

Impaired CD28-mediated Interleukin 2 Production and Proliferation in Stress Kinase SAPK/ERK1 Kinase (SEK1)/Mitogen-activated Protein Kinase Kinase 4 (MKK4)-deficient T Lymphocytes

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Summary

The dual specific kinase SAPK/ERK1 kinase (SEK1; mitogen-activated protein kinase kinase 4/Jun NH₂ terminal kinase [JNK] kinase) is a direct activator of stress-activated protein kinases ([SAPKs]/JNKs) in response to CD28 costimulation, CD40 signaling, or activation of the germinal center kinase. Here we show that SEK1^{-/-} recombination-activating gene (RAG)2^{-/-} chimeric mice have a partial block in B cell maturation. However, peripheral B cells displayed normal responses to IL-4, IgM, and CD40 cross-linking. SEK1^{-/-} peripheral T cells showed decreased proliferation and IL-2 production after CD28 costimulation and PMA/Ca²⁺ ionophore activation. Although CD28 expression was absolutely crucial to generate vesicular stomatitis virus (VSV)-specific germinal centers, SEK1^{-/-}RAG2^{-/-} chimeras mounted a protective antiviral B cell response, exhibited normal IgG class switching, and made germinal centers in response to VSV. Interestingly, PMA/Ca²⁺ ionophore stimulation, which mimics TCR-CD3 and CD28-mediated signal transduction, induced SAPK/JNK activation in peripheral T cells, but not in thymocytes, from SEK1^{-/-} mice. These results show that signaling pathways for SAPK activation are developmentally regulated in T cells. Although SEK1^{-/-} thymocytes failed to induce SAPK/JNK in response to PMA/Ca²⁺ ionophore, SEK1^{-/-}RAG2^{-/-} thymocytes proliferated and made IL-2 after PMA/Ca²⁺ ionophore and CD3/CD28 stimulation, albeit at significantly lower levels compared to SEK1^{+/+}RAG2^{-/-} thymocytes, implying that CD28 costimulation and PMA/Ca²⁺ ionophore-triggered signaling pathways exist that can mediate proliferation and IL-2 production independently of SAPK activation. Our data provide the first genetic evidence that SEK1 is an important effector molecule that relays CD28 signaling to IL-2 production and T cell proliferation.

Distinct and evolutionarily conserved signal transduction cascades mediate survival or death in response to developmental and environmental cues. Multiple stimuli for differentiation and cell growth activate the mitogen-

activated protein kinases (MAPKs)¹, also known as the extracellular signal-regulated kinases ERK1 and ERK2 (1–4),

¹Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; ES, embryonic stem; GCK, germinal center kinase; ICAM-1, intercellular adhesion molecule 1; JNK, Jun NH₂ terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK4, MAPK kinase; RAG, recombination-activating gene; SAPK, stress-activated protein kinase; SEK, SAPK/ERK kinase; slg, surface Ig; VSV, vesicular stomatitis.

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which translocate to the nucleus and regulate the activity of transcription factors (5). MAPKs are activated by the phosphorylation of a threonine and a tyrosine residue mediated by the dual specificity MAPK kinases MAPK/ERK kinase (MEK)1 and MEK2, which relay Ras and Raf signal transduction to MAPK activation (6–8).

A second signaling cascade exists in all cells that leads to the activation of stress-activated protein kinases (SAPKs) or Jun NH₂ terminal kinase (JNKs; 9,10). The SAPK signaling cascade is parallel and independent from MAPK activation (11, 12). SAPKs/JNKs are activated in response to a variety of cellular stresses such as changes in osmolarity and metabolism, DNA damage, heat shock, ischemia, inflammatory cytokines, or ceramide (13–18). Activated SAPKs/JNKs phosphorylate c-Jun, which leads to activation of the transcriptional complex AP-1 (19). SAPKs/JNKs are activated by the phosphorylation of tyrosine and threonine residues, which is catalyzed by the dual specificity kinase SAPK/ERK kinase (SEK)1 (also known as MAPK kinase [MKK4] and JNK kinase; 20–22). In addition to SEK1, a novel SAPK activator (SEK2 or MKK7) has been genetically identified but has not been cloned yet (23).

It has been proposed from transfection studies with dominant negative signaling mutants that the SEK1→SAPK/JNK→c-Jun signaling cascade is a common intracellular pathway required for the induction of apoptosis in response to many types of cellular stresses (16–18, 24–28). However, recent genetic evidence suggests that SEK1 and SEK1-mediated SAPK activation have no role in the induction of cell death in lymphocytes, but rather protect T cells from CD95 (FAS) and CD3-mediated apoptosis (23). The SAPK/JNK signaling cascade is also triggered by certain growth stimulating factors and phorbol esters (9, 14, 29, 30). In B cells, SEK1 and SAPK are activated in response to CD40 cross-linking (31, 32) and by the human STE20 homologue germinal center kinase (GCK) (33). The prominent expression of GCK in germinal centers (34) suggested that the GCK/SAPK pathway might be important for B cell differentiation or activation. Moreover, biochemical studies in T cells indicated that SAPKs/JNKs are involved in the integration of TCR–CD3 and CD28 costimulatory signals required for proliferation and IL-2 production (29, 35). Failure to activate SAPKs/JNKs in T cells may result in clonal anergy (36, 37).

To determine the role of SEK1 in B cell function and CD28-mediated costimulation, we reconstructed T (23) and B cell development in *SEK1* gene-deficient chimeras using recombination-activating gene (RAG)2 blastocyst complementation. We show that SEK1 is important for CD28-mediated costimulation for T cell proliferation and IL-2 production. B lymphocyte development was partially impaired. However, peripheral B cells displayed normal responses to IL-4 and to IgM and CD40 cross-linking, and exhibited normal IgG class switching after vesicular stomatitis virus (VSV) infections. Moreover, we show that CD28, but not SEK1, is crucial for VSV-specific germinal center formation. Interestingly, using the same activation regimen, i.e., PMA plus Ca²⁺ ionophore which mimics TCR–CD3- and CD28-mediated signal transduction (29),

SAPK activation was observed in peripheral T cells, but not in thymocytes, from *SEK1*^{-/-} mice. These data provide the first genetic evidence that SEK1-regulated stress signal transduction has a role in CD28 costimulation for IL-2 production and proliferation. These results also show that signaling pathways for SAPK activation are developmentally regulated in T cells.

Materials and Methods

Mice. The generation of embryonic stem (ES) cells homozygous for the SEK1 mutation, *SEK1*^{-/-} somatic chimeras using *RAG2*^{-/-} blastocyst complementation (23, 38), and *CD28*^{-/-} mice (39) have been previously described. Since E14 ES cells are derived from a 129/J mouse background, age-matched 129/J mice were used as wild-type controls. T and B cells from *SEK1*^{-/-} *RAG2*^{-/-} mice were tested for the SEK1 mutation using PCR (sense primer: 5'-ACAGCAAATTTTGGAAACAGC-3'; antisense primer: 5'-CTCCCCTACCCGGTAGAATTC-3'). All data presented throughout this study were obtained from two independently derived *SEK1*^{-/-} ES cell clones (No. 1–6 and No. 1–21), and all results were comparable between them. If not otherwise stated, all mice used for experiments were between 6 and 10 wk old. Mice were kept under pathogen-free conditions in accordance with guidelines of the Canadian Medical Research Council.

Immunocytometry. Single cell suspensions from thymocytes, spleen cells, mesenteric lymph node cells, and bone marrow cells from *SEK1*^{-/-} *RAG2*^{-/-} chimeric, *SEK1*^{+/+} *RAG2*^{-/-} chimeric, *RAG2*^{-/-}, and 129/J mice were prepared as described (40), re-suspended in immunofluorescence-staining buffer (PBS, 4% FCS, 0.1% NaN₃) and incubated with appropriate Abs. The following mAbs were used: anti-CD4 (FITC-, or PE-labeled), anti-CD8 (FITC-labeled, PE-labeled, or biotinylated), anti-TCRα/β (FITC-, or PE-labeled), anti-CD3-ε (FITC-labeled), anti-B220 (FITC-labeled, PE-labeled, or biotinylated), anti-CD43 (FITC-labeled), anti-CD25/IL-2R-α (biotinylated), anti-H2K^b (FITC-labeled), anti-CD44 (PE-labeled), anti-FAS (PE-labeled, or biotinylated), anti-intercellular cell adhesion molecule 1 (ICAM-1; biotinylated); anti-CD23 (PE-labeled), anti-CD28 (PE-labeled), anti-CTLA-4 (PE-labeled), anti-CD69 (FITC-labeled), anti-CD40L (gp39; biotinylated) (all above Abs were from PharMingen, San Diego, CA); anti-surface (s)IgM (clone B67; FITC-labeled, gift of C. Paige, Ontario Cancer Institute, Toronto, Canada), anti-sIgD (PE-labeled; gift of C. Paige), and anti-CD40 (FITC-labeled; Serotec, Toronto, Canada). All staining combinations were as indicated in the figure and table legends. Biotinylated Abs were visualized using Streptavidin-RED670 (Life Technologies, Burlington, Canada). Samples were analyzed using a FACScan® (Becton Dickinson, Mountain View, CA).

