition of 100 μl of Fix/Perm (BD Biosciences) for 20 min at room temperature. Samples were washed twice in BD Perm/Wash, resuspended in 50 μl of diluted pERK antibody (1:50; Cell Signaling Technology, catalog no. 4370) plus 0.5 μl of Fc block, and incubated at room temperature for 45 min. The samples were washed once and stained with APC-conjugated anti-rabbit secondary antibody (1:400; Invitrogen) and fluorochrome-conjugated anti-CD4 and anti-TCRβ for 30 min prior to flow cytometry.

Statistical analysis. All data are presented as averages ± the standard deviation. The significance between two data sets was determined by a two-tailed Student *t* test. *P* values of <0.05 were considered statistically significant. Tables containing *P* values for all pairwise comparisons shown in the manuscript are available on request.

RESULTS

Sos1 and RasGRP1 cooperate during pre-TCR-mediated development. To assess the combined roles of Sos1, Sos2, and RasGRP1 during thymocyte development, mice with Lck-Cre-driven, thymocyte-specific deletion of Sos1 [Sos1(T)$^{-/-}$] (12) were crossed with Sos2$^{-/-}$ (6) and RasGRP1$^{-/-}$ (5) mice to generate all double-knockout (DKO) and triple-knockout (TKO) combinations

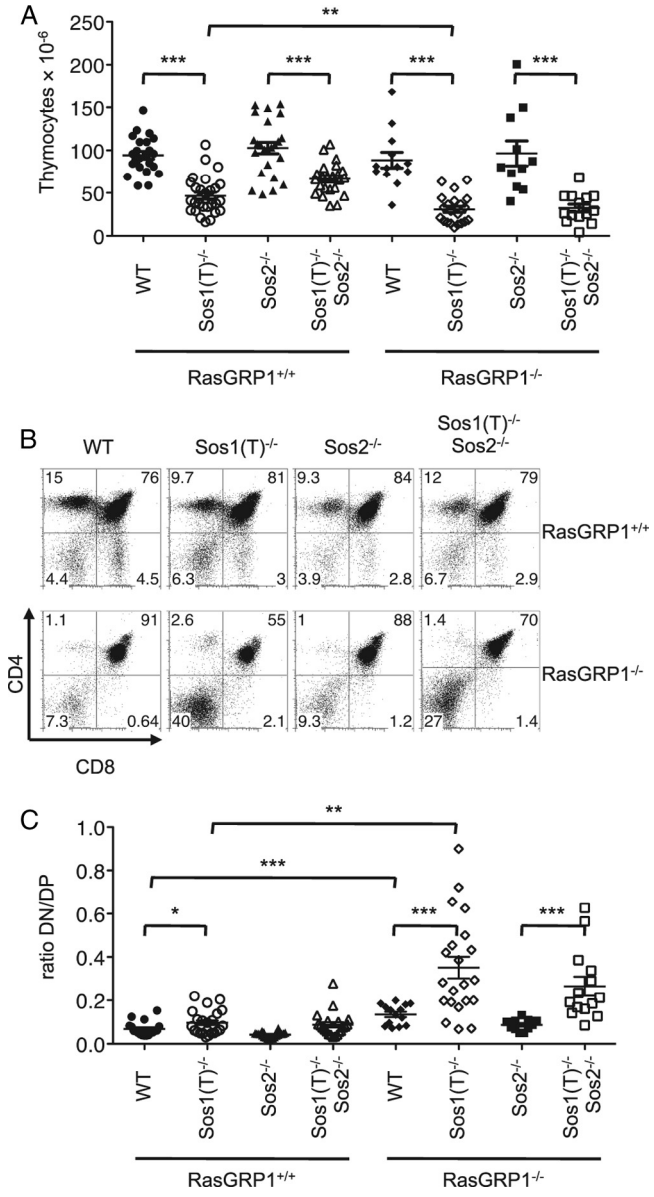

FIG 1 The combined actions of Sos1 and RasGRP1 are required at the pre-TCR checkpoint. (A) Total numbers of thymocytes isolated from 8-week-old mice from the indicated genotypes ($n = 8$ to 20 for each). Each symbol denotes an individual mouse, and the bar denotes the average for the group. **, $P < 0.01$; ***, $P < 0.001$. (B) Flow cytometry dot plots of total thymocytes stained with anti-CD4 and anti-CD8 from representative 8-week-old mice from panel A. (C) The DN/DP ratio from (B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

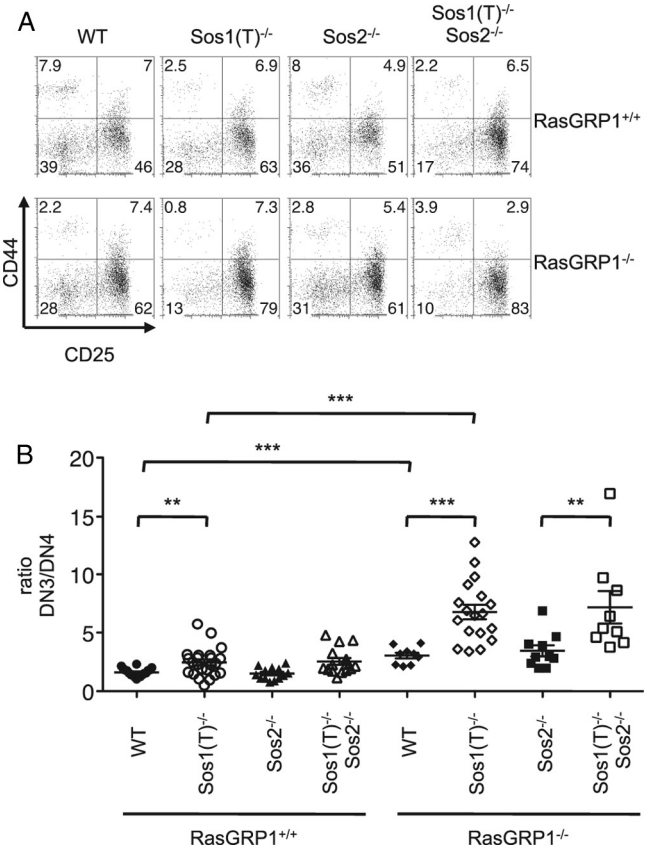

FIG 2 The combined actions of Sos1 and RasGRP1 are required at the DN3-DN4 transition. (A) Flow cytometry dot plots of gated lin$^-$ (CD4$^-$ CD8$^-$ CD11b$^-$ TCRβ$^-$ TCRγδ$^-$ Ter119$^-$ B220$^-$ NK1.1$^-$ [see Materials and Methods]) DN thymocytes stained with anti-CD44 and anti-CD25 to identify DN1-DN4 thymocytes from representative 8-week-old mice from Fig. 1. (B) The DN3/DN4 ratio from panel A. **, $P < 0.01$; ***, $P < 0.001$.

for these three RasGEFs. Whereas deletion of Sos2 or RasGRP1 did not significantly affect overall thymic cellularity, Sos1 deletion caused a 50% reduction in thymocyte numbers as previously reported (Fig. 1A) (12). This reduction was not exacerbated by combined deletion with Sos2; indeed, Sos1/Sos2 DKO thymi were slightly larger than Sos1(T)$^{-/-}$ thymi. In contrast, combined Sos1/RasGRP1 deletion caused a marked reduction in thymic cellularity that was not further affected in Sos1/Sos2/RasGRP1 TKO mice. These data suggest that, in addition to its required role in positive selection (Fig. 1B) (5), RasGRP1 may play another, previously unappreciated role in thymocyte development that is unmasked in the absence of Sos1.

Staining with anti-CD4 and anti-CD8 to assess thymocyte development revealed a marked block at the DN-to-DP transition in Sos1/RasGRP1 DKO mice, with an increase in the DN/DP ratio that was much more severe than that seen in either Sos1(T)$^{-/-}$ or RasGRP1$^{-/-}$ mice alone (Fig. 1B and C). These data indicate that Sos1 and RasGRP1 may cooperate at the pre-TCR developmental checkpoint. In contrast, deletion of Sos2 either alone or in combination with Sos1 and RasGRP1 did not exacerbate this early developmental block, indicating that Sos2 is dispensable for early thymocyte development. To confirm that Sos1 and RasGRP1 cooperate in development downstream of the pre-TCR, DN thymocytes were stained with anti-CD25 and anti-CD44 antibodies and analyzed by flow cytometry (Fig. 2). Sos1(T)$^{-/-}$ and RasGRP1$^{-/-}$ mice each showed a moderate decrease in the percentage of DN4 thymocytes (Fig. 2A) and a corresponding increase in the DN3/DN4 ratio (Fig. 2B), indicating that each of these RasGEFs plays a role at the pre-TCR checkpoint. Furthermore, Sos1/RasGRP1 DKO and Sos1/Sos2/RasGRP1 TKO mice showed a marked increase in the ratio of DN3/DN4 thymocytes (Fig. 2A and B), indicating cooperativity between these two RasGEFs downstream of the pre-TCR. This developmental block was not due to defective TCRβ rearrangement as assessed by intracellular TCRβ staining (data not shown). These data indicate a severe, but not complete,

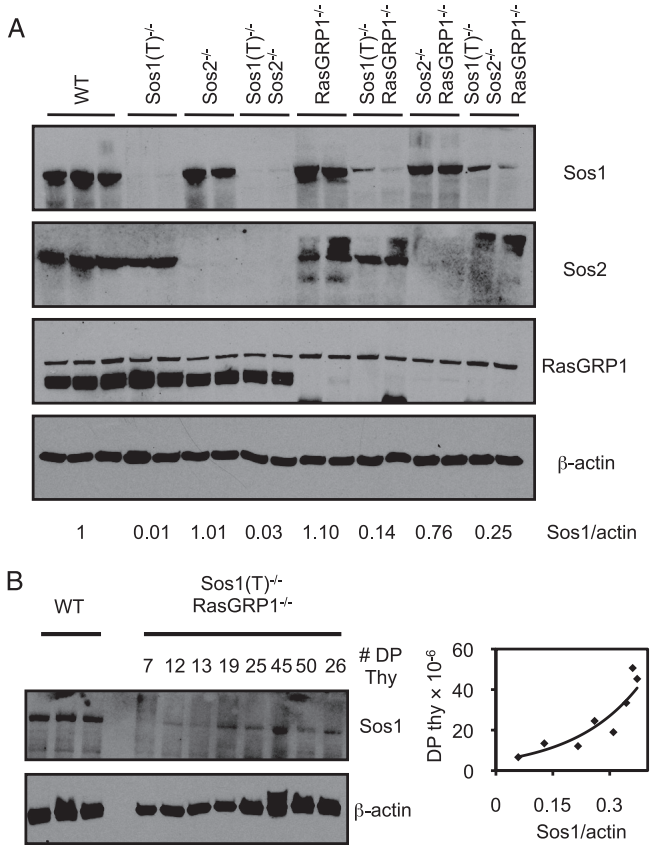

FIG 3 Sos1 expression is preferentially maintained upon RasGRP1 deletion. (A) Western blotting for Sos1, Sos2, RasGRP1, and β-actin in purified DP thymocytes pooled from two to five thymi from mice of the indicated genotypes. Quantitation of Sos1/actin is shown at the bottom relative to WT. Each lane represents an independently isolated population. (B) Western blotting for Sos1 and β-actin in purified DP thymocytes from individual WT or Sos1/RasGRP1 DKO mice and quantitation of the relative Sos1 expression level versus the number of DP thymocytes for each Sos1/RasGRP1 DKO mouse (the line represents the best-fit polynomial, $R = 0.86$).

developmental block at the pre-TCR checkpoint in the absence of both Sos1 and RasGRP1.

A partial (rather than complete) block in pre-TCR-mediated development can be seen in genetically engineered mice either when signaling is markedly, but not completely blocked (23) or if a floxed allele is incompletely deleted prior to the initiation of pre-TCR signaling (7). We had previously shown that in Sos1(T)$^{-/-}$ mice, Lck-Cre, but not CD4-Cre, allowed for efficient deletion of Sos1 protein in DP thymocytes (12). It was possible, however, that deletion of additional RasGEFs in a Sos1(T)$^{-/-}$ background created sufficient selective pressure so that those DN3/DN4 thymocytes expressing some residual Sos1 proliferated rapidly, thereby allowing the accumulation of DP thymocytes. To assess whether the incomplete early developmental block seen in Sos1/RasGRP1 DKO and Sos1/Sos2/RasGRP1 TKO mice was due to incomplete deletion of Sos1, Sos1 levels were measured in purified DP thymocytes from mice deleted for Sos1, Sos2, and/or RasGRP1 (Fig. 3). While Lck-Cre-mediated deletion was efficient in Sos1(T)$^{-/-}$ DP thymocytes, Sos1 expression was preferentially maintained in Sos1/RasGRP1 DKO and Sos1/Sos2/RasGRP1 TKO DP thymocytes, suggesting that Sos1 expression was required for development to the DP stage in the absence of RasGRP1 (Fig. 3A). To assess whether a direct correlation between the extent of DP cell development and retained Sos1 expression existed, DP thymocytes were isolated from eight independent Sos1/RasGRP1 DKO mice and Sos1 levels were assessed (Fig. 3B). These data showed a direct correlation between the number of DP thymocytes and the level of Sos1 expression, providing strong support that retained expression of Sos1 was driving development to the DP stage in these mice.

Combined Sos1/2 deletion does not affect TCR-mediated positive or negative selection. At the DP stage, thymocytes transit a second, TCR-dependent developmental checkpoint where the strength of signaling through the TCR is tested (positive and negative selection). Studies using RasGRP1$^{-/-}$ mice have shown that RasGRP1 is required for positive, but not negative, selection (5, 20). Consistent with these findings, we observed a significant block at the DP stage in RasGRP1$^{-/-}$ mice that was unaffected by further deletion of Sos1 and/or Sos2 (Fig. 1B). To further assess the role of Sos1 and Sos2 during positive selection, Sos1/2 DKO mice were crossed to HY (11), AND (10), OT-I (9), and OT-II (9) TCR-transgenic mice. These crosses revealed that Sos1/2 were not required for either MHC class I-mediated (HY females, OT-I) (Fig. 4A) or MHC class II-mediated (AND, OT-II) (Fig. 4B) positive selection.