Cell Sorting. Bone marrow cells were isolated from *RAG2*^{-/-}, *SEK1*^{-/-} *RAG2*^{-/-} chimeric, *SEK1*^{+/+} *RAG2*^{-/-} chimeric, and 129/J control mice and double stained for CD43 and B220 expression using anti-CD43-FITC and anti-B220-PE. CD43⁺B220⁺ and B220⁺CD43⁻ bone marrow B cell populations (Fig. 2) were sorted using a FACS® power sorter (FACS® Vantage). In all experiments, postsorting purity of CD43⁺B220⁺ and B220⁺CD43⁻ populations was >98%. Sorted cells were analyzed for the SEK1 mutation using PCR (see above).

B and T Cell Stimulation Assays. Lymph node T cells were negatively enriched from lymph nodes of *SEK1*^{-/-} *RAG2*^{-/-} chimeric and *SEK1*^{+/+} *RAG2*^{-/-} chimeric mice using affinity columns (R&D Sys. Inc., Minneapolis, MN) to avoid receptor

cross-linking during the purification process. Purified (>95%) T cells (10^4) and freshly isolated thymocytes were placed into round-bottom 96-well plates (Costar, Fisher Scientific, Unionville, Canada) in freshly prepared IMDM (10% FCS, 10^{-5} M β mercaptoethanol) and activated with PMA (12.5 ng/ml) plus Ca^{2+} ionophore A23617 (100 ng/ml), plate-bound anti-CD3- ϵ (clone 145-2C11, hamster IgG; PharMingen), soluble anti-CD3- ϵ (clone 145-2C11), and soluble anti-CD28 (clone 37.51, hamster IgG; gift of Dr. J. Allison, University of California, Berkeley, CA). PMA/ Ca^{2+} ionophore and mAbs were added at optimal concentrations determined in pilot studies. For CD3 cross-linking, plates were coated overnight (4°C) with 10 μg /well of rabbit anti-hamster IgG (Jackson Labs., West Grove, PA), and subsequently with anti-CD3- ϵ (37°C for 2 h, clone 145-2C11).

B cells were purified from $\text{SEK1}^{-/-}\text{RAG2}^{-/-}$ chimeric and $\text{SEK1}^{+/+}\text{RAG2}^{-/-}$ mice as described (41). In brief, erythrocyte-free spleen cells were treated with anti-Thy1.2, anti-CD4, and anti-CD8 followed by the addition of guinea pig complement (Cedarlane Hornby, Canada). The remaining cells were added to a Percoll gradient ($2.5 \times 10^6/10$ ml gradient). Recovered cells represented 10–30% of the cells placed on the gradient. FACS[®] analysis revealed that these cells were >90% sIg^+ . Cells were placed into a round-bottom 96-well plate (Costar, Fisher Scientific) in IMDM. B cells were then activated using soluble anti-Ig μ (mAb clone B76), recombinant murine IL-4 (Genzyme, Cambridge, MA), soluble anti-CD40 (Serotec), and LPS (Sigma Chemical Co., St. Louis, MO). Optimal conditions were determined in preliminary titration experiments. B and T cells were harvested at 1–4 d after a 12-h pulse with 1 μCi [³H]thymidine/well. T cell culture supernatants were assayed in triplicate for IL-2 by ELISA (Genzyme).

CD40 Cross-linking. For CD40-mediated upregulation of ICAM-1 and CD23 (42), purified B cells were activated with anti-CD40 (2 μg /ml; Serotec) in the absence or presence of IL-4 (50 U/ml) in IMDM (10% FCS, 37°C). After 24 h of activation, cells were harvested and triple stained with Abs reactive against B220 (PE), sIgM (FITC), and ICAM-1 (biotin) or CD23 (biotin). Biotinylated Abs were visualized using Streptavidin-RED670 and staining of cells was analyzed using a FACScan[®].

Detection of Ig-subclasses. Sera were collected from 6-wk-old individual $\text{SEK1}^{-/-}\text{RAG2}^{-/-}$ and $\text{SEK1}^{+/+}\text{RAG2}^{-/-}$ chimeric mice. The concentrations of Ig subclasses were determined by ELISA with isotype-specific, alkaline phosphatase-conjugated Abs (Southern Biotechnology Assoc. Birmingham, AL). Serum Ig concentrations were determined by fivefold serial dilutions and calculated according to standard charts as described previously (39).

VSV Infections and Detection of VSV-neutralizing Abs. Mice were immunized with VSV-Indiana (2×10^6 PFU, intravenously). After 4, 8, and 12 d, sera were collected and neutralizing IgM and IgG Ab titers determined as described (43). In brief, 1:2 dilutions of 40-fold prediluted serum were incubated with VSV for 90 min. The presence of remaining infectious virus was determined by incubating the VSV serum samples with fibroblasts for another 24 h. Serum dilutions that reduced the number of viral plaques by 50% were taken as specific titers. IgG titers were determined after preincubation of sera with 2 β mercaptoethanol, a procedure that eliminates IgM (43).

Germinal Center Formation and Immunohistochemistry. To determine formation of germinal centers, 6 wk old $\text{SEK1}^{-/-}\text{RAG2}^{-/-}$ and $\text{SEK1}^{+/+}\text{RAG2}^{-/-}$ chimeric mice and CD28^{-/-} mice were infected with VSV-Indiana as described above. Spleens from VSV-infected animals were harvested 12 d after the initial infection, frozen in liquid nitrogen, and processed for cryosections.

Cryostat sections (5 μm) were fixed in acetone (10 min). Sections were incubated with PNA (diluted 1:200) and bound PNA was detected by rabbit anti-PNA Abs (diluted 1:300; DAKO, Glostrup, Denmark). CD4 was detected by the rat mAb YTS191. Binding of primary Abs was detected by alkaline phosphatase-labeled goat Abs to rabbit or rat Ig (1:80 dilution; Jackson Labs.) followed by alkaline phosphatase-labeled donkey Abs against goat Ig (1:80 dilution; DAKO). Alkaline phosphatase was visualized using Naphthol AS-BI phosphatase and New Fuchsin as a substrate, which yields a red precipitate. Endogenous alkaline phosphatase activity was blocked by levamisole (44).

VSV-specific B cells were detected as described (44). In brief, dehydrated tissue sections were overlaid with a solution of UV-inactivated VSV (3×10^6 PFU/ml) for 4 h. Specifically bound virus was detected by incubation with polyclonal rabbit anti-VSV Indiana serum (diluted 1:1,500), followed by alkaline phosphatase-labeled goat Abs to rabbit Ig and rabbit anti-goat Ig (diluted 1:80; Jackson Labs.). Naphthol AS-BI phosphatase and New Fuchsin were used to develop the color reaction (44).

SAPK/JNK Activities. Thymocytes and purified lymph node T cells (5×10^6) were activated with PMA (50 ng/ml) and the Ca^{2+} ionophore A23617 (1 μg /ml) as previously described (23, 29). Cells were lysed in ice-cold lysis buffer (10 mM NaCl, 20 mM Pipes, pH 7.0, 0.5% NP-40, 5 mM EDTA, 0.05 β mercaptoethanol, 100 μM Na_3VO_4 , 50 mM NaF, 20 μg /ml leupeptin, and 1 mM benzamide). Cleared lysates were adjusted to equal protein concentrations (BioRad Protein Assay; Bio Rad Labs., Hercules, CA). SAPKs/JNKs were immunoprecipitated (1 h, 4°C) using polyclonal rabbit anti-SAPK/JNK Abs reactive against all SAPK/JNK isoforms (10). Immune complexes were harvested on protein A-Sepharose beads. For kinase assays, immune complexes were washed three times with PBS-Triton buffer (150 mM NaCl, Na_2HPO_4 , 4 mM NaH_2PO_4 , 0.1% Triton X-100, 100 mM Na_3VO_4 , 50 mM NaF, 20 μg /ml leupeptin, and 1 mM benzamide). SAPK/JNK kinase assays were performed in 20 μl of kinase buffer (10 mM MgCl_2 , 50 mM Tris-Cl, pH 7.5, 1 mM EGTA, pH 7.5) in the presence of 1.2 μCi [³²P] γ -ATP and 5 μg glutathione-S-transferase-c-Jun as in vitro substrate (30°C, 30 min). The reaction was stopped by the addition of 2 \times SDS sample buffer. Phosphoproteins were separated by SDS-PAGE and visualized by autoradiography as described (10). The levels of expression of SAPK/JNKs in thymocytes and lymph node cells were determined by immunoblotting using goat anti-JNK1 and rabbit anti-JNK2 polyclonal Abs (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA; 23).