The very strong effect of RasGRP1 deletion on positive selection, however, may overwhelm any effects of Sos1/2 deletion, and minor effects of Sos1 and or Sos2 might be revealed on a RasGRP1$^{+/-}$ or RasGRP1$^{-/-}$ background. Furthermore, although Sos1 was efficiently deleted in DP thymocytes isolated from both Sos1(T)$^{-/-}$ and Sos1(T)$^{-/-}$ Sos2$^{-/-}$ mice (Fig. 3A), it remains formally possible that a minority of cells could escape positive selection prior to Sos1 deletion, thereby masking any minor role Sos1 and Sos2 may play in positive selection.

To further assess whether Sos proteins are required for positive selection, the effects of Sos1 and/or Sos2 deletion on thymocyte development was examined in both a RasGRP1$^{+/-}$ and a RasGRP1$^{-/-}$ background. RasGRP1 heterozygosity did not significantly affect either overall thymocyte cellularity or the development of CD4SP and CD8SP thymocytes in Sos1 and/or Sos2 knockout mice (data not shown). Furthermore, positive selection of mice with a polyclonal TCR repertoire, as assessed by examining expression levels of the cell surface markers CD3ε, TCRβ, CD24, and CD69 during thymocyte development, was unaffected by Sos1 and/or Sos2 deletion on either a RasGRP1$^{+/+}$ or RasGRP1$^{+/-}$ background (Fig. 4C and D and data not shown). These data indicate that Sos1 and Sos2 are dispensable for positive selection.

In contrast, RasGRP1 deletion caused a marked block in positive selection, as assessed by loss of CD4SP and CD8SP thymocytes (Fig. 1B) and an inability to efficiently upregulate TCRβ and CD69 (Fig. 4C). Despite the relatively strong block in positive selection seen in these mice, a small number of peripheral T cells do accumulate in both the lymph nodes (Fig. 5A to C) and spleens (data not shown) of RasGRP1$^{-/-}$ mice, although the RasGRP1$^{-/-}$ T cells that accumulate show an activated phenotype indicative of altered thymic selection (19; data not shown). Although Sos1 deletion further reduced the numbers of lymphocytes seen in both a RasGRP1$^{+/+}$ and RasGRP1$^{-/-}$ background, this reduction could be due both to the early developmental block caused by Sos1 de-

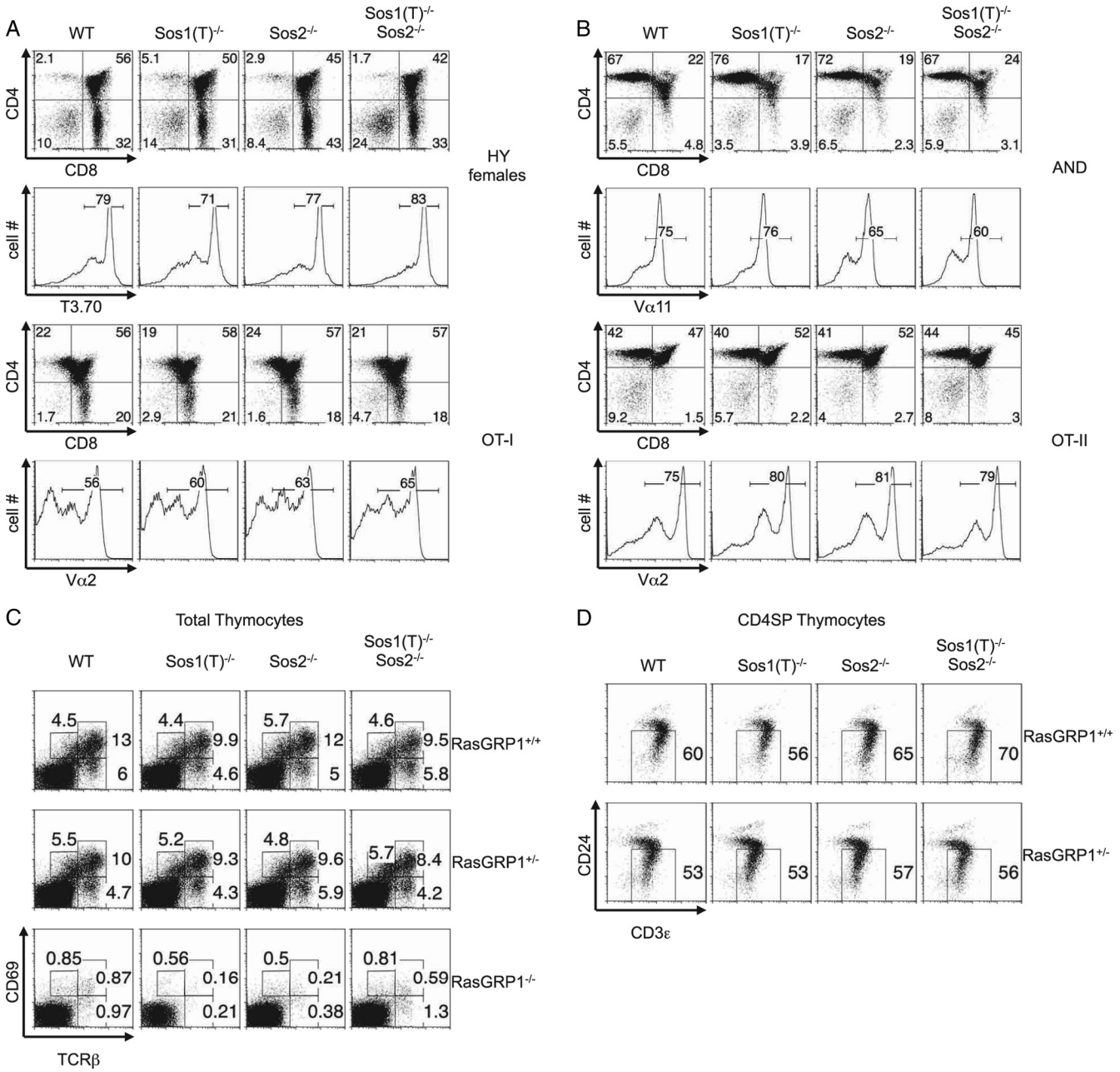

FIG 4 Sos1 and Sos2 are not required for positive selection at the TCR checkpoint. (A and B) Flow cytometry dot plots of gated, transgene$^+$ thymocytes from 8-week-old TCR-transgenic mice stained with anti-CD4 and anti-CD8 (above), and histograms showing gating for the transgenic TCR (below). (A) HY female and OT-I transgenic mice. (B) AND and OT-II TCR transgenic mice. $n \geq 4$ for each group. (C) Flow cytometry dot plots of total thymocytes stained with anti-CD69 and anti-TCRβ to assess the percentage of thymocytes entering into positive selection (CD69$^+$ TCRβ^{int}), immature SP cells (CD69$^+$ TCRβ^{hi}), and mature SP cells (CD69$^-$ TCRβ^{hi}) from 8-week-old mice with the indicated genotypes. (D) Flow cytometry dot plots of gated CD4SP thymocytes stained with anti-CD24 and anti-CD3ε to assess the percentages of mature (CD24$^-$ CD3ε^{hi}) SP thymocytes cells from 8-week-old mice from panel C. RasGRP1$^{-/-}$ mice are not shown, since they have few SP thymocytes.

letion (Fig. 1) (12) and to the effects of Lck-Cre expression on peripheral T cell expansion (Fig. 5) (7, 12).