Results

Impaired Proliferation and IL-2 Production of $\text{SEK1}^{-/-}$ Peripheral T Cells. Recent biochemical studies implied that the SAPK/JNK signaling pathway is operating in T cells, and that cell proliferation and IL-2 production induced by CD28 costimulation may be mediated via SAPK/JNK (29, 36, 37). $\text{SEK1}^{-/-}\text{RAG2}^{-/-}$ chimeric mice have a smaller thymus, but normal numbers of peripheral T cells (Table 1; reference 23). To test the role of SEK1 in CD28 costimulation, lymph node T cells were cultured in anti-CD3- ϵ Ab-coated plates in the absence or presence of various concentrations of soluble anti-CD28 Abs. Whereas $\text{SEK1}^{-/-}\text{RAG2}^{-/-}$ and $\text{SEK1}^{+/+}\text{RAG2}^{-/-}$ T cells responded in the same way to CD3- ϵ activation alone, CD28-mediated upregulation of

Table 1. T and B Cell Subpopulations in *SEK1*^{-/-} Chimeric Mice

Cell subset	Percentages ± SEM of positive cells per total cells			
	129/J	SEK1 ^{+/+}	SEK1 ^{-/-}	RAG2 ^{-/-}
Thymus				
CD4 ⁺ CD8 ⁺	81.8 ± 2.2	84 ± 0.9	57.7 ± 4.2	0
CD4 ⁺ CD8 ⁻	11.6 ± 0.8	7.7 ± 0.4	27.2 ± 2.8	0
CD4 ⁻ CD8 ⁺	3.2 ± 0.4	2.1 ± 0.7	7.5 ± 1.1	0
CD4 ⁻ CD8 ⁻	3.5 ± 0.6	6.1 ± 1.0	4.8 ± 0.4	100
Lymph node				
CD4 ⁺ CD8 ⁻	48.2 ± 3.2	44.2 ± 4.3	55.1 ± 3.1	0
CD4 ⁻ CD8 ⁺	19.0 ± 2.8	20.2 ± 3.7	22.1 ± 1.8	0
B220 ⁺ sIgM ⁺	30.5 ± 1.6	26.6 ± 3.5	12.0 ± 3.0	0
Spleen				
CD4 ⁺ CD8 ⁻	24.0 ± 5.3	22.6 ± 5.3	33.2 ± 2.8	0
CD4 ⁻ CD8 ⁺	11.3 ± 2.0	8.9 ± 2.7	13.5 ± 1.5	0
B220 ⁺ sIgM ⁺	33.5 ± 5.5	32.6 ± 3.5	16.7 ± 1.6	0
Bone marrow				
B220 ⁺ CD43 ⁺	11.9 ± 1.4	10.6 ± 2.5	13.4 ± 0.4	16.5 ± 3.5
B220 ⁺ CD43 ⁻	30.8 ± 2.5	34.5 ± 4.5	11.6 ± 1.6	<1
B220 ⁺ CD25 ⁺	30.5 ± 2.1	32.0 ± 3.3	10.2 ± 1.5	<1
B220 ⁺ CD25 ⁻	10.5 ± 0.9	15.1 ± 2.7	12.6 ± 1.2	14.6 ± 4.1

Cells from thymi, mesenteric lymph nodes, spleens, and bone marrow (one femur) from 129/J ($n = 6$), SEK1^{+/+} chimeric ($n = 3$), SEK1^{-/-} chimeric ($n = 6$), and RAG2^{-/-} ($n = 5$) mice were stained with the indicated Abs and populations determined using a FACScan®. Total cell numbers ($\times 10^6 \pm$ SEM) were: 129J: thymus, 80 ± 6.3 ; LN, 24 ± 2.2 ; spleen, 21 ± 3.4 ; BM, 7.8 ± 0.7 ; SEK1^{+/+}: thymus, 85 ± 10.1 ; LN, 22 ± 4.6 ; spleen, 32 ± 3.14 ; BM, 8.6 ± 2.5 ; SEK1^{-/-}: thymus, 14 ± 4.1 ; LN, 29 ± 4.2 ; spleen 27 ± 5.3 ; BM, 10.6 ± 0.4 ; RAG2^{-/-}: thymus, 1.1 ± 0.2 ; LN, 0.7 ± 0.2 ; spleen, 8.3 ± 1.6 ; BM 9.2 ± 0.9 (reference 23). Bold numbers indicate, statistically significant differences between 129/J or SEK1^{+/+} and SEK1^{-/-} subpopulations (Student's *t* test $P < 0.05$).

proliferation and IL-2 production were significantly reduced in SEK1^{-/-} T cells (Fig. 1, A and B). Reduced proliferation and IL-2 production were also observed in SEK1^{-/-} T cells after stimulation with PMA/Ca²⁺ ionophore (Fig. 1, A and B), which mimic TCR-CD3- and CD28-mediated signal transduction (29, 45).

Since the proliferative response to plate-bound anti-CD3- ϵ Abs alone was still very vigorous (Fig. 1 A), we analyzed T cell activation in response to suboptimal concentrations of soluble anti-CD3- ϵ Abs. As shown in Fig. 1, C and D, proliferation and IL-2 production of SEK1^{-/-}RAG2^{-/-} and SEK1^{+/+}RAG2^{-/-} chimeric T cells were minimal after stimulation with soluble anti-CD3- ϵ alone. Although the addition of anti-CD28 greatly enhanced the proliferation and IL-2 production of SEK1^{+/+}RAG2^{-/-} T cells, SEK1^{-/-}RAG2^{-/-} T cells were significantly impaired in proliferation and IL-2 production (Fig. 1, C and D). It should be noted that freshly isolated T cells from SEK1^{-/-} mice displayed upregulated expression of CD28, but normal surface expression of the TCR- α/β -CD3- ϵ complex, IL-2R- α chain (CD25), CD69, and adhesion molecules such as ICAM-1 (not shown). These data provide the first genetic evidence that SEK1 plays an important role in T

cell proliferation and IL-2 production in transmitting CD28 signals to downstream effector molecules.

Partial Defect in B Cell Maturation. To determine the effect of the SEK1 mutation on B cell development, single cell suspensions from spleen, lymph nodes, and bone marrow of SEK1^{-/-}RAG2^{-/-} chimeric, SEK1^{+/+}RAG2^{-/-} chimeric, RAG2^{-/-}, and control 129/J mice were stained with mAbs against B lineage-specific markers (Fig. 2, Table 1). The bone marrow of 129/J mice contained a relatively low number (12%) of B220⁺CD43⁺ pro-B cells and a larger population (30%) of B220⁺CD43⁻ pre-B cell precursors, and mature B cells in peripheral lymphatic organs expressed both IgM (Fig. 2) and IgD (not shown) on the cell surface (46). By contrast, B cell differentiation in the bone marrow of RAG2^{-/-} mice was blocked at the pro-B cell stage (B220⁺CD43⁺IgM⁻) and RAG2^{-/-} mice did not have any mature sIgM⁺ B cells (Fig. 2, Table 1; 47, 48). B-cell development and expression of sIgM and sIgD were restored in chimeras derived from injection with parental SEK1^{+/+} ES cells. In contrast, the relative and total numbers of B220⁺CD43⁻ bone marrow cells and B220⁺sIgM⁺ peripheral B cells were significantly reduced in SEK1^{-/-}RAG2^{-/-} chimeric mice (Fig. 2, Table 1). Pe-