In contrast to positive selection, several studies have hypothesized a role for Sos1 and/or Sos2 in generating high levels of Ras/ERK activation during negative selection (3, 18). To determine whether negative selection is affected by the absence of Sos, superantigen SEB-induced deletion of Vβ8$^+$ thymocytes was analyzed in Sos1/2 DKO mice (Fig. 6A and B). SEB-induced negative selection, measured by the selective loss of Vβ8$^+$ (but not Vβ6$^+$) SP thymocytes, was unaffected by deletion of either Sos1 and/or Sos2, indicating that Sos proteins were not required in this model of negative selection. Similarly, negative selection was unaffected by Sos1/2 deletion upon anti-CD3ε injection (Fig. 7A to C). Negative selection models that directly stimulate the TCR *in vivo*, such as SEB or anti-CD3ε injection, can kill DP thymocytes not only directly but also indirectly by stimulating lethal cytokine production

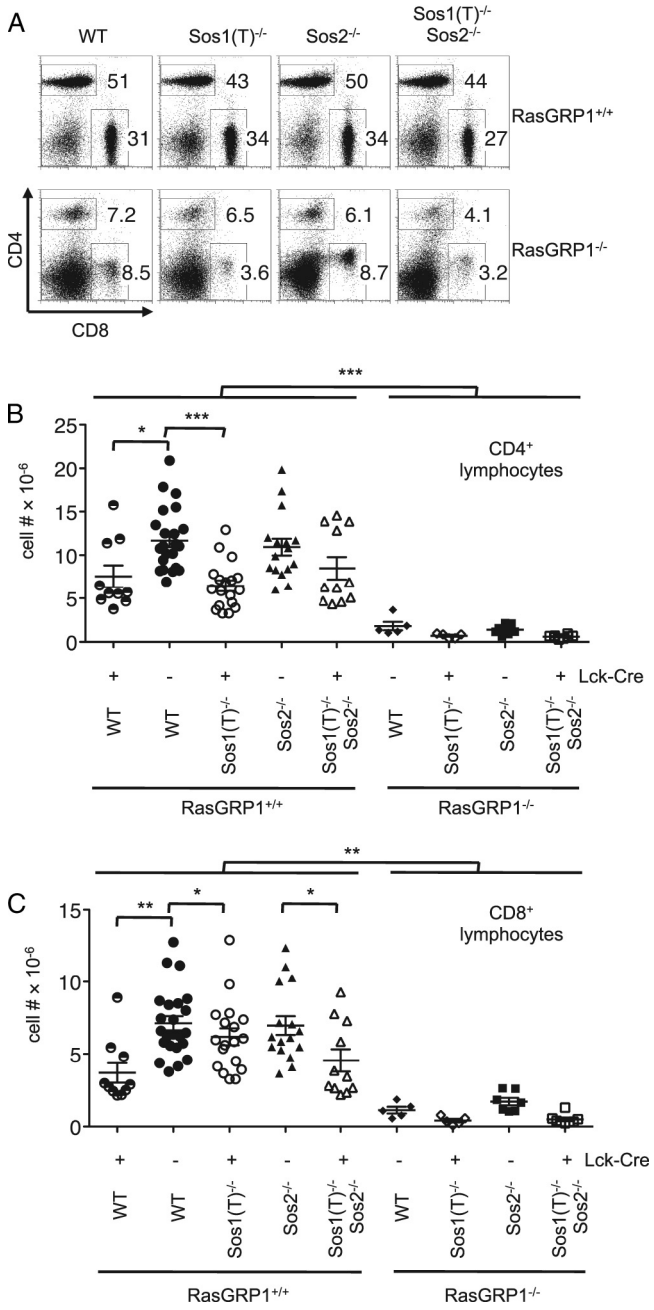

FIG 5 RasGRP1 deletion has a greater effect than Sos1/2 deletion on lymph node T cell numbers. (A) Flow cytometry dot plots of pooled axillary, brachial, and inguinal lymph nodes stained with anti-CD4 and anti-CD8 from 8-week-old mice from the indicated genotypes ($n = 5$ to 20 for each). (B and C) Total numbers of $CD4^+$ (B) and $CD8^+$ (C) LN T cells isolated from 8-week-old mice from the indicated genotypes ($n = 5$ to 20 for each). Each symbol denotes an individual mouse and the bar denotes the average for the group. Lck-Cre positive and negative WT control mice are shown, since early expression of Cre can affect peripheral T cell expansion. Lck-Cre status is given for each genotype. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in peripheral T cells (16). To assess self-peptide induced negative selection, Sos1 and/or Sos2 mice were crossed with HY^+ TCR-transgenic mice. HY mice express a high-affinity TCR early in thymocyte development. This transgenic TCR recognizes a male-specific peptide in the context of MHC class I, which causes strong signaling and negative selection at the DP stage. As was the case for SEB treatment and anti-CD3ε injection, Sos1 and/or Sos2 deletion did not alter negative selection in HY^+ male mice, even when crossed onto a RasGRP1$^{+/-}$ background to dampen RasGRP1-dependent effects on selection (Fig. 7E to G and RasGRP1$^{+/-}$ data not shown). These data indicate that expression of Sos1 and Sos2 are not required in three classical models of negative selection (Fig. 7, the experiments performed on a RasGRP1$^{-/-}$ background are discussed in greater detail below).

It is entirely possible, however, that the strong TCR signals generated by these models can generate sufficient Ras activation to trigger negative selection via RasGRP1 alone and that Sos1 and/or Sos2 play an accessory role in setting an hypothetical threshold of TCR activation required to trigger positive versus negative selection (18). If this were true, rather than being absolutely required for negative selection upon strong ligand stimulation, Sos1/2 might help shape Ras/ERK signaling at intermediate levels of TCR activation to determine the extent of TCR activation required to induce negative selection. To directly test this possibility, we assessed negative selection in OT-I TCR transgenic thymocytes. The OT-I TCR recognizes the octamer peptide SIINFEKL (OVAp) in the context of H-2K^b, and overnight stimulation of OT-I thymocytes *ex vivo* with increasing concentrations of OVAp presented by EL-4 cells causes a dose-dependent decrease in the percentage of viable DP thymocytes. In this system, a defect in negative selection is observed by survival of DP thymocytes at increasing peptide concentrations (1). Furthermore, the use of altered peptide ligands (APLs) with decreasing affinities to the OT-I TCR, which were previously used to correlate Sos engagement with the induction of negative selection (3), allows for the direct assessment of the role of Sos deletion in setting a threshold for negative selection. In our study, there were no differences in the dose of OVAp or any negatively selecting APL (Fig. 6C) required to induce loss of DP thymocytes regardless of Sos genotype. Assessment of moderate (anti-CD3 alone) or strong (anti-CD3 + anti-CD4)-induced ERK activation in DP thymocytes showed that deletion of either Sos1 alone or in combination with Sos2 led to a 30 to 40% reduction in ERK activation (Fig. 8A). However, deletion of Sos1/2 did not alter the digital nature of ERK activation, as single cell analysis revealed a bimodal pERK response at peak stimulation regardless of the Sos1/2 genotype (Fig. 8B).