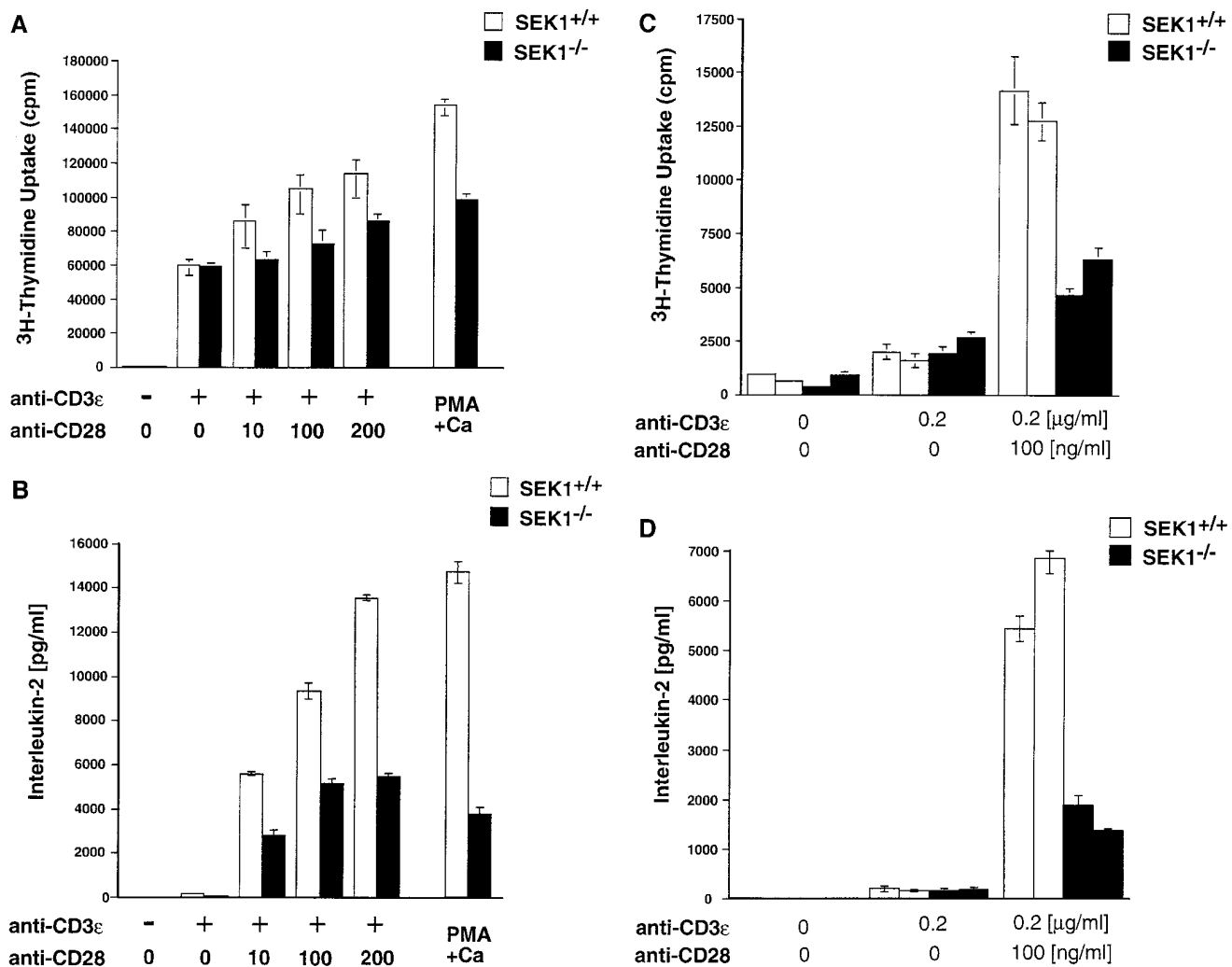


Figure 1. Proliferation (A and C) and IL-2 production (B and D) of SEK1^{-/-} chimeric (shaded bars) and SEK1^{+/+} chimeric (open bars) T cells. Purified lymph node responder T cells (10^5 T cells/well) were activated with (A and B) plate-bound anti-CD3- ϵ (1 μ g/ml) and different concentrations of soluble anti-CD28 Ab (10, 100, and 200 ng/ml) or PMA (12.5 ng/ml) plus Ca²⁺ ionophore (100 ng/ml) (PMA + Ca); and (C and D) soluble anti-CD3- ϵ and soluble anti-CD28 Abs at the indicated concentrations. Rabbit anti-hamster Ig-coated plates without addition of anti-CD3- ϵ (-) or CD28 (0) Abs are shown as controls in (A and B). (C and D) data from two individual SEK1^{-/-} and SEK1^{+/+} chimeric mice are shown. After 24 h of stimulation, proliferation was determined by [³H]thymidine uptake, and IL-2 production was determined by ELISA. Data of triplicate cultures \pm SD are shown. Similar results were obtained after 48 and 72 h of culture (not shown). One result representative of seven independent experiments is shown.

ripheral B cells from SEK1^{-/-} mice expressed normal levels of CD23, CD40, CD44, ICAM-1, CD95 (FAS), and H2K^b on the cell surface (not shown). The partial block in the development from B220⁺CD43⁺ pro-B cells to more mature B220⁺CD43⁻ pre-B cells was also observed by alterations in IL-2R- α chain (CD25) expression, an early B cell maturation marker that is expressed before sIgM expression (46). Although \sim 75% of 129/J or SEK1^{+/+} chimeric B220⁺ bone marrow B cells expressed CD25 on the cell surface, expression of CD25 was significantly reduced in SEK1^{-/-} bone marrow B cells (Table 1).

To analyze whether the observed block in B cell maturation was due to the SEK1 mutation and not due to low chimerism and contribution of RAG2^{-/-} cells to pro-B cells, we FACS[®] sorted B220⁺CD43⁺ and B220⁺CD43⁻ bone

marrow cells and analyzed the genotype of sorted cells by PCR (Fig. 3). Both B220⁺CD43⁺ pro-B cells and the more mature B220⁺CD43⁻ B cell populations in SEK1^{-/-} chimeras contained mutant, but not wild-type SEK1 alleles indicating that both populations were derived from SEK1^{-/-} ES cells. These data imply that SEK1-mediated signaling plays a role at the transition from B220⁺CD25⁻CD43⁺ pro-B cells to B220⁺CD25⁺CD43⁻ pre-B cells in the bone marrow.

B Cell Activation. Previously it has been shown that CD40 signaling in B cells leads to the induction of SAPK/JNK activity (31, 32). To determine the requirement of SEK1 for B cell activation, we measured proliferation of B cells in response to various stimuli. SEK1^{-/-}RAG2^{-/-} B cells responded normally to LPS, IL-4, anti-CD40, IL-4 plus anti-CD40, and

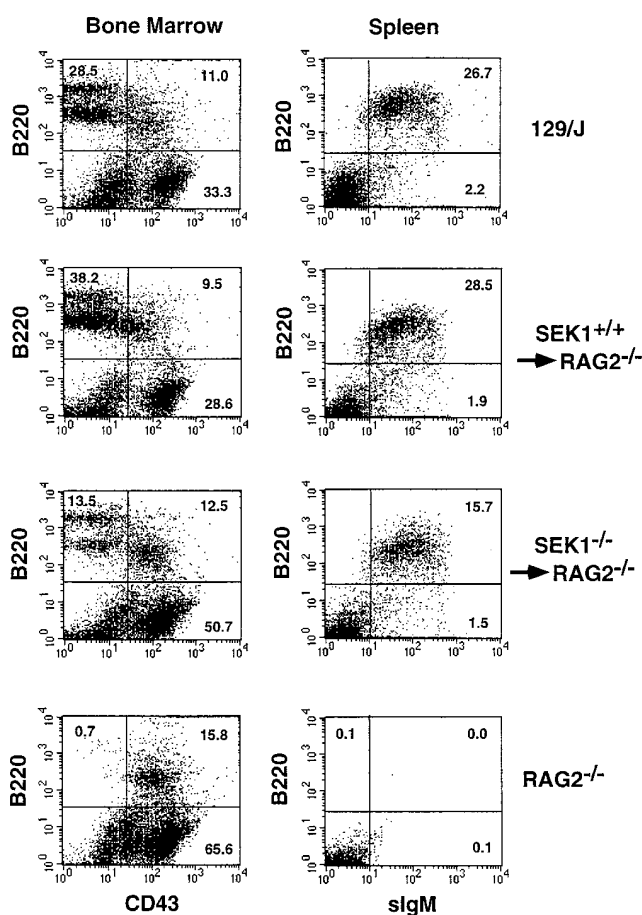


Figure 2. Immunocytometric analysis of B cell populations in the bone marrow (*left*) and spleen (*right*) of 129/J, SEK1^{+/+} chimeric, SEK1^{-/-} chimeric, and RAG2^{-/-} mice. Cells were isolated from 6-wk-old mice and double stained using anti-B220 (PE) and anti-CD43 (FITC), or anti-B220 (PE) and anti-sIgM (FITC). Percentages of positive cells within a quadrant are indicated. Note the partial block in the maturation from CD43⁺B220⁺ pro-B cells to CD43⁻B220⁺ pre-B cells in the bone marrow and the reduced number of sIgM⁺ B cells in the spleen of SEK1^{-/-} chimeric mice (see also Table 1). 10,000 viable cells were collected and analyzed on a FACScan[®]. Total cell numbers were: 129/J bone marrow (total lymphoid cells isolated from one femur), 7.9×10^6 ; 129/J spleen, 4.1×10^7 ; SEK1^{+/+} chimeric bone marrow, 8.3×10^6 ; SEK1^{+/+} chimeric spleen, 3.1×10^7 ; SEK1^{-/-} bone marrow, 7.7×10^6 ; SEK1^{-/-} chimeric spleen, 3.9×10^7 ; RAG2^{-/-} bone marrow, 9.8×10^6 ; RAG2^{-/-} spleen, 1.3×10^7 .

Ig μ cross-linking (Fig. 4 A). Moreover, SEK1^{-/-}RAG2^{-/-} B cells upregulated ICAM-1 and CD23 upon activation with anti-CD40 in the absence or presence of IL-4 (not shown; 42). The basal serum levels for the Ig subclasses IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were also comparable between SEK1^{-/-}RAG2^{-/-} and SEK1^{+/+}RAG2^{-/-} chimeric mice (Fig. 4 B).

VSV Infections and IgG Class Switching. VSV infections are exclusively controlled by neutralizing Abs (49). All neutralizing Abs are directed against the VSV glycoprotein which is present in a highly repetitive form in the viral envelope. Due to this repetitiveness, neutralizing IgM Abs are induced in complete absence of T cell help (49). However, the isotype switch from IgM to IgG is strictly T cell dependent

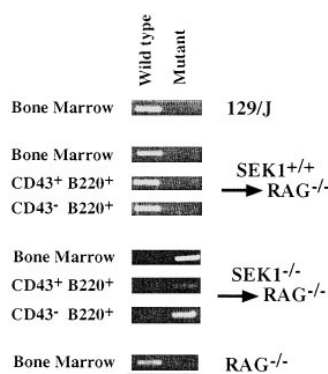


Figure 3. PCR analysis for SEK1 mutant and wild-type alleles in total bone marrow and sorted B220⁺CD43⁺ and B220⁺CD43⁻ bone marrow cells from SEK1^{-/-} and SEK1^{+/+} chimeric mice. Bone marrow cells were double stained with anti-B220 (PE) and anti-CD43 (FITC) and populations were sorted using a FACS[®] power sorter (Coulter). Postsorting purity of CD43⁺B220⁺ and B220⁺CD43⁻ cells was >98%. Purified B cell populations (5×10^4 cells) were subjected to PCR analysis as described in Materials and Methods. Total bone marrow cells (10^5) (*Bone marrow*) from 129/J, SEK1^{-/-} chimeric, SEK1^{+/+} chimeric, and RAG2^{-/-} mice are shown as controls.

dent (50). Recently, it has also been shown that the production of VSV-neutralizing IgG Abs is decreased in CD28^{-/-} mice (39). Since, SEK1^{-/-} T cells had reduced proliferation and IL-2 production in response to CD28 costimulation (Fig. 1), but SEK1^{-/-} B cells could be activated normally and produced normal levels of Ig subclasses (Fig. 4), we examined T cell help and IgG class switching in SEK1^{-/-}RAG2^{-/-} and SEK1^{+/+}RAG2^{-/-} mice after infection with VSV (Table 2). Neutralizing serum IgM was assessed 4 d and neutralizing serum IgG levels were measured 4, 8, and 12 d after VSV infection. VSV infection induced rapid, T cell-independent IgM production, followed by a T helper cell and CD28 costimulation-dependent IgG response (Table 2). Surprisingly, both early IgM production and IgG class switching were comparable between SEK1^{-/-}RAG2^{-/-} and SEK1^{+/+}RAG2^{-/-} mice (Table 2). Moreover, SEK1^{-/-} mice survived for more than 4 wk after infection, indicating that the B cell response was protective.

VSV-specific Germinal Center Formation. The prominent expression of the GCK in follicular germinal centers (34) and activation of SAPK through GCK (33) suggested that the GCK/SAPK pathway might be important for B cell differentiation within germinal centers. Moreover, mice lacking CD28 (51) or CD40 (52, 53) do not develop germinal centers. Since all of these receptors can activate SAPKs/JNKs (10, 29, 31, 32), we tested whether virus-specific germinal center formation was normal in SEK1^{-/-} mice. Although VSV-specific germinal centers were completely absent in CD28^{-/-} mice after challenge with VSV, SEK1^{-/-}RAG2^{-/-} chimeric mice developed germinal centers with normal morphology (Fig. 5) and at normal frequency (Table 3). Germinal center B cells were positive for PNA expression (Fig. 5, A–C). CD4⁺ T cells were mainly present in the T area, but were also observed within germinal centers (Fig. 5, D–F). Moreover, a light zone containing strongly VSV-binding germinal center B cells could be distinguished from a dark zone containing sIg-negative B lymphocytes (Fig. 5, G–I). It should be noted that VSV-specific plasma cells were detectable in the spleens of CD28^{-/-} mice (Fig. 5 J) and that CD28^{-/-} mice could still

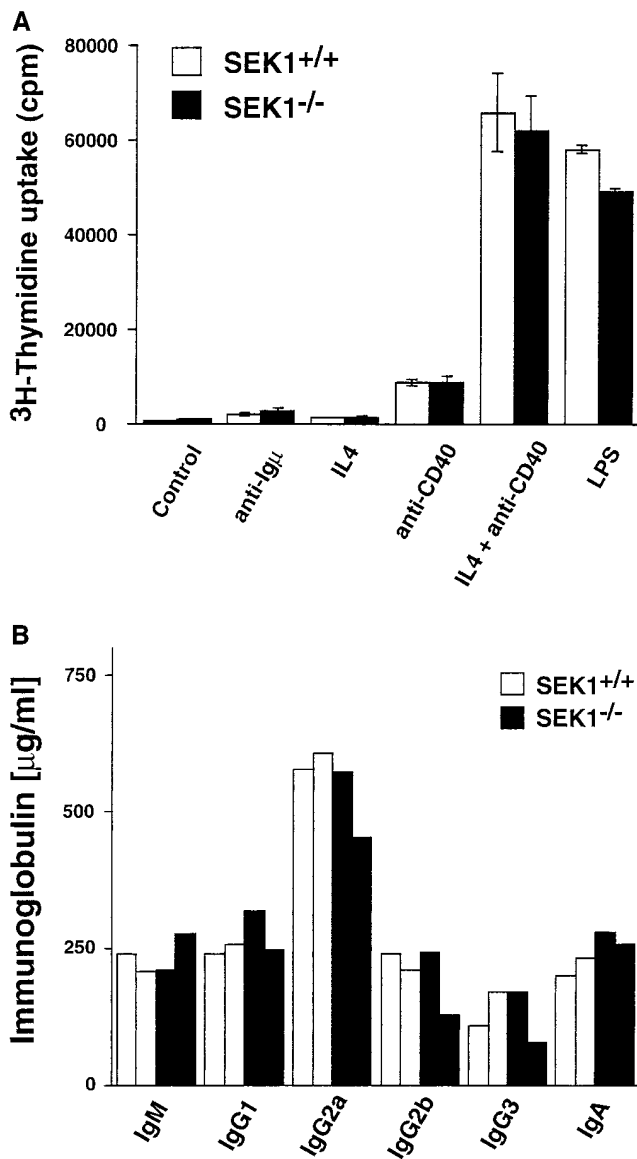


Figure 4. B cell activation and immunoglobulin production in SEK1^{-/-} mice. (A) Activation of splenic B cells. Purified splenic B cells (10⁵/well) from SEK1^{-/-} (shaded bars) and SEK1^{+/+} (open bars) control mice were seeded in medium containing no added stimulus (Control), soluble anti-Igµ Ab (10 µg/ml, clone B76), IL-4 (10 U/ml), soluble anti-CD40 (1 µg/ml), IL-4 (10 U/ml) plus soluble anti-CD40 (1 µg/ml), and 10 µg/ml LPS (LPS). After 24 h, the cells were pulsed for 12 h with 1 µCi [³H]thymidine/well. The experiment shown is one of four experiments in which conditions for stimulation varied (time, cell concentration, concentration of activators). No significant differences (Student's *t* test; *p* > 0.05) were observed in the [³H]thymidine uptake between SEK1^{-/-} and SEK1^{+/+} B cells in response to any of these conditions. [³H]thymidine uptake is shown in cpm ± SD. (B) SEK1^{-/-} mice produce normal levels of serum immunoglobulin subclasses. Sera were collected from two individual 6-wk-old SEK1^{-/-} (shaded bars) and two individual 6-wk-old SEK1^{+/+} (open bars) chimeric mice. The concentrations of Ig subclasses are shown in µg/ml and were determined by ELISA. Standard deviations were <25 µg/ml.

Table 2. Neutralizing Anti-VSV Response in SEK1^{-/-} and CD28^{-/-} Mice

Genotype	Titers of neutralizing activities (- log 2)*			
	Day 4		Day 8	Day 12
	IgM	IgG	IgG	IgG
SEK1 ^{+/+}	9	<1	9	11
	10	<1	9	11
SEK1 ^{-/-}	9	<1	7	10
	10	<1	8	11
CD28 ^{-/-}	9	<1	2	2
	9	<1	2	3

*Sera were isolated from mice after intravenous infection with VSV (2 × 10⁶ PFU). Neutralizing IgM and IgG titers were determined as described in Materials and Methods. Titers represent twofold dilution steps of sera starting with 1:40.

produce, albeit at low levels, neutralizing IgG Abs (Table 2). These data show that SEK1^{-/-}RAG2^{-/-} mice can mount biologically relevant responses against VSV and that SEK1 has no apparent role in CD28-dependent, virus-specific germinal center formation.