In contrast to the minor role for Sos1 in ERK activation at the DP stage, RasGRP1 deletion almost completely abrogated TCR-stimulated ERK activation. Stimulation with very high doses of anti-CD3ε (100 μg/ml) is required to appreciate TCR-stimulated ERK phosphorylation in RasGRP1$^{-/-}$ thymocytes by Western blotting (Fig. 8C), although the modest amount of pERK produced (>50-fold less than wild-type [WT] levels) was insufficient to be seen by flow cytometry (Fig. 8D). Combined Sos1/RasGRP1 deletion further reduced the level of TCR-stimulated pERK, although this reduction was variable between experiments, likely due to the high level of retained Sos1 in DP thymocytes isolated from Sos1(T)$^{-/-}$ RasGRP1$^{-/-}$ mice (Fig. 3).

These data indicate that although Sos plays a role in Ras/ERK signaling at the DP stage, the deletion of Sos1 and/or Sos2 was insufficient to affect negative selection in the presence of RasGRP1. Furthermore, the inability of Sos1/2 deletion (Fig. 6 and 7) or RasGRP1 deletion (20) to affect negative selection calls into question whether Ras signaling is at all required for negative selection, or if rather the observed correlation between high levels of

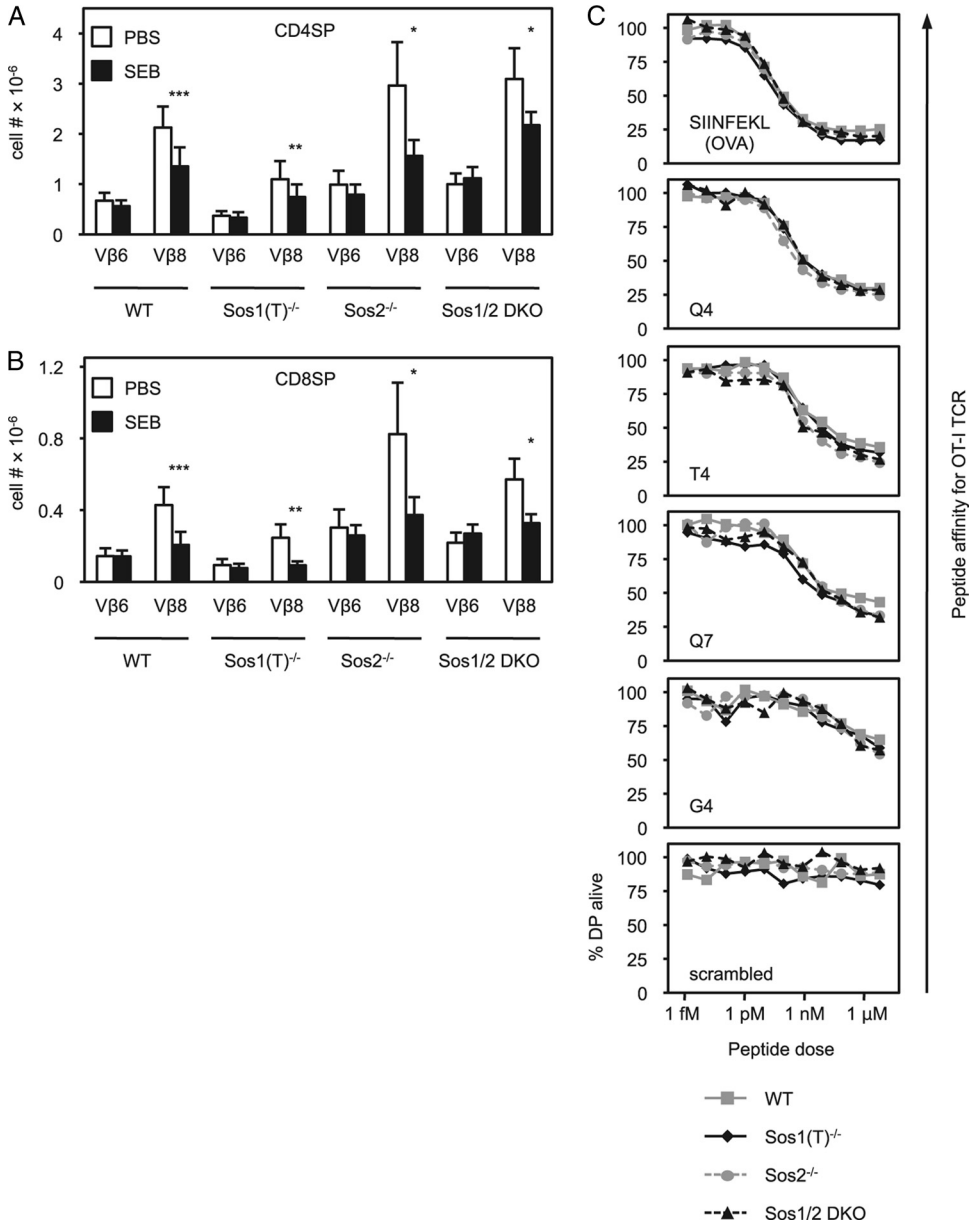

FIG 6 The Sos1 and Sos2 are not required for negative selection at the TCR checkpoint. (A and B) Total numbers of $V\beta6^+$ or $V\beta8^+$ CD4SP (A) or CD8SP (B) thymocytes from mice injected with PBS or SEB as described in Materials and Methods. $n = 5$ for each group. The data are represented as means ± the standard deviation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Relative percentage of live, OT-I^+ ($V\alpha2^+$) DP thymocytes after overnight incubation with increasing concentrations of the OVA peptide SIINFEKL or the indicated altered peptide ligand presented by EL4 cells. No peptide controls for each genotype were set to 100%. The data represent the mean of two independent experiments.

Ras activation and negative signaling is simply a by-product of the high levels of TCR activation required to stimulate TCR-dependent, Ras-independent apoptotic pathways.

Sos1 cooperates with RasGRP1 during TCR-mediated negative selection. To determine whether Ras signaling via the RasGEFs Sos1, Sos2, and RasGRP1 is indeed required for negative selection, all combinations of Sos1, Sos2, and RasGRP1 knockout mice were assessed in two classic models that cause strong signaling and early negative selection in DP thymocytes, *in vivo* anti-CD3ε injection (Fig. 7A to D) and HY^+ TCR-transgenic male mice (Fig. 7E to H). These models were chosen because they induce early negative selection in DP thymocytes, which is an absolute necessity when assessing negative selection in mice with a severe block in positive selection such as RasGRP1$^{-/-}$ mice.