SEK1-independent SAPK/JNK Activation in Peripheral T Cells. Although SEK1^{-/-} peripheral T cells displayed reduced proliferation and IL-2 production in response to CD28 in vitro, SEK1 expression was not an absolute requirement for T cell activation in vivo. Thus, similar to our data that shows that SEK1-dependent and SEK1-independent signaling pathways for SAPKs/JNKs activation exist in ES cells (23), it was possible that a SEK1-independent pathway for SAPK/JNK activation was operational in T cells. To test this hypothesis, we examined SAPKs/JNKs activation in peripheral T cells and thymocytes in response to PMA/Ca²⁺ ionophore (Fig. 6 A), which mimic TCR-CD3 and CD28-mediated signal transduction (29,45). SAPK activation, i.e., SAPK-mediated c-Jun phosphorylation, was observed in SEK1^{+/+}, but not in SEK1^{-/-}, thymocytes, indicating that SEK1 is the crucial genetic regulator of PMA/Ca²⁺ ionophore-triggered activation of SAPKs/JNKs in thymocytes (23; Fig. 6 A). Surprisingly, using the same PMA/Ca²⁺ ionophore activation regimen, SAPK activation was observed in peripheral lymph node T cells from both SEK1^{-/-} and SEK1^{+/+} mice (Fig. 6 A). The levels of SAPKs (JNK1 and JNK2) expression were comparable among SEK1^{-/-}RAG2^{-/-} and SEK1^{+/+}RAG2^{-/-} thymocytes and peripheral T cells (Fig. 6 B), suggesting that the observed differences in PMA/Ca²⁺ ionophore-mediated SAPK activation in SEK1^{-/-} thymocytes versus SEK1^{-/-} lymph node T cells were not the result of alterations in SAPK expression. Our data that SAPKs are activated in peripheral T cells, but not in thymocytes, from SEK1^{-/-} chimeras in response to the same stimulus PMA/Ca²⁺ ionophore indicate

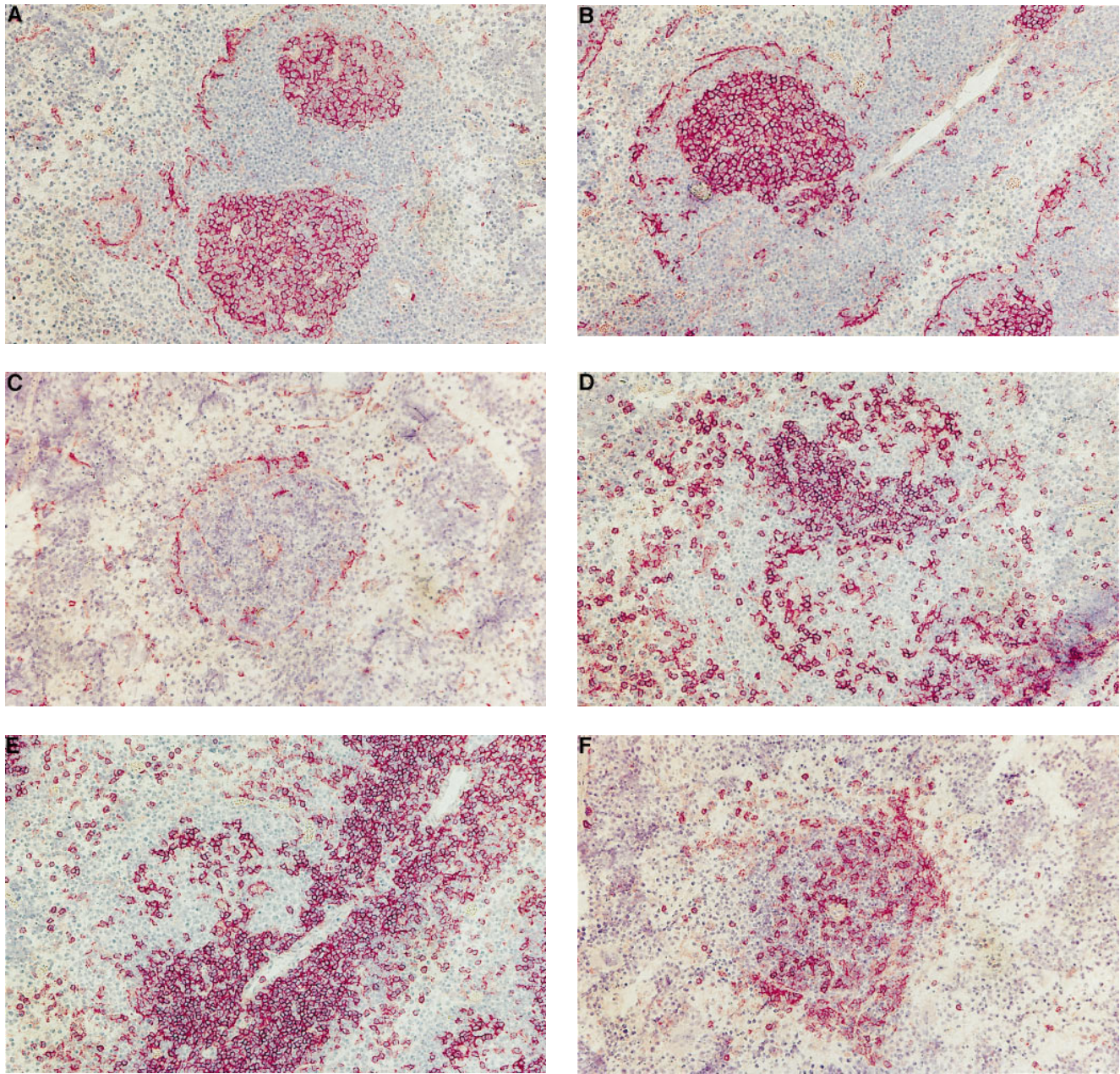


Figure 5. Germinal center formation in *SEK1*^{-/-} and *CD28*^{-/-} mice. *SEK1*^{-/-}, *SEK1*^{+/+}, and *CD28*^{-/-} mice were immunized with VSV Indiana (2×10^6 PFU). Serial spleen sections were processed for immunostaining 12 d after immunization as described in Materials and Methods. Original magnifications: (A–J) 200; (J) 400. (A–C) PNA⁺ cells localize to germinal centers and the marginal zone in (A) *SEK1*^{+/+} and (B) *SEK1*^{-/-} mice. (C) Absence of germinal center formation and germinal center PNA⁺ B cells in VSV-infected *CD28*^{-/-} mice. Some PNA⁺ B cells are present in the marginal zone and the red pulp of *CD28*^{-/-} mice. (D–F) CD4⁺ T cells localize mainly to the periarteriolar lymphatic sheaths, but are also present in germinal centers and the follicular mantle zone in (D) *SEK1*^{+/+} and (E) *SEK1*^{-/-} mice. (F) CD4⁺ T cells in the spleen of VSV-infected *CD28*^{-/-} mice. (G–I) VSV-specific B cells in germinal centers of (G) *SEK1*^{+/+} and (H) *SEK1*^{-/-} mice. Note the presence of VSV-specific B cells outside the germinal centers that show cytoplasmic staining. These cells are VSV-specific plasma cells (44). (I) VSV-specific germinal centers are absent in VSV-infected *CD28*^{-/-} mice. (J) VSV-specific plasma cells in VSV-infected *CD28*^{-/-} mice.

that signaling pathways for SAPK/JNK activation are developmentally regulated.