In either model, both the percentage and overall number of DP thymocytes is informative in assessing the extent of negative selection. However, since thymocyte numbers vary significantly between different RasGEF knockouts (Fig. 1A), assessment of the ratio between the experimental mice (anti-CD3ε injected or HY^+ male) and control (uninjected or nontransgenic) for each individual genotype can be as or more informative than the raw number of DP thymocytes. Therefore, analysis of both the number of DP

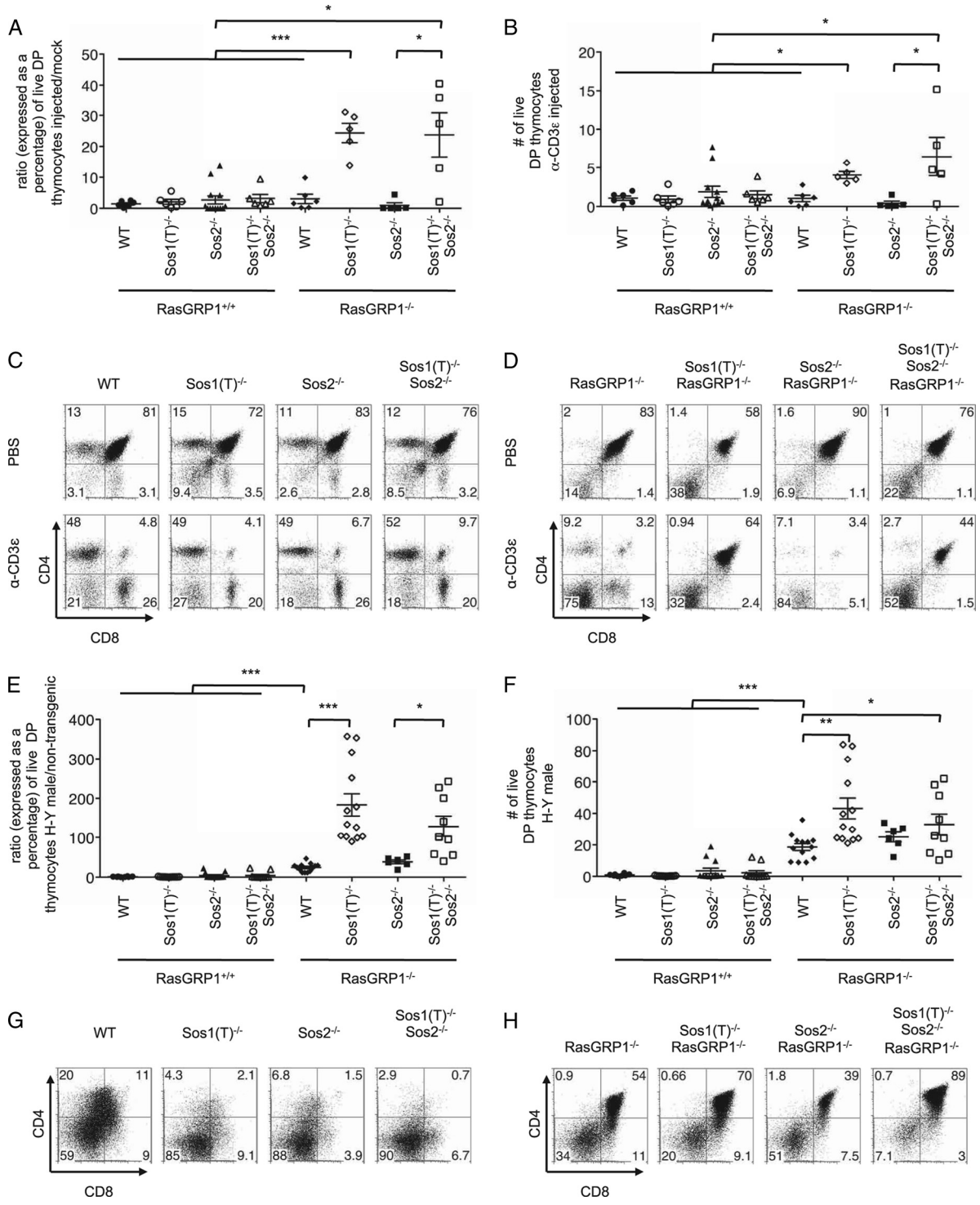

FIG 7 The combined actions of Sos1 and RasGRP1 are required for negative selection at the TCR checkpoint. (A to D) Quantification of the ratio (expressed as a percentage of the control) of surviving DP thymocytes after 2C11 injection/PBS injected controls (A), number of surviving DP thymocytes (B), and flow cytometry dot plots of total thymocytes stained with anti-CD4 and anti-CD8 from 8-week-old RasGRP1$^{+/+}$ (C) or RasGRP1$^{-/-}$ (D) mice 2 days after a single i.p. injection of 50 μg of anti-CD3ε (145-2C11). $n \geq 5$ for each time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (E to H) Quantification of the ratio (expressed as a percentage of control) of DP HY^{+} thymocytes/non-TCR-transgenic controls (E), number of DP HY^{+} thymocytes (F), and flow cytometry dot plots of total thymocytes stained with anti-CD4 and anti-CD8 from 8-week-old RasGRP1$^{+/+}$ (G) or RasGRP1$^{-/-}$ (H) HY TCR-transgenic male mice. $n \geq 6$ for each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