IL-2 Production in *SEK1*^{-/-} Thymocytes. The results in *SEK1*^{-/-} lymph node T cells indicated that *SEK1* has a role in *CD28*-mediated costimulation for proliferation and IL-2 production in peripheral T cells and that lymph node T cells use a second signaling pathway for SAPK activation

that is independent of *SEK1*. Since this second signaling pathway is not operational in *SEK1*^{-/-} thymocytes, we tested proliferation and IL-2 production of *SEK1*^{-/-} thymocytes in response to PMA/ Ca^{2+} ionophore and *CD3*/*CD28* activation. Surprisingly, *SEK1*^{-/-}*RAG2*^{-/-} thymocytes proliferated and made IL-2 after PMA/ Ca^{2+} ionophore and *CD3*/*CD28* stimulation, albeit at significantly

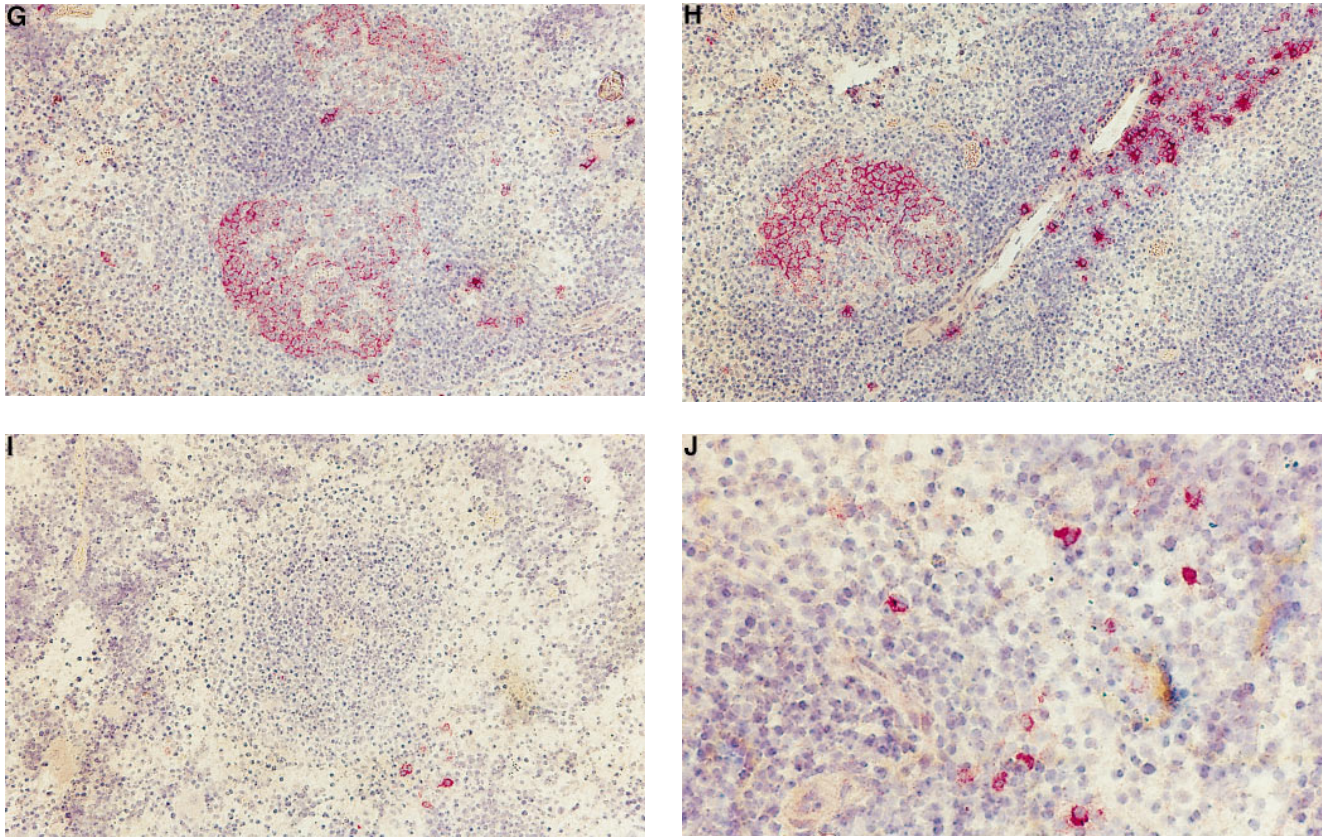


Figure 5. *Continued*

lower levels compared to $SEK1^{+/+}RAG2^{-/-}$ thymocytes (Fig. 7). These data further confirm that $SEK1$ relays $CD28$ costimulatory signals to $IL-2$ production in T cells. However, these results in thymocytes also indicate that $CD28$ costimulation and PMA/Ca^{2+} ionophore-triggered signaling pathways exist that can mediate proliferation and $IL-2$ production independently of $SAPK$ activation.

Discussion

$SAPKs/JNKs$ are activated in response to many cellular stresses such as osmolarity changes, metabolic poisons, DNA-damaging agents, heat shock, ischemia/reperfusion injury, UV-, or γ -irradiation (9, 10, 13, 14, 18, 25). The dual specificity kinase $SEK1$ (JNK kinase/MKK4) has been identified as a potent and direct activator of $SAPKs/JNKs$ in vitro and in cell lines in vivo (20–22). Although it has been shown genetically that a second $SAPK$ activator, $SEK2$, exists (23), $SEK1$ is the only cloned kinase that can directly activate $SAPKs/JNKs$ (11, 12).

In addition to the induction of $SAPK/JNK$ activity by many types of cellular stresses (10), $SAPKs/JNKs$ are activated in response to certain growth factors, heterotrimeric G proteins, phorbol esters, $CD40$ cross-linking, and $CD28$ -mediated costimulation in T cells (9, 10, 14, 29–32, 54). Moreover, activation of $SAPKs/JNKs$ leads to phosphorylation of $c-Jun$ and activation of Jun/Fos heterodimeric $AP-1$ complexes, which are involved in the coordinate ac-

tivation of $IL-2$ transcription (10, 19, 20, 55). In T cells, ligation of the TCR results in rapid activation of the $Ras \rightarrow Raf \rightarrow MEK \rightarrow MAPK$ signaling cascade (11, 56). However, activation of the $MAPK$ cascade is not sufficient for effective $IL-2$ production and cell proliferation for which T cells require a second signal (35). Recently, it has been shown that coordinate stimulation of the TCR- $CD3$ complex and the costimulatory receptor $CD28$ correlates with the activation of $SAPKs/JNKs$, phosphorylation of $c-Jun$, and induction of $AP-1$ activity (29). These bio-

Table 3. *Quantification of Germinal Centers in $SEK1^{-/-}$ and $CD28^{-/-}$ Mice*

Genotype	No. of germinal centers*	
	PNA ⁺	VSV specific
$SEK1^{+/+}$	53 ± 4.4	51 ± 5.0
$SEK1^{-/-}$	55 ± 2.0	49 ± 2.4
$CD28^{-/-}$	0	0

*The mean germinal center numbers (± SD) per section were determined on at least eight different spleen section levels from two individual mice/group. PNA-positive and VSV-specific germinal centers were visualized as described in Materials and Methods.

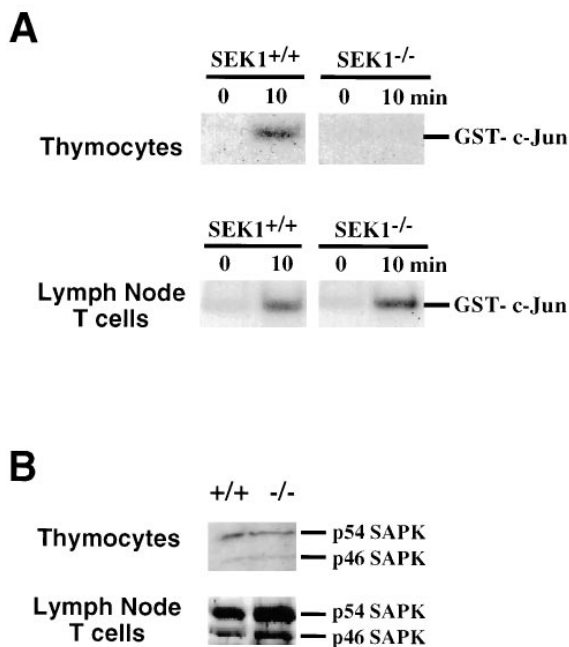


Figure 6. Activation of SAPKs/JNKs in thymocytes (*top*) and lymph node T cells (*bottom*). (A) Thymocytes (*top*) and purified mesenteric lymph node T cells (*bottom*) were isolated from SEK1^{+/+} and SEK1^{-/-} mice and cells (5×10^6 /lane) were activated with PMA (50 ng/ml) plus Ca²⁺ ionophore (1 μ g/ml) for 0 and 10 min as described in Materials and Methods. SAPK/JNK were immunoprecipitated and assayed for *in vitro* kinase activity using glutathione-S-transferase-c-Jun as a substrate. Peripheral T cells were purified using affinity columns and purity of CD3⁺ T cells was >98% as determined by cytometry. One result representative of three independent experiments is shown. (B) Western blotting for p46 and p54 SAPK/JNK isoform expression in SEK1^{+/+} (+/+) and SEK1^{-/-} (-/-) chimeric thymocytes and purified lymph node T cells. Thymocytes (10^6 /lane) and lymph node cells (5×10^6 /lane) were blotted for SAPK expression as described in Materials and Methods (23).

chemical data indicated that T cells use two distinct signaling cascades for antigen-specific activation, TCR-triggered MAPK activation and TCR-CD28-induced activation of SAPKs/JNKs. Importantly, it has been suggested that failure to activate SAPKs/JNKs in T cells might result in clonal anergy and the induction of immunological tolerance (36, 37).