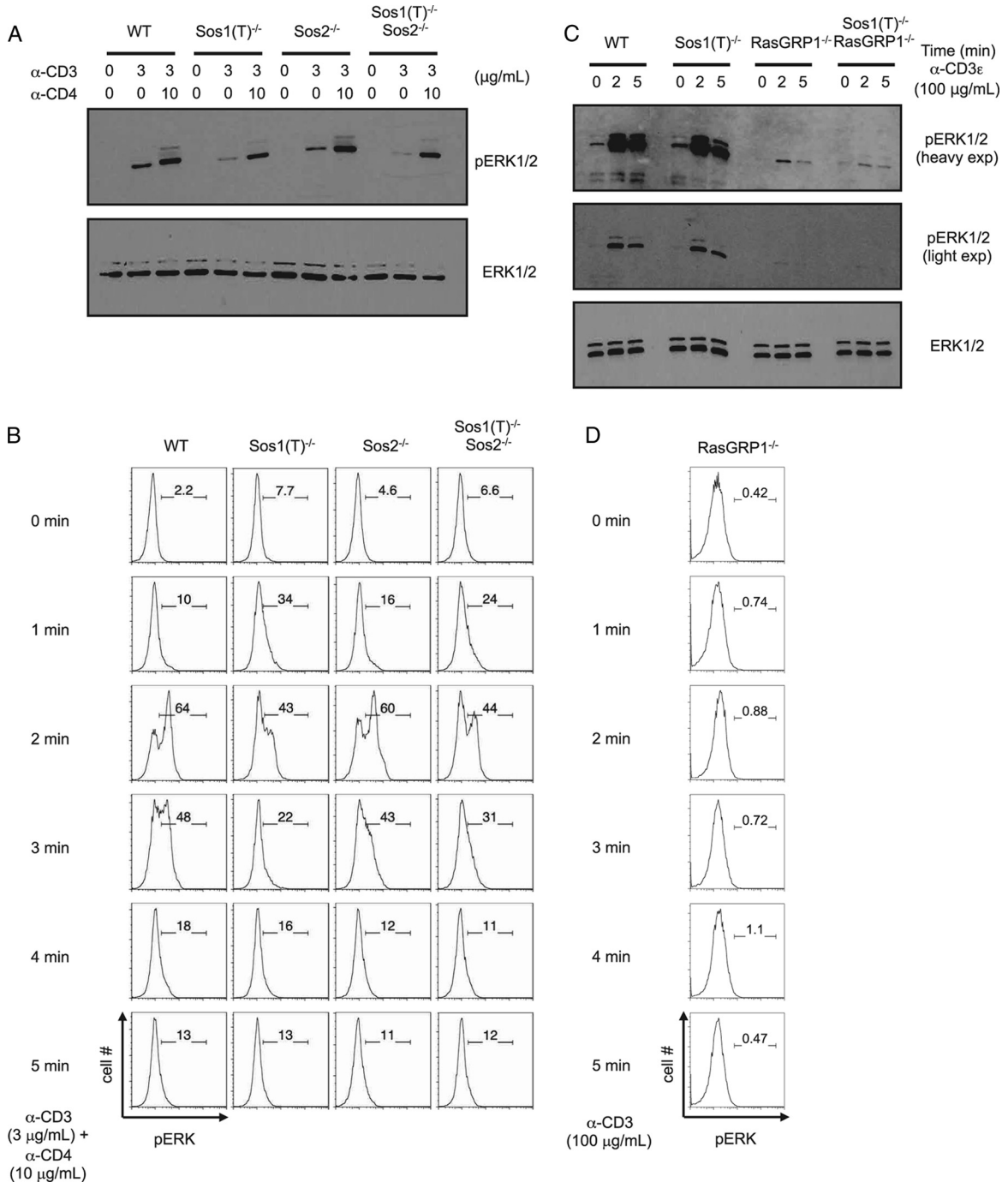

FIG 8 Sos1 and Sos2 have minor effects, whereas RasGRP1 is the major determinant, on ERK activation in DP thymocytes. (A) Western blotting for phospho-ERK and total ERK in purified DP thymocytes from mice stimulated with anti-CD3ε (3 μg/ml) with or without anti-CD4 (10 μg/ml) antibodies for 2 min. Representative data from one of two independent experiments are shown. (B) Histograms of phospho-ERK staining in gated DP thymocytes from 8-week-old mice following anti-CD3ε (3 μg/ml) plus anti-CD4 (10 μg/ml) stimulation for the indicated times. Representative data from one of three independent experiments are shown. (C) Western blotting for phospho-ERK and total ERK in purified DP thymocytes from mice stimulated with anti-CD3ε (100 mg/ml) for 2 or 5 min. Representative data from one of three independent experiments are shown. (D) Histograms of phospho-ERK staining in gated DP thymocytes from 8-week-old mice following anti-CD3ε (100 μg/ml) stimulation for the indicated times. Representative data from one of two independent experiments are shown.

thymocytes and this ratio forms the basis of examination of negative selection in the absence of Sos1, Sos2, and RasGRP1. Intriguingly, while DP thymocytes were efficiently deleted in either RasGRP1$^{-/-}$ or Sos1/2 DKO mice following anti-CD3ε injection *in vivo* (Fig. 7A to D), there was a selective survival of DP thymocytes in both Sos1/RasGRP1 DKO and Sos1/Sos2/RasGRP1 TKO mice, as assessed by both an increase in the ratio of live DP thymocytes after anti-CD3ε injection compared to PBS injected con-

trols (Fig. 7A) and the number (Fig. 7B) and percentage (Fig. 7C and D) of DP thymocytes remaining in the thymus after anti-CD3ε injection. These data suggest a built-in redundancy between RasGEFs for negative selection, since signaling through either Sos or RasGRP1 alone was sufficient to promote negative selection, but in the absence of both Sos1 and RasGRP1 negative selection was blocked.

To further assess the role of the RasGEFs Sos1, Sos2, and RasGRP1 in negative selection, thymocyte development in HY^+ male mice was analyzed (Fig. 7E to H). Here, a more graded effect of RasGRP1 and Sos during negative selection was observed. Deletion of Sos1 and/or Sos2 did not alter negative selection in HY^+ male mice (Fig. 7E to G). However, while HY^+RasGRP1$^{-/-}$ male mice still had small thymi compared to HY^-RasGRP1$^{-/-}$ controls (Fig. 7E), there was a moderate selective survival in both the number (Fig. 7F) and percentage (54%, Fig. 7H) of DP thymocytes, indicating a partial block in negative selection. This effect was markedly enhanced by deletion of Sos1 on a RasGRP1$^{-/-}$ background, such that both the percentage (70%, Fig. 7H) and the overall number (Fig. 7F) of DP thymocytes seen in HY^+Sos1/RasGRP1 DKO and HY^+Sos1/Sos2/RasGRP1 TKO male mice were equal to or higher than their nontransgenic controls (see ratio, Fig. 7E). These data indicate that Sos1 and RasGRP1 cooperate in a second, classical model of negative selection.

DISCUSSION

Our data define how Ras activation by the RasGEFs Sos1, Sos2, and RasGRP1 controls thymocyte development. In thymocytes, there is a reciprocal relationship between the levels of the RasGEFs Sos1 and RasGRP1 at the pre-TCR and TCR checkpoints. The relative expression of these two RasGEFs, as shown previously, defines their relative importance at each checkpoint (12). At the pre-TCR checkpoint, RasGRP1 plays a previously undetected role in supporting thymocyte development beyond the DN3 stage that is only appreciated in the absence of Sos1. At the TCR checkpoint, RasGRP1 is both necessary and sufficient for positive selection (5). However, while Sos1 deletion alone does not alter the threshold of signaling sufficiently to affect negative selection, RasGRP1 and Sos1 both play a previously undetected role in negative selection, which is only appreciated by the combined deletion of both RasGEFs. These data reveal a failsafe mechanism, via functional redundancy of Sos1 and RasGRP1 during negative selection, which ensures appropriate central tolerance at the TCR checkpoint. Sos2 deletion had no effect at either developmental checkpoint and was not further considered.

Genetic studies are not fully compatible with current models of RasGEF action during thymocyte development. Recently, two models have been proposed describing the determinants of Ras activation during thymocyte development. The first model (18), which addresses only the TCR checkpoint, predicts how Ras is activated to control positive and negative selection at the TCR checkpoint. In a peptide-titration system using OT-I thymocytes, RasGRP1 (but not Sos) colocalized with active Ras following stimulation with peptide ligands that trigger positive selection, whereas both RasGRP1 and Sos colocalized with active Ras following stimulation with negatively selecting peptides (3). Furthermore, Sos (but not RasGRP1) contains an allosteric Ras-GTP binding pocket that, when engaged, increases its catalytic activity 80-fold (15), allowing for the engagement of a RasGRP1-Ras-Sos-Ras positive-feedback loop (4, 21). Merging these ideas led the authors to hypothesize that low-potency ligands engage the Ras/ERK pathway in an analog manner through RasGRP1 alone to trigger positive selection. However, higher-potency ligands use both RasGRP1 and Sos to engage Ras, with Sos signaling causing a sharp, digital spike in Ras/ERK activation, which is required to define the threshold between positively and negatively selecting ligands (18).

Although the present study confirms two of the basic tenets of this model, i.e., RasGRP1 is required for positive selection and both Sos and RasGRP1 play a role in negative selection, many of the hypotheses regarding the role of Sos within this model were not confirmed. We found not only that Sos1/2 deletion does not alter the threshold at which negative selection occurs (Fig. 6C) but Sos1/2 deletion does not affect negative selection using four distinct experimental approaches (Fig. 6 and 7). Furthermore, we found that signaling through RasGRP1 alone to ERK, while decreased by 30%, remains digital in Sos1/2 DKO thymocytes (Fig. 8B). Based upon these data, this first model requires modification to describe the role of RasGEFs during positive and negative selection.