Our demonstration of defective IL-2 production and proliferation in SEK1^{-/-} T cells in response to CD28 costimulation and PMA/Ca²⁺ ionophore provides the first genetic evidence that the stress signaling kinase SEK1 is a downstream effector involved in TCR and CD28 coreceptor signaling. However, the impairments of proliferation and IL-2 production were not complete, and a strong activation signal via the TCR-CD3 complex alone triggered normal proliferation of SEK1^{-/-} T cells. Thus, although SEK1 appears to be necessary for adequate IL-2 production and proliferation in T cells, another activator(s) can compensate for the SEK1 deficiency in peripheral T cells. This hypothesis is in line with our biochemical data on SEK1-independent activation of SAPKs/JNKs in lymph node T cells in response to PMA/Ca²⁺ ionophore stimulation. In-

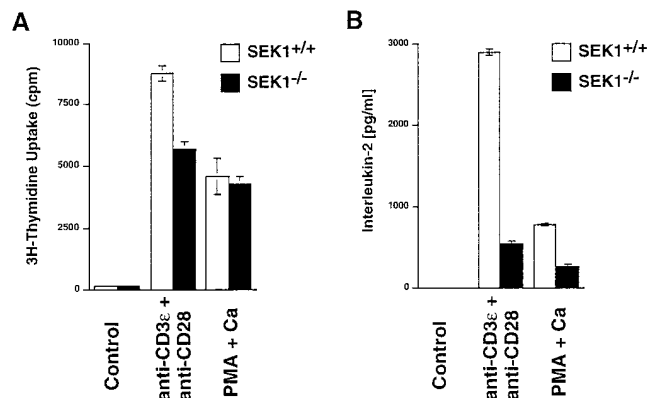


Figure 7. Proliferation (A) and IL-2 production (B) of SEK1^{-/-} (shaded bars) and SEK1^{+/+} chimeric thymocytes (open bars). Thymocytes (10^5 T cells/well) were activated with plate-bound anti-CD3- ϵ (1 μ g/ml) and soluble anti-CD28 Abs (100 ng/ml; CD3- ϵ + CD28) or PMA (12.5 ng/ml) plus Ca²⁺ ionophore (100 ng/ml) (PMA + Ca). Rabbit anti-hamster Ig-coated plates without addition of anti-CD3- ϵ /CD28 Abs are shown as controls. After 48 h of stimulation, proliferation was determined by [³H]thymidine uptake, and IL-2 production was determined by ELISA. Data of triplicate cultures \pm SD are shown. The low IL-2 production of thymocytes after PMA/Ca²⁺ ionophore stimulation is due the fact that PMA/Ca²⁺ ionophore also induces proliferation of CD4⁻CD8⁻TCR⁻ thymocytes, the majority of which does not produce IL-2. One result representative of three independent experiments is shown.

terestingly, this second pathway for SAPK/JNK activation is only operational in peripheral T cells but not in thymocytes, indicating that signaling pathways for SAPK/JNK activation are developmentally regulated in T cells. It has also been shown that proliferation and IL-2 production are normal in c-Jun^{-/-}RAG2^{-/-} T cells, suggesting that not only c-Jun, but also other Jun family members, i.e., JunD and JunB, may have a role in T cell activation (57). The exact role of distinct SAPK/JNK activators, SEK1 versus SEK2, and of different Jun family transcription factors in CD28-mediated IL-2 production and T cell activation needs to be determined.

SEK1^{-/-}RAG2^{-/-} thymocytes failed to induce SAPK/JNK in response to PMA/Ca²⁺ ionophore. Interestingly, SEK1^{-/-}RAG2^{-/-} thymocytes still proliferated and produced IL-2 after PMA/Ca²⁺ ionophore and CD3/CD28 stimulation, albeit at significantly lower levels compared to SEK1^{+/+}RAG2^{-/-} thymocytes. These data further confirmed that SEK1 relays CD28 costimulatory signals to IL-2 production in T cells. However, these results also indicate that, at least in thymocytes, CD28 and PMA/Ca²⁺ ionophore-triggered signaling pathways exist that can mediate proliferation and IL-2 production independently of SAPK activation. Besides activation of SEK1 and SAPKs/JNKs, additional downstream effectors for CD28 signaling have been identified including PI3'K, PLC γ 1, Raf-1, and Vav (see review in reference 58). In particular, it has been shown that Vav, Ras, and the Vav-associated tyrosine phosphoprotein SLP76 can cooperate to induce nuclear factor of activated T cells activity and IL-2 secretion after activation of the TCR (59-61). Nevertheless, a growing

body of evidence suggests that Vav functions as a guanine nucleotide (GDP/GTP)-exchange factor for members of the Rho family of small GTPases that regulate activation of the SAPK pathway (62–64). Whether Vav can relay TCR-mediated signals to proliferation and IL-2 production independently of SAPK activation needs to be determined.

Similar to the reported reduction in CD4⁺CD8⁺ thymocyte numbers (23), SEK1^{-/-}RAG2^{-/-} chimeric mice had a partial block in the transition from B220⁺CD25⁻CD43⁺ pro-B cells to B220⁺CD25⁺CD43⁻ pre-B cells in the bone marrow. This effect was due to the SEK1 mutation and not due to a low chimerism and contribution of RAG2^{-/-} cells to the CD43⁺ population (Fig. 3). However, splenic B cells from SEK1^{-/-}RAG2^{-/-} chimeras displayed normal proliferation in response to Ig μ , CD40, IL-4, or LPS activation. Moreover, basal Ig levels of all subclasses and IgG class switching after viral challenge were comparable between SEK1^{-/-} and SEK1^{+/+} B cells. These results imply that SEK1 has a role in early differentiation of B cell precursors, but SEK1 is not necessary for proliferation and Ig secretion of peripheral B cells.

The prominent expression of the GCK within germinal centers (34) and activation of SAPK through GCK (33) suggested that the GCK/SAPK pathway might be important for B cell differentiation within germinal centers. Moreover, mice lacking CD28 (51) or CD40 (52, 53) do not develop germinal centers. Both CD28- and CD40-triggered signaling cascades lead to the activation of SAPKs/JNKs (10, 29, 31, 32). Although SEK1 is an important component of CD28-mediated IL-2 production and T cell proliferation and VSV-specific germinal center formation is dependent on CD28 expression (Fig. 5), our results clearly indicate that SEK1 is not involved in germinal center formation in response to VSV infection. SEK1-independent activation of peripheral T cells might explain the fact that SEK1^{-/-} chimeric mice can mount CD28 costimulation dependent responses against VSV infections. Since multiple

downstream effectors for CD28 signaling have been identified including SEK1/SAPK, PI3'K, PLC γ 1, Vav, or Raf-1 kinase (see reviewed in reference 58), it is possible that CD28-mediated signaling for IL-2 production and CD28-dependent signaling for germinal center formation are biochemically different.

Conclusion. SEK1 (MKK4, JNK kinase) is a direct activator of stress-activated protein kinases (SAPK/JNK) in response to CD28 costimulation, CD40 ligation, and activation of the GCK. SEK1^{-/-}RAG2^{-/-} chimeric mice have a partial block in B cell maturation, but peripheral B cells displayed normal responses to IL-4, IgM, and CD40 cross-linking and normal IgG class switching of neutralizing Abs after viral challenge. T cells from chimeric mice showed decreased proliferation and IL-2 production in response to CD28 costimulation and PMA/Ca²⁺ ionophore activation. Although CD28 was absolutely crucial to generate VSV-specific germinal centers, SEK1^{-/-} chimeras made normal germinal centers in response to VSV. Interestingly, PMA/Ca²⁺ ionophores' stimulation, which mimic TCR-CD3 and CD28-mediated signal transduction, induced SAPK/JNK activation in peripheral T cells, but not in thymocytes, from SEK1^{-/-} mice. These results demonstrate that signaling pathways for SAPK activation are developmentally regulated in T cells. Although SEK1^{-/-} thymocytes failed to induce SAPK/JNK in response to PMA/Ca²⁺ ionophore, SEK1^{-/-}RAG2^{-/-} thymocytes proliferated and made IL-2 after PMA/Ca²⁺ ionophores and CD3/CD28 stimulation, albeit at significantly lower levels compared to SEK1^{+/+}RAG2^{-/-} thymocytes, indicating that CD28 costimulation and PMA/Ca²⁺ ionophore-triggered signaling pathways exist that can mediate proliferation and IL-2 production independently of SAPK activation. These data provide the first genetic evidence that SEK1 is an important effector molecule that relays CD28 signaling to IL-2 production and T cell proliferation.

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