Combined knockout studies reveal complexity within the system that cannot be appreciated by single knockout or transgenic studies. We have recently proposed an alternative model that describes RasGEF/Ras signaling during thymocyte development (12). Single-knockout studies showed that Sos1 (and not RasGRP1) was required at the pre-TCR checkpoint, whereas RasGRP1 (and not Sos1) was required for TCR-driven positive selection (5, 12). Furthermore, there is a marked downregulation of Sos1 and upregulation of RasGRP1 expression between DN3 and DP thymocytes (12). These data led us to hypothesize that the level of RasGEF expression was the major determinant of Ras activation during thymocyte development. Furthermore, since negative selection occurred normally in both Sos1(T)$^{-/-}$ mice (12) and RasGRP1$^{-/-}$ mice (20), we further hypothesized that perhaps negative selection was independent of Ras activation, as has previously been postulated (1, 24).

While the present study confirms the basic tenet that RasGEF expression level is the major determinant of Ras activation during thymocyte development, several hypotheses based upon this model were not confirmed. First, RasGRP1 did indeed play a previously unappreciated role in pre-TCR-driven development, which was best revealed on a Sos1(T)$^{-/-}$ background (Fig. 2). RasGRP1 deletion placed tremendous selective pressure on cells to delay Lck-Cre mediated Sos1 deletion [Sos1(T)$^{-/-}$] and maintain Sos1 expression through the pre-TCR checkpoint to the DP stage (Fig. 3). Second, negative selection requires signaling via either Sos1 or RasGRP1. If one assumes that much more Ras activity is required during negative (and not positive) selection, one might hypothesize that deletion of either Sos1 or RasGRP1 would affect negative selection and leave positive selection intact. However, positive selection, and not negative selection, was defective in RasGRP1$^{-/-}$ mice. This preference for using RasGRP1 (and not Sos1) for Ras activation by low potency ligands may be due to the biochemical properties of RasGRP1 compared to Sos1, as previously hypothesized (4, 18, 21). Here, the relatively low levels of Ras-GTP generated may be insufficient to activate Sos1. However, during negative selection high-potency ligands may generate sufficiently high upstream signaling through LAT not only to induce high levels of Ras activation by RasGRP1 in the absence of Sos1 but also to sufficiently engage Sos1 and induce a positive-feedback

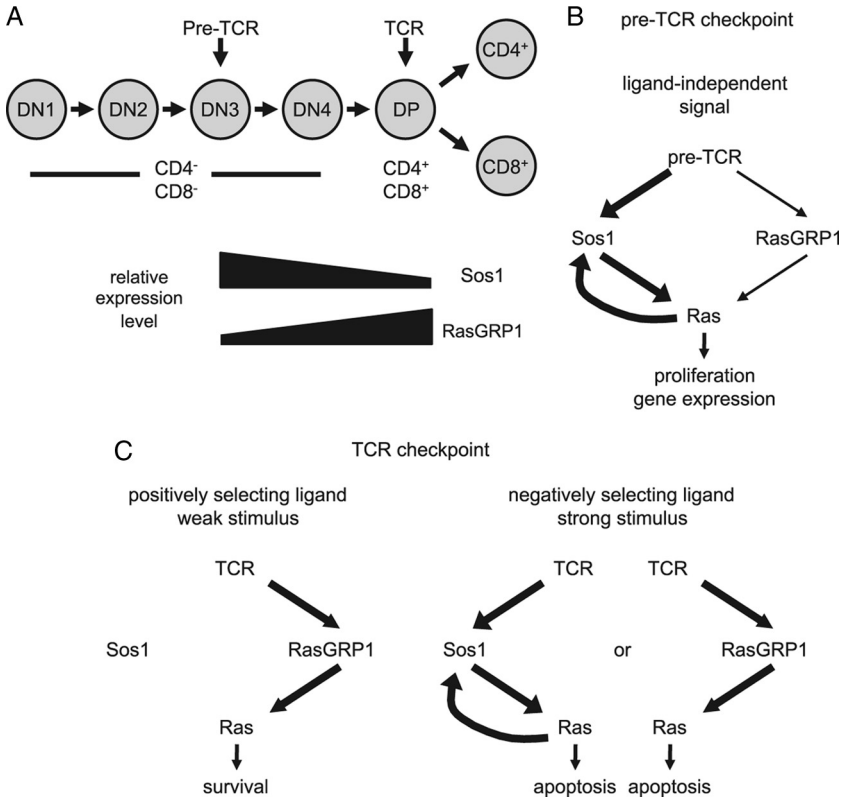

FIG 9 Model for Ras activation during thymocyte development. (A) Schematic representation of thymocyte development. The developmental checkpoints requiring signaling through the pre-TCR and TCR are shown above the respective developmental stages where these checkpoints occur. A graphical representation of the relative expression of Sos1 and RasGRP1 during thymocyte development based upon earlier data (12) are shown below. (B) At the pre-TCR checkpoint, ligand-independent developmental signaling through Ras occurs primarily via Sos1 (darker line), although RasGRP1 does make a significant contribution, as evidenced by the enhanced DN3 block observed in Sos1/RasGRP1 DKO mice. (C) At the TCR checkpoint, low-potency ligands signal entirely via RasGRP1 to induce positive selection. Conversely, high-potency ligands signal to Ras using both Sos1 and RasGRP1, with each RasGEF capable of independently inducing negative selection.

loop capable of generating high levels of Ras activation in the absence of RasGRP1 (15).

Synthesizing a new model describing RasGEF signaling during thymocyte development. Our new data require the rewriting of existing hypotheses into a new, more complete model describing the roles of Sos1 and RasGRP1 during thymocyte development. Although there is functional redundancy between Sos1 and RasGRP1 downstream of both the pre-TCR and TCR during thymocyte development, their expression profiles (Fig. 9A) dictate their relative importance at the two receptor-dependent intrathymic checkpoints. Ras is preferentially activated via Sos1 for ligand-independent pre-TCR signaling, although RasGRP1 functions as a back-up to ensure cells can transit the β-selection checkpoint (Fig. 9B). Conversely, Ras is primarily activated via RasGRP1 for ligand-dependent TCR signaling at low potencies, although Sos1 functions as a back-up as ligand potency increases to ensure appropriate negative selection (Fig. 9C).

Understanding how the Ras/ERK pathway is regulated developmentally is a central biologic question. Here, by defining the requirements for Ras activation by RasGEFs during thymocyte development, we describe how a fundamental signaling pathway can be temporally regulated by both ubiquitous (Sos1) and tissue-specific (RasGRP1) enzymes to control a complex developmental program. While the levels of RasGEF expression are the dominant factor in determining developmental signaling through Ras in the thymus, the simultaneous expression of multiple RasGEFs with unique signaling properties has the potential to both temporally and spatially integrate multiple signaling inputs for fine control of Ras signaling. The genetic models described here will allow future experiments aimed to understand how RasGEFs could potentially act to integrate the multiple inputs required for peripheral immune cell function. Furthermore, these and other mouse models will allow one to assess the therapeutic potential of targeting these RasGEFs to shape Ras signaling to both alter normal physiologic responses and target pathological conditions.

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